ORIGINAL ARTICLE

Presence of D4 dopamine receptors in human prefrontal cortex: a postmortem study

Presença de receptores dopaminérgicos D4 em córtex cerebral humano: um estudo post-mortem

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Abstract

Objective: The aim of our study was to explore the presence and the distribution of D4 dopamine receptors in postmortem human prefrontal cortex, by means of the binding of [^{3}H]YM-09151-2, an antagonist that has equal affinity for D2, D3 and D4 receptors. It was therefore necessary to devise a unique assay method in order to distinguish and detect the D4 component. Method: Frontal cortex samples were harvested postmortem, during autopsy sessions, from 5 subjects. In the first assay, tissue homogenates were incubated with increasing concentrations of [^{3}H]YM-09151-2, whereas L-745870, which has a high affinity for D4 and a low affinity for D2/D3 receptors, was used as the displacer. In the second assay, raclopride, which has a high affinity for D2/D3 receptors and a low affinity for D4 receptors, was used to block D2/D3. The L-745870 (500 nM) was added to both assays in order to determine the nonspecific binding. Results: Our experiments revealed the presence of specific and saturable binding of [^{3}H]YM-09151-2. The blockade of D2 and D3 receptors with raclopride ensured that the D4 receptors were labeled. The mean maximum binding capacity was 88 ± 25 fmol/mg protein, and the dissociation constant was 0.8 ± 0.4 nM. Discussion and conclusions: Our findings, although not conclusive, suggest that the density of D4 receptors is low in the human prefrontal cortex.

Descriptors: Dopamine; Receptors, dopamine; Receptors, dopamine D4; Brain; Prefrontal cortex

Resumo

Objetivo: O objetivo deste estudo foi quantificar a presença e a distribuição de receptores dopaminérgicos do tipo 4 (D4) no córtex cerebral humano em amostras post-mortem através do bloqueio com ^{3}H-YM-09151-2 – um antagonista com afinidade equivalente pelos receptores D2, D3 e D4 – e do desenvolvimento de um método para a detecção específica do componente D4. Método: Foram obtidas amostras de córtex cerebral de cinco cadáveres. Em um primeiro ensaio, os homogeneizados de tecido cerebral foram incubados em concentrações crescentes de ^{3}H-YM-09151-2, enquanto que o L-745,870, ligante que apresenta grande afinidade pelo receptor D4 e baixa afinidade por D2 e D3, foi utilizado como controle. Em um segundo ensaio, a racloprida, que apresenta alta afinidade por receptores D2 e D3, mas baixa afinidade por D4, foi usada para bloquear D2 e D3. O L-745,870 foi adicionado em ambos os ensaios para determinar o bloqueio não específico. Resultados: Os resultados do experimento demonstraram a presença de um bloqueio específico e saturável com ^{3}H-YM-09151-2. O bloqueio de receptores D2 e D3 com racloprida confirmou que apenas os receptores D4 livres foram avaliados. A Bmax (média ± DP) foi de 88 ± 25 fmol/mg de proteínas, enquanto que a Kd (média ± DP) foi de 0,8 ± 0,4 nM. Discussão e conclusões: Tais achados, ainda que não definitivamente conclusivos, sugerem a presença de uma baixa densidade de receptores D4 no córtex pré-frontal humano.

Descritores: Dopamina; Receptores dopaminérgicos; Receptores dopaminérgicos do tipo D4; Cérebro; Córtex pré-frontal

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Introduction

The synthesis of the so-called 'atypical' antipsychotics, in particular that of clozapine, represented a fundamental advance in the management of psychotic disorders and promoted new research strategies to explore their pathophysiology.[1] All atypical antipsychotics are characterized by the blockade of dopamine receptors, especially in the mesolimbic and mesocortical pathways, coupled with specific antagonism of serotonin type 2 (5-HT2) receptors and diverse effects on various other receptors.[2-5]

Dopamine receptors are currently classified in five subtypes that are grouped in two main families: the D1 family, which includes the D1 and D5 receptors; and the D2 family, which includes the D2, D3 and D4 receptors.[6] Clozapine has a 20-times higher binding affinity for D4 receptors than for the other dopamine receptors. Therefore, this receptor subtype seems to be relevant to the mechanism of action of clozapine and, perhaps to a larger extent, to that of all atypical antipsychotics.[2,7] The D4 receptors, like all of those belonging to the D2 family, have a high affinity for dopamine, as well as for adrenaline and noradrenaline, and inhibit adenylate-cyclase activity. Since there is no selective ligand capable of labeling D4 receptors, there is little information available on their distribution in the human brain. Although the different techniques employed, such as the methods used for binding subtraction, autoradiography and determination of mRNA expression, make it difficult to draw direct comparisons among studies, D4 receptors seem to be mainly distributed in the frontal cortex, limbic areas (such as the amygdala and hippocampus) and the mesencephalon, as well as (at a very low density) in the basal ganglia and olfactory tubercle. Nevertheless, the distribution of D4 receptors appears to be meager and quite unique in comparison with that of the other dopamine receptors, which are mainly found in striatal areas.[8-10]

The aim of our study was, therefore, to explore the presence and distribution of D4 receptor in postmortem human prefrontal cortex by means of the direct binding of [^{3}H]YM-09151-2. Since this radioligand is an antagonist that has equal affinity for D2, D3 and D4 receptors, it was therefore necessary to devise a specific experimental assay in order to selectively distinguish and detect the D4 component. The prefrontal cortex was chosen because it has been reported to be involved in the so called 'negative symptoms' of psychoses, for which clozapine is particularly effective.

Method

1. Subjects

Prefrontal cortex samples were taken postmortem, during autopsy sessions, from five subjects (three male and two female; mean age at time of death: 62 ± 4 years). The samples were immediately packed in dry ice and stored in a -80° freezer. Autolysis time (i.e., the time between death and the freezing of the samples) ranged from 7 h to 25 h. All subjects had died from causes not primarily involving the brain (2 from myocardial infarction, 2 from pulmonary embolism and 1 from respiratory failure) and had not suffered from any psychiatric or neurological disorders. Nor had any received psychotropic drugs, according to their medical charts. The study was approved by the Ethics Committee of the University of Pisa, in Pisa, Italy.

2. Preparation of prefrontal cortex homogenate

The prefrontal cortex were defrosted and separated from the white matter. In order to achieve the original 4 mg/ml wet weight, the tissue was re-suspended in D4 buffer (120 mM NaCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, EDTA 1 mM, pH 7.4) according to the method described by Seeman et al.[11-12] The tissue was then homogenized in an Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany) in D4 buffer. The homogenate was not washed and centrifuged, since these procedures can result in the loss of 15 to 60% of the receptors.

3. Binding of [^{3}H]YM-09151-2

The radioligand employed has an affinity for D2, D3 and D4 receptors. Therefore, in order to determine the effective D4 component labeling for each sample, two separate binding assays were performed, each involving more than one ligand.

In the first assay, 500 µl of cortex homogenate were incubated with six increasing concentrations of [^{3}H]YM-09151-2 (specific activity, 85.5 Ci/mmol; NEN, Milan, Italy) ranging from 0.015 nM to 2.6 nM, in a final volume of 1.5 ml. The D4 receptor density was determined by calculating the difference between the [^{3}H]YM-09151-2 total binding (Figure 1A) and the binding obtained using a ligand that has a high affinity for D4 (Kd = 0.4 nM) and a low affinity for D2/D3 receptors (Kd = 0.9 µM and 2.3 µM for D2 and D3 receptors, respectively),[12] namely L-745870 (Sigma-Aldrich, Milan, Italy) at 500 nM (Figure 1B).

In the second assay, total binding was determined using the same concentrations of [^{3}H]YM-09151-2 described above, and selective D4 binding was estimated in the presence of 100 nM raclopride (Sigma-Aldrich), which has high affinity for D2/D3 receptors and low affinity for D4 receptors (Figure 1C). The L-745870 (500 nM) was used to determine the nonspecific binding (Figure 1D). All samples were assayed in triplicate.

The incubation with [^{3}H]YM-09151-2 was performed for 2 h at 22°C. The bound ligand was separated from the free ligand by vacuum filtration through GF/C fiber filters (Whatman, Maidstone, UK), which were pre-soaked in polyethyleneimine (2%) to minimize nonspecific binding to the filters. Radioactivity was counted by a liquid scintillation counter (LS-1600, Beckman, Fullerton, CA, USA).

Using this procedure, we expected to obtain, for each subject, two comparable values that would correspond to D4 receptor density.

The equilibrium-saturation binding parameters, i.e., the maximum binding capacity (Bmax, expressed in fmol/mg protein) and the dissociation constant (Kd, expressed in nM), were analyzed using the EBDA iterative curve-fitting computer program.[13]

4. Statistical analysis

The differences between the two groups of binding parameter values obtained in the two assays were analyzed by conducting Student's t-tests (paired, two-tailed) using the computer program Statview V for Macintosh.

Results

We identified specific and saturable binding for the D2/D3/D4 receptor antagonist [^{3}H]YM-09151-2 in postmortem human prefrontal cortex. Figure 2 shows the representative saturation curve and Scatchard plot obtained in the second assay from subject no. 5 (a 63-year-old male).

The overall mean Bmax and Kd values obtained in the two assays were 88 ± 25 fmol/mg protein and 0.8 ± 0.4 nM, respectively. The mean Bmax and Kd values obtained in the first assay were 90 ± 30 fmol/mg protein and 0.8 ± 0.4 nM,

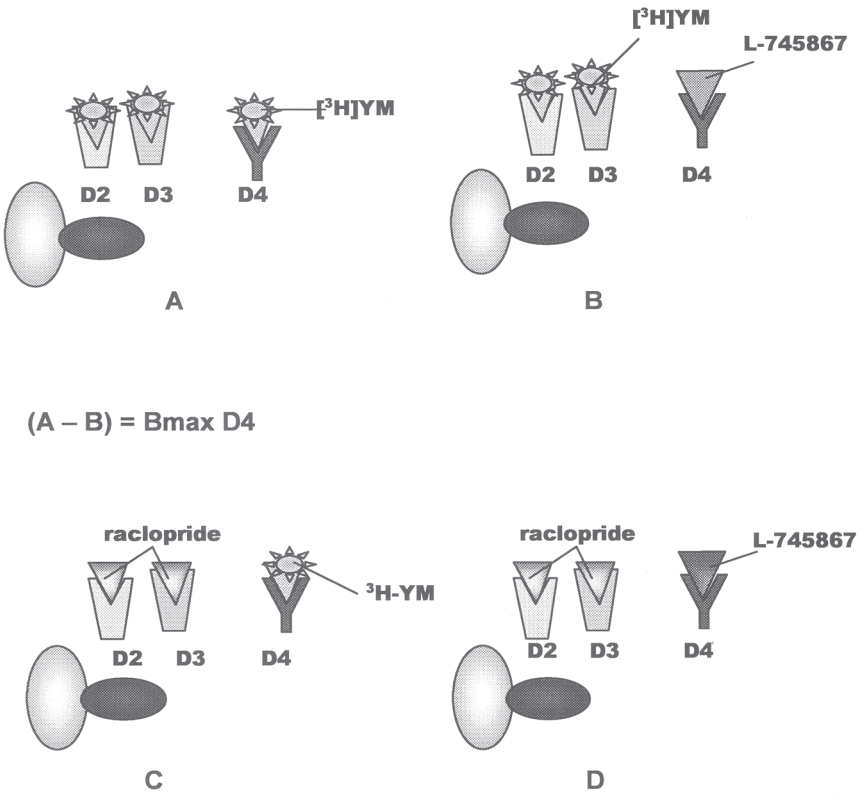

Figure 1 - Binding assay schemes: in the first assay, the D4 receptor density was calculated as the difference between the [^{3}H]YM-09151-2 total binding (A) and the binding obtained using 500 nM L-745870 (B). In the second assay, total binding was determined using the same concentrations of [^{3}H]YM-09151-2 described above, and selective D4 binding was estimated in the presence of 100 nM raclopride (C). L-745870 (500 nM) was used to determine the nonspecific binding (D).
Using this procedure, it was expected that, for each sample, two comparable values would be obtained, and that these values would correspond to D4 receptor density: (A – B) = Bmax D4; (C – D) = Bmax D4.

respectively. The mean Bmax and Kd values obtained from the second assay were 86 ± 23 fmol/mg protein and 0.8 ± 0.4 nM, respectively. There were no significant differences between the two groups of assays regarding Bmax or Kd ($p > 0.05$). The Bmax and Kd values for each subject are reported in Table 1. The blockade of D2 and D3 receptors with raclopride ensured that the D4 receptors were labeled.

Discussion and conclusions

The main finding of the present study is that D4 receptors were identified in postmortem human prefrontal cortex using direct binding techniques. The majority of the data available previously was obtained from studies performed in the rat or human brain with ligands such as [^{3}H]nemonapride, [^{3}H]YM-09151-2 or [^{3}H]raclopride.[11-12,14] The authors of such studies typically extrapolated the presence of D4 receptors by using subtraction methods. The ensuing findings revealed that D4 receptors were essentially distributed in various limbic areas, in the cerebral cortex and (perhaps) in cortical pyramidal cells, as well as, to a lesser extent, in the basal ganglia, probably on GABAergic neurons.[15] By means of radioactive probes, such as specific messenger RNAs, D4 receptors have been labeled, mainly in the nucleus accumbens, cerebral cortex and basal ganglia:[8-9] This is consistent with the results of quantitative analyses of autoradiographs, which also revealed that the density of D4 receptors is low in comparison to that the other subtypes.[16] Only one group of authors studied human and rat brains and identified D4 receptors in both.[10] The authors used [^{3}H]NGD 94-1, a selective D4 antagonist, and described some differences between the two species, especially in terms of affinity. However, the density of the sites labeled in the prefrontal cortex in the present study was 8-times higher than that reported by those authors.[10]

The principal limitation of our study is that, due to the lack of sufficient tissues, we could not perform a complete analysis of various brain areas. For the same reason, we could not

Table 1 - Binding parameters of the two assays

		Bmax (fmol/mg protein)	Kd (nM)
Subject 1 (M, 62)	(A – B)	60	1.52
	(C – D)	55	1.65
Subject 2 (F, 58)	(A – B)	87	0.51
	(C – D)	85	0.39
Subject 3 (F, 68)	(A – B)	92	0.92
	(C – D)	94	0.89
Subject 4 (M, 59)	(A – B)	71	0.56
	(C – D)	78	0.61
Subject 5 (M, 63)	(A – B)	140	0.67
	(C – D)	120	0.72

Bmax: maximum binding capacity; Kd: dissociation constant; M: male; F: female; A: [^{3}H]YM-09151-2; B: L-745870; C: [^{3}H]YM-09151-2 + raclopride; D: L-745870
The number following M or F indicates the age (in years)

carry out the pharmacological characterization of the binding, which is considered essential. Therefore, we cannot even suggest an explanation for the fact that the Kd values obtained were considerably higher than those reported in the literature.

The characteristic distribution of D4 receptors in the human brain, coupled with the observation that clozapine, the first atypical antipsychotic introduced into clinical practice, has a high affinity for these receptors (approximately 20-times higher than its affinity for the other dopamine receptors),[2] suggests that D4 receptors are involved in at least some symptoms of schizophrenia and psychosis. Following this line of thought, various authors have reported alterations in the D4 receptors in the frontal cortex of schizophrenic patients, although these reports are questionable in terms of the previously mentioned methodological limitations, as well as because the patients involved had been treated for several years with antipsychotics.[17-20] It has been also proposed that the polymorphic variants of D4 receptors are responsible for the heterogeneous patient response to clozapine, as evidenced by the fact that a segment of the population is resistant to clozapine. However, none of the genetic studies conducted to date have demonstrated any correlation between polymorphism of the D4 receptor gene and the response to clozapine.[21-22]

Further studies are necessary in order to draw a comprehensive map of D4 receptors in the human brain and to clarify their roles in the physiology of the central nervous system, as well as in the pathophysiology of neuropsychiatric disorders.

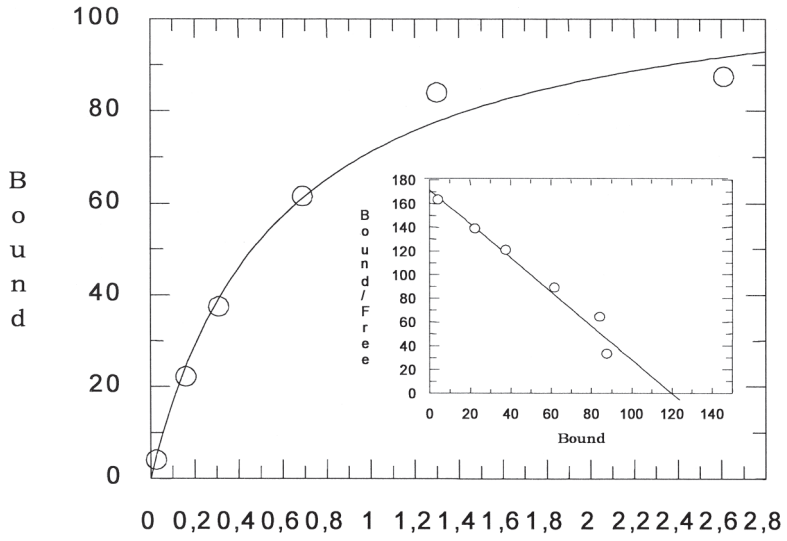

Figure 2 - Representative saturation curve and Scatchard plot of specifically bound [^{3}H]YM-09151-2 obtained from subject no. 5 (second assay). Cortex homogenates were incubated in triplicate with six increasing concentrations of [^{3}H]YM-09151-2 in the presence of raclopride (100 nM). L-745870 (500 nM) was used to determine the non-specific binding (D). The values are the means of three determinations with SEM of less than 3%.

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