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Synaptic Concentration of Dopamine in Rat Striatal Slices in Relationship to [^{3}H]Raclopride Binding to the Dopamine D_2 Receptor

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The *in vivo* binding of dopamine (DA) radioligands to D_2 receptors can be affected by competition with endogenous dopamine. In the present study, we used a brain slice preparation that provides more controlled conditions than *in vivo* preparations in order to examine the relationship between synaptic DA and the binding of [^{3}H]raclopride to D_2 receptors. We also estimated the synaptic DA concentration in rat striatal slices by determining the changes in [^{3}H]raclopride binding. To correlate the changes in [^{3}H]raclopride binding with the concentration of synaptic DA, the kinetic parameters were determined. [^{3}H]Raclopride reached equilibrium binding conditions within two hours. The K_i value for DA in inhibiting [^{3}H]raclopride binding was about 2.2 nM. The increase in synaptic DA evoked by electrical stimulation decreased the striatal binding of [^{3}H]raclopride in a frequency-dependent manner. Increases in the DA concentration evoked by amphetamine (AMPH) or cocaine decreased [^{3}H]raclopride binding by 74% or 20%, respectively, corresponding to increases in the synaptic DA concentrations of 1.6 nM or 0.6 nM, respectively. Pargyline also decreased [^{3}H]raclopride binding by 36% corresponding at a concentration of 1.2 nM. In contrast, the depletion of synaptic DA by α-methyl-p-tyrosine (α-MpT) increased the specific binding of [^{3}H]raclopride by 43% when the DA concentration was decreased to 0.7 nM. The changes in the DA concentration at the synapse were responsible for the changes in the striatal binding of [^{3}H]raclopride. The values calculated in this study may therefore approximate the changes in the synaptic DA concentration in rat striatal slices following manipulation.

Key words: Dopamine, [^{3}H]Raclopride, Striatal slice, Electrical stimulation, Amphetamine, α-Methyl-p-tyrosine

INTRODUCTION

In vivo studies of positron emission tomography (PET) in primates and humans have shown that the binding of radioligands to the D_2 receptor can be affected by competition with endogenous dopamine (DA). The D_2 radioligands with relatively lower affinity, such as [^{11}C]raclopride, have been shown to be sensitive to changes in the synaptic DA concentration when treated with amphetamine (AMPH) or cocaine (Laruelle *et al.*,1995; Volkow *et al.*, 1999). On the other hand, a decrease in the synaptic DA concentration by the inhibition of DA synthesis with α-methyl-p-tyrosine (α-MpT) produced an increase in the binding of radioligands (Laruelle *et al.*, 1997). In rodent studies, it has been reported that the binding of [^{3}H] and [^{11}C] raclopride to D_2 receptors was influenced by changes in the synaptic DA concentration (Hume *et al.*, 1992; Young *et al.*, 1991).

Competition between endogenous DA and D_2 ligands can be used to estimate the synaptic DA concentration by measuring the changes in the *in vivo* binding of radioligands. However, the *in vivo* binding studies of [^{3}H]raclopride have shown relatively small effects. For example, PET studies in primates and humans have shown that treatment with AMPH decreases the binding of DA receptor radioligands by 10-30% (Carson *et al.*, 1997; Volkow *et al.*, 1994). However, in microdialysis studies equivalent doses of AMPH produced 10- to 20-fold increases in extracellular DA concentration (Butcher *et al.*, 1988; Laruelle *et al.*, 1997). In addition, the effect of endogenous DA on the *in vivo* binding of radioligands is

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complicated by factors including changes in the rate of radioligand delivery resulting from the effects of the pharmacological treatments on blood flow or on radioligand metabolism, and changes in the number of receptors (Logan *et al.*, 1994).

It is possible to examine the binding of radioligands to the receptors in the living cells of a brain slice without the influences that arise *in vivo*. In addition, the morphology and the synaptic connections between the neurons are similar to living brain tissue, whereas the receptors in the homogenate tissues used in *in vitro* binding studies may have different ionic and chemical environments than in the living tissues. In recent studies the factors that may influence the binding of radioligands to D_2 receptors have been examined using a superfused living brain slice preparation (Gifford *et al.*, 1998).

In the present study, we first examined the kinetic parameters of [^{3}H]raclopride binding in living rat striatal slices to obtain fundamental information on [^{3}H]raclopride binding, as well as to estimate the synaptic DA concentration. To our knowledge, no studies on the binding parameters of [^{3}H]raclopride using brain slice preparation have been performed before. The K_i value was used to estimate the synaptic DA concentration based on changes in the binding of [^{3}H]raclopride. Second, we measured the changes in [^{3}H]raclopride binding following manipulations such as electrical stimulation or treatment with pharmacological agents. Finally, we estimated the synaptic DA concentrations by calculating the relationship between the K_i value and the corresponding changes in [^{3}H]raclopride binding in rat striatal slices.

MATERIALS AND METHODS

Material

[^{3}H]Raclopride (76.3 Ci/mmol) was obtained from Dupont NEN (Boston, MA, USA). *d*-Amphetamine sulfate and α-methyl-*dl*-*p*-tyrosine methyl ester hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pargyline hydrochloride was obtained from Abbott Laboratories (North Chicago, IL, USA). Haloperidol was purchased from Research Biochemicals International (Natick, MA, USA). Cocaine hydrochlorides were obtained from the National Institute on Drug Abuse (Rockville, MD, USA). All of the other chemicals used were reagent grade. Male Sprague-Dawley rats were purchased from Taconic Farms, NY, USA. The animals were kept two to a cage under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C). Food and tap water were allowed *ad libitum*. USA Federal guidelines for the care and use of laboratory animals were strictly followed.

Preparation of brain slices

Male Sprague-Dawley rats (150-250 g) were sacrificed by decapitation.The brains were then immediately removed and immersed in ice-cold 0.9% saline solution. The striatum was dissected, glued to a plastic block and placed in ice-cold Krebs buffer (millimolar: NaCl 119.5, KCl 3.3, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, tyrosine 0.02, EDTA 0.03, pH 7.4), and saturated with 95% O_2 -5% CO_2. 300 μm slices were cut with a vibratome and incubated with oxygenated Kreb's buffer at 37°C for 90 minutes.

Electrically-evoked DA release

Striatal slices were subsequently transferred to ten superfusion chambers (Fig. 1). The slices were sandwiched between two wire mesh screens that were positioned midway between two platinum electrodes. The electrodes were connected to a stimulation apparatus that was able to stimulate each of the ten chambers at separate frequencies. The slices were superfused at 37°C with oxygenated Krebs buffer at a rate of 0.5 ml/min for approximately 30 min before the period of stimulation. The electrical stimulation consisted of unipolar, square-shaped pulses (25 mA, 2 msec) at frequencies of 0.5, 1, 2.5, 5, and 10 Hz. Five of the chambers were left unstimulated as controls, and to determine nonspecific binding. Only

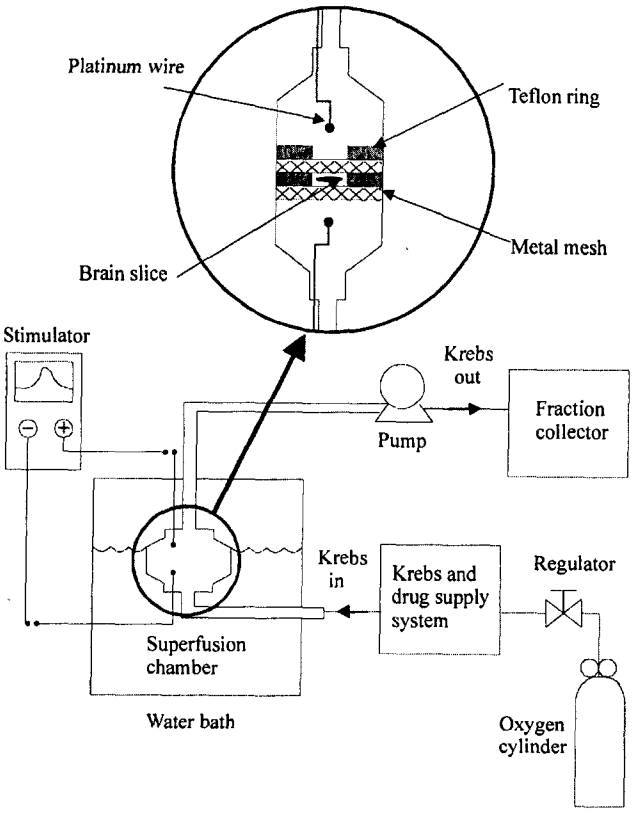

Fig. 1. Schematic diagram of the superfusion system.

one period of stimulation was applied per experiment. [^{3}H]Raclopride and α-MpT or cocaine was added to the superfusion medium simultaneously with the initiation of stimulation. Superfusion in the presence of the radioligand was continued for 30 min. A five-minute washout period followed, during which the slices were superfused in the absence of the radioligand to remove the unbound ligand. Electrical stimulation of the slices was continued during washout. The slices were thus exposed to electrical stimulation for a total of 35 min.

Pharmacologically-induced DA release

Striatal slices were prepared exactly as described above. The tissues were transferred to beakers containing 80 ml of oxygenated Krebs buffer along with [^{3}H]raclopride and various pharmacological agents (AMPH, cocaine, pargyline, or α-MpT). This mixture, including the drugs that were used to change the synaptic DA concentration, was incubated at 37 C while undergoing mild shaking for 30 min. At the end of the incubation, the slices were briefly washed in fresh Kreb's buffer before being transferred into scintillation vials containing 1 ml of 0.5 N NaOH to digest the tissues. After solubilization, a 50 μl aliquot was removed for the determination of protein using a Pierce BCA protein assay (Pierce, Rockford, IL). The remaining radioactivity in the scintillation vials was measured using a scintillation counter.

Data analysis

In order to correct for variations in each experiment, the radioactivity bound to the slices was divided by the total activity added to the incubation medium. It was then divided by the protein content of each slice, to correct for variations in slice size. Nonspecific binding was determined by incubating several of the slices in each experiment in the presence of 1 μM haloperidol. Data were normalized by expressing the specific binding as a percentage of the value in the control group in each experiment. Specific binding was obtained by subtracting the nonspecific binding from the total binding. IC_{50} and K_i values were determined by fitting a sigmoid curve to the data using the non-linear regression routine in Graphicpad Prism (San Deigo, CA). The Student's t-test for unpaired observation between the control and treated groups was carried out for the evaluation of the statistically significant differences. P values of 0.05 or less were considered as statistically significant.

Calculation of the synaptic DA concentration

Under equilibrium conditions the concentrations of specifically bound radioligand (B) can be determined from the Michaelis-Menten equation using an unbound radioligand (L):

$$B = \frac{LB_{max}}{K_d + L} \quad [1]$$

where B_{max} indicates the total receptor concentration for maximal binding and K_d is the equilibrium dissociation constant for the receptor

In the presence of a competitive inhibitor this equation [1] becomes:

$$B^* = \frac{LB_{max}}{K_d(1 + I/K_i) + L} \quad [2]$$

where the concentration of bound radioligand (B*) in the presence of an inhibitor is related to the concentration of the inhibitor (I) and the K_d of the inhibitor (K_i).

For the fraction of receptor occupied by the radioligand with or without the inhibitor these two equations can be combined to give the following relationship:

$$B/B^* = 1 + (I/K_i) \quad [3]$$

The concentration of an inhibitor (I) can thus be determined if the K_i value is known, and if the relative concentrations of specifically bound radioligands in the presence and absence of the inhibitor (B*/B) are also known.

RESULTS

The time course of [^{3}H]raclopride binding is shown in Fig. 2. The steady state level of [^{3}H]raclopride was reached

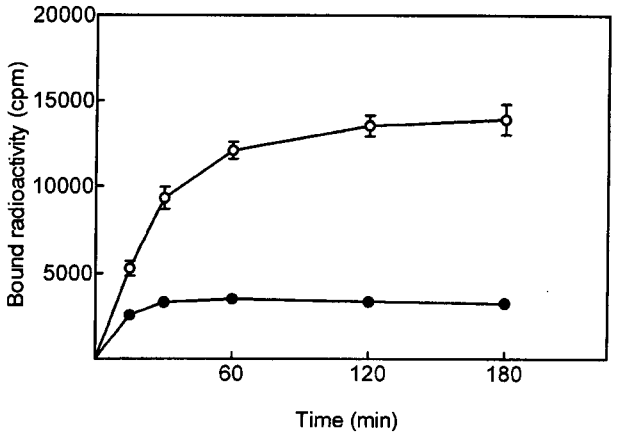

Fig. 2. Time course of the accumulation of [^{3}H]raclopride in rat striatal slices. Slices were incubated at 37°C in the presence of 0.3 nM [^{3}H]raclopride for increasing periods before being washed for 15 seconds. The Krebs buffer containing 0.3 nM [^{3}H]raclopride was changed every 30 min in order to minimize the accumulation of endogenous dopamine in the buffer. The nonspecific binding was determined by the addition of 1 mM haloperidol. Values represent the cpm of radioactivity per milligram of protein. (– ○ –: Total binding, – ● –: Nonspecific binding).

approximately two hours after the start of the incubation. [^{3}H]Raclopride was bound in a saturable manner to the slices of rat striatum, while the nonspecific binding, which was determined using 1 μM haloperidol, increased linearly over the entire range of concentrations tested (Fig. 3). Scatchard analysis indicated the presence of a single binding site with a K_d of 1.5 nM and a B_{max} of 0.64 pmoles/g protein. The apparent K_i value determined from the displacement of [^{3}H]raclopride by unlabeled raclopride in the rat striatal slices is shown in Fig. 4. The K_i value, which was determined using unlabeled raclopride, was 2.2 nM.

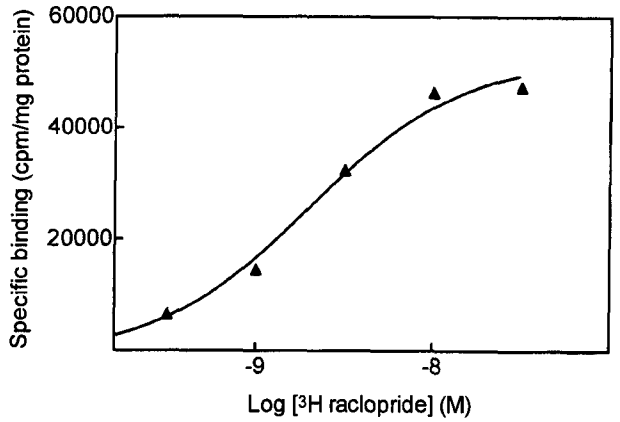

Fig. 3. Saturation of [^{3}H]raclopride binding in rat striatal slices. Slices were incubated at 37°C for two hours in increasing concentrations of [^{3}H]raclopride from 0.3 nM to 30 nM. Kreb's buffer. Fresh Kreb's buffer was used after one hour of incubation in order to minimize the accumulation of endogenous dopamine in the buffer. A K_d value of 1.5 nM and a B_{max} value of 0.64 pmoles/g protein were obtained by Scatchard analysis. Values represent the cpm of radioactivity per milligram of protein.

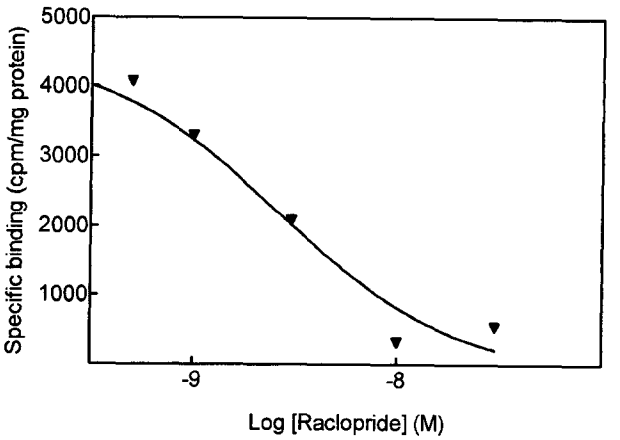

Fig. 4. Competition of [^{3}H]raclopride binding by unlabeled raclopride in rat striatal slices. Slices were incubated at 37°C for two hours in the presence of 0.3 nM [^{3}H]raclopride following increases in the concentrations of unlabeled raclopride from 0.3 nM to 30 nM. Kreb's buffer containing each concentration of unlabeled raclopride was switched with fresh Krebs buffer after one hour of incubation in order to minimize the accumulation of endogenous dopamine in the buffer. A K_i value of 2.2 nM was obtained by fitting a sigmoid curve to the data using non-linear regression. Values represent the cpm of radioactivity per milligram of protein.

As a result of the increases in the synaptic DA evoked by electrical stimulation (0.5 Hz-10 Hz), the specific binding of [^{3}H]raclopride in the striatal slices decreased in a frequency-dependent manner. Electrical stimulation above 2.5 Hz completely prevented [^{3}H]raclopride from binding to the D_2 receptors. This corresponded to an increase in synaptic DA concentration of approximately 26.0 nM (Fig. 5).

Changes in the binding of [^{3}H]raclopride in the striatal slices were examined following treatment with various pharmacological agents (Table I). The synaptic DA released by AMPH decreased the specific binding of [^{3}H]raclopride by 59%, corresponding to an increase in the synaptic DA concentration of 1.6 nM. A decrease in the [^{3}H]raclopride

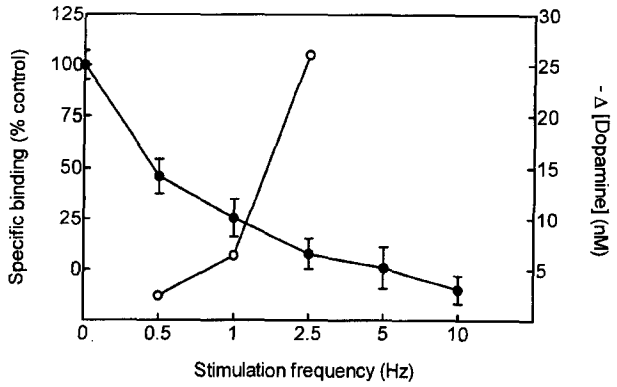

Fig. 5. Inhibition of [^{3}H]raclopride binding in rat striatal slices by electrical stimulation. Slices were superfused at 37°C with Kreb's buffer for 60 min before initiating electrical stimulation. Superfusion in the presence of 0.3 nM [^{3}H]raclopride was continued for 30 min with electrical stimulation (0.5 Hz to 10 Hz). Values represent the means ± SEM of ten experiments. (- ● -: Specific binding, - ○ -:Δ[Dopamine]).

Table I. Effects of amphetamine, cocaine, pargyline and α-methyl-p-tyrosine on [3H]raclopride binding and on changes in the synaptic dopamine concentration in rat striatal slices

Drugs	Dose (mM)	[^{3}H]Raclopride binding[a] (% of control)	-Δ [Dopamine] (nM)
Control		100	
Amphetamine	3	58.5 ± 3.9**	1.6
Cocaine	3	79.7 ± 7.2*	0.6
Pargyline	20	64.4 ± 5.1**	1.2
a-Methyl-p-tyrosine	100	143.3 ± 12.4*	-0.7

Slices were incubated at 37°C for 30 min in the presence of 0.3 nM [^{3}H]raclopride including each drug at the concentrations shown above.

[a]Values represent the means ± SEM of nine to twelve slices. *p< 0.05, **p< 0.01

binding was observed using a DA uptake blocker cocaine. This corresponded to an increase in the DA concentration of 0.6 nM. Pargyline inhibiting monoamine oxidase decreased the [^{3}H]raclopride binding by 36%, corresponding to an increase in synaptic DA concentration of 1.2 nM. In contrast, inhibiting the DA synthesis by α-MpT increased [^{3}H]raclopride binding by 43% according to a decrease at a synaptic DA concentration of 0.7 nM.

DISCUSSION

In vivo studies have demonstrated that the [^{11}C]raclopride binding to D_2 DA receptors is sensitive to synaptic DA concentration (Dewey *et al.*, 1993; Laruelle *et al.*,1995; Volkow *et al.*,1999). In the present study, we used a brain slice preparation, which provides a more controlled environment than *in vivo*, to examine the competition between endogenous DA and [^{3}H]raclopride for binding to D_2 receptors. We also estimated the synaptic DA concentration based on the changes in the binding of [^{3}H]raclopride in rat striatal slices. To our knowledge, no studies on the kinetic parameters of [^{3}H]raclopride binding in a brain slice preparation have previously been performed. In the present study the kinetic parameters of [^{3}H]raclopride binding in rat striatal slices were examined for fundamental information, and were also used as the basis of a calculation to estimate the synaptic DA concentration from changes in the binding of [^{3}H]raclopride. Raclopride has been found to have a relatively low affinity for D_2 DA receptors compared to some other D_2 receptor radioligands *in vivo*. Thus, its equilibrium binding conditions are reached relatively quickly (Farde *et al.*, 1989).

To ensure an equilibrium binding conditions, [^{3}H]raclopride was incubated in the presence of 0.3 nM raclopride using striatal slice preparation. Under these conditions, the [^{3}H]raclopride binding in brain slices reached an equilibrium state within two hours, whereas in homogenate tissue a steady state was reached approximately 40 min after the start of incubation (Hall *et al.*, 1988; Köhler *et al.*, 1985). When converting the changes in [^{3}H]raclopride binding into the DA concentration, this finding supports the assumption-that equilibrium binding conditions are obtained, and the interaction between DA and radioligand binding is competitive, with no change in receptor density. Gifford *et al.*, (1998) has shown that a D_1 receptor radioligand [^{3}H]SCH 23390 failed to reach equilibrium binding conditions in striatal slices, while in homogenate tissue [^{3}H]SCH 23390 reached a steady state in six minutes. The difference between the two preparations is that the receptors located in the brain slices can be exposed to the normal cytoplasmic fluids in their intracellular spaces, whereas the receptors in the homogenate tissues can be exposed to the binding buffer. It also appears that the radioligands may repeat the binding to DA receptors because of their slow diffusion. The specific binding of [^{3}H]raclopride to D_2 receptors in striatal slices followed saturation kinetics. The B_{max} and K_d values calculated from the Scatchard plot were 0.64 pmoles/g protein and 1.5 nM, respectively. These values are relatively lower than those from an *in vivo* binding study (Ross and Jackson, 1989). The K_i value was also found to be substantially lower than the K_i value obtained from homogenate tissues (Ross, 1991), which has been shown to vary widely (Seeman *et al.*, 1989).

In the present study, both electrical stimulation and pharmacological agents were employed to induce changes in the accumulation of [^{3}H]raclopride in the brain slices. This means that the effects of competition with endogenous DA on [^{3}H]raclopride binding can be demonstrated in an *in vitro* brain slice preparation following controlled manipulations. A decrease in [^{3}H]raclopride binding in the striatal slices was observed by electrical stimulation in a frequency-dependent manner. [^{3}H]Raclopride binding above 90% was reduced by electrical stimulation at 2.5 Hz, and corresponded to a decrease in the synaptic DA concentration of 26 nM. However, this is not consistent with the results of a microdialysis study, in which electrical stimulation at 3 Hz increased the synaptic DA concentration in rat striatal slices approximately threefold (Herdon and Nahorski, 1987). The microdialysis technique is an alternative approach to measure the basal level of DA in the extracellular space. Extracellular DA concentration is measured over a relatively large volume of tissue surrounding the microdialysis probe. Therefore, this technique may show a higher than actual concentration of DA than measured in the present study. In rat striatal slices [^{3}H]raclopride binding was significantly decreased by AMPH at the lower concentration. This suggests that the change in [^{3}H]raclopride binding, at least at the lower concentration of AMPH, is mediated through released DA rather than by direct competition with AMPH. Parker and Cubeddu (1986) observed that the potency of AMPH in inhibiting [3H]raclopride binding in brain slices was consistent with its potency in releasing DA from brain slices. Similar results have also been demonstrated in *in vivo* experiments measuring the binding of several radioligands (Carson *et al.*, 1997; Mukherjee *et al.*, 1997). However, our data showed that the potency of AMPH in the brain slices was greater than its potency in inhibiting [^{3}H]raclopride binding in homogenate tissue. Cocaine, known to be a DA uptake blocker, was used to increase the synaptic DA concentration (Ritz *et al.*, 1987). Cocaine and methylphenidate significantly reduced [^{11}C]raclopride binding in an *in vivo* study (Dewey *et al.*, 1993; Volkow *et al.* 1994). On the other hand, the present data showed that cocaine decreased [^{3}H]raclopride binding slightly. The lack of any effect on the brain slice by cocaine may reflect the fact that the DA terminals in the brain slice are electrically silent, whereas *in vivo* DA neurons are tonically active. This may be involved in the basal levels of

DA release, which is less in the slices than in *in vivo*. Thus, a DA uptake blocker such as cocaine may not be able to increase the extracellular DA to the level where radioligand binding would be significantly reduced. Pargyline, an inhibitor of monoamine oxidase, produced significant increases in the efflux of endogenous DA and DA content (Parker and Cubeddu, 1986). In agreement with this finding, our data showed that pargyline decreased [^{3}H]raclopride binding, corresponding to an increase in the synaptic DA concentration of 1.2 nM in rat striatal slices. In contrast, the depletion of synaptic DA following α-MpT administration significantly increased the specific binding of [^{3}H]raclopride by 43%, corresponding to a decrease in the synaptic DA concentration of 0.7 nM. α-MpT has been known to be a specific competitive inhibitor of tyrosine hydroxylase, an enzyme essential in DA synthesis (Parker and Cubeddu, 1986). Therefore, it was used to determine if the DA released through increasing DA synthesis was involved in the changes in [^{3}H]raclopride binding. The determination of normal synaptic DA concentration by examining the effect of DA depletion on *in vivo* radioligand binding has been previously employed by Ross (1991).

In conclusion, the results obtained in the present study demonstrate that the relationship between synaptic DA concentration and [^{3}H]raclopride binding which was found in *in vivo* experiments was also observed in *in vitro* rat striatal slice preparation. Brain slice preparation represents a more controlled environment than *in vivo* experiments, and simplifies the *in vivo* situation without the complications resulting from metabolism and changes in radioligand delivery depending on the blood flow. Synaptic DA concentration can also be estimated in rat striatal slice preparation by measuring the changes in [^{3}H]raclopride binding.

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