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Interaction of [^{3}H]Spiperone with Rat Striatal Dopamine D-2 Receptors: Kinetic Evidence for Antagonist-Induced Formation of Ternary Complex

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SUMMARY

The characteristics of [^{3}H]spiperone interactions with rat striatal dopamine D-2 receptor were investigated. Although the association of [^{3}H]spiperone occurred monoexponentially, the pseudo-first order rate constant of association showed a hyperbolic dependence on ligand concentration. The data were therefore analyzed with the assumption of a two-step binding reaction leading to ligand-induced receptor isomerization. For the first equilibrium, the dissociation constant (K_D) was 1.2 nM, while for the second equilibrium, the association and the dissociation rate constants were 71.6×10^{-3} sec^{-1} and 0.9×10^{-3} sec^{-1}, respectively. The dissociation rate constant of the overall binding reaction, as determined by inducing the dissociation of [^{3}H]spiperone from its binding sites by 1 μM (+)-butaclamol, was 0.92×10^{-3} sec^{-1}. However, the kinetically derived K_D (15 pM) of the binding reaction differed significantly from the K_D (218 pM) obtained from equilibrium binding experiments. This inconsistency between the two K_D values appeared to have arisen from using different receptor concentrations in deriving kinetic and equilibrium data. The K_D of the equilibrium binding reaction indeed showed significant variation with the receptor concentrations in an inverse way, implicating the involvement of a third component in the two-step binding reaction to form a high affinity ternary complex rather than a simple ligand induced receptor isomerization. Pretreatment of the membrane with 0.1 mM guanosine 5′-imidodiphosphate [Gpp(NH)p] reduced the affinity of the equilibrium binding reaction to a value (K_D = 1.2 nM) which corresponded to the kinetically derived K_D of the first step of the binding reaction, indicating the involvement of a guanine nucleotide-binding protein or G protein in inducing the formation of the high affinity ternary complex. The affinity of the binding reaction in Gpp(NH)p-pretreated membranes, however, increased with the duration of incubation, indicating that the ligand receptor complex still can couple with the G protein even in the presence of Gpp(NH)p. Pretreatment of the membrane with pertussis toxin irreversibly decreased the affinity of the binding reaction without significantly affecting the total number of binding sites, implying the involvement of the G_i subclass of G protein in the interaction of [^{3}H]spiperone with D-2 receptors. Inhibition of the [^{3}H]spiperone binding by a dopamine receptor agonist, bromocriptine, also yielded a monophasic dose response curve both in the presence and in the absence of Gpp(NH)p. However, dopamine inhibited the binding with a multiphasic dose response curve in the presence as well as in the absence of Gpp(NH)p. Furthermore, dopamine accelerated [^{3}H]spiperone dissociation by infinite dilution and inhibited [^{3}H]spiperone binding noncompetitively, characteristics sharply in contrast to those of bromocriptine. We propose that the dopamine D-2 receptor population in striatum does not exist predominantly in association with G proteins; [^{3}H]spiperone interacts with a homogeneous population of the free form of the receptor, with low affinity to promote receptor coupling with G protein to form a high affinity ternary complex, and this ternary complex, induced by antagonist occupancy of the receptor sites, is not destabilized by Gpp(NH)p. These results also allow us to suggest that the anamolous observations for dopamine inhibition of [^{3}H]-spiperone binding may be mediated, in part, by an interaction of dopamine with a site linked allosterically with [^{3}H]spiperone-binding sites.

Based on pharmacological criteria of recognition and the regulation of adenylate cyclase activity, dopamine receptors have been classified into D-1 and D-2 subclasses (1, 2). Agonist occupancy of D-1 receptor mediates the stimulation of adenylate cyclase activity, while D-2 receptor occupancy by agonist is correlated either with a decrease in, or no effect on, the activity of this enzyme.

In membrane preparations of rat pituitary and striatum, the inhibitory coupling of D-2 receptors with adenylate cyclase is now well documented (3–6). This inhibitory coupling is mediated through a guanine nucleotide-binding protein (G protein) which is distinct from the G protein involved in the stimulation of adenylate cyclase (7). Based on radioligand binding experiments, it has been suggested that the D-2 recep-

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ABBREVIATIONS: Gpp(NH)p, guanosine 5′-imidodiphosphate; EDTA, ethylenediaminetetraacetate.

tor population exists coupled with a G protein (RG) as well as in the free form (R) (8–10). Agonists recognize only the coupled form of the receptor with high affinity, whereas antagonist affinity does not differ for these two forms of D-2 receptors (8–10).

The findings that the affinity of agonists for the D-2 receptor is markedly reduced in the presence of the guanine nucleotide, whereas antagonist binding is 1) insensitive to such treatment, 2) monophasic in competition with other antagonists and characterized by a linear Scatchard plot, and 3) labels nearly twice as many receptor sites as those labeled by agonists provided support for the currently accepted model of the D-2 receptor (8, 10). According to this model, the agonist high (RG) and low affinity (R) forms of D-2 receptors exist in equal proportion; nM concentrations of agonists label only the high affinity form of the receptor (RG form), whereas pM concentrations of antagonists label both forms (RG and R forms) with equal affinity (8, 10). From this model it follows that the receptor population which exists in the low affinity (R) form does not undergo complex formation with G protein even in the presence of an agonist.

Such a model of D-2 receptor, however, does not seem to be adequate to appropriately explain several reports on the interactions of agonists and antagonists with rat striatal D-2 receptors. According to Hancock and Marsh (11), the binding sites for agonists and antagonists for D-2 receptors are different in rat striatum. In contrast, Seeman and Grigoriadis (12) suggest the opposite. However, the studies on striatal D-2 receptors using protein-modifying agents, such as phenoxybenzamine (13, 14) and radiation treatments (15), indicate the existence of agonist- and antagonists-binding sites with different characteristics. Although phenoxybenzamine treatments eliminate all the [^{3}H]spiperone-binding sites in the striatum, they leave the ^{3}H-agonist binding in this tissue virtually unaffected; radiation produces a differential inactivation of [^{3}H]spiperone- and [^{3}H]*N*-propylnorapomorphine-binding sites. Also, the agonist and antagonist binding to striatal D-2 receptors is differentially affected by Gpp(NH)p. When labeled with agonists, the high affinity form of the D-2 receptor showed marked sensitivity to Gpp(NH)p (16, 17), whereas, upon labeling with the antagonist, the high affinity form of D-2 receptor, as manifested by agonist competition studies, showed poor sensitivity to Gpp(NH)p (10, 18–20).

The anamoly in binding characteristics not only exists between the binding of agonists and antagonists to the D-2 receptor as described above, but also in the antagonist/^{3}H-antagonist competition for this receptor in the striatum. Several laboratories have reported the antagonist/[^{3}H]spiperone competition curves to be "shallow" (18–20) suggesting a complex binding phenomenon. [^{3}H]Spiperone also binds to 5HT-2 receptor sites, and such binding may contribute to the complexity of the binding characteristics. Huff and Molinoff (20), however, indicated that the shallow antagonist/[^{3}H]spiperone competition was more consistent with the existence of two affinity states of the D-2 receptor than with any contribution of [^{3}H]spiperone binding to 5HT-2 receptors. Grigoriadis and Seeman (10), in contrast, suggest that the shallow competition of antagonist/[^{3}H]spiperone can be explained by assuming a significant interaction of [^{3}H]spiperone with 5HT-2 receptors. They proposed that 80 pM [^{3}H]spiperone, a concentration used in their experiment, labels a significant proportion of 5HT-2 receptor sites even in the presence of 50 nM ketanserin, a concentration about 100-fold in excess of the K_D (0.42 nM) concentration of ketanserin for 5HT-2 receptor (21). If [^{3}H]spiperone labels a significant proportion of 5HT-2 receptor sites along with D-2 receptor sites, one would expect a significant deviation from linearity of the kinetic characteristics of [^{3}H]spiperone binding with rat striatal tissue under conditions used to label D-2 receptor sites, performed either in the presence or absence of a 5HT-2 receptor blocker. The preliminary findings from this laboratory (22) on kinetics of association and dissociation of [^{3}H]spiperone at rat striatal D-2 receptors did not reveal any such deviation from linearity.

The inconsistencies between various studies and the current model of the D-2 receptor raise doubts about the validity of the model. Therefore, we undertook a thorough reevaluation of the characteristics of binding of both agonists and antagonists with the D-2 receptor. The results of the antagonist binding studies allow us to suggest that, in contrast to the widely held view, the D-2 receptor population exists without association with a G protein; [^{3}H]spiperone (L) interacts with a homogeneous population of the free form of the receptor (R), with low affinity to promote receptor coupling with a G protein to form a high affinity ternary complex (LRG), and this ternary complex, induced by occupancy of the receptor by an antagonist, is not destabilized by Gpp(NH)p. The results of agonist binding studies indicate that dopamine, unlike bromocriptine, inhibits [^{3}H]spiperone binding with more than one affinity in the absence as well as in the presence of 0.1 mN Gpp(NH)p. Inhibition of [^{3}H]spiperone binding by dopamine is mediated, in part, through an interaction of dopamine with a site linked allosterically to the D-2 receptors, whereas the inhibition of [^{3}H]spiperone binding by bromocriptine, in contrast, is mediated via direct (competitive) interactions of bromocriptine with D-2 receptor sites.

Materials and Methods

[^{3}H]Spiperone (specific activity, 20–23 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA). Gpp(NH)p, thymidine, ATP, GTP, dithiothrietol, creatine phosphate, creatine phosphokinase, 1,10-*o*-phenanthroline, NAD^+, dopamine, pargyline, and polyethylenimine were obtained from Sigma Chemical Co. (St. Louis, MO). Pertussis toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA) and ketanserin bitartrate from Research Biochemical Inc. (Wayland, MA). (+)-Butaclamol was generously supplied by Dr. D. J. Marshall (Ayerst Research Laboratories, Montreal, Canada). All other chemicals used in the present study were obtained from Fisher Scientific Co. (Chicago, IL).

Preparation of membranes. Male Sprague-Dawley rats (Harlan Laboratory, Madison, WI) (200–300 g) were used. Rats were killed by decapitation, the brain was rapidly removed, and the corpus striatum was dissected out over a cold Petri dish and pooled. Tissue was homogenized in 1:100 volumes (wet weight, g/volume) of ice-cold 50 mM Tris·HCl buffer (pH 7.4 at 25°) containing 5 mM KCl, 1 mM EDTA, and 4 mM $MgCl_2$ using a Brinkmann Polytron homogenizer for 20 sec at setting 7. The homogenate was centrifuged for 10 min in the cold (4°) at 48,000 × *g* and the resulting pellet was resuspended in an identical volume of fresh buffer and incubated for 10 min at 37°. The suspension was then centrifuged again at 48,000 × *g* for 10 min and the resulting pellet was washed three more times by intermediate rehomogenization in fresh buffer and recentrifugation. The resulting pellet was suspended in an assay buffer of 50 mM Tris·HCl (pH 7.4 at 25°) containing 5 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 150 mM NaCl, 0.1% ascorbic acid, and 10 μM pargyline to a final

concentration of 10 mg wet weight/ml, which yielded approximately 0.5 mg of protein/ml as determined by the method of Lowry *et al.* (23). In some experiments assay buffer also contained, in addition, 50 nM ketanserin.

Association kinetics. To study the kinetics of association of [^{3}H] spiperone with the D-2 receptor, 6.0 ml of the striatal membrane suspension and 22.5 ml of assay buffer, containing an appropriate concentration of [^{3}H]spiperone, were brought to 37° by incubating them separately in a 37° water bath for 30 min. The association reaction was initiated at 37° by the addition of 2.5 ml of the tissue suspension to the assay buffer containing the radioligand; at various time intervals 2.0-ml aliquots were collected over Whatman GF/B filter discs and washed three times with 5.0 ml of cold assay buffer. Filters were placed in glass counting vials containing 10.0 ml of Ready-Solv (Beckman, Irvine, CA) and counted in a liquid scintillation spectrometer at an efficiency of 46–51%. Nonspecific association of [^{3}H]spiperone was determined for each time point in a parallel experiment in which 1 μM (+)-butaclamol was present prior to the initiation of the association reaction. In some experiments 50 nM ketanserin was included in the assay buffer so that the initial tissue preincubation and the association reaction occurred in the presence of 5HT-2 receptor blocker.

Dissociation kinetics. To study the kinetics of dissociation of [^{3}H] spiperone from the D-2 receptor, 6.0 ml of the striatal membrane suspension, either in a concentration of 0.5 mg of protein/ml or a specific dilution thereof, and 22.5 ml of assay buffer containing the appropriate concentration of [^{3}H]spiperone were brought to 37° by incubating them separatly in a 37° water bath for 30 min. The ligand-receptor binding reaction was initiated by adding 2.5 ml of the tissue suspension to the assay buffer containing the radioligand, and the reaction was continued for 30 min at 37°. Dissociation was initiated at 37° by adding 1 μM (+)-butaclamol; 2.0-ml aliquots were withdrawn at specified times after initiation of dissociation and collected over Whatman GF/B filters, which were washed and counted as previously described. Nonspecific binding was monitored in parallel experiments in which 1 μM (+)-butaclamol was present in the assay medium prior to the addition of the tissue suspension. The effect of 5HT-2 receptor blocker on dissociation was examined by including 50 nM ketanserin in the assay buffer.

In experiments where dissociation was initiated by infinite dilution, a modified procedure was followed. Striatal membrane suspensions of 3.0 mg of protein/ml in assay buffer and 2.0 ml of the assay buffer containing 0.4 nM [^{3}H]spiperone were brought to the same temperature by incubating them separately in a 37° water bath for 30 min; the binding reaction was initiated by the addition of 2.0 ml of the membrane suspension to the buffer containing the radioligand and incubated for 30 min at 37°. The dissociation reaction was initiated by adding 396 ml of prewarmed (37°) assay buffer or the same volume of buffer containing either 1 μM (+)-butaclamol or indicated agents in specific concentrations. This procedure was sufficient to prevent any reassociation of [^{3}H]spiperone with the receptor. At appropriate times after the initiation of dissociation, 10.0-ml aliquots were collected over Whatman GF/B filters, washed, and counted as previously described. Dissociation from nonspecific sites was monitored similarly, except that 1 μM (+)-butaclamol was added prior to initiation of the binding reaction. The effect of 5HT-2 receptor blocker on dissociation was examined by including 50 nM ketanserin in the assay buffer.

Equilibrium binding. Equilibrium binding experiments were performed in a final assay volume of 20.0 ml; 1.0 ml of radioligand of appropriate dilution in assay buffer was added to 18.8 ml of assay buffer in 50.0-ml capacity polypropylene tubes. This mixture and the tissue suspension were kept separately at 37° for 30 min before adding 0.2 ml of the tissue suspension. The binding reaction was continued for 30 min at 37° and the entire assay mixture was vacuum filtered over Whatman GF/B discs, washed, and counted as previously described. Where indicated, the tissue suspension was diluted to yield an appropriate receptor concentration in the binding reaction medium when added in 0.2 ml volume. In some experiments, dopamine or bromocriptine, at appropriate concentrations, were included in the binding assay medium to ensure measurement of equilibrium binding parameters in the presence of these agents.

Pertussis toxin treatment. Pertussis toxin treatment of the tissue was performed according to a method described earlier (24). Striatal tissue was homogenized in 1:100 volumes (wet weight/volume) of ice-cold 50 mM Tris·HCl buffer (pH 7.4 at 25°) containing 5 mM KCl, 1 mM EDTA, and 4 mM $MgCl_2$ using the Polytron for 20 sec at setting 7. The homogenate was centrifuged for 10 min in the cold at 48,000 × *g*, and the resulting pellet was resuspended in an identical volume of fresh buffer and incubated for 10 min at 37°. The homogenate was then centrifuged in the cold at 48,000 × *g* for 10 min and the resulting pellet was suspended in 1:25 volumes (original wet weight/volume) of 40 mM Tris·HCl buffer (pH 8.0 at 25°) containing 16 mM thymidine, 0.4 mM ATP, 16 μM GTP, 4 mM $MgCl_2$, 0.8 mM EDTA, 4 mM dithiothrietol, 16 mM creatine phosphate, 4 units of creatine phosphokinase, 0.8 mM 1,10-*o*-phenanthroline, 10 μM NAD^+, and 10 μg/ml pertussis toxin. This suspension was then incubated for 45 min at 37°. This treatment schedule was found to be optimal to induce maximum possible effects of pertussis toxin on binding. In an earlier study (25) a lack of an effect of pertussis toxin treatment on [^{3}H]spiperone binding in bovine striatum was reported. However, using a different source of pertussis toxin in the present experiments and the method of Law *et al.* (24) for treatment of membranes with pertussis toxin, we obtained consistent effects of this treatment on [^{3}H]spiperone binding. Control tissue was similarly treated except that pertussis toxin was omitted. At the end of the incubation, the suspension was diluted 1:100 with ice-cold 50 mM Tris·HCl buffer (pH 7.4 at 25°) containing 5 mM KCl, 1 mM EDTA, and 4 mM $MgCl_2$ and centrifuged in the cold at 48,000 × *g* for 10 min. The pellet was washed three more times with 50 mM Tris·HCl buffer (pH 7.4) containing 5 mM KCl, 1 mM EDTA, and 4 mM $MgCl_2$ before final suspension in the assay buffer.

Competition. The assay mixture (5.0 ml final volume) contained 2.7 ml of assay buffer (in some experiments, assay buffer also contained 50 nM ketanserin), 1.0 ml of [^{3}H]spiperone in assay buffer to yield a final concentration of 0.5 nM and competing agents in 0.8 ml volume of assay buffer. The reaction was initiated by the addition of 0.5 ml of the tissue suspension (1:100 volumes in assay buffer and preincubated at 37° for 30 min) and terminated after 30 min of incubation at 37° by vacuum filtration over Whatman GF/B filters. The filters were washed and counted as previously described. In some experiments 0.1 mM Gpp(NH)p was included in the binding assay medium.

Analysis of data. Data from experiments studying equilibrium binding and competition were analyzed by using the LIGAND program of Munson and Rodbard (26) with the modification made by McPherson (27) for adoption to microcomputers without constraining the values of nonspecific binding. The points are weighted using the model described by Munson and Rodbard (26). The goodness of fit of the data was examined by using the runs test (28), and the appropriateness for selecting a particular fit was determined by using the approximate F test as previously described (26, 29). The kinetics of association and dissociation were evaluated by least squares regression analysis using the appropriate equation. A runs test (28) and an approximate F test (26, 29) were also applied to kinetic data to determine whether the transformed data points were randomly distributed around the regression line. The runs test provides a more appropriate indicator of the linearity of a set of points than does the correlation coefficient of the regression analysis.

Results

Kinetics of [^{3}H]spiperone association. The kinetics of [^{3}H]spiperone association with the D-2 receptor in rat striatal membrane preparations were followed at 37° as a function of ligand concentrations under pseudo-first order conditions involving a greater than 5- to 58-fold excess of the ligand over the receptor concentration. The semilogarithmic transforma-

tion of the first order association plot is depicted in Fig. 1. It is evident from the figure that the association of [³H]spiperone with the D-2 receptor is monophasic at all ligand concentrations examined. The characteristics of the association plots were not affected when 50 nM ketanserin was included in the association reaction medium as a measure to block [³H]spiperone binding to 5HT-2 receptor sites (data not shown). The slopes of the association plots gave k_{obs} values at different ligand concentrations. The plot of k_{obs} values over ligand concentrations showed a hyperbolic dependence of k_{obs} on ligand concentrations (Fig. 2). As indicated elsewhere (30), this characteristic can best be described by assuming a two-step binding reaction where ligand (L) rapidly combines with receptor (R) to form a precomplex RL which then slowly isomerizes to the final complex $R'L$ according to the following scheme:

$$L + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL \underset{k_4}{\overset{k_3}{\rightleftharpoons}} R'L$$

Under equilibrium conditions where $k_2 > k_3$, we have for this reaction (30):

$$k_{obs} = \frac{k_3\,[L]}{K_A + [L]} + k_4 \qquad (1)$$

where $K_A = \frac{k_2}{k_1}$ represents the dissociation constant of the first equilibrium. Eq. 1 can be rearranged to:

$$1/(K_{obs} - k_4) = 1/k_3 + K_A/k_3 \cdot 1/[L] \qquad (2)$$

As described elsewhere (30), from the limiting ordinate intercept of the data in Fig. 2, $k_4 = 0.9 \times 10^{-3}$ sec^{-1} can be computed. Using this estimate of k_4, it is possible to construct a $1/(K_{obs} - k_4)$ versus $1/L$ plot. According to Eq. 2, such a plot ought to be linear with an ordinate intercept of $1/k_3$ and a slope of K_A/k_3. When such a plot was constructed, it was indeed found to be linear over the entire concentration range examined (Fig. 3). The linear least squares regression analyses of the data presented in Fig. 3 yielded a dissociation constant (K_A) of 1.27 ± 0.02 nM for the first equilibrium and a k_3 value of $71.63 \times 10^{-3} \pm 6.60 \times 10^{-3}$ sec^{-1} (Table 1). Upon computation, the ratio of k_4/k_3 yielded a value of 1.2×10^{-2}, implying that at equilibrium only 1% of the total receptor-ligand complex remained in the RL form. In equilibrium binding experiments the net reaction that would be observed can be represented by the following equation:

$$R + L \overset{(K_D)_{app}}{\longrightarrow} R'L$$

where $(K_D)_{app} = K_A \times k_4/k_3$. From the values of K_A and k_4/k_3, the overall K_D was estimated to be 15 pM. The absence of a lag phase in the primary semilogarithmic plots in Fig. 1 supports the validity of the assumption that the equilibrium has been established (30).

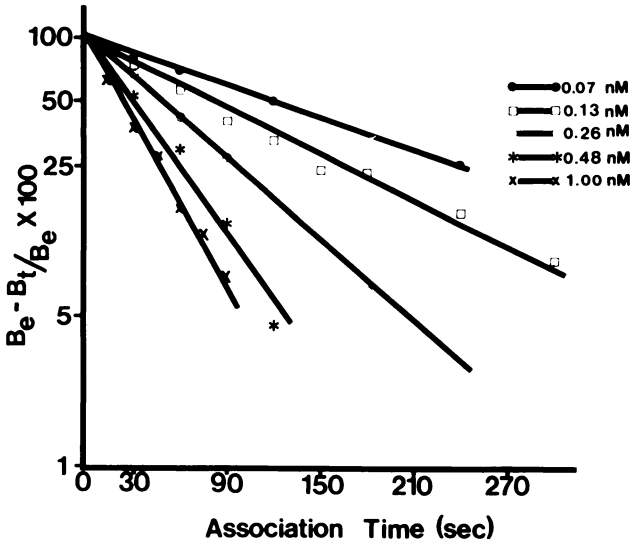


Fig. 1. Kinetics of association of [³H]spiperone with rat striatal membrane binding sites. Rat striatal membranes were prepared, and kinetics of association were determined as described under Materials and Methods. Association reaction was initiated at 37° by adding 2.5 ml of the prewarmed (37°) tissue suspension to 22.5 ml of prewarmed (37°) assay medium which contained appropriate concentrations of [³H]spiperone. At various time intervals, 2.0-ml aliquots were collected over Whatmann GF/B filters. Kinetics of association at pseudo-first order conditions with [³H]spiperone concentrations of 0.07 nM, 0.13 nM, 0.26 nM, 0.48 nM, and 1.00 nM are shown. For clarity, data with other concentrations of [³H] spiperone are not shown. The characteristics of the association plots were not affected when 50 nM ketanserin was included in the binding reaction medium (data not shown). Lines drawn are the weighted linear least squares fit of the experimental data. Data are representative of three such experiments. Note that the *ordinate* is a logarithmic scale.

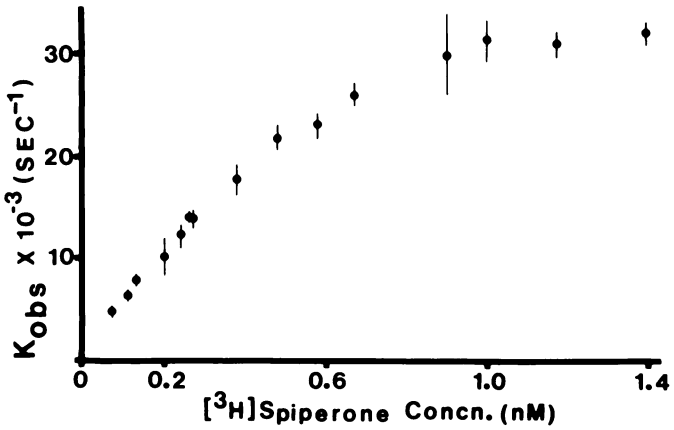


Fig. 2. Dependence of K_{obs} of [³H]spiperone association with rat striatal membranes on [³H]spiperone concentrations. Rat striatal membranes were prepared, and kinetics of association were determined as described in the legend of Fig. 1. The association rate constant (K_{obs}) for each concentration of [³H]spiperone is calculated and plotted against the concentration of [³H]spiperone. Data are the mean of three to six separate determinations. *Bars* represent standard errors.

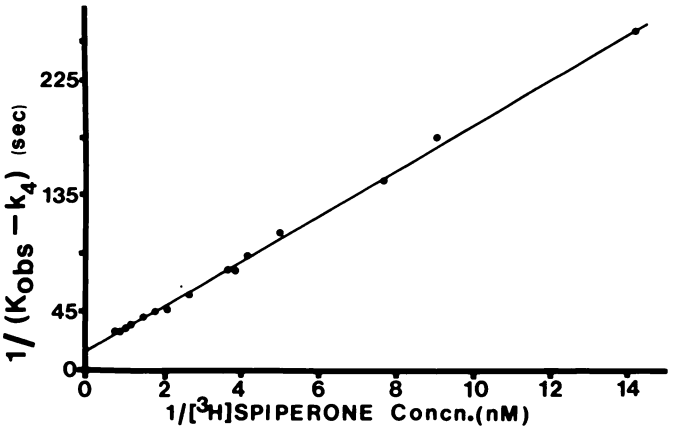


Fig. 3. The plot of $(K_{obs} - k_4)^{-1}$ versus the reciprocal of [³H]spiperone concentration. The rationale behind this plot is discussed under Results as well as in Ref. 30. The line drawn represents the weighted linear least squares fit of the experimental data.

TABLE 1

Kinetic constants of [^{3}H]spiperone binding to rat striatal membrane preparation

The kinetic constants of the binding reaction were determined as described under Results. The association kinetics data were analyzed assuming a two-step binding reaction. The equilibrium dissociation constant (K_A) of the first equilibrium and the association rate constant (K_3) of the second equilibrium were calculated using 0.9 $\times 10^{-3}$ sec^{-1} as an estimate for the dissociation rate constant (K_4) value of the second equilibrium. k_{diss} represents the dissociation rate constant value of the binding reaction determined from the direct dissociation experiment when dissociation reaction was initiated by the addition of 1 μM (+)-butaclamol, and $(K_D)_{app}$ represents the product of the dissociation constant of the two equilibria. Where appropriate, values are means ± standard errors.

K_A	K_3	K_4	k_{diss}	$(K_D)_{app}$
nM		(sec^{-1}) $\times 10^{-3}$		pM
1.2 ± 0.02	71.6 ± 6.6	0.9	0.9 ± 0.15	15

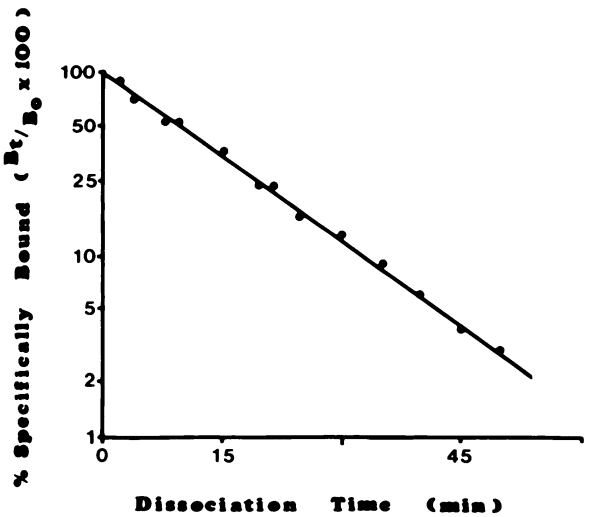


Fig. 4. Kinetics of dissociation of [^{3}H]spiperone from rat striatal membrane binding sites. Rat striatal membranes were prepared, and kinetics of dissociation were determined as described under Materials and Methods. The ligand receptor binding reaction was performed at 37° for 30 min in a 25.0-ml assay volume containing 0.4 mM [^{3}H]spiperone. The binding reaction medium also contained 50 nM ketanserin. Dissociation was initiated at 37° by adding 1 μM (+)-butaclamol, and 2.0-ml aliquots were withdrawn at the indicated times. The data were analyzed by using a weighted nonlinear iterative curve-fitting program, as described by McPherson (27) for kinetic studies. The data are from a representative experiment repeated at least three times. Note that the *ordinate* is a logarithmic scale.

Kinetics of [^{3}H]spiperone dissociation. The rate constant (k_{diss}) of dissociation of receptor-ligand complex was determined at 37° from the release of [^{3}H]spiperone from its receptor sites, initiated by the addition of 1 μM (+)-butaclamol, after establishment of equilibrium of the receptor-ligand binding reaction. The semilogarithmic plot of dissociation is presented in Fig. 4. This plot shows that the dissociation of [^{3}H]spiperone from its receptor complex is monoexponential with a rate constant of 0.97 $\times 10^{-3}$ ± 0.12 $\times 10^{-3}$ sec^{-1} (n = 3). In the absence of 50 nM ketanserin this value was 0.92 $\times 10^{-3}$ ± 0.15 $\times 10^{-3}$ sec^{-1} (n = 7). This dissociation rate constant value does not depend upon the degree of receptor saturation ([^{3}H]spiperone concentration varied from 0.1 to 1.0 nM) or on the methods of dissociation (infinite dilution versus (+)-butaclamol) (data not shown).

Equilibrium binding of [^{3}H]spiperone. The equilibrium binding of [^{3}H]spiperone was determined at 37° by incubating the membrane preparations with [^{3}H]spiperone (50–2000 pM concentrations) until equilibrium was reached. At all ligand concentrations studied, less than 5% of [^{3}H]spiperone was bound. The results can best be fitted to a model of a single class of binding sites with a dissociation constant (K_D) of 218 ± 8.2 pM and a maximum number of binding sites (B_{max}) of 1.9 ± 0.2 pM (approximately 250 fmol/mg of protein) (n = 7). The Hill coefficient did not differ significantly from unity. A representative experiment is depicted as a Scatchard plot in Fig. 5. Inclusion of 50 nM ketanserin in the binding assay medium as a measure to block any interaction of [^{3}H]spiperone with 5HT-2 receptor sites caused no appreciable change either in the total number of binding sites labeled by [^{3}H]spiperone or its affinity for the labeled sites (data not shown).

Receptor concentration and equilibrium dissociation constant. The equilibrium binding of [^{3}H]spiperone was determined at 37° at different receptor concentrations by incubating varying concentrations of membrane preparations in a fixed assay volume of 20.0 ml with varying concentrations of [^{3}H]spiperone ranging from 10 pM to 2000 pM. In all cases, less than 10% of the added ligand was bound.

The equilibrium binding isotherms obtained using different receptor concentrations were consistent with a single homogeneous population of binding sites. K_D and B_{max} values obtained from a Scatchard plot of the data from experiments done with different receptor concentrations are plotted in Fig. 6. The equilibrium dissociation constant (K_D) varied inversely as a function of concentration of binding sites.

Effects of Gpp(NH)p on [^{3}H]spiperone association. The time course of association of [^{3}H]spiperone (0.25 nM) with striatal D-2 receptors was followed at 37° using membrane preparations that had been preexposed to 0.1 mM Gpp(NH)p for 30 min at 37°, and the results are plotted in Fig. 7. The preincubation with Gpp(NH)p markedly reduced equilibrium binding and also resulted in a much faster association.

Effects of Gpp(NH)p on equilibrium binding of [^{3}H] spiperone. The equilibrium binding of [^{3}H]spiperone (ligand concentrations ranging from 0.5 to 15.0 nM) was done at 37° in the presence of 0.1 mM Gpp(NH)p with membrane preparations that were preincubated with 0.1 mM Gpp(NH)p for 30 min at 37°. Since use of high concentrations of [^{3}H]spiperone resulted in the appearance of a ketanserin-sensitive binding component, we routinely used 50 nM ketanserin in all of the binding experiments described here. Ketanserin, even at 20 nM concentration, totally eliminated this component of binding and a further increase in the ketanserin concentration to 50 nM did

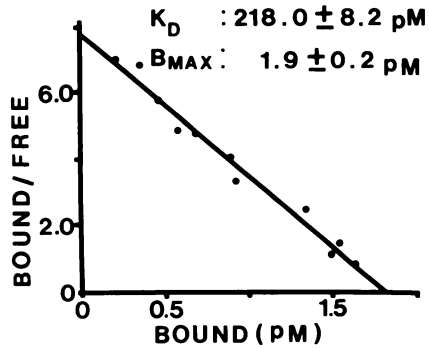


Fig. 5. Scatchard plot of [^{3}H]spiperone binding with rat striatal membranes. The binding study was performed in a final assay volume of 20.0 ml and the final concentration of radioligand utilized in this experiment ranged from 0.05 to 2.00 nM. The reaction was continued for 30 min at 37°. Data are representative of seven experiments. The line shown is the best fit of the experimental data drawn using the LIGAND program.

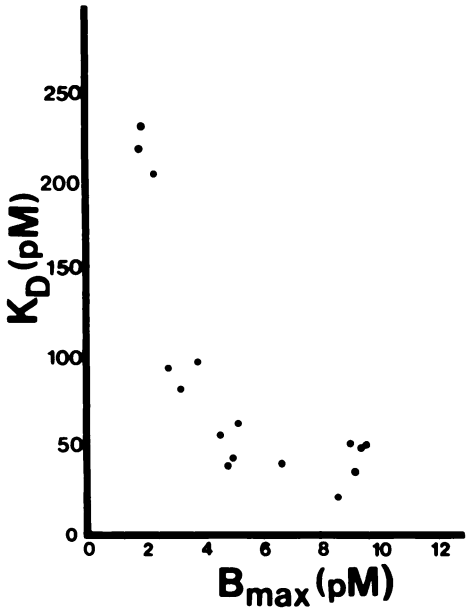


Fig. 6. Dependence of K_D of [^{3}H]spiperone binding in rat striatal membrane on receptor concentrations. The binding reactions were performed in 20.0-ml assay volumes with specific radioligand concentrations to achieve at least 10–90% saturation of the receptor sites. The reaction was initiated by the addition of 0.2 ml of tissue suspension to 18.8 ml of assay buffer containing radioligand, and the reaction was continued for 30 min at 37°. The concentration of the receptor in the assay medium was varied by diluting the tissue suspension appropriately to achieve the desired receptor concentration in the assay medium when added in 0.2-ml volumes. Data from each experiment were analyzed by the LIGAND program and the K_D and B_{max} values were determined. The values for the respective K_D and B_{max} from different experiments are plotted with K_D on the *ordinate* and B_{max} on the *abscissa*. Scatchard plot of binding reaction, in each case, can best be fitted to a one-site model. Each point represents data from a single experiment.

not further affect [^{3}H]spiperone binding. However, use of much higher concentrations of ketanserin (500 or 1000 nM) resulted in a marked decrease in the affinity of [^{3}H]spiperone for its binding sites without changing the maximum number of binding sites labeled by spiperone. Thus, 50 nM ketanserin is a reasonable concentration to block [^{3}H]spiperone interactions with 5HT-2 receptor sites without compromising its interaction with D-2 receptor sites. Also, use of high concentrations of [^{3}H]spiperone resulted in significant binding of the radioligand to the filter paper, yielding high blank values. Thus, to increase the sensitivity of the assay and reduce the binding of the radioligand to filter paper, the filters were presoaked for 2 hr in a 0.5% aqueous solution of polyethylenimine (pH adjusted to 7.4 with HCl) containing 0.1% bovine serum albumin. This treatment was used in all binding experiments described here. Preincubation of membrane preparation with 0.1 mM Gpp(NH)p yielded a K_D of 1.2 ± 0.1 nM with B_{max} of 3.4 ± 0.4 pM (n = 3) when the binding reaction was stopped 5 min after initiation of the formation of the ligand-receptor complex (Fig. 8). When the reaction was allowed to proceed for 60 min, the K_D and not the B_{max} of the receptor decreased to a value close to that observed in control experiments (data not shown). In control experiments the K_D obtained was 74.5 ± 2.5 pM, with a B_{max} of 3.2 ± 0.3 pM (n = 3); this value did not differ significantly when formation of the ligand-receptor complex was performed in the presence of 0.1 mM Gpp(NH)p without prior preincubation of the tissue with Gpp(NH)p (K_D = 72.2 ± 3.1 pM; B_{max} = 3.4 ± 0.5 pM). None of these treatment conditions significantly affected the total number of binding sites labeled by [^{3}H]spiperone.

Effects of pertussis toxin treatment on equilibrium binding of [^{3}H]spiperone. The equilibrium binding of [^{3}H]spiperone (ligand concentrations ranging from 0.5 to 15.0 nM) was done at 37° for 30 min with membrane preparations pretreated with pertussis toxin. In both control and pertussis toxin-treated experiments, 50 nM ketanserin was included in the binding assay medium to block [^{3}H]spiperone binding with 5HT-2 receptor sites and, also, in both cases, filter paper was treated with polyethylenimine as described earlier to reduce ligand binding to filter paper. [^{3}H]Spiperone binding in pertussis toxin-treated preparations shows a K_D of 1.1 nM with a B_{max} of 3.5 pM, while untreated control tissue yielded a K_D of 72.5 pM with a B_{max} of 3.2 pM (Fig. 9). Similar K_D and B_{max} were obtained with the pertussis toxin-treated tissue when the time for ligand-receptor complex formation was 5 min (data not shown).

Dopamine agonist and antagonist inhibition of [^{3}H] spiperone binding. The inhibition of [^{3}H]spiperone (0.5 nM)

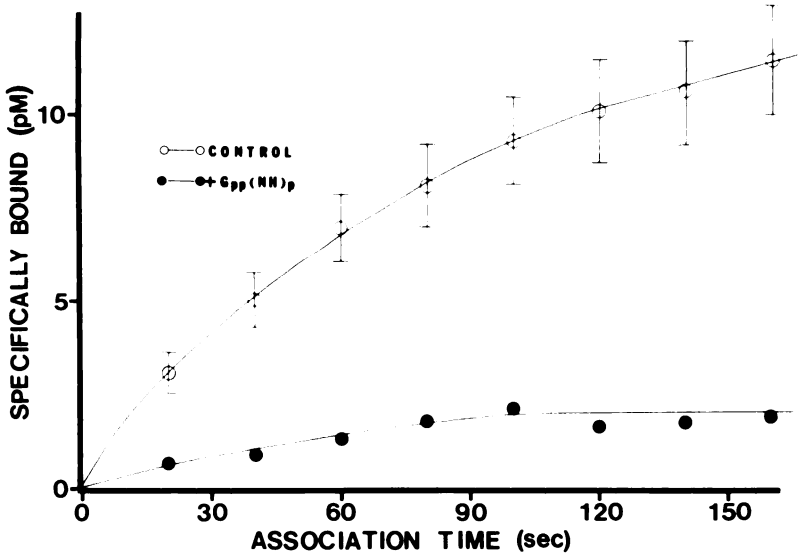


Fig. 7. Time course of association of [^{3}H]spiperone with rat striatal membrane pretreated with Gpp(NH)p. The tissue suspension in assay buffer containing 0.1 mM Gpp(NH)p was incubated for 30 min at 37°. Assay buffer containing [^{3}H]spiperone was also brought to the same temperature by preincubating at 37°. The association reaction was initiated at 37° by the addition of 2.5 ml of the Gpn(NH)p-pretreated tissue suspension to 22.5 ml of the assay medium. The concentration of Gpp(NH)p in the assay medium was also maintained at 0.1 mM. The time course of association of 0.25 nM [^{3}H]spiperone was followed as described under Materials and Methods. Points represent the mean of three separate experiments. Bars represent standard errors. Where not visible, the size of the bars is smaller than the size of the symbols.

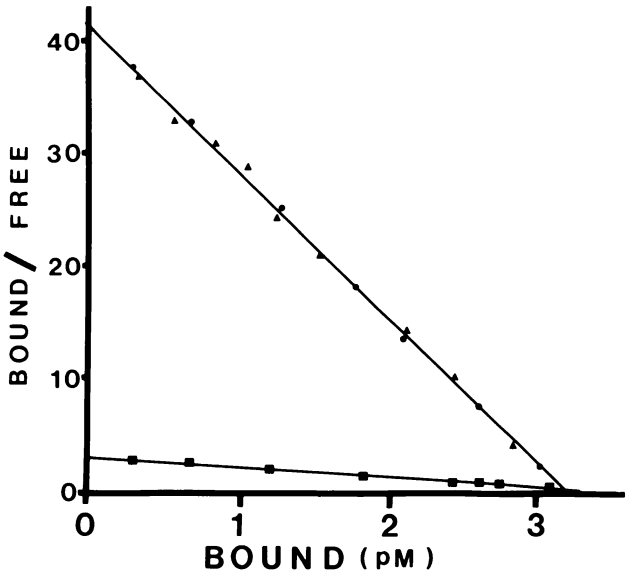


Fig. 8. Scatchard plot of [^{3}H]spiperone binding in rat striatal membranes pretreated with Gpp(NH)p. The tissue was pretreated with Gpp(NH)p by preincubating with 0.1 mM Gpp(NH)p at 37° for 30 min, and the equilibrium binding reaction was also performed in the presence of 0.1 mM Gpp(NH)p (■). Control tissue was similarly treated but without Gpp(NH)p, and the binding reaction was followed in the absence of Gpp(NH)p (●). In some experiments, the binding reaction medium of the control tissue also contained 0.1 mM Gpp(NH)p (▲). Data are from a representative experiment. Lines drawn are the best fit of the experimental data using the LIGAND program.

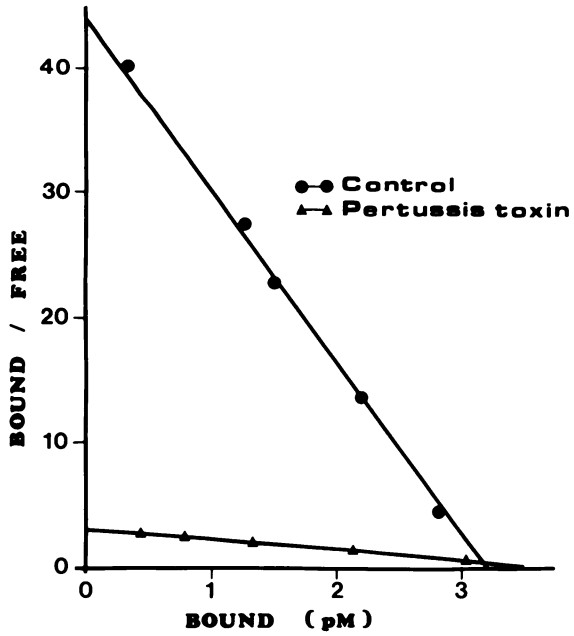


Fig. 9. Scatchard plot of [^{3}H]spiperone binding in rat striatal membranes pretreated with pertussis toxin. Control and pertussis toxin-treated data were obtained from the same tissue preparations treated identically except in the presence and absence of pertussis toxin. Equilibrium binding of [^{3}H]spiperone was done as described under Materials and Methods. Data are from a representative experiment replicated twice. Lines drawn are the best fit to the experimental data using the LIGAND program.

binding with rat striatal membranes by (+)-butaclamol, bromocriptine, and dopamine was conducted at 37° in the presence of 50 nM ketanserin. The data were subjected to a one-, two-, and three-site fit using the LIGAND program. A particular fit was chosen only when a statistically better fit was achieved. Inhibition of [^{3}H]spiperone binding by (+)-butaclamol could best be described by assuming a (+)-butaclamol interaction with a homogeneous population of binding sites existing in a single affinity state (Fig. 10), and a one-site fit of the data yielded a K_i of 285 pM. Similarly, inhibition of [^{3}H]spiperone by bromocriptine could also be best described by assuming its interaction to a homogeneous population of binding sites existing in a single affinity state (Fig. 11). Analysis based on such an assumption yielded a K_1 value of 1.4 nM. Inclusion of 0.1 mM Gpp(NH)p in the reaction medium did not significantly affect this K_1 value. However, as shown in Fig. 12, inhibition of [^{3}H]spiperone binding by dopamine deviated from a one-site model. When fitted to the two-site model, the dopamine affinities for high and low affinity sites were 200 nM and 2.4 μM, respectively, with 48% of the receptor sites existing in the high affinity state. Further improvement of fit was obtained when the data were fitted to a three-site model and yielded the affinities of dopamine for the superhigh (K_{SH}), high (K_H) and low (K_L) affinity sites of 12 nM, 256 nM, and 3 μM, respectively (Fig. 12). The fractions of the total binding sites existing in superhigh (R_{SH}), high (R_H), and low affinity states for dopamine were 4%, 46% and 50%, respectively. Whether done in the presence or in the absence of 50 nM ketanserin, the three-site fit represents the best description of the inhibition data. The superhigh affinity component varied from 2% to 8% in three different experiments done in the presence of 50 nM ketanserin and from 6% to 20% in two separate experiments done in the absence of ketanserin. A three-site fit for inhibition of [^{3}H] spiperone binding by dopamine was also reported by Grigoriadis and Seeman (10). However, when 0.1 mM Gpp(NH)p was included in the binding assay medium, the inhibition of [^{3}H] spiperone binding by dopamine could best fit to a two-site

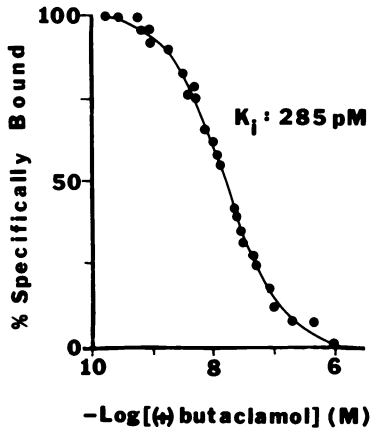
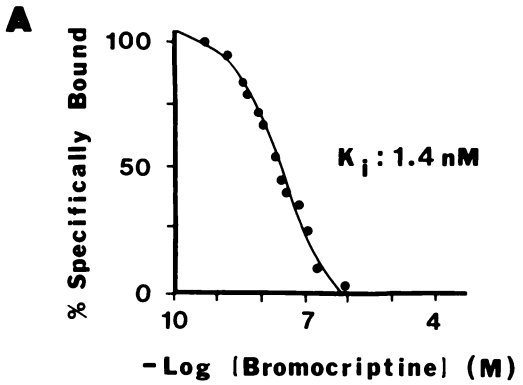


Fig. 10. Inhibition of [^{3}H]spiperone binding in rat striatal membranes by (+)-butaclamol. The binding reaction mixture contained [^{3}H]spiperone in 1.0 ml of assay buffer, specified concentrations of (+)-butaclamol in 0.8 ml of assay buffer, and 0.5 ml of tissue suspension (1:100 volume in assay buffer and preincubated for 30 min at 37°) to give a final volume of 5.0 ml. The binding reaction was continued for 30 min at 37°. Data are from a representative experiment replicated twice. The line drawn is the best fit of the experimental data according to the LIGAND program when the K_D of [^{3}H]spiperone for its binding sites was constrained to 20 pM.



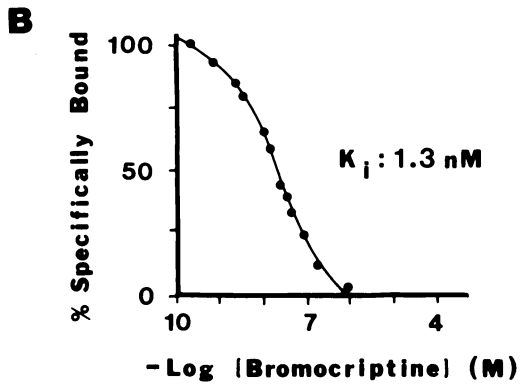
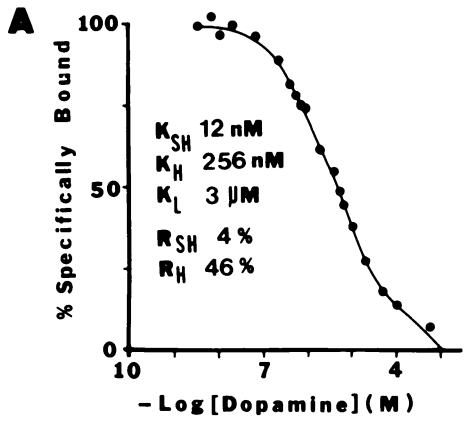


Fig. 11. Inhibition of [3H]spiperone binding in rat striatal membranes by bromocriptine in the absence and presence of 0.1 mM Gpp(NH)p. The inhibition of [3H]spiperone (0.5 nM) binding by bromocriptine in the absence (A) and presence (B) of Gpp(NH)p was done as described in the legend to Fig. 10. Where indicated, Gpp(NH)p was included in the assay medium to yield a final concentration of 0.1 mM. Data presented were generated in an assay medium which contained 50 nM ketanserin and were from a representative experiment repeated three times. Lines drawn are the best fits of experimental data according to the LIGAND program. The K_D of [3H]spiperone for its binding sites was constrained to 20 pM. The curves plateau at 0% specific binding; the points falling on the *abscissa* are not shown.



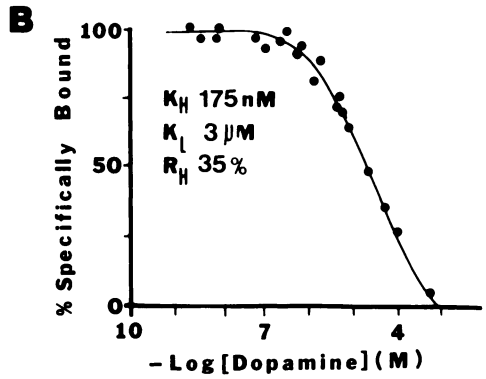


Fig. 12. Inhibition of [3H]spiperone binding in rat striatal membranes by dopamine in the absence and presence of 0.1 mM Gpp(NH)p. The inhibition of [3H]spiperone (0.5 nM) binding by dopamine in the absence (A) and presence (B) of Gpp(NH)p was done as described in the legend to Fig. 10. Where indicated, Gpp(NH)p was included in the assay mixture to yield a final concentration of 0.1 mM. Data presented were generated in an assay medium which contained 50 nM ketanserin and were from a representative experiment repeated three times. Lines drawn are the best fits of experimental data according to the LIGAND program. The K_D of [3H]spiperone for its binding sites was constrained to 20 pM. The curves plateau at 0% specific binding; the points falling on the *abscissa* are not shown.

model, with dopamine affinities for the high and low affinity states being 175 nM and 3 μM, respectively, and the fraction of the total binding sites remaining in high and low affinity states were 35% and 65%, respectively (Fig. 12B).

Equilibrium binding of [3H]spiperone in the presence of bromocriptine and dopamine. The equilibrium binding of [3H]spiperone (ligand concentrations ranging from 8 pM to 1.95 nM) was done at 37° in the presence of bromocriptine (2.5 nM and 5.0 nM) (Fig. 13) or dopamine (0.5 μM and 2.0 μM) (Fig. 14).

In all cases, the equilibrium binding data could best be fitted to a model of a single class of binding sites. In the presence of bromocriptine, the affinity (K_D) of the binding reaction significantly changed from 36.6 pM (control) to 214.4 pM (in the presence of 2.5 nM bromocriptine) and to 419.7 pM (in the presence of 5.0 nM bromocriptine), whereas the number of binding sites (B_{max}) did not differ significantly from control (6.84 pM) in the presence of either concentration of bromocriptine. In contrast, dopamine significantly affected the number of binding sites (B_{max}) labeled by [3H]spiperone; the B_{max} of 7.27 pM in the control condition was reduced to 5.79 pM (in the presence of 0.5 μM dopamine) and to 4.25 pM (in the presence of 2.0 μM dopamine). The dissociation constant (K_D) of the binding reaction also changed from 32.1 pM (control) to 67.0 pM (in the presence of 0.5 μM dopamine) and to 97.1 pM (in the presence of 2.0 μM dopamine).

Effects of dopamine receptor agonist and antagonist on [3H]spiperone dissociation by infinite dilution. The dissociation of [3H]spiperone-receptor complex was determined at 37° by subjecting the ligand-receptor complex to infinite dilution in the presence and absence of various test drugs (Fig. 15). The dissociation of ligand-receptor complex at various time intervals was measured and plotted against time. The dissociation initiated by dilution alone was linear and did not differ significantly from that when dissociation medium contained, in addition, 1 μM bromocriptine or 1 μM (+)-butaclamol. However, the presence of 20 mM dopamine in the diluting medium significantly accelerated the dissociation and caused the dissociation plot to deviate significantly from linearity. The rate constant of dissociation by dilution only was 0.87×10^{-3} sec^{-1}, whereas in the presence of bromocriptine and (+)-butaclamol these values were 0.89×10^{-3} sec^{-1} and 0.91×10^{-3} sec^{-1}, respectively. The curvilinear dissociation, observed in the presence of dopamine, was fitted to the equation where [3H]spipe-

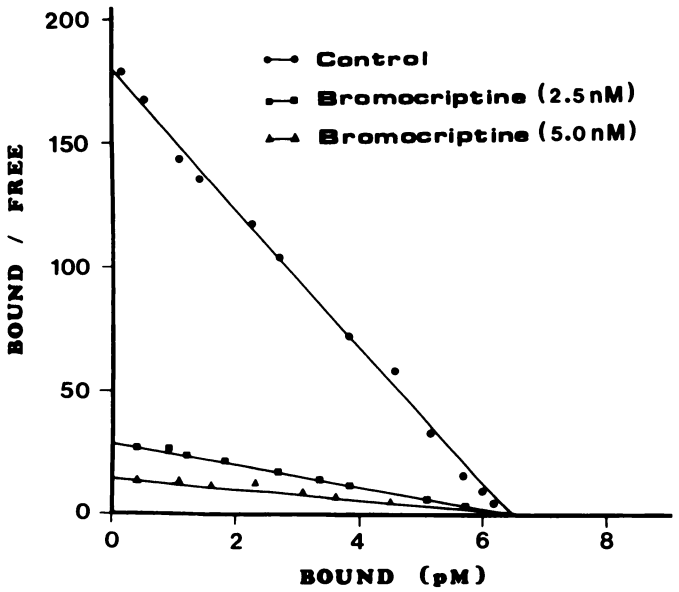


Fig. 13. Scatchard plots of [^{3}H]spiperone binding inhibition by bromocriptine in rat striatal membranes. The equilibrium binding assay was performed in a 20-ml assay volume and the radioligand concentration ranged from 10 pM to 2000 pM. Where indicated, the binding reaction medium contained bromocriptine in a final concentration of either 2.5 nM or 5.0 nM. The control assay was performed in the absence of bromocriptine. In all cases, the reaction medium contained 50 nM ketanserin. The binding reaction was done at 37° for 30 min. Lines drawn are best fit of the experimental data points using the LIGAND program.

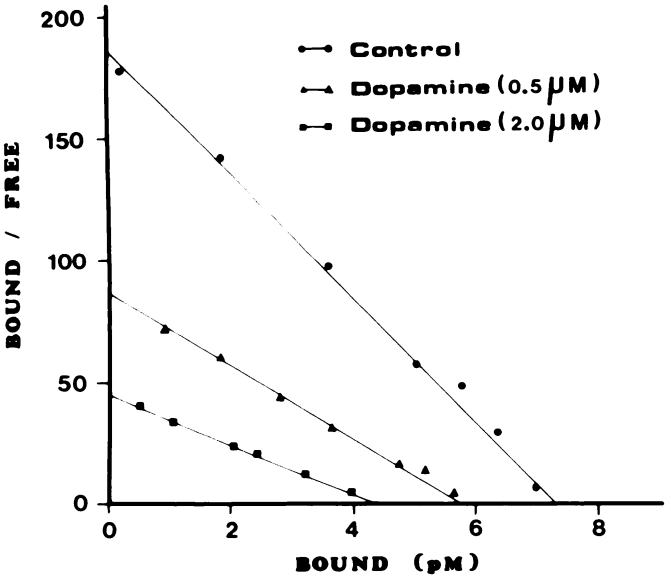


Fig. 14. Scatchard plots of [^{3}H]spiperone binding inhibition by dopamine in rat striatal membranes. The equilibrium binding assay was performed in a 20-ml assay volume and the radioligand concentration ranged from 10 pM to 1500 pM. Where indicated, the binding reaction medium contained dopamine in a final concentration of either 0.5 uM or 2.0 uM. The control assay was performed in the absence of dopamine. In all cases, the rection medium contained 50 nM ketanserin. The binding reaction was done at 37° for 30 min. Lines drawn are best fit of the experimental data points using the LIGAND program.

rone dissociation was assumed to occur from two independent affinity states or sites of the receptor. Analysis based on such an assumption revealed that the dissociation of the ligand from the high (~70%) and the low affinity (~30%) states or sites

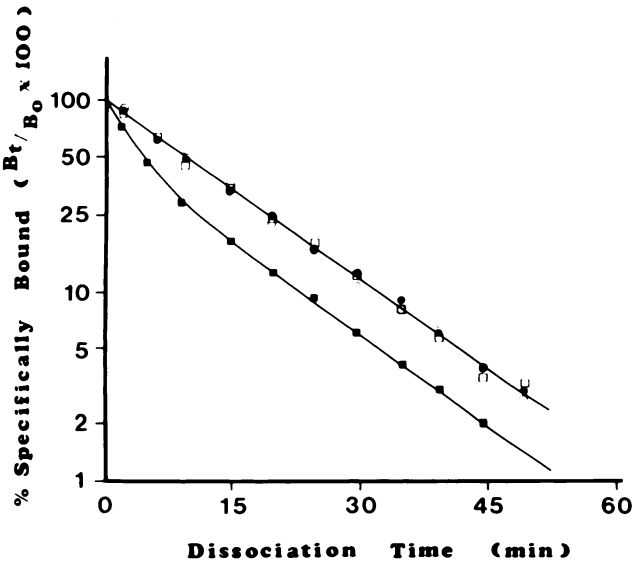


Fig. 15. Kinetics of dissociation of [^{3}H]spiperone from rat striatal membrane binding sites when dissociation was initiated by infinite dilution in the presence and absence of various dopamine agonists and antagonists. The kinetics of dissociation at infinite dilution in the presence and absence of various drugs were performed as detailed under Materials and Methods. The initial reaction of ligand-receptor complex formation was performed in an assay buffer containing 50 nM ketanserin, and the ligand-receptor complex was subsequently subjected to dissociation at 37° by 100-fold dilution of the reaction medium with the assay buffer; at the appropriate time after initation of dissociation, a 5.0-ml aliquot was withdrawn and filtered. In the experiments presented here, the diluting buffer contained no drug (●), 1 uM (+) butaclamol (○), 1 uM bromocriptine (□), and 20 uM dopamine (■). The data were analyzed by asuming ligand dissociation from a single affinity site or two discreet affinity states or sites of the receptor. The dissociation data were subjected to a weighed nonlinear iterative curve-fitting program, as described by McPherson (27) for kinetic studies. The data are representative of four separate experiments. Note that the *oridinate* is a logarithmic scale.

occurs with rate constants of 1.1×10^{-3} sec^{-1} and 12.0×10^{-3} sec^{-1}, respectively, for the high and low affinity states or sites.

Discussion

The most important finding of the present study is the demonstration that antagonist binding with dopamine D-2 receptors induces receptor coupling with a guanine nucleotide-binding protein (or G protein), resulting in the formation of a high affinity ligand-receptor-G protein ternary complex. This phenomenon has previously been shown to occur only in cases of agonist occupancy of receptor sites (6). However, unlike the characteristics of agonist-receptor binding, the formation of a ternary complex induced by an antagonist is not destabilized by Gpp(NH)p.

The kinetics of association of [^{3}H]spiperone with D-2 receptors proceeded in a monoexponential manner at all concentrations of [^{3}H]spiperone tested. The linearity of the pseudo-first order association implies that [^{3}H]spiperone interacts with a single class of binding sites in rat striatal membrane preparations. The monophasic association of [^{3}H]spiperone with D-2 receptors and the inability of ketanserin to affect the association also indicates the lack of a significant interaction of [^{3}H] spiperone with 5HT-2 receptor sites. The linearity in the pseudo-first order association of [^{3}H]spiperone with D-2 receptors has also been reported by others (8, 31, 32). However, the relationship between K_{obs} of association and the concentration

of [^{3}H]spiperone has not previously been tested. We found that K_{obs} of association had a hyperbolic dependence on the concentration of [^{3}H]spiperone. The simplest binding scheme that can account for such a hyperbolic dependence of K_{obs} on ligand concentration is a two-step binding reaction where a fast binding step of formation of a ligand-receptor complex is followed by a slow isomerization of this complex (30). This type of two-step binding reaction has been observed with antagonist binding to muscarinic receptors (33–35), agonist binding to acetylcholine receptors (36–38), and benzodiazepine binding to its brain receptor sites (39, 40). Based on such an assumption, a dissociation constant value of 1.2 nM for the first equilibrium and an approximate K_D of the overall binding reaction of 15 pM were obtained. The analysis also revealed that 99% of the total ligand-receptor complex remains in the high affinity isomerized conformation. Consistent with this, the dissociation of [^{3}H]spiperone from its binding sites appears to occur from a single affinity conformational state of the receptor (Fig. 4). The close agreement between the dissociation rate constant, determined directly, and the calculated k_4, suggests that the overall rate of dissociation of the binding reaction is limited by the dissociation of the ligand from the isomerized state of the receptor. The dissociation rate constant of the overall binding reaction, as observed in the present study, is in excellent agreement with previous reports where a similar monophasic dissociation has also been noted (32).

It should be emphasized here that the dissociation kinetics experiment is a powerful experimental tool to demonstrate receptor heterogeneity, even when the tracer selectivity is very low. This type of experimental design has been used to demonstrate the existence of two opioid receptors with the same affinity for the tracer but different kinetic characteristics (41), the different subtypes of benzodiazepine receptors having different kinetic constants (42), and the three classes of binding sites for [^{3}H]-*N*-methylscopolamine in rat brain (43). It is therefore reasonable to conclude that monoexponential dissociation of [^{3}H]spiperone, as observed in the present study, not only indicates the absence of a receptor heterogeneity but also rules out the possibility of labeling of 5HT-2 receptors by [^{3}H] spiperone. Moreover, ketanserin did not influence the dissociation of [^{3}H]spiperone. Thus, based on kinetic and equilibrium binding data, we rule out the possibility of any significant contribution of 5HT-2 receptor sites to our binding data. The lack of a significant effect of 50 nM ketanserin on [^{3}H]spiperone binding, under conditions used to label D-2 receptors in rat striatum, was also reported by Grigoriadis and Seeman (10). Huff and Molinoff (20) could not detect any appreciable interaction of [^{3}H]spiperone with 5HT-2 receptors in rat striatum under conditions where spiperone was employed to label D-2 receptor population in this tissue. In contrast, Hamblin *et al.* (44) suggested that nearly half of the total binding sites labeled by [^{3}H]spiperone in rat striatum represent 5HT-2 sites. Their suggestion was based on the finding that a Scatchard plot of [^{3}H]spiperone binding was curvilinear with a high and a low affinity component of binding and that the low affinity component of binding was eliminated when ketanserin was included in the binding assay medium. The reason for the difference between their study and ours or between this study and that of Grigoriadis and Seeman (10) or Huff and Molinoff (20) is readily apparent from the fact that Hamblin et al. (44) have used a very high (100 μM) concentration of (+)-butaclamol to define nonspecific binding. The use of such a high concentration of (+)-butaclamol, as shown by George *et al.* (45), would artifactually produce a nonlinear Scatchard plot, presumably by displacing [^{3}H]spiperone from nonspecific sites. Our study and that of Grigoriadis and Seeman (10), as well as that of Huff and Molinoff (20), used 1 μM (+)-butaclamol to define nonspecific binding. Under conditions of the present study, neither the kinetic nor the equilibrium binding data provide any support for any significant interaction of [^{3}H]spiperone with 5HT-2 receptor sites.

The estimated K_D in the equilibrium binding experiment was 14 times higher than the K_D derived from the kinetic experiments, a difference well beyond the limit of experimental error. We surmised that this discrepancy might stem from using a protein concentration in the equilibrium binding experiment 10 times lower than that used in the kinetic experiments. The use of a lower protein concentration in the equilibrium binding experiment was necessary to keep the receptor concentration below the expected K_D to obtain a reasonably true estimate of K_D (46).

Examination of the relationship between receptor concentration and K_D revealed, however, that K_D varied with receptor concentration in an inverse manner. At lower receptor concentrations, K_D is higher, whereas at higher receptor concentrations, K_D is lower. The lowest K_D of the binding reaction was noted at receptor concentration between 8 and 10 pM; with further increases in the receptor concentration, however, the K_D of the binding reaction starts to increase (not shown in the figure). This increase is indeed expected in view of the relationship between the apparent K_D and the true K_D. The use of a very high receptor concentration in the binding assay will also result in spurious estimates of K_D (47).

This phenomenon of an inverse relationship between receptor concentration and affinity of the binding reaction does not support the validity of the assumption of ligand-induced receptor isomerization as a mechanism for a two-step binding reaction; in contrast, it suggests the involvement of a third component in inducing the second reaction, leading to the formation of a high affinity ternary complex (48). The possible reaction scheme for a two-step binding reaction involving a third component (X) can be:

$$R + L \rightleftharpoons LR \overset{+X}{\rightleftharpoons} LRX$$

where R represents D-2 receptor, L represents [^{3}H]spiperone, and LR is ligand-receptor complex. It is presumed that X will be a membrane-bound component. Although the increase in receptor concentration will not affect the relative concentration of R to X, it will nonetheless increase the rate of the second reaction leading to the formation of a high affinity LRX complex (48, 49). As a result, an increase in receptor concentration, in essence, will increase the overall affinity of the binding reaction. Thus, depending upon the concentration of the receptor in the binding assay medium, the ligand-receptor complex can exist either predominantly in a low affinity LR state or a high affinity LRX state. It is predictable, therefore, that at high receptor concentrations, the dissociation of ligand will be solely from the high affinity LRX state, leading to a monophasic and slow dissociation (since k_4 seems to be rate limiting), whereas progressive decreases in the concentration of the receptor will lead to curvilinearity in the dissociation plot, with the progressively increasing component having a faster dissociation rate. Although not shown, similar dependence was noted in the dissocation characteristics in relation to receptor concentration. Thus, the curvilinearity in dissociation, observed in some earlier studies (31, 51), might well be due to the reasons indicated above. It can also be predicted that, at lower receptor

concentration, the displacement of labeled by unlabeled antagonist will be shallow, with the unlabeled antagonist displacing the labeled antagonist with high and low affinity, respectively, from *LRX* and *LR* states of the receptor-ligand complex. In fact, in some earlier studies (18–20) a shallow displacement of [^{3}H]spiperone by unlabeled antagonists was noted.

The induction of formation of a ternary complex by an antagonist is surprising. This finding predicts that, instead of passively occupying receptor sites, binding of the antagonist actively induces receptor coupling with a third component to stabilize the antagonist-receptor complex. However, the nature of this third component is unknown. In light of our existing knowledge of the association of striatal D-2 receptors with the adenylate cyclase system through a G protein, we tested whether this third component bears the characteristics of a G protein. Gpp(NH)p pretreatment of tissue, which is now known to interact with G protein and thereby influence its interaction with the receptor-ligand complex, caused the association of [^{3}H]spiperone to occur faster with a markedly lower equilibrium binding value. This sensitivity of binding to Gpp(NH)p makes the involvement of G proteins in the interaction of [^{3}H]spiperone with D-2 receptors highly probable. The decrease in the equilibrium binding in tissue pretreated with Gpp(NH)p might result from a decrease in receptor affinity. If, in the presence of Gpp(NH)p, the second reaction of receptor coupling with a G protein cannot occur, we would predict a low affinity binding in tissue pretreated with Gpp(NH)p and a K_D of the binding reaction that would be the same as the K_D (~1.2 nM) we derived kinetically for the first step of the binding reaction. The analyses of equilibrium binding in tissue pretreated with Gpp(NH)p yielded a K_D of 1.2 nM without showing any appreciable difference in the total number of binding sites labeled, indicating a strong internal consistency in the proposed binding model. It should be noted, however, that prolonging the duration of incubation of the receptor-ligand complex well after equilibrium (5 min) caused the affinity to increase, and after 60 min of incubation, the affinity of the binding reaction did not appreciably differ from that of the control. This increase in affinity of the binding reaction was not due to degradation of Gpp(NH)p during prolonged incubation, since 90 min of preincubation of the tissue with Gpp(NH)p could still elicit low affinity binding in the 5-min incubation schedule. We suggest, therefore, that the *LR* complex can still couple with a G-protein, even in the presence of Gpp(NH)p, albeit with a slower rate. Thus, the absence of any effect of Gpp(NH)p on [^{3}H]spiperone binding, observed in all previous studies, is entirely consistent with the model we propose for interaction of [^{3}H]spiperone with the D-2 receptor.

The inhibitory coupling of striatal D-2 receptors with adenylate cyclase is now well established (3–6). It is also known that the G_i subclass of G protein is involved in D-2 receptor-mediated inhibition of striatal adenylate cyclase (3, 52). Therefore, the G_i subclass of G protein can be presumed to be the prime candidate for inducing the formation of this high affinity ternary complex. Since the G_i protein is selectively and irreversibly inactivated by pertussis toxin treatment (3, 52, 53), one would predict that pertussis toxin-treated tissue would show the same low affinity K_D as that observed with Gpp(NH)p-pretreated tissue and, in addition, that the low affinity K_D of pertussis toxin-treated tissue, unlike that observed with Gpp(NH)p-pretreated tissue, would not change with the increase in the duration of incubation. The equilibrium binding study with pertussis toxin-treated tissue supports the above assumption.

On the basis of these converging lines of evidence, we propose the following model for the interaction of [^{3}H]spiperone with D-2 receptors:

$$L + R \rightleftharpoons LR \overset{+G_i}{\rightleftharpoons} LRG_i$$

Thus, under conditions where the receptor remains predominantly in the LRG_i state, both agonist and antagonist competing ligands will displace labeled [^{3}H]spiperone from its binding sites monophasically. Our finding of monophasic inhibition of [^{3}H]spiperone binding by agonist ligand bromocriptine and by antagonist ligand (+)-butaclamol supports such a conjecture. However, inhibition of [^{3}H]spiperone binding by dopamine indicates that this agonist displaces [^{3}H]spiperone with more than one affinity. This characteristic inhibition of [^{3}H]spiperone binding by agonists such as dopamine was also noted by several other investigators using dopamine and its different analogs (8, 10, 16, 20, 32, 34). This anomaly in the interaction of dopamine with D-2 receptor made us question how dopamine can inhibit [^{3}H]spiperone binding with more than one affinity from a seemingly homogeneous population of binding sites existing in a single affinity state. To accommodate such apparently contradictory experimental findings, we suggest that a part of dopamine-induced inhibition of [^{3}H]spiperone binding, unlike that of bromocriptine, is mediated through interactions of dopamine with a site other than that labeled by [^{3}H]spiperone.

The existence of a second site for the interaction of dopamine with D-2 receptor is supported by our demonstration that dopamine inhibited [^{3}H]spiperone binding by a mixed noncompetitive and competitive mechanism (change in both K_D and B_{max}), whereas inhibition of [^{3}H]spiperone binding by bromocriptine was purely competitive in nature, and that dopamine but not bromocriptine or (+)-butaclamol accelerated the dissociation of [^{3}H]spiperone from its binding sites. We propose that acceleration of dissociation of [^{3}H]spiperone by dopamine from D-2 receptors results from the interaction of dopamine with a site that is allosterically coupled to D-2 receptors; bromocriptine mediates its agonist action at the dopamine receptor system exclusively by interacting with D-2 receptor sites, whereas dopamine action is mediated, in part, through a yet unidentified site which is linked allosterically with D-2 receptor sites. The biochemical and physiological correlates of the activation of these dopamine recognition sites is yet to be elucidated; however, recent reports implicating the dopamine receptor systems in the modulation of inositol phosphate metabolism (54–57) make it likely that there might exist yet another subset of dopamine receptors, other than those mediating the modulation of the cAMP system (D-1 and D-2 subtypes), which is involved in the modulation of phosphoinositide turnover.

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