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Properties of D_2 Dopamine Receptor Autoradiography: High Percentage of High-Affinity Agonist Sites and Increased Nucleotide Sensitivity in Tissue Sections

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[^{3}H]Spiroperidol (spiperone) binding in the presence of mianserin, a serotonin (5-HT_2) receptor antagonist, was characterized in rat brain using quantitative autoradiography. All binding parameters were directly determined from film densities. Competition and kinetic studies revealed that [^{3}H]spiroperidol binds to a site having characteristics of the dopamine, D_2, receptor in striatum. The general binding parameters were similar to values obtained in homogenate and swabbed section studies except as related to agonist binding and guanine nucleotide sensitivity. Competition studies with dopamine revealed biphasic competition curves with a K_h of 8.23 nM and a K_l of 12.3 μM. The percentage of high-affinity sites was 90%. Guanine nucleotides (1 μM guanylyl-imidodiphosphate) completely converted the high-affinity site to a low-affinity site. Quantitative regional distribution studies revealed high binding in striatum, olfactory tubercle and nucleus accumbens, with lower binding in other dopamine innervated regions including frontal and cingulate cortex. [^{3}H]Spiroperidol was also found to bind to a spirodecanone site with an anatomical localization distinct from the dopamine and serotonin systems and in a region (entorhinal cortex) not previously reported. This report provides a detailed pharmacologic and regional characterization of [^{3}H]spiroperidol binding to D_2 receptor in rat brain using quantitative autoradiography to determine all binding parameters. This report also demonstrates an increased percentage of sites in the high-affinity state of the D_2 receptor in tissue sections and increased affinity of the guanine regulatory protein for guanine nucleotides.

INTRODUCTION

[^{3}H]Spiroperidol, a butyrophenone dopamine antagonist, has been used extensively to label the D_2 dopamine receptor subtype in homogenate[6,30], intact cell[33] and autoradiographic studies[1,2,27]. The D_2 receptor is thought to be linked to adenylate cyclase in an inhibitory manner[34]. Agonists recognize both high- and low-affinity forms of the receptor which can be interconverted by guanine nucleotides[7,32,36,38]. The recent use of quantitative autoradiography with other radiolabeled ligands[13,17,28,29,35,37] prompted the adaptation of this technique to study [^{3}H]spiroperidol binding to the D_2 receptor. The ability to perform quantitative autoradiography of the D_2 receptor in which all quantitative measurements were taken directly from the film would allow determination of binding parameters in many anatomically small and distinct regions in a single animal. Experiments modifying the dopamine receptor could then be analyzed autoradiographically in the different dopamine systems including nigrostriatal, mesocortical and mesolimbic. The goal of this report is to describe appropriate kinetic and binding conditions for using [^{3}H]spiroperidol to study the D_2 receptor with quantitative autoradiography. In addition, we report properties relating to agonist binding and guanine nucleotide regulation which are different from those found with the homogenate and swabbed tissue section methods.

MATERIALS AND METHODS

Tissue preparation

Male Sprague–Dawley rats averaging 200 g were decapitated and their brains rapidly removed. Brains

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were mounted on chucks using Lipshaw embedding matrix and rapidly frozen in crushed dry ice. After warming to −20 °C, 20-μm cryostat sections were cut, thaw-mounted onto gelatin-coated microscope slides, dehydrated at room temperature and stored at −20 °C until used in assays. All slides were used within 5 days of sectioning.

Binding assay

All assays were performed at room temperature. The preincubation wash and postincubation rinse were performed at 4 °C. All assays used the same buffer consisting of 170 mM Tris-HCl at a pH of 7.70, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.001% ascorbate and 1 μM pargyline. All slides were given two 5-min washes in buffer and a 3-s dip in distilled water after incubation. After the dip in distilled water the slides were fan-dried at room temperature. Mianserin (100 nM) was present in the buffer of all experiments except for mianserin competition studies when the concentration was varied.

Saturation studies were performed with concentrations of [^{3}H]spiroperidol from 0.2 to 4 nM and an incubation time of 120 min to ensure equilibrium binding at the lowest concentration. Ligand concentrations in the incubation medium were measured before and after incubation to ensure that free ligand concentration was constant. Competition and distribution studies were performed at a [^{3}H]spiroperidol concentration of 0.50 nM. Incubation time was 60 min for competition and distribution studies and varied from 1 to 120 min for kinetic studies.

Autoradiography

Dried slides were placed in an X-ray cassette with ^{14}C plastic standards previously calibrated with ^{3}H brain paste sections[28] and exposed to LKB Ultrofilm ^{3}H at 4 °C for 21 days for all studies except the distribution studies which were exposed for 28 days to allow imaging of regions with a low density of D_2 receptors. The Ultrofilm ^{3}H was developed at room temperature with Kodak D19 for 3 min, stopped and fixed with Kodak rapid fix for 3.5 min. All binding data were determined directly from film densities in regions of interest. Films were analyzed using a photographic enlarger and a computer to determine optical densities[10]. A minimum of 20 readings from each area were averaged and the radioactivity determined by a computer-generated polynomial regression analysis which compared film densities produced by tissue sections with those produced by radioactive standards.

Materials

[^{3}H]Spiroperidol (19–26 Ci/mmol) was obtained from New England Nuclear (Boston) and Amersham Corp. (Arlington Heights, IL), mianserin from Organon (Oss, The Netherlands), haloperidol from McNeil Pharmaceutical (Spring House, MA), (+)- and (−)-butaclamol from Research Biochemicals Inc. (Wayland, MA) and dopamine from Sigma (St. Louis, MO).

RESULTS

Kinetic studies were performed to determine appropriate incubation and postincubation rinse times. Kinetic studies were performed at several [^{3}H]spiroperidol concentrations between 0.5 and 1 nM. The association rate constant as determined by a pseudo first-order method[3] was 0.055 ± 0.002 min^{-1} (Fig. 1A). The dissociation rate constant, as determined by infinite dilution after equilibrium binding, was 0.004 ± 0.0003 min^{-1} (Fig. 1B). Equilibrium for com-

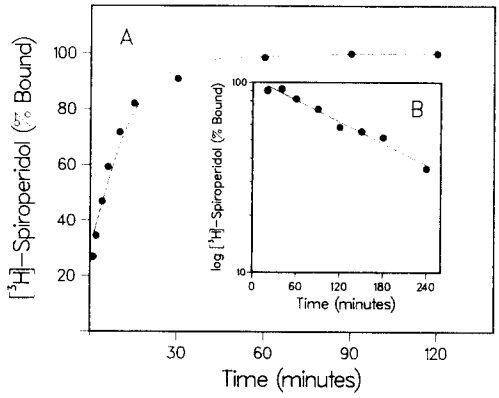


Fig. 1. A: association curve of [^{3}H]spiroperidol binding to striatum. Representative association curve performed at a ligand concentration of 0.50 nM. k_{ob} was determined from the slope of ln (Be–B) vs time where Be is binding at equilibrium (curve not shown). k_1 was determined as described by Bylund[3]. The average k_1 from 3 experiments was 0.055 ± 0.002 min^{-1}·nM^{-1}. B: dissociation curve of [^{3}H]spiroperidol binding to striatum. Representative dissociation curve at a ligand concentration of 0.50 nM. k_{-1} was determined from the slope of the curve of ln B/Bo vs time where Bo is the amount bound at equilibrium. The average k_{-1} from 3 experiments was 0.004 ± 0.0003 min^{-1}.

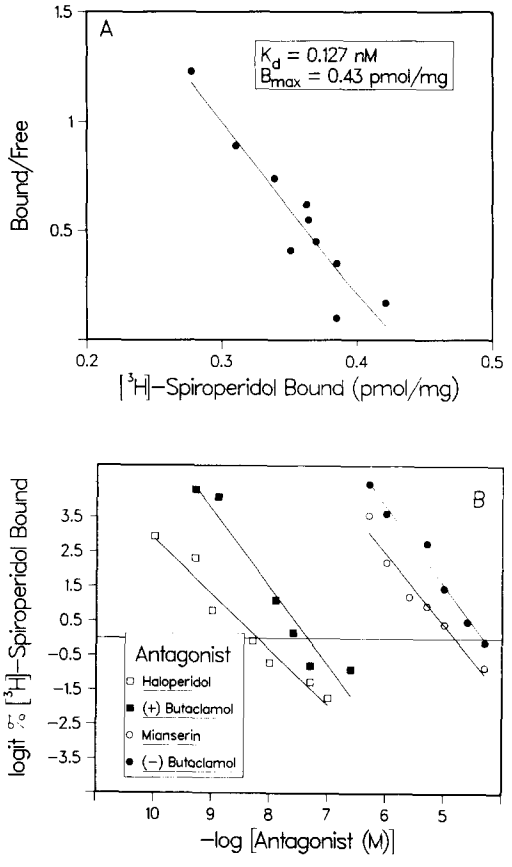


Fig. 2. A: Scatchard plot of [^{3}H]spiroperidol binding to striatum. Representative Scatchard plot of binding with ligand concentrations from 0.20 to 4.0 nM in the presence to 100 nM mianserin. Specific binding was determined by subtracting binding in the presence of 10 μM dopamine from total binding. The K_d and B_{max} from two experiments was 0.170 ± 0.006 nM and 0.440 ± 0.010 pmol/mg protein, respectively. B: antagonist competition curves for [^{3}H]spiroperidol binding to striatum. Representative competition curves from a single experiment plotted as logit % bound vs log competitor. The logit values were obtained by the formula logit (% bound) = ln (% bound/(100–% bound)). The K_i values were determined from the IC_{50} by the equation of Cheng and Prusoff[4]. The average K_is from 3 experiments were as follows: haloperidol, 7.62 ± 0.916 nM; (+)-butaclamol, 11.28 nM; mianserin, 5.88 ± 2.56 μM; (–)-butaclamol, 11.84 ± 1.65 μM.

petition and distribution studies occurred by 60 min for [^{3}H]spiroperidol concentrations above 0.5 nM. The equilibrium dissociation constant (K_d) as determined by kinetic relationship (k_{-1}/k_{+1}) was 0.170 ± 0.002 nM.

Saturation studies revealed a K_d of 0.170 ± 0.006 nM and a maximum number of binding sites (B_{max}) of 0.44 ± 0.01 pmol/mg protein in striatum (Fig. 2A). Hill plots yielded a slope of 1.06 ± 0.11, indicating a

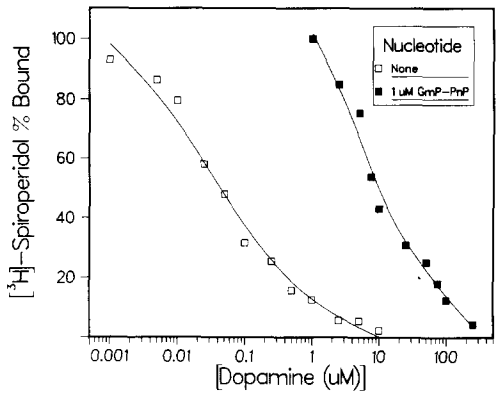
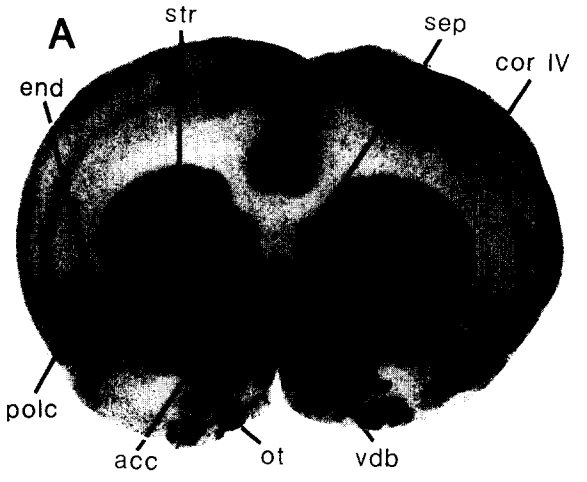


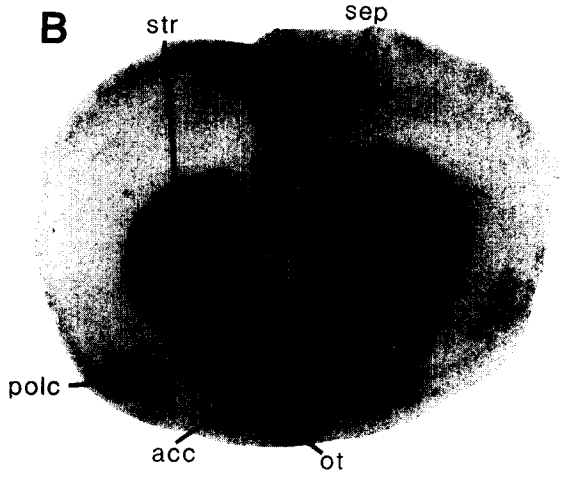
Fig. 3. Dopamine competition curves for [^{3}H]spiroperidol binding to striatum. Representative dopamine competition curves from a single experiment in the absence and presence of 1 μM GmP-PnP. Dopamine competed biphasically for [^{3}H]spiroperidol binding with a Hill slope of 0.63 ± 0.042. In the presence of 1 μM GmP-PnP the Hill slope was changed to 0.95 ± 0.035. The values represent the average of 3 experiments.

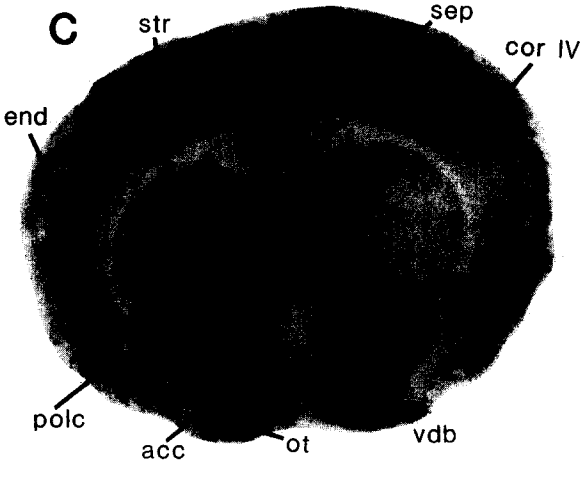
single binding site in striatum in the presence of mianserin. Ligand concentrations measured before and after incubation revealed that zone 'A' conditions were maintained at all ligand concentrations[12].

Competition curves using various antagonists revealed a rank order of potency for binding to the D_2 receptor in tissue sections similar to that found in homogenate studies (Fig. 2B). Haloperidol had the lowest K_i of 7.62 ± 3.16 nM. Butaclamol demonstrated stereospecificity with the (+) isomer having a K_i (11.3 ± 1.6 nM) more than a thousand-fold lower than the (–) isomer (K_i 11.8 ± 1.7 μM). Mianserin had a K_i of 5.88 ± 2.6 μM for the D_2 site. The Hill slopes for the antagonists did not vary significantly from one.

Dopamine had a biphasic pattern of competition with a high affinity (K_h) of 8.2 ± 6.3 nM and a low affinity (K_l) of 12.3 ± 8.4 μM (Fig. 3). K_h was higher than that seen in most homogenate studies. The percentage of high-affinity sites was 90%. Agonist binding was very sensitive to low concentrations of guanylyl-imidodiphosphate (GmP-PnP), with 1 μM GmP-PnP causing a complete shift to the low-affinity state (Fig. 3). The Hill slope for dopamine competition changed from 0.63 ± 0.04 to 0.95 ± 0.35 in the presence of 1 μM GmP-PnP. A complete shift to the same low-affinity state was seen in the presence of 100 μM GTP. There was no change in affinity states in the presence of 10 μM adenylyl-imidodiphosphate (AmP-PnP).







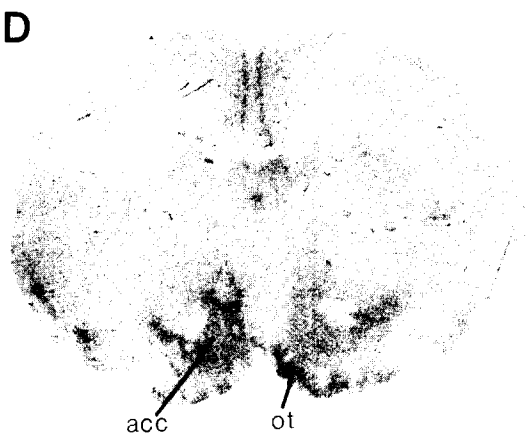


Fig. 4. [^{3}H]Spiroperidol autoradiograms of coronal sections through striatum. All incubations were done at a concentration of approximately 0.8 nM [^{3}H]spiroperidol. A: binding without competing drug present to display binding to the D_2, 5-HT_2 and spirodecanone sites in various structures. B: binding in the presence of 100 nM mianserin to block binding to the 5-HT_2 receptor. This eliminates binding to layer IV of cortex which has no dopamine or spirodecanone sites, and reduces binding in nucleus accumbens, olfactory tubercle and septal nuclei which have both D_2 and 5-HT_2 sites. C: binding in the presence of 10 μM dopamine to block the D_2 receptor. This leaves binding to the 5-HT_2 and spirodecanone sites. D: binding in the presence of 10 μM dopamine and 100 nM mianserin. This leaves residual binding in olfactory tubercle and nucleus accumbens. ot, olfactory tubercle; acc, nucleus accumbens; cor IV, layer IV cerebral cortex; end, endopiriform nucleus; polc, primary olfactory cortex; sep, lateral dorsal septal nucleus; str, striatum; vdb, vertical limb of the nucleus of the diagonal band.

The autoradiograms in Fig. 4 show that binding to either the D_2 (Fig. 4B) or the 5-HT_2 (Fig. 4C) receptor can be selected with the use of an appropriate concentration of a competitor. In addition, [^{3}H]spiroperidol labels a spirodecanone site which is insensitive to dopamine and mianserin. Binding to this site is found in the CA1 region of hippocampus with a high density in the pyramidal cell layer and a lower density in entorhinal cortex (Fig. 5). In the presence of excess of dopamine and mianserin there is still a small degree of residual binding in nucleus accumbens and olfactory tubercle which may represent the spirodecanone site as well. The distribution of the D_2 site is summarized in Table I in terms of amount of [^{3}H]spiroperidol bound and the amount bound relative to striatum.

DISCUSSION

The data in this report support the use of quantita-

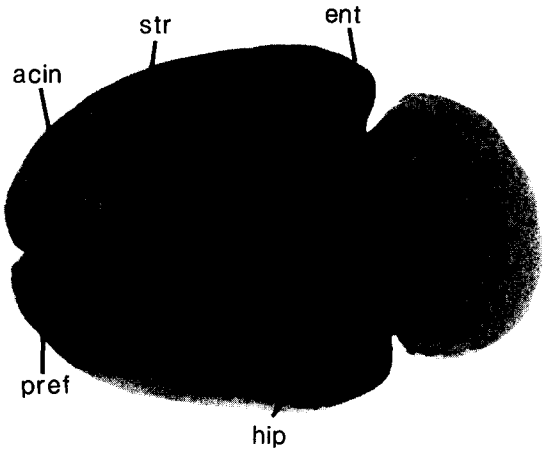


Fig. 5. [^{3}H]Spiroperidol autoradiogram of a horizontal section incubated at a [^{3}H]spiroperidol concentration of 0.8 nM in the presence of 100 nM mianserin. This demonstrates binding to the D_2 receptor in striatum, frontal cortex and anterior cingulate cortex, as well as to the spirodecanone site in hippocampus and entorhinal cortex. acin, anterior cingulate cortex; ent, entorhinal cortex; hip, hippocampus; pref, prefrontal cortex; str, striatum.

tive autoradiography to determine binding parameters directly from film density. Others have demonstrated the ability to perform autoradiography using [^{3}H]spiroperidol of the D_2 receptor[1,2,27]. We have expanded this methodology to determine additional properties of [^{3}H]spiroperidol binding to tissue sections. Saturation studies reveal that [^{3}H]spiroperidol binding is saturable with an affinity and B_{max} similar to that found in a quantitative autoradiographic study[21] using a different D_2 receptor ligand. In two studies using swabbed tissue sections[26,27] the K_d (0.14 and 0.92 nM) and B_{max} (63 and 447 fmol/mg protein) differ from each other and may be related to differences in technique[26,27]. In addition, when using swabbed sections to determine binding parameters, anatomically discrete regions having different binding parameters are averaged together. Tissue sections in the region containing striatum may also contain other regions having dopamine receptors including nucleus accumbens, olfactory tubercle and septum. By determining values directly from film densities each area can be evaluated independently.

TABLE I

Regional distribution of [^{3}H]spiroperidol binding to the D_2 receptor

Binding was performed at a [^{3}H]spiroperidol concentration of 0.5 nM in the presence of 100 nM mianserin to eliminate 5-HT_2 binding. Values represent the average of 3 separate experiments.

Region	*Bound (pmol/mg)*	*% Bound (relative to striatum)*
Striatum	0.286 ± 0.063	100
Nucleus accumbens	0.112 ± 0.017	39
Olfactory tubercle	0.218 ± 0.076	76
Mesial frontal cortex	0.060 ± 0.003	21
Anterior cingulate cortex	0.089 ± 0.006	31
Piriform cortex	0.077 ± 0.038	27
Septum	0.028 ± 0.003	10
Central amygdaloid nucleus	0.046 ± 0.019	16
Substantia nigra	0.093 ± 0.004	32

Kinetic studies established that binding is reversible, with kinetic constants producing a $K_d = k_{-1}/k_{+1} = 0.071$ nM which is in close agreement with saturation experiments ($K_d = 0.170$ nM). The kinetic constants determined in this study differ from those obtained using swabbed tissue sections[26] which suggests that kinetic parameters may vary with technique. If other binding values are to be determined autoradiographically, kinetic parameters should be obtained densitometrically first. The competition studies established that the rank order of potency for the various antagonists agrees with that found in homogenate studies and that binding is stereospecific with (+)-butaclamol having a K_i more than a thousand times lower than (−)-butaclamol. Mianserin was shown to block both the 5-HT_2 and D_2 receptor. A concentration of mianserin (100 nM) was selected that blocked all the 5-HT_2 sites as demonstrated by the absence of all cortical binding in layer IV (which is specific for serotonin sites) but was 100-fold lower than the IC_{50} for the D_2 receptor in striatum. This concentration agrees with the value found by others to displace serotonin binding without effecting dopamine binding in homogenate studies using [^{3}H]spiroperidol[24].

This study also reports quantitative D_2 receptor density measurements in multiple brain regions which range from a low of 0.028 pmol/mg protein in septum to 0.286 pmol/mg protein in striatum at a [^{3}H]spiroperidol concentration of 0.5 nM. Determination of values in some of these brain regions would be technically difficult using homogenates or tissue section swabs. The distribution of binding of [^{3}H]spiroperidol to D_2 receptors in rat brain is consistent

with what is known of the dopamine systems. A sparse projection from substantia nigra to globus pallidus and entopeduncular nucleus has been reported[25]; however, in this study binding was not found in those areas in films exposed for 4 weeks and would have a density less than 0.028 pmol/mg protein which is the lowest density seen in septum. The mesocortical system has been reported[18,23] to project to the nucleus accumbens, amygdaloid complex, anterior cingulate cortex, prefrontal cortex and septal nuclei. Significant binding to these areas was seen in this quantitative autoradiographic study.

The binding of [^{3}H]spiroperidol to the spirodecanone site has been reported by others[19,27]. This autoradiographic study demonstrates the unique laminar distribution of that site in the hippocampus, as well as, an additional site, not previously reported, found in entorhinal cortex. In addition, there is a small amount of binding present in nucleus accumbens and olfactory tubercle in the presence of high concentrations of dopamine and mianserin which may also represent this spirodecanone site. The significance of this site remains unknown, however, its location in only mesolimbic areas is of interest. At high [^{3}H]spiroperidol concentrations binding was also observed in the superficial layer next to cortex. This binding may represent receptors on the arachnoid membrane.

The major pharmacological difference between [^{3}H]spiroperidol binding to tissue sections and to homogenates or swabbed sections seen in this study is the interaction of D_2 receptor agonists and guanine nucleotides. In general, the behavior of the D_2 receptor has been analogous to the β-adrenergic system[16,22]. In the β-adrenergic system, antagonists bind to one homogeneous state of the receptor with a single affinity, while agonists bind to two distinct states; one with high affinity and one with low affinity. Guanyl nucleotides are able to shift the high-affinity state to a low-affinity state. In the D_2 system, agonists have been shown to manifest heterogeneous binding also. However, there remains question as to whether agonist binding is best modeled as a two-site[11,32] or 3-site[36] model. Guanine nucleotides have been shown to decrease the high-affinity binding of agonists to the D_2 receptor[8,11,36,39].

In this report, we find that the use of quantitative autoradiography to study agonist and guanine nucleotide interactions involving the D_2 receptor demonstrates several advantages over other techniques. First, the percentage of D_2 sites in the high-affinity agonist state is approximately 90% in tissue sections. This contrasts with homogenate binding where the percentage is usually much lower, varying from 28%[36] to 56%[11] using dopamine as a competitor. There is also wide variation depending on the agonist used. In addition, the high-affinity site of the D_2 receptor had a K_i for dopamine of 8 nM. This contrasts with the high-affinity K_is found in homogenate studies which vary from 16 nM[16] to 66 nM[11] and 190 nM[32].

A second difference between this report and others is the ability of GmP-PnP to cause a complete shift to the low-affinity state in a buffer that does not reduce the high-affinity state. Several reports[20,36] failed to find a complete shift in the presence of excess guanine nucleotides. A recent report demonstrated a complete shift in the presence of 120 mM NaCl. However, the percentage of high-affinity sites was only 15% in that situation[14].

Another difference between tissue sections and homogenates is the concentration of guanine nucleotide required to convert the D_2 receptor from high to low affinity. Most of the homogenate studies demonstrating this shift used a concentration of GmP-PnP of 100 μM. In this study, a concentration of 1 μM was able to cause a complete shift from high to low affinity. The complete shift seen in the presence of 100 μM GTP, but not with 10 μM AmP-PnP verifies the selectivity of the regulation.

In summary, in tissue sections it appears that 90% of the D_2 receptor sites are in the high-affinity state and bind dopamine with a K_i of 8 nM. These sites are also very sensitive to guanine nucleotides since 1 μM GmP-PnP can shift all of the high-affinity sites of the D_2 receptor to a low-affinity state for which dopamine has a K_i of 12 μM. These differences may be due to factors that preserve certain membrane properties in the tissue sections. It may reflect preservation of G_i protein units that are damaged during homogenization, or it may reflect a stoichiometric relationship between subunits that is altered by membrane disruption.

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