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Quantitative determination of dopamine receptor subtypes not linked to activation of adenylate cyclase in rat striatum

([^{3}H]spiroperidol/[^{3}H]domperidone)

RITA M. HUFF AND PERRY B. MOLINOFF

Department of Pharmacology, School of Medicine/G3, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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ABSTRACT **The binding of [^{3}H]domperidone and [^{3}H]spiroperidol was examined in membranes prepared from rat striatum. Scatchard analysis of the binding of [^{3}H]domperidone resulted in curvilinear plots consistent with the presence of multiple classes of binding sites. Nonlinear regression analysis of untransformed data showed that the curvature was best explained by the presence of two populations of binding sites. Scatchard plots of the binding of [^{3}H]spiroperidol were linear, suggesting that this radioligand binds to a single class of receptors. However, results obtained in studies of the inhibition of [^{3}H]spiroperidol binding by a number of competing ligands were not consistent with the interaction of these agents with a single class of binding sites. Computer-assisted analysis of the Hofstee plots of six competing ligands gave the same relative proportion for two classes of sites as determined by analysis of the binding of [^{3}H]domperidone. The two classes of receptors labeled with [^{3}H]spiroperidol had affinities for domperidone that were similar to those of the two populations of binding sites for [^{3}H]domperidone. Furthermore, the number of binding sites for [^{3}H]spiroperidol was equal to the total number of binding sites for [^{3}H]domperidone. These findings suggest that the two radioligands bind to the same two classes of binding sites. It is unlikely that either of the two classes of striatal sites are receptors for serotonin. The approach described will make it possible to assess the effects of physiological or pharmacological manipulations on the densities or properties of subtypes of dopamine receptors.**

The striatum is thought to contain multiple subtypes of dopamine receptors. Kebabian *et al.* (1) characterized a dopamine-sensitive adenylate cyclase activity in the striatum. *In vitro* binding assays using the butyrophenones [^{3}H]haloperidol and [^{3}H]spiroperidol also revealed the presence of dopamine receptors in this brain region (2–4). However, the properties and distribution of the binding sites for butyrophenones differed from those expected based on studies of dopamine-sensitive adenylate cyclase activity (5–7). A classification scheme was developed such that the subtypes of dopamine receptors whose effects involved activation of adenylate cyclase were called D-1 receptors and receptors not linked to stimulation of the enzyme were called D-2 receptors (8).

Guanine nucleotides decreased the affinity of [^{3}H]spiroperidol binding sites in the striatum for agonists (9). By analogy to other receptor systems that are linked to activation of adenylate cyclase (10–12), we suggested that some of the sites labeled by [^{3}H]spiroperidol were also linked to stimulation of this enzyme. High concentrations of spiroperidol are required to inhibit dopamine-stimulated adenylate cyclase activity (ref. 13; unpublished data) but binding assays are routinely carried out with concentrations of the ligand that are lower by a factor of 2 × 10^{-3} (14–16). Although the binding sites for [^{3}H]spiroperidol appear to interact with a guanine nucleotide binding protein, this radioligand probably does not label the D-1 receptor. Preliminary experiments carried out in our laboratory gave results consistent with the existence of multiple classes of dopamine receptors labeled by [^{3}H]spiroperidol. Inhibition of the binding of [^{3}H]spiroperidol by antagonists and agonists, even in the presence of GTP, were associated with Hill coefficients of <1.

In the study reported here, curvilinear Hofstee plots were observed in experiments on the inhibition of [^{3}H]spiroperidol binding sites by various competing ligands. Curvilinear Scatchard plots were observed in studies of the binding of [^{3}H]domperidone, another radioligand used for labeling dopamine receptors that are not linked to activation of adenylate cyclase (17–19). Computer-assisted analysis of these results made it possible to quantitate and characterize two types of binding sites labeled by these radioligands.

METHODS

The striatum or frontal cortex from male Sprague–Dawley rats was homogenized in 35 ml of 20 mM Hepes, pH 7.5/154 mM NaCl/5 mM EDTA. After centrifugation (20,000 × *g* for 10 min at 4°C) pellets were resuspended in 20 mM Hepes, pH 7.5/154 mM NaCl, incubated at 37°C for 15 min, and recentrifuged. Striatal membranes were resuspended in 20 mM Hepes/154 mM NaCl at a concentration of 2–4 mg of tissue per ml for studies of [^{3}H]domperidone binding, 1–2 mg/ml for studies of [^{3}H]spiroperidol binding in the striatum, and 4 mg/ml for studies of [^{3}H]spiroperidol binding in frontal cortex. [^{3}H]Domperidone (59.7 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels), [^{3}H]spiroperidol (29.9 Ci/mmol), drugs, and GTP (final concentration, 0.3 mM) were diluted in 2.6 mM ascorbic acid containing 20 μg of bovine serum albumin per ml.

Binding reactions were carried out for 45 min at 37°C and then terminated by the addition of 10 ml of ice-cold 10 mM Tris, pH 7.5/154 mM NaCl. Samples were filtered through glass fiber filters (Schleicher & Schuell, no. 30) and washed with an additional 10 ml of the same buffer. Specific binding was defined as the difference between the amount of radioligand bound in the presence and absence of 2 μM (+) butaclamol. Protein content was determined by the method of Bradford (20).

The untransformed data obtained in studies of the binding of [^{3}H]domperidone were analyzed by nonlinear regression analysis using the computer modeling program SAAM27. The data were compared to model curves describing one, two, and three sites, and *F*-test analysis was used to determine the most appropriate model (21). Regression analysis of linear Scatchard, Hill, and Hofstee plots was carried out by the least squares method. Analysis of nonlinear Hofstee and Scatchard plots was carried out with a computer-based method that uses an iterative

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Abbreviations: 5-HT, serotonin; NPA, *N*-propylnorapomorphine.

procedure to provide B_{max} and EC_{50} values for each component (22). EC_{50} values were converted to K_d values for each competing ligand (23).

RESULTS

[^{3}H]Domperidone. Saturation data of the binding of [^{3}H]-domperidone was best fit to a two-site model (Fig. 1A). The nonlinear analysis provided the following parameters for the best fit model: 23% of the sites had a K_d of 167 pM, and 77% of the sites had a K_d of 1,250 pM. Scatchard transformation of the data was markedly curvilinear (Fig. 1B). The affinities and capacities of two classes of binding sites detected in each of eight independent experiments are shown in Table 1. The high-affinity binding sites represented 21% of the total number of sites and had a K_d value for [^{3}H]domperidone of 130 pM. The remaining 79% of the sites had 1/10th the affinity for [^{3}H]domperidone.

[^{3}H]Spiroperidol. Scatchard plots of binding data obtained with [^{3}H]spiroperidol were linear (Fig. 2B), apparently reflecting the interaction of this radioligand with a single class of high-affinity binding sites. The mean K_d value obtained from several such experiments was 50 ± 4 pM and the density of binding sites was 1,223 ± 87 fmol/mg of protein (Table 1). Another approach to assess the homogeneity of [^{3}H]spiroperidol binding sites is to examine the Hill coefficient obtained from studies of the displacement of bound radioligand by unlabeled spiroperidol (Fig. 2). The slope of Hill plots of these data were very close to 1, again consistent with the presence of a single class of high-

Table 1. Scatchard analysis of the binding of [^{3}H]domperidone and [^{3}H]spiroperidol

	K_d, nM	B_{max}, fmol/mg	%
[^{3}H]Domperidone			
Site A	0.13 ± 0.03	292 ± 72	21 ± 4
Site B	1.3 ± 0.2	1,038 ± 76	79 ± 4
Total		1,330 ± 105	
[^{3}H]Spiroperidol	0.05 ± 0.004	1,223 ± 87	

Curvilinear Scatchard plots of domperidone binding to rat striatal membranes (n = 8) were dissected into two components by using a computer-assisted analysis. The values obtained by linear regression analysis of Scatchard plots of [^{3}H]spiroperidol binding in rat striatal membranes (n = 14) are also shown. All values are mean ± SEM.

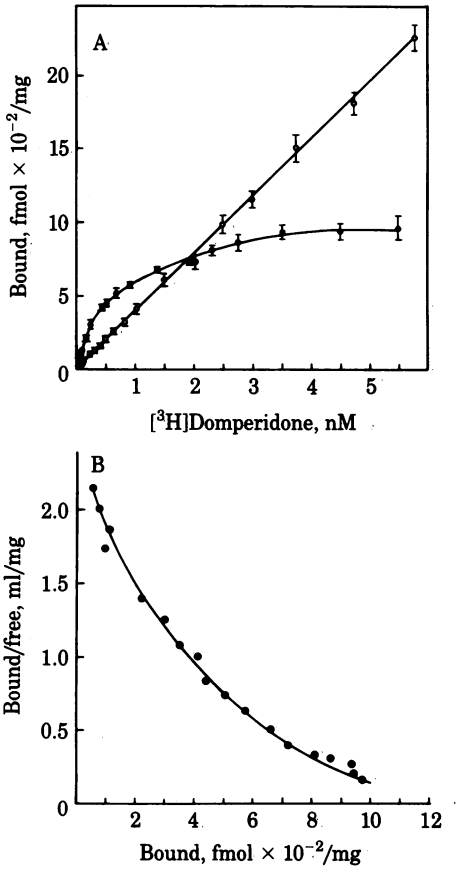

FIG. 1. Binding of [^{3}H]domperidone to dopamine receptors in rat striatum. Rat striatal membranes were incubated with varying concentrations of [^{3}H]domperidone (0.04–6 nM). (A) The mean (± SEM) amounts of specifically bound radioligand are plotted versus the free concentration of [^{3}H]domperidone (●). Nonspecific binding values (mean ± SEM) of four determinations were also plotted versus the free concentration of [^{3}H]domperidone (○). The curved line represents the theoretical saturation plot for two populations of binding sites having the affinities and relative proportions determined by nonlinear regression analysis. (B) The Scatchard plot is the transformation of saturation data averaged from four tissue preparations analyzed simultaneously. The curved line represents the theoretical curve for two populations of binding sites having the affinities and proportions determined by computer-assisted analysis of the transformed data.

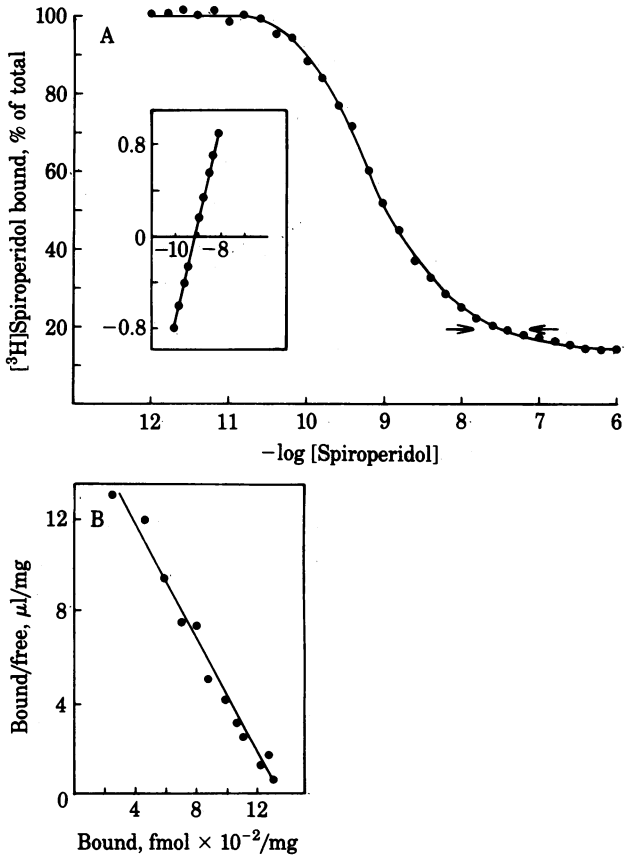

FIG. 2. Binding of spiroperidol to dopamine receptors in rat striatum. (A) The inhibition of the binding of [^{3}H]spiroperidol (300–600 pM) to rat striatal membranes was determined in the presence of 30 concentrations of unlabeled spiroperidol. The data are mean values from four individual experiments. The arrows indicate the amount [^{3}H]spiroperidol binding inhibited by 2 μM (+) butaclamol (specific binding). The K_i value, 47 pM, was determined (24) by using a K_d of 50 pM for [^{3}H]spiroperidol (Table 1). (*Inset*) Hill transformation of the data plotted as log bound/(B_{max}-bound) vs. log (drug concentration) gave n_H = 0.95. (B) Scatchard transformation of data obtained after incubation of rat striatal membranes with varying concentrations of [^{3}H]spiroperidol (0.01–1 nM); K_d = 36 pM.

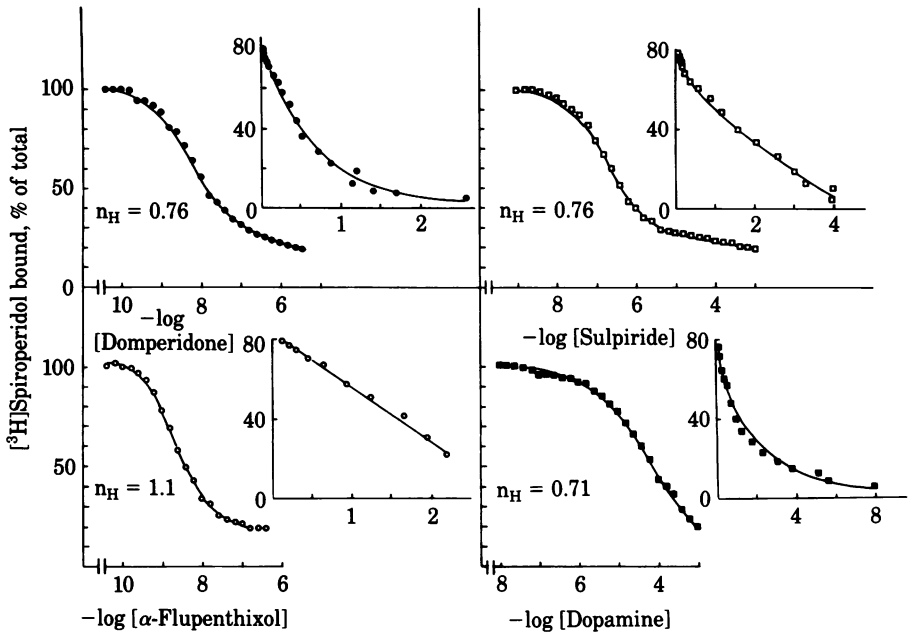

FIG. 3. Inhibition of [³H]spiroperidol binding by competing ligands. The amount of [³H]spiroperidol (300–600 pM) bound was determined in the presence of varying concentrations of competing ligands. The values shown are the means of three to seven determinations for each drug. The Hill coefficients of each dose–response curve are shown. (*Insets*) Hofstee transformation (bound vs. bound/concentration of drug) of the same data.

affinity binding sites (Fig. 2*A Inset*). The K_i value (47 pM) for unlabeled spiroperidol was similar to the K_d value (50 pM) determined from Scatchard analysis of the binding of [³H]-spiroperidol.

Competing Ligands. Inhibition of [³H]spiroperidol binding by agonists and antagonists was examined. Effects of the agonists dopamine and *N*-propylnorapomorphine (NPA) were studied in the presence of 300 μM GTP. Domperidone, sulpiride, and dopamine in the presence of GTP displaced [³H]spiroperidol with Hill coefficients <1, resulting in nonlinear Hofstee plots. α-Flupenthixol, however, generated competition curves with Hill coefficients of 1 (Fig. 3).

Computer-assisted analysis of domperidone displacement of [³H]spiroperidol binding in the striatum showed that 28% of the sites had a K_d for domperidone of 93 pM and 72% of the sites had a K_d of 1,800 pM (Table 2). These values are in good agreement with those obtained from analysis of Scatchard plots of the binding of [³H]domperidone. Similar analysis of nonlinear Hofstee plots observed in studies with the other drugs resulted in nearly the same proportions of two classes of sites as determined in studies with [³H]domperidone: approximately 3:1. Site A, defined as the high-affinity site for domperidone, also had a higher affinity for bromocriptine and dopamine than did site B; the reverse was true for sulpiride, fluphenazine, and NPA.

Serotonin Receptors. [³H]Spiroperidol has been reported to label serotinin-2 (5-HT_2) receptors (25–27). The binding of [³H]spiroperidol was examined in the frontal cortex, a tissue thought to contain a high density of 5-HT_2 receptors. Scatchard analysis of the binding of [³H]spiroperidol in this tissue resulted in curvilinear plots. Ten percent of the sites had a high affinity

Table 2. Quantitative determination of dopamine receptor subtypes by analysis of curvilinear Hofstee plots

		Site A		Site B	
Competing drug	*n*	K_d, nM	%	K_d, nM	%
Selective:					
Domperidone	7	0.093 ± 0.001	28 ± 2	1.8 ± 0.2	72 ± 2
Sulpiride	6	450 ± 70	20 ± 3	16 ± 3	80 ± 3
Bromocriptine	3	0.042 ± 0.018	30 ± 3	1.1 ± 0.2	70 ± 3
Fluphenazine	5	1.4 ± 0.4	25 ± 4	0.097 ± 0.008	75 ± 4
Dopamine + GTP	3	130 ± 6	23 ± 3	5,300 ± 200	77 ± 3
NPA + GTP	7	420 ± 210	26 ± 2	6.5 ± 1.4	74 ± 2
Nonselective:					
α-Flupenthixol	3	0.25 ± 0.02			
Spiroperidol	4	0.047 ± 0.005			

Hofstee plots obtained from studies of the inhibition of the binding of [³H]spiroperidol by several drugs were examined in membranes prepared from rat striatum. Inhibition of the binding of [³H]spiroperidol by selective competing ligands resulted in curvilinear Hofstee plots that were analyzed by a computer-based iterative program to give the affinities of each of two classes of sites for each drug and the relative proportions of the two sites. Nonselective competing ligands resulted in linear Hofstee plots, and a single K_d value was determined. *n*, Number of individual experiments. Results are mean ± SEM.

for [^{3}H]spiroperidol (K_d = 59 ± 6 pM) and 90% of the sites had a low affinity (K_d = 1,012 ± 113 pM; n = 7). The affinity of the small population of binding sites for [^{3}H]spiroperidol was similar to that determined for the [^{3}H]spiroperidol binding sites in the striatum. In the caudate, 5-HT was less potent than dopamine as an inhibitor of [^{3}H]spiroperidol binding but in the frontal cortex the opposite order of potency was observed (data not shown). R41-468, a compound reported to be selective for 5-HT$_2$ receptors (28), was 100 times more potent at displacing [^{3}H]spiroperidol binding in the cortex than in the striatum. Analysis of the inhibition of [^{3}H]spiroperidol binding in the frontal cortex by domperidone resulted in a K_d value of 110 nM. This value is 60-fold greater than the K_d value of either of the two classes of [^{3}H]spiroperidol binding sites in the striatum for domperidone. Attempts to measure the binding of [^{3}H]domperidone in frontal cortex were unsuccessful because the percentage of specific binding was very low.

DISCUSSION

The present results suggest that [^{3}H]spiroperidol and [^{3}H]-domperidone label multiple classes of binding sites in the striatum. Nonlinear regression analysis of the binding of [^{3}H]domperidone showed that fitting the data to a two-component model significantly increased the goodness of fit compared to a one-component model but postulating the presence of a third component did not improve the fit. The same relative proportions of the two putative classes of sites were obtained from analysis of results of the nonlinear Hofstee plots in studies of the inhibition of binding of [^{3}H]spiroperidol by various drugs. In addition, this same relative proportion of the two populations of receptors was obtained from analysis of the curvilinear Scatchard plots observed in studies of the binding of [^{3}H]-domperidone. The affinities of the two classes of sites for domperidone—whether obtained from Scatchard plots of [^{3}H]-domperidone binding or from studies of the inhibition of the binding of [^{3}H]spiroperidol by domperidone—were similar. The total number of receptors defined with [^{3}H]spiroperidol and [^{3}H]domperidone also were similar. These findings support the conclusion that these two radioligands are labeling the same two classes of receptors.

Scatchard analysis of saturation binding of [^{3}H]domperidone in the striatum has been studied by Martres *et al.* (24) and by Lazareno and Nahorski (19). The former investigators reported a K_d value of 900 pM for a single class of binding sites labeled with high concentrations of [^{3}H]domperidone. Lazareno and Nahorski (19), on the other hand, used low concentrations of [^{3}H]domperidone and observed a single class of receptors with a K_d value of about 80 pM. Failure of these groups to use a sufficiently large range of concentrations of [^{3}H]domperidone may account for the fact that only a single class of receptors was observed in each case.

A potential problem with the use of [^{3}H]spiroperidol to study dopamine receptors stems from the fact that this ligand also labels 5-HT$_2$ receptors (25, 26). The receptor population defined as site A comprises only 25% of the total, making these sites the more likely candidates to be receptors for 5-HT. Site A, however, had a higher affinity for dopamine than site B, making this suggestion improbable. Comparison of the properties of the binding sites for [^{3}H]spiroperidol in the striatum to those in the frontal cortex suggests that [^{3}H]spiroperidol does not bind to 5-HT$_2$ receptors in the striatum under the conditions used in our laboratory. Scatchard analysis of the binding of [^{3}H]spiroperidol was linear in the striatum, and the affinity of the receptors for this ligand was much higher than the affinity of the majority of the receptors in the frontal cortex for [^{3}H]spiroperidol. The small population of high-affinity binding sites for [^{3}H]spiroperidol probably represents dopamine receptors present in the frontal cortex (27). In addition, receptors in the frontal cortex have 1/60th the affinity for domperidone compared to either of the two classes of striatal sites.

At this time it is not possible to fit these two classes of receptors into the existing classification schemes for subtypes of dopamine receptors except to exclude their possible relationship to so-called D-1 receptors. The low potencies of domperidone and spiroperidol for inhibition of dopamine-stimulated adenylate cyclase activity (13, 18) make it unlikely that any of the sites labeled by these ligands are associated with stimulation of this enzyme. Sokoloff *et al.* (30) classified dopamine receptors into four subtypes. The D-2 and D-4 sites were defined by biphasic displacement curves of dopamine and apomorphine for [^{3}H]domperidone binding sites. [^{3}H]Domperidone bound with a high affinity to the D-2 and D-4 sites; there were half as many D-2 sites as D-4 sites. Bromocriptine had a higher affinity for the D-2 sites; sulpiride had a higher affinity for the D-4 sites. The agonist displacement curves were not studied, however, in the presence of guanine nucleotides. Agonist interactions with many receptors involve a two-step/three-component binding reaction yielding results that are similar to those obtained if multiple classes of receptors are present. In the presence of guanine nucleotides, agonist interactions with these receptors obey simple principles of mass action (Hill coefficients = 1) in tissues with a single class of receptors (31). Thus, for quantitative analysis of dopamine receptors it is imperative to utilize antagonists or to study the binding of agonists only in the presence of guanine nucleotides. The two classes of receptors identified here resemble the D-2 and D-4 sites defined by Sokoloff *et al.* (30), but the absence of GTP in the studies of those authors makes it impossible to interpret the results in a quantitative way (see ref. 29). Creese *et al.* (32) reported that, following kainate lesions of the rat striatum, only half of the binding sites for [^{3}H]spiroperidol remained and these sites were no longer affected by GTP. It is possible that these findings are indicative of the same heterogeneity of [^{3}H]spiroperidol binding sites observed in the present studies. Rosenfield *et al.* (33) also reported findings suggesting heterogeneity of the binding sites for [^{3}H]spiroperidol in rat striatum; however, their findings were not analyzed quantitatively.

At least one of the classes of [^{3}H]spiroperidol binding sites described here is affected by guanine nucleotides, yet neither class of sites is linked to stimulation of adenylate cyclase activity. The presence, in the intermediate lobe of the pituitary (34) and the striatum (35), of dopamine receptors that mediate inhibition of adenylate cyclase activity has been reported. The observed effects of GTP on binding sites for [^{3}H]spiroperidol in the rat striatum may reflect an interaction of some of these receptors with a guanine nucleotide binding protein that mediates inhibition of adenylate cyclase activity.

A large number of pharmacological agents are needed for the classification of receptor subtypes. For further characterization of dopamine receptor subtypes in the striatum and in other tissues it will be useful to identify agents that have a high degree of selectivity for each of the classes of sites. In any case, the fundamental criterion that must be fulfilled in the delineation of receptor subtypes is that the properties of the receptor must be conserved. This means that the pharmacological specificity of a particular receptor subtype must be shown to be identical in every tissue that is examined. This criterion is yet to be tested for the proposed scheme. Additional studies using other drugs and various tissues will be required to test the proposed classification. This scheme may make it possible to implicate specific subtypes of dopamine receptors with identifiable dopamine-mediated physiological responses.

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