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In Vivo Evidence for Dopamine-Mediated Internalization of D_2-Receptors after Amphetamine: Differential Findings with [^{3}H]Raclopride versus [^{3}H]Spiperone

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ABSTRACT

Competition with endogenous dopamine (DA) is usually invoked to explain changes in [^{11}C]raclopride binding observed after amphetamine administration in animals and humans. This account has recently been questioned because a number of inconsistencies have been reported that contradict it. In the present study, we investigated whether the decrease in [^{3}H]raclopride binding observed in the rat striatum after an amphetamine challenge reflects true competition with endogenous DA or agonist-mediated internalization of D_2-receptors. We found that the amphetamine-induced decrease in [^{3}H]raclopride binding is caused by a decrease in D_2-receptor density (B_{max}) with no change in affinity (K_d). In contrast, in the same tissue, neither the B_{max} nor the K_d were affected when measured with [^{3}H]spiperone. Challenge with amphetamine not only decreased the number of D_2-receptors but also eliminated the proportion (22%) of receptors usually in the high-affinity state. The addition of Gpp(NH)p had no effect on B_{max}, suggesting that these receptors were not just noncompetitively bound with dopamine at the cell-surface. Subcellular fractionation studies showed that amphetamine treatment led to a decrease in radioligand binding in the cell-surface fraction for both [^{3}H]raclopride and [^{3}H]spiperone; however, in the case of [^{3}H]spiperone, this was accompanied by a compensatory increase in binding in the intracellular compartment, whereas no increase was seen with [^{3}H]raclopride. These data suggest that amphetamine releases dopamine, which binds to the high-affinity state of the D_2-receptor, leading to its sequestration in some intracellular compartment; in this compartment, sequestered receptors are inaccessible to [^{3}H]raclopride binding but can still be bound by [^{3}H]spiperone.

In vivo imaging techniques such as positron emission tomography (PET) and single-photon emission computed tomography (SPECT) have been applied to assess the endogenous levels of dopamine (DA) in both basic and clinical investigations. These techniques have been used to show that behavioral tasks, such as playing video games or writing, induce an increased release of striatal DA (Koepp et al., 1998; de la Fuente-Fernandez et al., 2001) and that patients with schizophrenia show an abnormally high release of DA when challenged with amphetamine (Laruelle et al., 1996; Breier et al., 1997). Although these techniques are being extensively used, PET and SPECT do not provide a direct measurement of endogenous DA levels. Changes in endogenous DA levels are inferred from changes in the binding of [^{11}C]raclopride and [^{123}I]IBZM to DA D_2-receptors. Binding of [^{11}C]raclopride and [^{123}I]IBZM is consistently decreased by drugs that elevate synaptic DA (Innis et al., 1992; Volkow et al., 1994; Laruelle et al., 1995; Smith et al., 1997), whereas the opposite effect is observed with drugs that decrease synaptic DA, such as reserpine and α-methyl-paratyrosine (Ginovart et al., 1997; Laruelle et al., 1997a). The precise mechanism whereby DA release leads to decrease in the binding of [^{11}C]raclopride and [^{123}I]IBZM is still a matter of debate.

The conventional explanation for this phenomenon is that increased competition between released endogenous DA and radioligand for binding to D_2-receptors leads to a decrease in [^{11}C]raclopride and [^{123}I]IBZM bindings. In the last few years, a number of inconsistencies have emerged, however, that questioned the validity of this model. First, not all radioligands show this effect. Although benzamide radioligands (such as raclopride, IBZM, fallypride, clebopride) are always affected by endogenous DA in a manner consistent with the model (Innis et al., 1992; Volkow et al., 1994; Laruelle et al., 1995; Ginovart et al., 1997; Hartvig et al., 1997; Mach et al., 1997; Mukherjee et al., 1997), in vivo and ex vivo binding of butyrophenone compounds (such as spiperone, NMSP, pimozide) to D_2-receptors show either no change or

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ABBREVIATIONS: PET, positron emission tomography; SPECT, single photon emission computed tomography; DA, dopamine; IBZM, iodobenzamide; NMSP, *N*-methyl-spiperone; GPP(NH)p, 5′-guanylylimidodiphosphate.

changes in the direction opposite that expected (Niehoff et al., 1979; Saelens et al., 1980; Bischoff et al., 1991; Onoe et al., 1994; Kobayashi et al., 1995). Second, the amphetamine-induced changes in [11C]raclopride and [123I]IBZM bindings far outlive the drug-induced changes in DA levels as measured with microdialysis (Laruelle et al., 1997b; Carson et al., 2001), calling into question a simple competition model.

To account for these discrepancies, Laruelle (2000) has proposed that the decrease in [11C]raclopride binding observed after amphetamine reflects a D_2 receptor internalization triggered by the release of DA. According to this hypothesis, released DA promotes a shift (i.e., internalization) of D_2-receptors from the cell membrane to the intracellular endosomal compartment. Because of their low lipophilicity, benzamide-like ligands (raclopride, IBZM) lose access to the shifted receptors, which translates into a decreased radioligand binding. In contrast, butyrophenone-like ligands (spiperone, *N*-methyl-spiperone) are lipophilic enough to cross the cell membrane and have access to both the cell surface and internalized receptors and thus do not show any response to amphetamine. By delinking [11C]raclopride binding decreases from direct DA competition, the internalization model can explain the temporal discrepancy that has been observed after amphetamine challenge (Laruelle et al., 1997b; Carson et al., 2001).

Although this hypothesis has good heuristic value, there are few data to support it. In the present study, we first standardized in vivo conditions in rats that give rise to a decrease in [3H]raclopride binding similar to that seen in patients. We then confirmed that this finding in rats was long-lasting, thus replicating the temporal discrepancy seen in vivo imaging studies. Then we used this model to harvest striatal tissue and investigate (ex vivo) the molecular mechanisms underlying the decrease in binding. We first examined the B_{max} and K_d of DA D_2-receptors in the same tissue using [3H]raclopride and [3H]spiperone. We demonstrated that the amphetamine challenge affected only [3H]raclopride binding and did so by decreasing B_{max} (not K_d), thus showing that the decrease in binding was not competitive. We then showed that this decrease in binding reflected a temporarily irreversible loss of the high-affinity state of DA D_2-receptors. Subcellular distribution study showed a translocation of these receptors from the cell-surface fraction to the intracellular endosomal fraction after amphetamine. Thus, our studies reject the simple-competition model and suggest that the decrease in [11C]-raclopride and [123I]IBZM binding observed after an amphetamine challenge are more compatible with an internalization model.

Materials and Methods

Animals. Animal studies and experimental procedures conformed to the guidelines established by the Canadian Council on Animal Care, and were approved by the Animal Care Committee at the Centre for Addiction and Mental Health. Adult male Sprague-Dawley rats, 200 to 225 g (Charles River, Montréal, Canada) were housed two per cage under reversed light/dark conditions using a 12-h on/off schedule (lights off at 8 AM). Room temperature was maintained at 21 ± 1°C with a relative humidity of 55 to 60%. Food and water were available ad libitum. The animals were allowed 1 week of adaptation to laboratory conditions before being used in experiments.

Drugs. [3H]Raclopride (78 Ci/mmol) was purchased from PerkinElmer Life Science (Boston, MA) and [3H]spiperone (101 Ci/mmol) was purchased from Amersham Bioscience (Piscataway, NJ). (*S*)-Sulpiride, 5′-guanylylimidodiphosphate [GPP(NH)p], and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical (St. Louis). Amphetamine was purchased from US Pharmacopeia (Rockville, MD). Pepstatin and leupeptin were purchased from Boehringer Mannheim (Mannheim, Germany).

Dose-Effect of Amphetamine on in Vivo Binding of [3H]Raclopride. Four groups of rats (n = 8 per group) were preinjected with either 0.9% saline or increasing doses of amphetamine (2, 4, and 8 mg/kg; s.c.) 20 min before [3H]raclopride administration. Rats were then injected in the tail vein with [3H]raclopride and sacrificed by decapitation 30 min after radiotracer injection. Their brains were quickly removed and dissected for striatum and cerebellum tissues and then processed as previously published (Wadenberg et al., 2000). Briefly, tissues were solubilized in 2 ml of Solvable (Canberra Packard, Mississauga, ON, Canada) for 24 h at 23°C. Five ml of Aquasure (Canberra Packard) scintillation fluid was then added and their radioactivity concentration measured using liquid-scintillation counting system (LS5000 CE; Beckman Coulter, Fullerton, CA). The ratio of counts per milligram of tissue in the striatum provided a measure of the total number of binding sites (specific and nonspecific) for [3H]raclopride. The cerebellum, a brain region relatively devoid of D_2-receptors (Cortes et al., 1989), was used to estimate the nonspecific binding. The ratio of radioactivity level measured in the striatum to that measured in the cerebellum provided an index of the concentration of D_2-receptors available for [3H]raclopride binding (Farde et al., 1988; Kapur et al., 1999). By comparing the index obtained in amphetamine-treated rats to that obtained in control rats, a measure of the number of receptors that became inaccessible to [3H]raclopride after amphetamine was obtained.

Time Course of Amphetamine Effect on the in Vivo Binding of [3H]Raclopride. Rats were preinjected subcutaneously with either 0.9% saline (n = 12; s.c.) or amphetamine (8 mg/kg; n = 24; s.c.) and were then injected in the tail vein with [3H]raclopride at different times (1 h, 3 h, 6 h) after the preinjection. Rats were sacrificed by decapitation at 30 min after [3H]raclopride injection, their brain was quickly removed and their brain tissues processed as described above.

Membrane Homogenate Preparation and in Vitro Receptor Binding. Rats were injected with either amphetamine (n = 8, 8 mg/kg, s.c.) or 0.9% saline (n = 8, s.c.) and decapitated at 50 min after drug administration. The striata were dissected out on ice and stored at −70°C until used. For each rat, one striatum was used for [3H]raclopride binding assays, and the contralateral striatum was used for [3H]spiperone binding assays. The striatum side (left and right) used for the binding assays ([3H]raclopride or [3H]spiperone) was counterbalanced from one rat to the other. The assays were performed essentially as described earlier (Seeman et al., 1992). Briefly, the tissues were thawed and homogenized in a motor-driven glass Teflon homogenizer by 10 up and down strokes at 500 rpm at 0°C in 5 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, and 1 mM EDTA. The tissues were incubated with 8 to 12 increasing concentrations of [3H]raclopride (0.2 to 20 nM) or [3H]spiperone (0.01 to 0.5 nM). The binding of each concentration of radioligand was determined in duplicate. Incubations were carried out at room temperature (22°C) for 120 min. The final concentration of the tissue in each binding assay was 1 mg/ml. (*S*)-Sulpiride (10 μM) was used to define the nonspecific binding. The incubations were terminated by rapid filtration using a 12-well cell harvester (Titertek; Skatron, Sterling, VA) and buffer-presoaked glass fiber filter mats (No. 7034; Skatron). The filters were then quickly rinsed with 7.5 ml of 50 mM Tris buffer, pH 7.4, and dissolved in 4 ml of scintillant (Ready Solve; Beckman Coulter) at room temperature for overnight. The radioactivity of the filters was counted in a scintillation spectrometer (Packard 4660) at 55% efficiency.

For binding studies performed in the presence of GPP(NH)p,

which was expected to abolish the high-affinity states of D_2-receptors, membrane homogenates were preincubated with 200 μM GP-P(NH)p at room temperature for 60 min. The membranes were not further washed, and binding assays were then carried out on the preincubated membranes directly as described above.

The results presented were obtained from the total binding and nonspecific binding. The estimates of B_{max} (the number of available binding receptors) and K_d (the dissociation constant) were calculated by fitting the specific binding data with nonlinear regression using Prism data analysis software (GraphPad Software, San Diego, CA). Statistical comparisons between groups were made using Student's two-tailed t test.

Competition Studies of [^{3}H]Raclopride in Vitro Binding by DA. Rats were injected with either amphetamine (n = 8, 8 mg/kg; s.c.) or 0.9% saline (n = 8, s.c.) and decapitated at 50 min after drug administration. Striata from control and amphetamine-treated rats were obtained and homogenized as described above and incubated with increasing concentrations of DA (10^{-12} to 10^{-3} M) in 50 mM, pH 7.4, Tris-HCl buffer containing 10 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, and 1 mM EDTA. Incubations were initiated after the addition of 2 nM [^{3}H]raclopride and conducted at room temperature for 120 min. The incubates were then filtered and rinsed as described above, and the radioactivity remaining on the mat was counted. The percentage of [^{3}H]raclopride-specific binding remaining in the presence of DA was calculated and plotted versus the concentration of the DA. Nonlinear least-squares fitting of the data were performed using GraphPad Prism data analysis software and the goodness of fit was judged with one-site fit or two-site fit. A two-site was selected only if the F-test comparing the sum of square for errors with that for one-site fit indicated that the sum of squares for errors was significantly reduced using the two-site model ($p < 0.05$).

Subcellular Fractionation Studies. Rats were injected with either amphetamine (n = 12, 8 mg/kg; s.c.) or 0.9% saline (n = 12, s.c.) and decapitated at 50 min after drug administration. Subcellular fractionation was performed at 4°C according to a method developed by Clement-Cormier and George (1979) and slightly modified. Briefly, striata were rapidly dissected after decapitation and pooled from the control or amphetamine-pretreated rats. The tissue was homogenized in 20 vol (w/v) of 0.32 M sucrose containing 5.0 mM Na-HEPES, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 1 μM leupeptin. Aliquots were taken for protein assay (100 μl) and receptor binding (0.5 ml) in total homogenate. The remaining homogenate was centrifuged at 900g for 10 min to remove cellular debris and nuclei. The pellet was rehomogenized in 15 vol of 0.32 M sucrose and recentrifuged. The supernatants were pooled and centrifuged at 12,000g for 20 min to yield the crude mitochondria pellet. The supernatant was further centrifuged at 100,000g for 90 min to yield the microsomal fraction. The final pellets were not further washed, but directly resuspended in 50 mM Tris-HCl binding buffer, pH 7.4 (containing 120 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, and 1 mM EDTA). Binding assays were conducted immediately on all fractions including total homogenate, nuclear fraction (nuclei and few sheets of plasma membrane), mitochondrial fraction (synaptic plasma membrane fragments, mitochondria), microsomal fraction (endosomes), and final supernatant. Incubations were performed as described above but at a single radioligand concentration using 10 nM [^{3}H]raclopride or 0.5 nM [^{3}H]spiperone. The radioactivity for each fraction was counted and presented as a specific binding of radioactivity to the protein concentration. Protein content of each fraction (micrograms) was determined using the bicinchoninic acid protein assay kit from Pierce Chemicals (Rockford, IL).

Results

Effect of Amphetamine Administration on in Vivo Binding of [^{3}H]Raclopride. The dose-effect of amphetamine administration on in vivo [^{3}H]raclopride binding was determined using three different doses of the drug. As shown in Fig. 1, at 2 and 4 mg/kg, amphetamine induced 10 and 18% decreases in [^{3}H]raclopride binding, but this effect was not statistically significant. At a dose of 8 mg/kg, amphetamine induced a significant ($p < 0.001$) 45% decrease in [^{3}H]raclopride binding. This dose was used in further studies. Nonspecific binding in cerebellum was not statistically different in treated rats compared with control rats, at any of the amphetamine doses used. Nonspecific binding mean values in cerebellum were 15.5 ± 3.5 dpm/mg of tissue in controls and 14.4 ± 1.0, 16.3 ± 3.5, and 13.4 ± 4.6 dpm/mg of tissue after an injection of amphetamine at a dose of 2, 4, and 8 mg/kg, respectively. The time-course of the effect of amphetamine at 8 mg/kg is shown in Fig. 2. A single administration of amphetamine at 8 mg/kg s.c. significantly decreased [^{3}H]raclopride binding by 33% at 1 h, 42% at 3 h, and 59% at 6 h after injection—thus replicating the long-lasting effect of the drug seen in PET and SPECT studies.

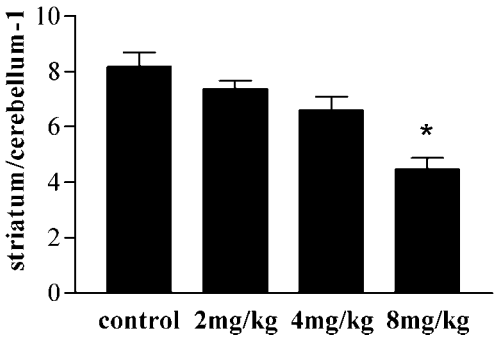

Fig. 1. Effects of increasing doses of amphetamine on in vivo [^{3}H]raclopride binding. Rats were pretreated with 2, 4, or 8 mg/kg amphetamine (s.c.), respectively, injected with [^{3}H]raclopride (7.5 μCi, i.v.) at 20 min after treatment, and sacrificed at 50 min after the amphetamine injection. There is a significant decrease in binding of [^{3}H]raclopride after the 8 mg/kg amphetamine administration (*, $p < 0.001$).

Effect of Amphetamine Administration on in Vitro Binding of [^{3}H]raclopride and [^{3}H]spiperone. In this set of experiments, the receptor binding characteristics of [^{3}H]raclopride and [^{3}H]spiperone were compared under control conditions and after pretreatment with amphetamine. The D_2-receptors' K_d and B_{max} values determined using each radioligand are summarized in Table 1. A significant 30% decrease ($p < 0.05$) in D_2-receptor density with no significant

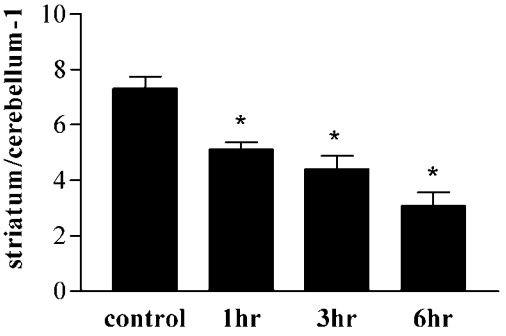

Fig. 2. Time course of [^{3}H]raclopride accumulation in the rat striatum after an amphetamine administration. Rats were pretreated with 8 mg/kg amphetamine (s.c.) and sacrificed 1, 3, or 6 h after treatment. [^{3}H]Raclopride (7.5 μCi, i.v.) was injected in the tail vein 30 min before sacrifice. [^{3}H]Raclopride binding showed a significant decrease after amphetamine pretreatment (*, $p < 0.001$).

TABLE 1

Effect of amphetamine administration on B_{max} and K_d values of [3H]raclopride and [3H]spiperone binding in the rat striatum

Rats were pretreated with either saline or 8 mg/kg amphetamine (s.c.) and sacrificed 50 min after treatment. B_{max} and K_d were determined using GraphPad Prism software. Values are presented as means ± S.E. of seven to eight experiments.

	B_{max}	K_d
	pmol/g tissue	*nM*
[3H]Raclopride		
Control	21.3 ± 1.3	1.9 ± 0.1
Amphetamine	14.9 ± 0.5[a]	1.7 ± 0.2
[3H]Spiperone		
Control	12.6 ± 0.8	0.07 ± 0.005
Amphetamine	11.1 ± 0.6	0.07 ± 0.005

[a] $p < 0.05$ compared with control values (Student's t test).

change in affinity was observed in amphetamine-treated rats compared with controls when using [3H]raclopride. In contrast, no significant effect of amphetamine administration was observed on either B_{max} or K_d when using [3H]spiperone. Thus, we find a "loss" of receptors only with [3H]raclopride, and not [3H]spiperone, in the same tissue.

Evaluation for High- and Low-Affinity States of D_2-Receptors with [3H]Raclopride. To examine whether this loss of receptors was related to the high- or low-affinity state of the DA D_2-receptors, the percentage of D_2-receptors in their high- and low-affinity states for DA was evaluated at control conditions and after a single administration of amphetamine (8 mg/kg, s.c.). As shown in Fig. 3, at control conditions, a two-site model fits the data significantly better than a one-site model ($F = 38.24$, $p < 0.001$), and about 22% of DA D_2-receptors labeled by [3H]raclopride were in the high-affinity state. The K_d of DA for the high- and low-affinity state of the receptor was 0.11 nM and 118 nM,

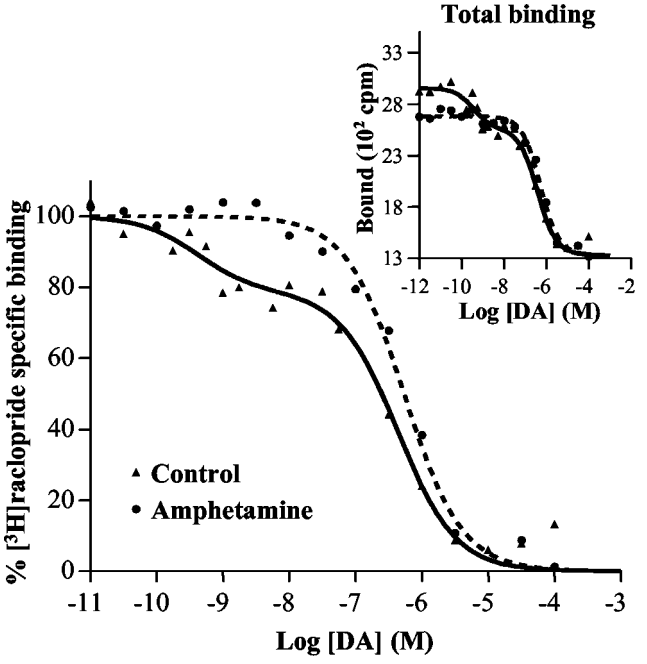

Fig. 3. Competitive-inhibition curves illustrating high-affinity state DA receptors lost in rat striatal membranes after amphetamine administration. Rats were pretreated with 8 mg/kg amphetamine (s.c.) and decapitated 50 min after treatment. The curves shown are representative of three independent experiments for each group. The data were analyzed using GraphPad Prism software. [3H]Raclopride competitive curves exhibited the following computer-derived binding parameters: control, K_H, 0.11 nM; K_L, 118 nM; R_H, 22%; amphetamine, K_L, 147 nM.

respectively. In contrast, in amphetamine-treated rats, the results show that a one-site model fit the data significantly better than a two-site model ($F = 0.31$, $p = 0.74$). The K_d of DA for this single population of binding site was 147 nM. Thus, the data showed that the apparent loss of receptors after amphetamine challenge was related more to the high-affinity state of the DA D_2-receptors.

Effect of Amphetamine Administration on in Vitro Binding of [3H]Raclopride and [3H]Spiperone in the Presence of GPP(NH)p. Because this apparent loss of receptors was related more to the high-affinity state of the DA D_2-receptors, we wondered whether this reflected the fact that a greater amount of endogenous DA was noncompetitively bound to, and hence obscuring, DA D_2-receptors. Our previous work has demonstrated that GPP(NH)p converts all receptors to their low-affinity state and can reverse such an apparent loss in binding. However, when the in vitro binding assays were performed using [3H]raclopride in the presence of GPP(NH)p, no difference was seen versus the standard measurement conditions (Table 2). The B_{max} values measured using [3H]raclopride in the presence of GPP(NH)p were decreased by 31% in amphetamine-treated rats compared with controls, which is similar to the decreases measured in the absence of GPP(NH)p (Table 1). Thus, the apparent loss of receptors was not just a reflection of noncompetitive binding of the receptors to endogenous dopamine.

Subcellular Fractionation. Fractionation studies by differential centrifugation were performed on rat striatal homogenates to examine whether amphetamine treatment led to a differential distribution of the DA D_2-receptors. Data on [3H]raclopride and [3H]spiperone binding in subcellular fractions of striata from control and amphetamine-treated rats are show in Fig. 4. The specific D_2-receptor bound radioactivity was measured in each fraction. In control rats, the distribution profiles of radioligand binding were similar for [3H]raclopride and [3H]spiperone. The highest radioactive binding was observed in the mitochondrial fraction. Amphetamine treatment significantly reduced the binding of [3H]raclopride in the total homogenate, the nuclear fraction, and the mitochondrial fraction by 20 ($p < 0.01$), 34 ($p < 0.05$), and 37% ($p < 0.05$), respectively. In contrast, [3H]spiperone binding was only reduced in the mitochondrial fraction (30%, $p < 0.05$). In addition, amphetamine pretreatment had a differential effect on [3H]raclopride and [3H]spiperone bindings in the microsomal fraction; it did not alter [3H]raclopride bind-

TABLE 2

Effect of amphetamine administration on B_{max} and K_d values of [3H]raclopride and [3H]spiperone binding to D_2 receptors in the rat striatum in the presence of 200 μM GPP(NH)p

Rats were pretreated with either saline or 8 mg/kg amphetamine (s.c.) and sacrificed 50 min after treatment. Membrane preparations were preincubated with 200 μM GPP(NH)p for 60 min at 22°C before receptor binding assay. B_{max} and K_d were determined using GraphPad Prism software. Values are presented as means ± S.E. of seven to eight experiments.

	B_{max}	K_d
	pmol/g tissue	*nM*
[3H]Raclopride		
Control	28.3 ± 2.0	1.5 ± 0.2
Amphetamine	19.5 ± 1.0[a]	1.2 ± 0.1
[3H]Spiperone		
Control	15.5 ± 0.5	0.05 ± 0.001
Amphetamine	15.8 ± 0.6	0.06 ± 0.002

[a] $p < 0.05$ compared with control values (Student's t test).

ing in the microsomal fraction but significantly increased [^{3}H]spiperone binding by 25% ($p < 0.05$).

Discussion

In agreement with previous in vivo studies using [^{3}H]raclopride in rodents (Ross and Jackson, 1989; Seeman et al., 1989), we also found that increasing endogenous DA levels with amphetamine leads to a decrease in [^{3}H]raclopride binding to striatal D_2-receptors. Besides, our data showed that this effect was long-lasting and persisted for up to 6 h after amphetamine. This long-lasting decrease in [^{3}H]raclopride binding is also in agreement with previous studies showing that [^{123}I]IBZM and [^{11}C]raclopride bindings are still reduced at 2 and 5.5 h, respectively, after an amphetamine challenge in primates (Laruelle et al., 1997b; Carson et al., 2001).

The classical explanation for the decreased [^{3}H]raclopride observed after amphetamine relies on the DA competition model, which predicts that increased levels of endogenous DA exerts a competitive inhibition on [^{3}H]raclopride binding. However, the long-lasting nature of this effect cannot be explained by increased DA levels competing with [^{3}H]raclopride because DA levels are known to return to baseline at 2 h after amphetamine (Laruelle et al., 1997a).

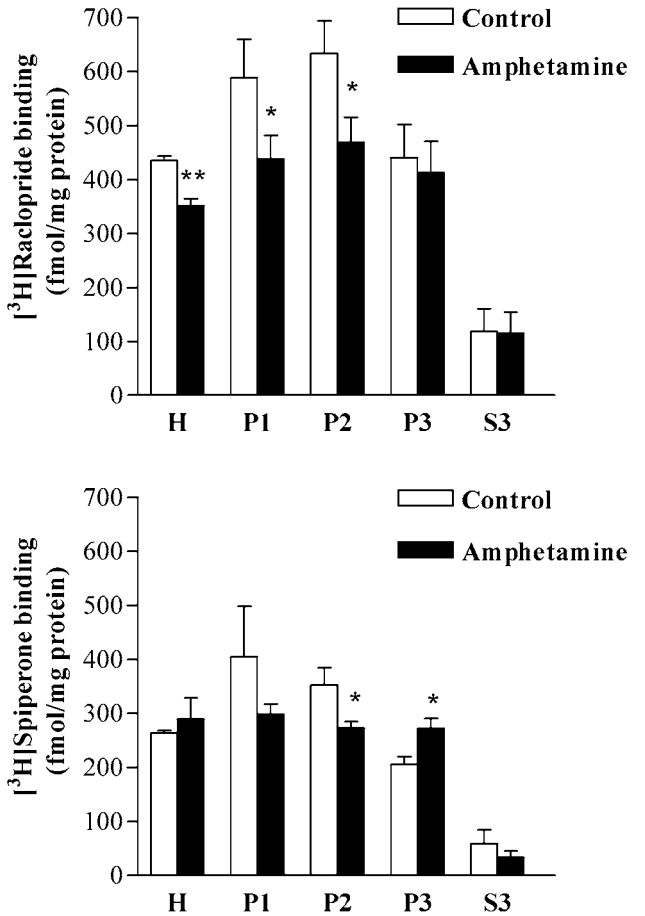

Fig. 4. Comparisons of [^{3}H]raclopride and [^{3}H]spiperone bindings to subcellular fractions of rat striata after an administration of amphetamine. Rats were pretreated with 8 mg/kg amphetamine (s.c.), and decapitated at 50 min after amphetamine administration. The averages of protein content for each fraction were 22.12 mg (H), 5.69 mg (P1), 8.38 mg (P2), 1.11 mg (P3), and 6.58 mg (S3) for control rats; and 22.35 mg (H), 6.27 mg (P1), 8.35 mg (P2), 1.06 mg (P3), and 7.11 mg (S3) for amphetamine rats. Data represent the mean ± S.E. of four experiments (*, $p < 0.05$, **, $p < 0.01$). H, total homogenate; P1, nuclear pellet; P2, mitochondrial pellet; P3, microsomal pellet; S3, final supernatant.

The DA competition model predicts that the amphetamine effect on [^{11}C]raclopride binding reflects a change in affinity but not in receptor density. Scatchard analyses of [^{3}H]raclopride and [^{3}H]spiperone bindings were performed to determine which, if any, of these parameters was affected by amphetamine. In these experiments, the use of (*S*)-sulpiride to define [^{3}H]raclopride and [^{3}H]spiperone nonspecific bindings could presumably seem problematic, because it is a rather lipophilic ligand that should only block cell surface D_2-receptors. However, data in our laboratory have consistently shown that using 10 μM (*S*)-sulpiride to define nonspecific binding in vitro gives identical values as 1 μM (+)-butaclamol for both radioligands (data not shown). Similarly, using either 10 μM (*S*)-sulpiride or 1 μM (+)-butaclamol gave identical B_{max} and K_d values for both [^{3}H]spiperone and [^{3}H]raclopride, suggesting that because of the high concentration we used (10 μM), (*S*)-sulpiride can penetrate cell membranes and has access to both the cell surface and intracellular receptors. In accordance with previous studies, the B_{max} measured in control rats using [^{3}H]raclopride was about twice that measured using [^{3}H]spiperone (Niznik et al., 1985; Terai et al., 1989; Seeman et al., 1992). The "monomer-dimer theory" has been evoked to account for these differences. Indeed, it has been shown that D_2-receptors can exist in both monomer and dimer forms and that butyrophenones bind primarily to the monomer form, whereas benzamides bind to both receptor forms (Seeman et al., 1992; Zawarynski et al., 1998). Differences in the monomer-dimer equilibrium could thus explain the differences in D_2-receptor densities revealed by the two classes of compounds.

Scatchard analyses revealed that the amphetamine-induced reduction in [^{3}H]raclopride binding was attributable to a decreased B_{max} with no change in K_d. This suggests that the mechanism is not competitive, because competition should have led to a change in K_d with no change in B_{max}. Although amphetamine pretreatment induced a reduction in [^{3}H]raclopride B_{max}, no change in either B_{max} or K_d was detected with [^{3}H]spiperone. This result is also in agreement with previous studies showing no change in [^{3}H]spiperone binding in the striatum of amphetamine-treated rats (Niehoff et al., 1979; De Jesus et al., 1986). Because both [^{3}H]raclopride and [^{3}H]spiperone binding measurements were performed on membrane preparations obtained from the same animals, it suggests that the reduction in [^{3}H]raclopride B_{max} rather reflects a diminished capacity of [^{3}H]raclopride to access D_2-receptors than a "real" receptor loss.

It seems, given the above results, that the decrease in [^{3}H]raclopride binding is caused by a noncompetitive "apparent loss" of receptors instigated by the release of DA. Two possible mechanisms may explain this. First, dopamine that is released after amphetamine treatment could have bound to the high-affinity state of the receptors in a noncompetitive fashion, and although the receptors were still present on the cell surface, they were not available for [^{3}H]raclopride binding. Second, the amphetamine-induced dopamine-release could have bound to the high-affinity state of the receptors and then internalized them so that the receptors were no longer on the cell surface but translocated to a compartment in which [^{3}H]raclopride cannot bind to them but [^{3}H]spiper-

one still can. The homogenates consist of newly formed and largely spherical vesicles. These "inside-out" vesicles are not disrupted by the homogenization procedure used in our study (500 rpm, 10 strokes). Thus, even though the cells are disrupted, [^{3}H]raclopride, because of its lower lipophilicity, may not permeate these vesicles as readily as [^{3}H]spiperone. As we argue below, the majority of facts favor the latter possibility. Although the first mechanism could account for the fact that we observe a change in [^{3}H]raclopride B_{max} and not K_d, other findings in our experiments make it unlikely. Indeed, it has been shown that when DA binds to its receptors noncompetitively, the addition of GPP(NH)p converts the DA-occupied high-affinity receptors to their low-affinity states, releasing DA bound on the receptors and restoring the apparently lost receptors (Seeman et al., 1989). This was not observed in our study. The addition of GPP(NH)p did not reverse the apparent loss of [^{3}H]raclopride binding sites observed after amphetamine. Also, if DA bound to the receptors noncompetitively and led to a lower B_{max}, it should also have occurred for [^{3}H]spiperone—and that was not the case. Finally, if noncompetitive binding of DA were the complete explanation, we should not have observed a differential distribution of D_2-receptors in subcellular fractions—thus making this explanation unlikely.

The most likely explanation to the reduction in [^{3}H]raclopride B_{max} is that amphetamine administration induced a DA-promoted internalization of D_2-receptors from the cell membrane to the intracellular compartment. Indeed, agonist-promoted internalization of D_2-receptors is a well-established phenomenon that has been shown to occur in vitro (Ng et al., 1997; Ito et al., 1999). Consistent with this hypothesis, subcellular fractionation studies showed that amphetamine pretreatment induced a redistribution of D_2-receptors between different cell compartments. Amphetamine administration caused a significant and parallel decrease in both [^{3}H]raclopride and [^{3}H]spiperone bindings in the mitochondrial fraction, indicating a loss of cell-surface receptors. A differential effect of the treatment on [^{3}H]raclopride and[^{3}H]spiperone bindings was observed in the microsomal fraction. [^{3}H]Raclopride binding was unaffected in the microsomal fraction and closed to that measured in control rats. In contrast, [^{3}H]spiperone binding was increased in the microsomal fraction, and this increase paralleled the decrease in radioligand binding measured in the mitochondrial fraction. These results thus indicate that the amphetamine-induced reductions in [^{3}H]raclopride binding do not result from either a receptor degeneration or a competition with released DA but rather from a translocation of receptors from the cell surface to the endosomes. The extent of [^{3}H]spiperone binding transfer from the mitochondrial to the microsomal compartment was similar to the loss of [^{3}H]raclopride binding from the cell surface, suggesting that once translocated, D_2-receptors were not accessible to [^{3}H]raclopride but were still accessible to [^{3}H]spiperone.

A common explanation for this finding is that, because of their low lipophilicity, benzamide ligands such as [^{3}H]raclopride cannot penetrate cell membranes and only bind to cell-surface receptors, whereas butyrophenone ligands such as [^{3}H]spiperone, which are rather lipophilic, bind both to cell-surface and internalized receptors. Indeed, Barton et al. (1991) were the first to propose that agonist-mediated D_2-receptor internalization affects radioligand binding differently depending on lipophilicity. These authors showed that pre-exposure of retinoblastoma cells to DA resulted in decreased binding of the hydrophilic benzamide [^{3}H]iodosulpiride with no change in the binding of the lipophilic butyrophenone [^{3}H]NMSP. They suggested that [^{3}H]iodosulpiride detects only receptors present on the cell surface, whereas [^{3}H]NMSP detects both the receptors present on the cell surface and those sequestered. Since this pioneering study, several studies have shown that DA-induced internalization of D_2-receptors can be measured in vitro by measuring the loss of binding of [^{3}H]sulpiride from the cell surface (Itokawa et al., 1996; Ito et al., 1999). Evidence also exists that D_2-receptors internalization occurs in vivo. Chugani et al. (1988), using [^{3}H]spiperone, have demonstrated that agonist-mediated internalization and recycling of D_2-receptors occur in the rat striatum after amphetamine. Although our data confirm the transfer of [^{3}H]spiperone binding to intracellular compartment reported by these authors, our data did not support their finding of an increased number of [^{3}H]spiperone binding sites in amphetamine-treated rats. This may be because, in our experimental conditions, the receptor internalization process could have been stopped by tissue homogenization and that, consequently, no more [^{3}H]spiperone trapping into endosomes occurred.

In summary, our results demonstrate that the reduction in [^{3}H]raclopride binding observed in vivo after amphetamine is caused by an apparent decrease in D_2-receptor density with no change in affinity. Subcellular patterns of receptor distribution were consistent with an internalization mechanism occurring after amphetamine pretreatment and resulting in a selective accumulation of [^{3}H]spiperone in endosomes and a parallel loss of [^{3}H]raclopride and [^{3}H]spiperone bindings at the cell surface receptors. DA-promoted internalization of D_2-receptors, rather than competition with endogenous DA, thus seems to represent a reasonable explanation to the decreased [^{3}H]raclopride binding as well as to the differential outcome obtained with benzamide and butyrophenone radioligands after amphetamine. The so called "internalization model" can account for the two major problems of the competition model. First, by de-linking [^{3}H]raclopride binding decreases from on-line DA competition, it can explain the temporal persistence of the [^{3}H]raclopride effect that has been observed after amphetamine challenge. Indeed, as DA internalizes receptors, they become unavailable to the radiotracer, not because of competition, but because of shift in compartment. Thus, even if DA levels returned to baseline, [^{3}H]raclopride binding levels would not be able to return to baseline until the receptors were recycled to the cell surface. Thus receptor dynamics, not DA competition, might explain the prolonged decrease in [^{3}H]raclopride binding. Second, it can also explain why radioligands exhibiting different lipophilicities, and thereby exhibiting different capabilities to permeate membranes and access internalized receptors, could show different pattern of binding changes after amphetamine administration. Our study provides indirect proof for the internalization hypothesis. Direct studies aimed at visualizing the translocation of receptors [as have been done for the dopamine D_1 receptors (Dumartin et al., 1998)] are now indicated.

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