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Determination of dopamine D_4 receptor density in rat striatum using PB12 as a probe

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Abstract

N-[2-[4-(4-Chlorophenyl)piperazin-1-yl]ethyl]-3-methoxybenzamide (PB12), a potent and selective dopamine D_4 receptor ligand, was used as a probe for the direct determination of the dopamine D_4 receptor density in rat striatum as an alternative to the subtraction method. The experiment was performed using [^{3}H]spiroperidol to label D_2, D_3 and D_4 receptors and PB12 to determine directly dopamine D_4 receptor specific binding. The determined B_{max} value was 82 fmol/mg protein. The contribution of the dopamine D_4 receptor to the overall population of D_2-like receptors was 63%; however, this value cannot be considered reliable because of the observed difference in the kinetic profiles of D_2, D_3 and D_4 receptors.

Keywords: Dopamine D_4 receptor; Striatum, rat

1. Introduction

In the early 1990s, five dopamine receptor subtypes were identified and classified in D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, D_4) receptor families (Baldessarini and Tarazi, 1996) on the basis of their molecular and neuropharmacological similarities.

The mRNA for the dopamine D_4 receptor was localized mainly in limbic and cortical areas rather than in the nigrostriatal pathway (O'Malley et al., 1992; Meador-Woodruff et al., 1994). This evidence led to the hypothesis that dopamine D_4 receptors might be involved in the pathophysiology of psychoses, and therefore, that they constitute a potential target for antipsychotic drugs. Furthermore, it has been hypothesized that the atypical profile of the antipsychotic clozapine (Van Tol et al., 1991, 1992) depends on its tenfold higher affinity for dopamine D_4 receptor than for dopamine D_2 receptor (Seeman et al., 1997).

The absence of highly selective dopamine D_4 receptor ligands hampers the localization and determination of D_4 receptor proteins in brain tissue, and therefore, characterization of their action. Two different methodologies have been used to localize and measure dopamine D_4 receptor protein: immunocytochemistry and tritiated ligand binding (Kula et al., 1999).

Many indirect labelled ligand binding approaches have been used. For example, the subtraction of D_2 and D_3 receptors labelled with [^{3}H]raclopride from D_2-like total binding obtained with [^{3}H]nemonapride, a potent but non-selective dopamine D_2-like receptor ligand (Seeman et al., 1993a,b; Sumiyoschi et al., 1995). An alternative binding method uses cold raclopride to mask D_2 and D_3 receptors in the [^{3}H]nemonapride binding assay: the experimental results, according to autoradiographic studies, revealed the localization of dopamine D_4 receptors in the hippocampus, frontal and enthorinal cortex and nucleus accumbens, both in rat and in human brains (Tarazi et al., 1997). These observations were confirmed with an autoradiographic method performed with [^{3}H]NGD 94-1, (2-phenyl-4(5)-[4-(2-pyrimidinyl)-piperazin-1-yl)methyl]imidazole dimaleate), a selective dopamine D_4 ligand (Primus et al., 1997).

However, the subtraction methodology in human and rat brains showed a wide variety of D_4 dopamine receptor levels because this analysis can be reliable only with selective radioligands for dopamine D_2-like receptors. In fact, [^{3}H]nemonapride binds to other non-dopamine receptors such as sigma and 5-HT receptors (Tang et al., 1997).

At the moment, the experimental method to estimate the density of dopamine D_4 receptors consists of displacing

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[^{3}H]nemonapride from D_2 and D_3 receptors with cold raclopride. Nevertheless, in this way, the calculated abundance of dopamine D_4 receptors is overestimated because raclopride is unable to displace [^{3}H]nemonapride from non-dopamine D_2-like receptors (Defagot et al., 2000).

In spite of all the work done over the last years, the quantification of dopamine D_4 receptors with the subtraction method has yielded controversial results both in rats and in humans (Reynolds and Mason, 1995; Murray et al., 1995; Sumiyoschi et al., 1995; Defagot and Antonelli, 1997).

Our research group has recently published the binding profile of *N*-[2-[4-(4-chlorophenyl)piperazin-1-yl]ethyl]-3-methoxybenzamide (PB12), a potent and selective dopamine D_4 receptor ligand (Perrone et al., 1998).

As an alternative to the subtraction method, we used PB12 as a probe for the direct measurement of dopamine D_4 receptor specific binding in the rat striatum. Both in rat and in human striatum, high D_2-like receptor density was detected and the contribution of the dopamine D_4 receptor was found to be 10% (Seeman et al., 1993a).

Our experiment was performed using [^{3}H]spiroperidol to label the D_2, D_3 and D_4 receptors, and PB12 to determine directly dopamine D_4 receptor specific binding.

2. Materials and method

[^{3}H]Spiroperidol was purchased from New England Nuclear; haloperidol was purchased from Sigma RBI; and PB12 was synthesized in our laboratory as described (Perrone et al., 1998).

Adult male Wistar Hannover rats (200–300 g; Harlan-Italy) were anesthetized and then decapitated. Brains were quickly removed, and striatum membranes were stored at −80 °C for no more than 48 h.

2.1. Preparation of rat striatum membranes

Corpora striata (1.0 g) was homogenized in 50 volumes of Tris–HCl buffer (50 mM, pH 7.4) with a Brinkmann Polytron (setting 5 for 15 s); the homogenate was then centrifuged at 50,000 × *g* for 10 min. The supernatant was discarded and the pellet was washed once (Creese and Snyder, 1978). Protein concentration was determined by the method of Lowry et al. (1951).

2.2. Rat striatum D_2-like saturation binding assay

Saturation binding experiments were performed according to Creese and Snyder (1978) with minor modifications. Each tube received in a final volume of 3 ml of 50 mM Tris–HCl (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid, pH 7.4) 500 μg of rat striatum membranes and [^{3}H]spiroperidol over a concentration range of 0.03–1.00 nM. Non-specific binding was determined in the presence of 10 μM haloperidol. Samples were incubated at 37 °C for 20 min and then filtered on Whatman GF/B glass microfiber filters. Then the filters were washed with 50 mM Tris–HCl pH 7.4 (2 × 3 ml). After addition of scintillation cocktail, samples were allowed to equilibrate for at least 6 h. The amount of bound radioactivity was determined by liquid scintillation spectrometry using a Beckman LS 6500 scintillation counter with an approximately 60% efficiency for tritium.

2.3. Rat striatum D_4 saturation binding assay

Saturation binding experiments were performed according to Creese and Snyder (1978). Each tube received in a final volume of 3 ml of 50 mM Tris · HCl (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid, pH 7.4) 500 μg of rat striatum membranes and [^{3}H]spiroperidol over a concentration range of 0.03–1.00 nM. Non-specific total binding was determined in the presence of 10 μM haloperidol, and D_4 specific binding was determined in the presence of 10 μM PB12. Samples were incubated at 37 °C for 20 min and then filtered on Whatman GF/B glass microfiber filters. Then the filters were washed with 50 mM Tris–HCl pH 7.4 (2 × 3 ml). The amount of bound radioactivity was determined as described above.

2.4. D_2-like association experiments

The association experiment was initiated by addition of rat striatum membranes in a sample containing incubation buffer (50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid, pH 7.4), and 0.2 nM [^{3}H]spiroperidol in a total volume of 3 ml. The incubation was performed at 37 °C for various lengths of time before filtration. Samples were filtered and washed as described above. The specific binding was determined with 10 μM haloperidol.

2.5. Dissociation experiments

A sample containing 0.2 nM [^{3}H]spiroperidol and 500 μg of rat striatum membranes was equilibrated in incuba-

Table 1
Receptor affinity binding data (IC_{50}, nM)[a]

$D_{4.4}$	D_{2L}	D_3	D_1	D_5	α_1	5-HT_{1A}	σ_1	σ_2
0.057	3800	> 1000	> 1000	> 1000	270	220	> 1000	> 1000

[a] For each receptor the following radioligand and tissue were used. $D_{4.4}$: [^{3}H]nemonapride; human cloned in CHO cells; D_{2L}: [^{3}H]spiroperidol; human cloned in CHO cells; D_3: [^{3}H]spiroperidol; rat cloned in Sf9 cells; D_1: [^{3}H]SCH-23390; human cloned in Sf9 cells. D_5: [^{3}H]SCH-23390; human cloned in Sf9 cells. α_1: [^{3}H]prazosin, rat cortex membranes; 5-HT_{1A}: [^{3}H]-8-OH-DPAT; hippocampus rat membranes; σ_1: [^{3}H]-(+)-pentazocine, guinea-pig brain membranes; σ_2: [^{3}H]DTG, rat liver membranes.

Table 2
Kinetics and Scatchard binding data of [^{3}H]spiroperidol in rat striatum

Receptor	K_{obs} (min^{-1})	K_{on} (min^{-1} nM^{-1})	K_{off} (min^{-1})	K_d (K_{off}/K_{on}) (nM)	K_d Scatchard (nM)	B_{max} (fmol/mg)
D_2-like	0.140	0.317	0.07660	0.24	0.13	130
D_4	0.180	0.719	0.03616	0.050	0.081	82

tion buffer (50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid, pH 7.4) for 20 min at 37 °C. At equilibrium, an excess of haloperidol (10 μM) was added in a total volume of 3 ml. The incubation was performed at 37 °C for various lengths of time before filtration. Samples were filtered and washed as described above.

2.6. Data analysis

Scatchard parameters (K_d and B_{max}), inhibition constants (IC_{50} value) and kinetic constants (K_{obs}, K_{on} and K_{off}) were calculated using GraphPad Prism® software. The K_{on} was calculated with the formula $K_{on} = K_{obs} - K_{off}/[Rx]$.

3. Results

3.1. Competitive binding data

An extended binding profile of PB12 is reported in Table 1. Experimental conditions have been already reported (Perrone et al., 1998).

3.2. Saturation studies

Equilibrium dissociation constants (K_d) and maximal density of receptors (B_{max}) were obtained from Scatchard analysis (Table 2) with a non-linear regression least-squares curve-fitting program. The saturation experiment with rat striatum D_2-like receptors was performed using [^{3}H]spiroperidol in the range 0.03–1.00 nM and 10-μM haloperidol to determine non-specific binding (Fig. 1). The K_d value was 0.13 ± 0.08 nM and B_{max} was 130 ± 12 fmol/mg protein. A one-binding site model was the best fit for the experimental data. The saturation experiment with rat striatum dopamine D_4 receptors was performed using [^{3}H]spiroperidol in the range 0.03–1.00 nM and 10 μM PB12 to determine specific binding (Fig. 2). The K_d value was 0.081 ± 0.007 nM and B_{max} was 82 ± 7 fmol/mg protein.

3.3. Kinetic studies

Kinetic data for rat striatum dopamine D_4 receptors (Table 2) revealed that the association rate was 20-fold greater than the dissociation rate for [^{3}H]spiroperidol (Fig. 3). The concentration of [^{3}H]spiroperidol used was 0.20

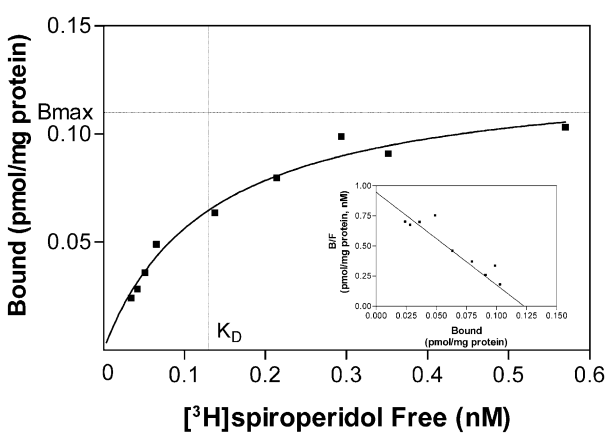

Fig. 1. Representative saturation curve for [^{3}H]spiroperidol binding to D_2-like receptors in membranes prepared from rat striatum corpora. Each concentration was tested in triplicate with six to nine concentrations of [^{3}H]spiroperidol (range: 0.03–1.00 nM). The average K_d and B_{max} values, as determined by computer analysis of the saturation isotherm data for three independent experiments, were 0.13 ± 0.08 nM and 130 ± 12 fmol/mg protein, respectively. The inset shows the corresponding Scatchard plot of the data.

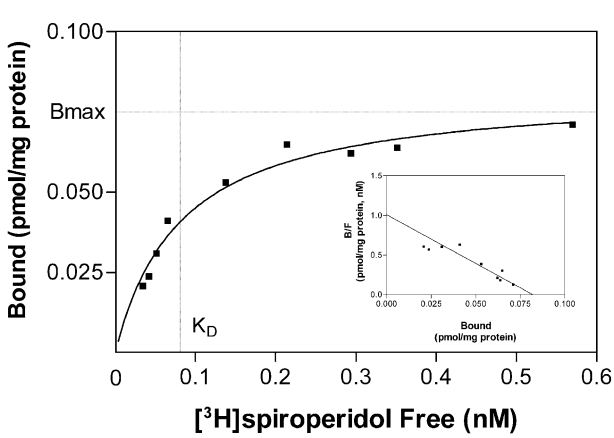

Fig. 2. Representative saturation curve for [^{3}H]spiroperidol binding to D_4 receptors in membranes prepared from rat striatum corpora. Each concentration was tested in triplicate with six to nine concentrations of [^{3}H]spiroperidol (range: 0.03–1.00 nM). The average K_d and B_{max} values, as determined by computer analysis of the saturation isotherm data for three independent experiments, were 0.081 ± 0.007 nM and 82 ± 7 fmol/mg protein, respectively. The inset shows the corresponding Scatchard plot of the data.

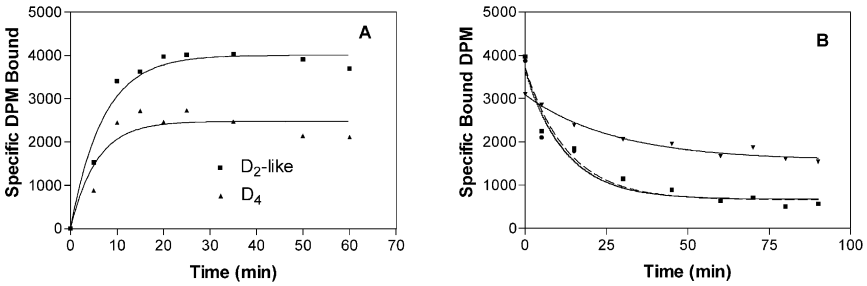

Fig. 3. Kinetics of [^{3}H]spiroperidol binding to membranes of rat striatum. (A) Association experiments. [^{3}H]spiroperidol (0.2 nM) was coincubated with rat striatum membranes and specific binding was measured for 60 min. (B) Dissociation experiments: specific binding was measured for 90 min (nine points for each curve). ■ An excess of haloperidol (10 μM) was added at equilibrium. ▲ An excess of PB12 (10 μM) was added at equilibrium. ● An excess of haloperidol (10 μM) and of PB12 (10 μM) was added at equilibrium (dotted line).

nM: 8% of the total concentration was bound at steady-state after addition. Therefore, pseudo-first order kinetics can be applied. The K_{obs} was 0.18 ± 0.027 min^{-1}, corresponding to a calculated K_{on} of 0.719 ± 0.012 min^{-1} nM^{-1}. The K_{off} was 0.0362 min^{-1} and the $t_{1/2}$ for dissociation of binding was 19.2 ± 3.1 min. A $K_d = 0.050$ nM calculated from kinetic rate constants was in agreement with the K_d value of 0.081 nM determined in the saturation experiment. The K_{obs} of D_2-like receptors in rat striatum was 0.140 min^{-1}, and the K_{on} was 0.317 min^{-1} nM^{-1} (Fig. 3). The K_{off} was 0.0766 ± 0.0015 min^{-1}, and the $t_{1/2}$ for dissociation of binding was 9.0 ± 4.0 min. A $K_d = 0.24$ nM, calculated from the kinetic rate constants, was in agreement with the K_d value of 0.13 nM determined in the saturation experiment. The concentration of [^{3}H]spiroperidol used was 0.20 nM.

In order to check that the addition of 10 μM PB12 did not produce more displacement when added with 10 μM haloperidol, we measured under these conditions the K_{off} and the $t_{1/2}$ for dissociation (Fig. 3B). The K_{off} was 0.08135 ± 0.0010 min^{-1}, and the $t_{1/2}$ for dissociation of binding was 8.5 ± 3.0 min. The K_{off} and the $t_{1/2}$ for dissociation showed that there were no differences between binding measured in the presence of haloperidol and PB12, and binding measured in the presence of haloperidol alone ($K_{off} = 0.08135$ min^{-1} and $t_{1/2} = 8.5$ min compared to $K_{off} = 0.0766$ min^{-1} and $t_{1/2} = 9.0$ min, respectively).

Association and dissociation data were modeled to both one- and two-site fits; no statistical improvement was observed with the two-site fit. Additional lack of evidence for two different sites was revealed by the linear Scatchard plot.

4. Discussion

The quantitative determination of dopamine D_4 receptors in rat striatum by means of radioligand binding assay requires the availability of a potent and selective dopamine D_4 receptor ligand, such as PB12.

The B_{max} results from Scatchard plots revealed a value of 130 fmol/mg protein for total D_2-like receptors and a value of 87 fmol/mg protein for dopamine D_4 receptors. Therefore, dopamine D_4 receptors represent 63% of total D_2-like receptors in rat brain. However, some differences can be observed between the Scatchard data and the kinetic results. Indeed, the D_2-like/D_4 K_d ratio was 1.6 in the Scatchard analysis, whereas, a ratio of 4.8 was determined by kinetic experiments. This inconsistency can be explained by the observation that the D_2-like K_{off} value was twofold greater than the D_4 K_{off} value, whereas, the D_2-like K_{obs} and D_4 K_{obs} had similar values.

The dissociation rate of [^{3}H]spiroperidol from D_2-like receptors can be estimated as the mean of D_2, D_3 and D_4 dissociation rates. The high value resulting from the D_2-like K_{off}/D_4 K_{off} ratio yielded an incorrect determination of the B_{max} value. Since [^{3}H]spiroperidol dissociates quickly from the receptor, the equilibrium is reached faster for D_2-like receptors than for dopamine D_4 receptors but with less specific binding. Therefore, the B_{max} value of 130 fmol/mg protein must be considered an apparent B_{max} value, and is underestimated. These considerations led us to believe that the 63% contribution of the dopamine D_4 receptor to the overall population of D_2-like receptors in rat striatum is an overestimate. In conclusion, PB12 allowed the determination of the D_4 B_{max} value (82 fmol/mg protein) in rat striatum with a direct approach which seems to be more accurate than a subtraction method. However, the value cannot be considered as reliable because of a difference in the kinetic profiles of D_2, D_3 and D_4 receptors. Therefore, the determination of dopamine D_4 receptors requires not only a potent and selective dopamine D_4 receptor ligand, but also knowledge of the B_{max} values for D_2 and D_3 receptors, calculated throughout a direct approach.

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