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α_2-Adrenoceptor subtypes in the human brain: a pharmacological delineation of [^{3}H]RX-821002 binding to membranes and tissue sections

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Abstract

In order to study the characterization and localization of [^{3}H]RX-821002 (2-methoxy-idazoxan) binding to α_2-adrenoceptor subtypes in several regions of the human brain, we have carried out competition studies using both autoradiography and membrane binding assays. The α_{2A}-adrenoceptor subtype was found to be predominant in the different layers of the frontal cortex, cerebellum and hippocampal formation, while in the neostriatum it was the non-α_{2A}- (α_{2B}- and α_{2C}-) adrenoceptor subtype. In the frontal cortex, in addition to binding to the α_{2A}-adrenoceptor subtype, [^{3}H]RX-821002 bound also to a small portion of α_{2B}- and α_{2C}-adrenoceptors in layer III, and to an unidentified binding site in the external layers. In the hippocampus, both α_{2A}- and non-α_{2A}- (α_{2B}- and α_{2C}-) adrenoceptors were labelled in the dentate gyrus and the CA_1 field, together with 5-HT_{1A} receptors. 5-HT_{1A} receptors were labelled predominantly in the stratum pyramidale layer. These results, in addition to delineate the relative presence of α_2-adrenoceptor subtypes, indicate that caution is needed when analyzing RX 821002 binding to human brain tissue.

Keywords: α_2-Adrenoceptor subtype; Brain, human; RX 821002; Autoradiography; Frontal cortex; Hippocampus

1. Introduction

α_2-Adrenoceptors, a group of G protein-linked receptors, are present in a wide range of tissues, including the central nervous system, where their properties and functional significance have been subject of particular interest (Bylund et al., 1994). Early indications of α_2-adrenoceptors heterogeneity came from the existence of different rank order of potencies for several adrenergic compounds, and two subtypes were identified as α_{2A}- and α_{2B}-adrenoceptors, based on their respectively high and low affinity for oxymetazoline and their low and high affinity for prazosin (Bylund et al., 1988). Further studies more recently have resulted in the division of these receptors into four different subtypes, α_{2A}-, α_{2B}-, α_{2C}- and α_{2D}-adrenoceptors, also on the basis of their differences in affinity for several drugs (Murphy and Bylund, 1988; Blaxall et al., 1991; Simonneaux et al., 1991). Moreover, tissues and cell lines expressing only one subtype have been identified (Bylund et al., 1988). Molecular cloning has recently confirmed the existence of three distinct α_2-adrenoceptor genes in human and rat tissues (Kobilka et al., 1987; Zeng et al., 1990; Chalberg et al., 1990; Voigt et al., 1991, Lanier et al., 1991; see also Bylund et al., 1994). In the human genome these genes are designated α_2-C10, α_2-C2 and α_2-C4, according to their chromosomal localization, and they correspond to the previously defined α_{2A}-, α_{2B}- and α_{2C}- adrenoceptors, respectively (Kobilka et al., 1987; Regan et al., 1988; Lomasney et al., 1990). It has been recently demonstrated that the pharmacologically defined α_{2D}-adrenoceptor subtype in rats and mice genetically belongs to the human α_{2A}-adrenoceptor subtype (Kurose et al., 1993; O'Rourke et al., 1994b).

The human brain contains the three subtypes of α_2-adrenoceptors. The α_{2A} type appears to be predominant, at least in the cerebral cortex (De Vos et al., 1992; Sastre and García-Sevilla, 1994). However, the relative presence of the three subtypes throughout the human brain, in terms of pharmacological profile and anatomical localization, is still not well defined. Although a number of studies have

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addressed this issue (Petrash and Bylund, 1986; Ordway et al., 1993; Sastre and García-Sevilla, 1994), its full clarification requires the use of subtype-selective drugs and procedures with high anatomical resolution. In this regard, a number of compounds, in addition to oxymetazoline and prazosin, have been demonstrated to present subtype selectivity for human α_2-adrenoceptors. Among them, guanfacine (Uhlén and Wikberg, 1991) and the recently developed drugs BRL-44408 (2-[2*H*-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole HCl) and ARC-239 (2-[2-[4-(*o*-methoxyphenyl)piperazin-1-yl]-ethyl]-4,4-dimethyl-1,3-(2*H*,4*H*)-isoquinolinedione HCl) exhibit high selectivity for α_{2A}-, α_{2B}- and α_{2C}- adrenoceptors (Bylund et al., 1988; Young et al., 1989; Devedjian et al., 1994). In the present paper, we have studied the competition profile of such compounds against the binding of [^{3}H]RX-821002 (2-methoxy-idazoxan), a mixed α_{2A}- α_{2B}- α_{2C} adrenoceptor antagonist (Langin et al., 1989; Hudson et al., 1992), in several regions of the human brain by both binding in homogenates and autoradiographic assays. In addition to exploiting the selectivity of those drugs to further characterize the presence and localization of the different receptor subtypes, this combined experimental approach has allowed the re-analysis of the nature of the sites recognized by RX-821002, BRL-44408, ARC-239 and other related compounds in the human brain.

2. Materials and methods

2.1. Drugs and chemicals

[^{3}H]RX-821002 (2-methoxy-idazoxan) (53–60 Ci/mmol) was obtained from Amersham International plc (UK) and stored at 4°C. Other drugs (and their sources) included: 2-[2-[4-(*o*-methoxyphenyl) piperazin-1-yl]-ethyl]-4,4-dimethyl-1,3-(2*H*,4*H*)-isoquinolinedione HCl (ARC-239) (Thomae GmbH, Biberach, Germany); 2-[2*H*-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole HCl (BRL-44408) (Smith Kline Beecham, Essex, UK); (−)adrenaline bitartrate and corynanthine (Sigma, St. Louis, USA), clonidine (Boehringer Ingelheim, Germany), oxymetazoline (Pensa Laboratorios, Spain), prazosin (Pfizer, Spain), sumatriptam (Glaxo, UK), MK-912 (Merck, Sharp and Dohme, West Point, USA), tizanidine (Sandoz, Basel, Switzerland), efaroxan (RBI, Natick, MD, USA), guanfacine (Sandoz, Barcelona) and 5-HT and (±)8-OH-DPAT (RBI, Natick, MD, USA). RX 821002 HCl was synthesized by Dr F. Geijo at SA Lasa Laboratorios (Barcelona, Spain). All other compounds and reagents were purchased from either Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Tissue preparation

Human brains were obtained at autopsy from subjects without history of neurological or psychiatric disorders. A total of 31 specimens were used for the binding studies in

Table 1
Parameters for the displacement of [^{3}H]RX-821002 (2-methoxy-idazoxan) binding (1 nM) in human prefrontal cortex

Drug	pK_{i1}	pK_{i2}	B_{max1} (%)	B_{max2} (%)	n
RX-821002	9.12	10.84	91±12 (85.0)	16±2 (15.0)	3
Serotonin	4.33	10.80	111± 9 (88.9)	14±2 (11.1)	5
ARC-239	6.20	9.20	105± 5 (84.0)	10±1 (16.0)	6
BRL-44408	7.28	9.12	153±19 (89.5)	18±3 (10.5)	4
8-OH-DPAT	5.09	8.51	143±12 (97.3)	4±1 (2.7)	3
Yohimbine	8.30		113±18		3
(−)Adrenaline	6.35		170±14		3
MK-912	8.28		43± 6		4
Oxymetazoline	7.85		93±11		5
Tizanidine	7.28		56± 8		4
Clonidine	7.00		176±21		3
Prazosin	5.32		131± 9		3
Corynanthine	5.17		122±11		3
Sumatriptam	4.49		173±12		3

Cortical membranes from 22 human postmortem brains were incubated at 25°C for 30 min with [^{3}H]RX-821002 (1 nM) in the absence or presence of competing drugs (10^{-12} M–10^{-3} M, 19 concentrations). Binding parameters were estimated by simultaneous analysis of independent competition experiments for each drug and [^{3}H]RX-821002 saturation assays (0.06–8 nM) using the EBDA-LIGAND programs. pK_{i1} and pK_{i2} are the negative logarithms of the inhibition constants for each competing drug for the low and the high affinity binding sites of [^{3}H]RX-821002, obtained in a co-analysis of multiple experiments. B_{max1} and B_{max2} are the densities for each [^{3}H]RX-821002 binding site in fmol/mg protein. A two-site fit was accepted only if it was significantly better than a one-site binding model.

Table 2
Parameters for the displacement of [^{3}H]RX-821002 (2-methoxy-idazoxan) binding (1 nM) in human caudate

Drug	pK_{i1}	pK_{i2}	B_{max1} (%)	B_{max2} (%)	n
RX-821002	9.89	8.67	5±4 (25.0)	15±6 (75.0)	8
Efaroxan	9.62	7.30	4±2 (12.1)	29±4 (87.9)	4
Guanfacine	9.20	6.11	7±1 (16.7)	35±6 (83.3)	3
ARC-239	6.58	8.94	14±2 (42.4)	19±2 (57.6)	5
BRL-44408	8.63	6.23	5±1 (13.9)	31±5 (86.1)	4
Tizanidine	6.59	5.10	10±6 (25.0)	30±1 (75.0)	6
Clonidine	8.99	6.68	3±1 (7.5)	37±3 (92.5)	3
Prazosin	5.75	7.85	6±1 (28.6)	15±1 (71.4)	5
Oxymetazoline	8.16	6.33	14±3 (21.5)	51±3 (78.5)	3
Yohimbine	9.14		13±1		8
(−)-Adrenaline	6.36		28±2		3
MK-912	9.14		12±2		4
Corynanthine	6.33		22±2		4
Serotonin	5.11		20±2		5
8-OH-DPAT	6.08		18±2		5
Sumatriptam	4.67		18±3		3
Glibenclamide	4.82		12±1		5
Cimetidine	4.49		21±2		3
Diazoxide	4.21		17±2		3

Caudate membranes from 28 human postmortem brains were incubated at 25°C for 30 min with [^{3}H]RX-821002 (1 nM) in the absence or presence of competing drugs (10^{-12} M–10^{-3} M, 19 concentrations). Binding parameters were estimated by simultaneous analysis of independent competition experiments for each drug and [^{3}H]RX-821002 saturation assays (0.06–8 nM) using the EBDA-LIGAND programs. pK_{i1} and pK_{i2} are the negative logarithms of the inhibition constants for each competing drug for the high and the low affinity binding sites of [^{3}H]RX-821002. B_{max1} and B_{max2} are the densities for each [^{3}H]RX-821002 binding site in fmol/mg protein. A two-site fit was accepted only if it was significantly better than a one-site binding model.

homogenates of membranes and 5 were used for the autoradiographic competition studies (28 males and 8 females, mean age ± S.E.M. of 36 ± 2.7 years). The time interval between death and autopsy (postmortem delay at 4°C) was 32.0 ± 2.3 h. Brains were promptly removed, dissected and stored at −80°C (binding studies in homogenates) or −25°C (autoradiography).

For the binding studies in homogenates, membranes were isolated as previously described (Meana et al., 1989) from prefrontal cortex (Brodmann's area 9) and caudate head. After thawing, tissue samples of approximately 300 mg were homogenized in 5 ml of ice-cold Tris-sucrose buffer (5 mM Tris-HCl, 250 mM sucrose, 1 mM $MgCl_2$, pH 7.4). The homogenates were centrifuged at 1 100 × g for 10 min at 4°C, and the supernatants were then recentrifuged at 40 000 × g for 10 min at 4°C. The resulting pellet was washed with 2 ml of fresh incubation buffer (50 mM Tris-HCl, pH 7.5) and recentrifuged at 40 000 × g for 10 min at 4°C. The resulting pellet was washed again with 2 ml of fresh incubation buffer and subsequently recentrifuged in the same conditions. The final pellet was resuspended in incubation buffer to a final protein content of 0.71 ± 0.13 mg/ml for the prefrontal cortex membranes and 0.88 ± 0.18 mg/ml for the caudate head membranes.

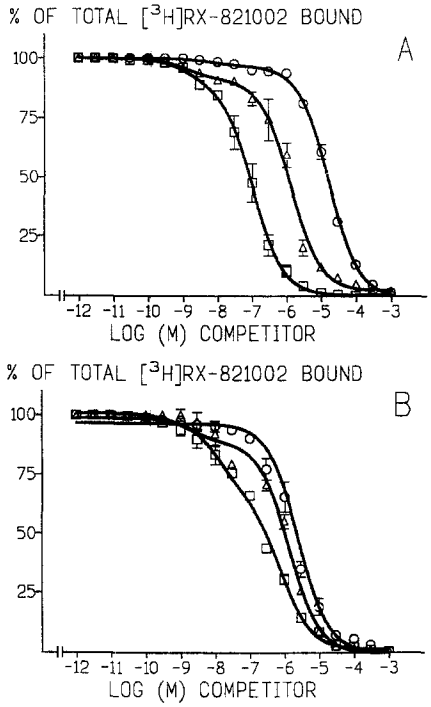

Fig. 1. Inhibition of total [^{3}H]RX-821002 (1 nM) binding by BRL-44408 (□), ARC-239 (△) and 8-OH-DPAT (○) in human prefrontal cortex (A) and caudate (B) membranes. The points are means ± S.E. of 3 (○), 4 (□) or 6 (△) experiments, each performed in duplicate. The curved lines represent the computer drawn fits obtained from the simultaneous fitting of all data in each experiment to a model that assumed that ligands bound to one or two independent sites according to the law of mass action. The selection between a one-site or two-site model was made statistically by means of an F-test.

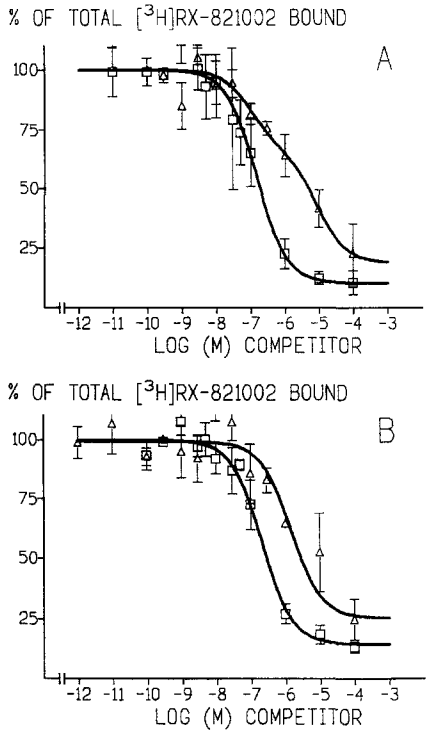

Fig. 2. Inhibition of total [^{3}H]RX-821002 (6 nM) binding by BRL-44408 (□) and ARC-239 (△) in human frontal cortex tissue sections. (A) Layer I; (B) layers IV–VI. The points are means ± S.E. of 3 experiments. The curved lines represent computer drawn fits obtained from the simultaneous fitting of all data in each experiment to a model that assumed that ligands bound to one or two independent sites according to the law of mass action. The selection between a one-site or two-site model was made statistically by means of an F-test.

Table 3
Inhibition of [^{3}H]RX-821002 binding by BRL-44408 and ARC-239 in human tissue sections

Area	BRL-44408		ARC-239	
	pK_H [a]	pK_L [a]	pK_H [a]	pK_L [a]
Frontal cortex				
Layer I	7.78		7.86 (42%)	5.96 (58%)
Layer II	7.65		8.41 (64%)	6.00 (36%)
Layer III	7.79 (90%)	5.40 (10%)	8.52 (67%)	5.84 (33%)
Layer IV }				
Layer V }	7.64		7.02	
Layer VI }				
Hippocampus				
CA_1 lac-mol	7.68 (60%)	6.40 (40%)	8.76 (46%)	6.02 (54%)
CA_1 pyr	7.93 (74%)	6.23 (26%)	8.85 (82%)	6.28 (18%)
DG gran	7.68 (90%)	6.15 (10%)	8.94 (36%)	6.03 (64%)
Cerebellum				
Granular layer	7.59		5.35	
Molecular layer	7.47		5.32	

[a] Values are expressed in pK_i; pK_H is the pK_i value for the high affinity site and pK_L is the value for the low affinity site. H and L sites do not correspond with sites 1 and 2 in membrane binding experiments (see text); the corresponding percentage of each site is expressed in parentheses. lac-mol = lacunosum-moleculare; pyr = pyramidale; DG gran = dentate gyrus, granular.

Protein content was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

For autoradiography, 10 μm sections from frontal cortex, hippocampus, putamen and cerebellar cortex were cut at −22°C using a cryostat (2800 Frigocut E, Reichert-Jung, Germany), mounted on gelatin-coated slides and stored at −25°C.

2.3. Binding assays in homogenates

Binding experiments were performed in duplicate at 25°C with shaking. Radioligand binding was done by incubating, for 30 min, 500 μl of membrane preparation, 10 μl of radioligand and 40 μl of either incubation buffer (50 mM Tris-HCl, pH 7.5) or drugs. In competition studies with agonists, the incubation buffer contained also 1 mM EDTA, 100 μM Gpp(NH)p (guanyl-5′-yl-imido-diphosphate) and 140 mM NaCl to preclude the high-affinity state of α_2-adrenoceptors for agonists. For the saturation studies, the final concentrations of radioligand ranged from 0.06 nM to 8 nM (eight points). Drug competition studies were performed with 1 nM [^{3}H]RX-821002 in the absence or presence of various concentrations of competing drugs (10^{-12} M–10^{-3} M; 19 concentrations). Incubations were terminated by diluting the samples with 5 ml of ice-cold incubation buffer (4°C). Membrane-bound [^{3}H]RX-821002 was measured by rapid filtration under vacuum (650 mm Hg) through Whatman GF/C glass fiber filters, that had been presoaked with 0.5% polyethylenimine. Then, the filters were rinsed twice with 10 ml of ice-cold incubation buffer, air-dried, transferred to minivials containing 3 ml of OptiPhase HiSafe II cocktail (LKB, UK) and counted for radioactivity for 5 min each in a liquid scintillation spectrometry (Packard model 2200 CA). Non-specific binding, as estimated in the presence of 10^{-5} M (−)-adrenaline, ranged from 5 to 37%.

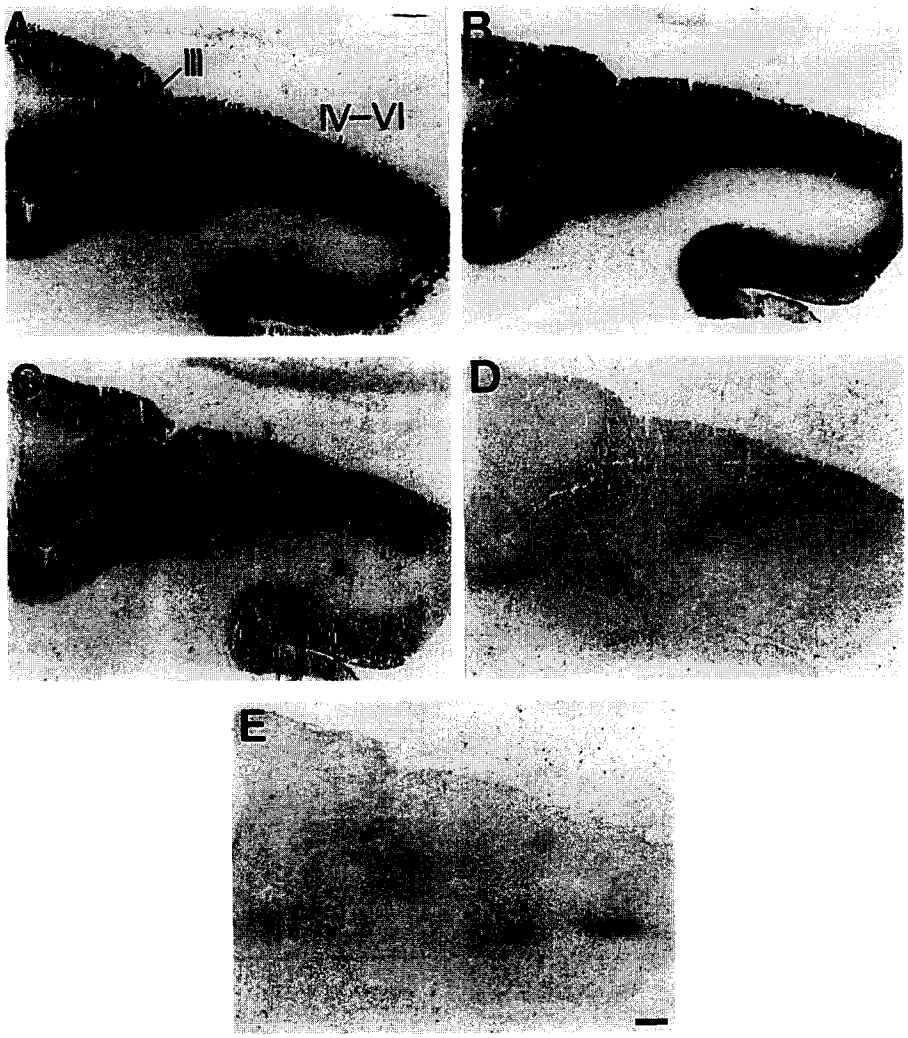

Fig. 3. Autoradiography of [^{3}H]RX-821002 (6 nM) binding in human frontal cortex. (A) Total binding; (B) binding in the presence of 0.3 μM 8-OH-DPAT; C: binding in the presence of 100 nM BRL-44408; (D) binding in the presence of 1 μM BRL-44408; (E) binding in the presence of 10 μM (−)-adrenaline. III = layer III, IV–VI = layers IV–VI. Bar = 2 mm.

2.4. Autoradiography

After a 30 min preincubation at room temperature in 50 mM Tris-HCl buffer containing 1 mM $MgCl_2$ and 1 g/l of ascorbic acid (pH 7.4), tissue sections were incubated with 6 nM [^{3}H]RX-821002 for 30 min in the same buffer, also at room temperature. Following the incubation, sections were washed twice for 20 s in ice-cold buffer, dipped in ice-cold distilled water and then dried in a cold air stream. Non-specific binding ($25.0 \pm 3.2\%$) was defined as that remaining in the presence of 10^{-5} M (−)-adrenaline. Sections were exposed at 4°C for 75 days to tritium-sensitive film (Hyperfilm-^{3}H, Amersham International) together with the appropriate standards (autoradiographic ^{3}H micro-scales, Amersham International). The films were developed in Kodak D-19 developer and the resulting autoradiograms were quantified using a computerized densitometry (Microm, Barcelona, Spain). Optical densities were converted into amount of ligand bound by comparison with the optical densities of the standards. Competition studies were performed in sections of frontal cortex, hippocampus and cerebellar cortex with ARC-239 and BRL-44408 (10^{-11} M–10^{-4} M; 13 concentrations). Sections of the putamen were incubated in the presence of a masking concentration of ARC-239 (3×10^{-7} M) and BRL-44408 (10^{-7} M). To study the binding of [^{3}H]RX-821002 to 5-HT_{1A} receptors, tissue sections of frontal cortex and hippocampus were also incubated in the presence of 3 concentrations of 8-OH-DPAT (10^{-9} M, 10^{-8} M and 3×10^{-7} M).

2.5. Data analysis

Competition and saturation studies were analyzed by non-linear least-square curve fitting with the program LIGAND (Munson and Rodbard, 1980), allowing curve-fitting to a one-site or a two-site inhibition model, to determine the maximal number of sites (B_{max}) and the equilibrium dissociation constants (K_d and K_i). The selection between the one-site or two-site binding model was made statistically by means of an F-test, as outlined by Munson and Rodbard (1980). Instead of presenting the mean value of n cases analyzed individually, co-analysis values of multiple experiments are presented. Drug affinities are expressed as negative logarithms of K_i (pK_i values). B_{max} values are expressed as mean ± estimated error values as determined in the co-analyses.

3. Results

3.1. Binding assays with [^{3}H]RX-821002 in human frontal cortex and caudate nucleus membranes

In the frontal cortex, competition experiments with MK-912, clonidine, tizanidine, oxymetazoline (α_{2A}-adrenoceptor-selective) and prazosin (α_{2B}- and α_{2C}-adrenoceptor-selective) against [^{3}H]RX-821002 binding were monophasic, the best fits being to single populations of high affinity (with the exception of prazosin) binding sites (Table 1), suggesting a very predominant presence of α_{2A}-adrenoceptors. In contrast, inhibition parameters for RX-821002, BRL-44408 and ARC-239, as well as for the serotonergic compounds 5-HT and 8-OH-DPAT, were readily analyzed and resolved in terms of two binding sites. The clearly predominant site (B_{max1}) displayed nanomolar affinity for RX-821002 and BRL-44408 (α_{2A}-adrenoceptor-selective) and lower affinity for ARC-239 (α_{2B}- and α_{2C}-adrenoceptor-selective), serotonin and 8-OH-DPAT. The minority site (B_{max2}), which represented between 5% and 20% of the specific binding, was recognized with high affinity by RX 821002, BRL-44408, ARC-239, as well as by 5-HT and 8-OH-DPAT (Table 1 and Fig. 1). Interestingly, a lower proportion of the minority site was found for 8-OH-DPAT as compared with other compounds (Table 1). After the addition of 100 nM 8-OH-DPAT to cortical homogenates the competition curves for RX 821002, ARC-239 and 5-HT still remained biphasic (pK_{i2} values 9.45, 7.69 and 11.0, respectively) with a

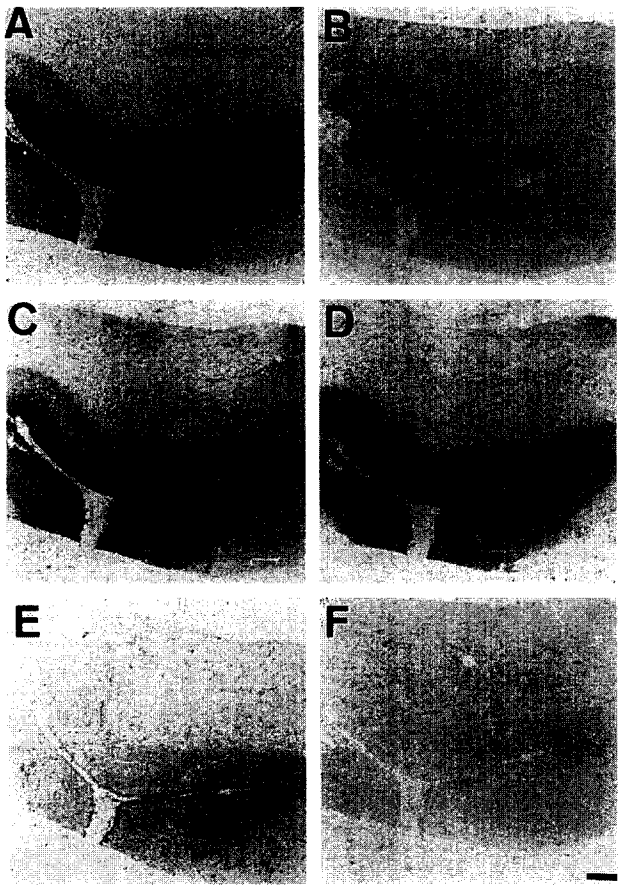

Fig. 4. Autoradiography of [^{3}H]RX-821002 (6 nM) binding in human frontal cortex. (A) Total binding; (B) binding in the presence of 10 μM (−)-adrenaline; (C, D, E and F) Binding in the presence of 10 nM, 100 nM, 10 μM and 100 μM ARC-239, respectively. Bar = 2 mm.

decrease in the proportion of the minority site (B_{max2} 10%, 5% and 9%, respectively). This finding indicates that [^{3}H]RX-821002 labels a further population of non-adrenoceptor non-5-HT_{1A} sites in the frontal cortex.

In the caudate nucleus, while MK-912 inhibited [^{3}H]RX-821002 binding with high affinity in a monophasic manner, competition experiments resulted in clearly biphasic (shallow) curves for a number of drugs, including RX-821002, efaroxan, clonidine and most of the reported so-called 'subtype-selective' compounds, such as BRL-44408, oxymetazoline, ARC-239 and prazosin, among others (Table 2 and Fig. 1), thus suggesting a mixed population of α_2-adrenoceptor subtypes in this brain region. High affinity components were predominant for ARC-239 and prazosin (α_{2B}- and α_{2C}-adrenoceptor-selective), while low affinity binding sites were predominant for BRL-44408, efaroxan, guanfacine and oxymetazoline (α_{2A}-adrenoceptor-selective), indicating that most α_2-adrenoceptors in the caudate nucleus are of the non-α_{2A}- (α_{2B}- and α_{2C}-) adrenoceptor subtype.

The serotonergic compounds 5-HT, 8-OH-DPAT and sumatriptan showed low or very low affinities for [^{3}H]RX-821002 binding in caudate membranes (Table 2).

3.2. Autoradiographic assays with [^{3}H]RX-821002 in tissue sections from human brain regions

In the frontal cortex, competition autoradiographic experiments with the α_{2A}-adrenoceptor-selective antagonist BRL-44408 were monophasic and of high affinity along the different layers analyzed (with the exception of a very small component of low affinity in layer III) (Table 3 and Figs. 2 and 3). In contrast, the α_{2B}- and α_{2C}-adrenoceptor-selective antagonist ARC-239 showed a differential pattern of inhibition of [^{3}H]RX-821002 depending on the layer studied: while in the internal layers (IV, V, VI) the competition was monophasic and of low affinity, the curves obtained in the external layers (I, II, III) were clearly biphasic, with a relevant component of high affinity for ARC-239 (Table 3 and Figs. 2 and 4). The 5-HT_{1A} receptor-selective compound 8-OH-DPAT showed a very exiguous component of high affinity for [^{3}H]RX-821002 bind-

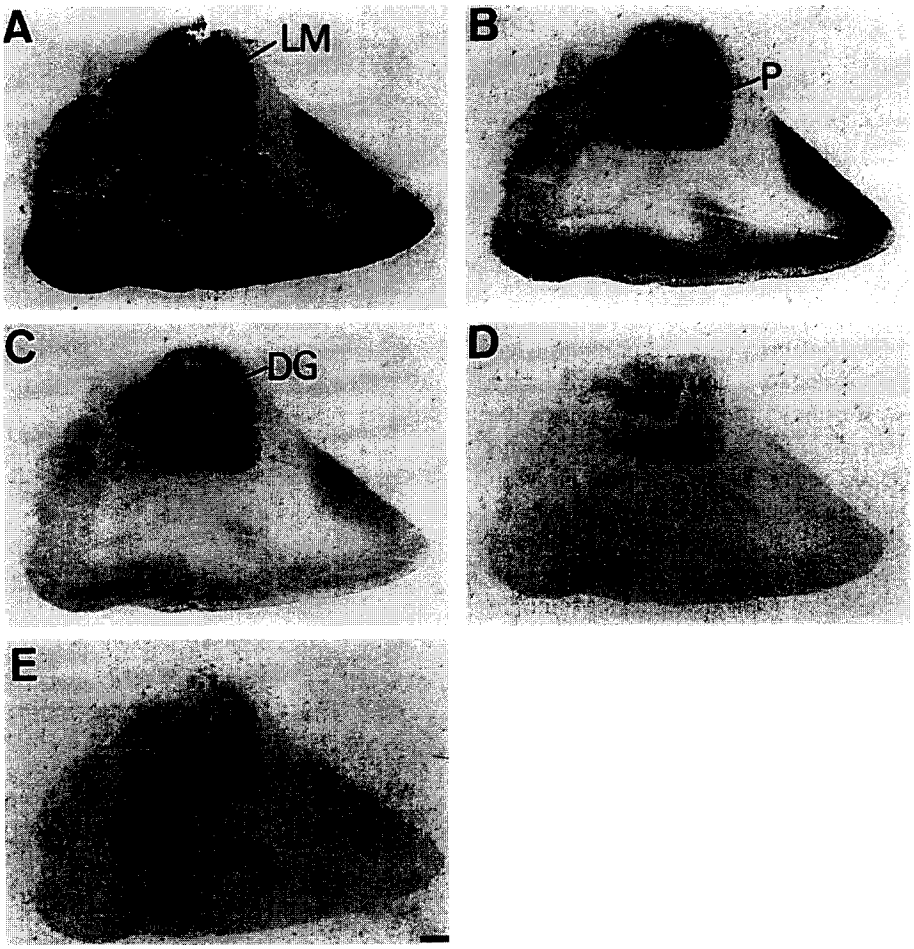

Fig. 5. Autoradiography of [^{3}H]RX-821002 (6 nM) binding in human hippocampus. (A) Total binding; (B) binding in the presence of 0.3 μM 8-OH-DPAT; (C and D) binding in the presence of 100 nM and 1 μM BRL-44408, respectively; (E) binding in the presence of 10 μM (−)-adrenaline. DG = dentate gyrus, LM = stratum lacunosum-moleculare, P = stratum pyramidale. Bar = 2 mm.

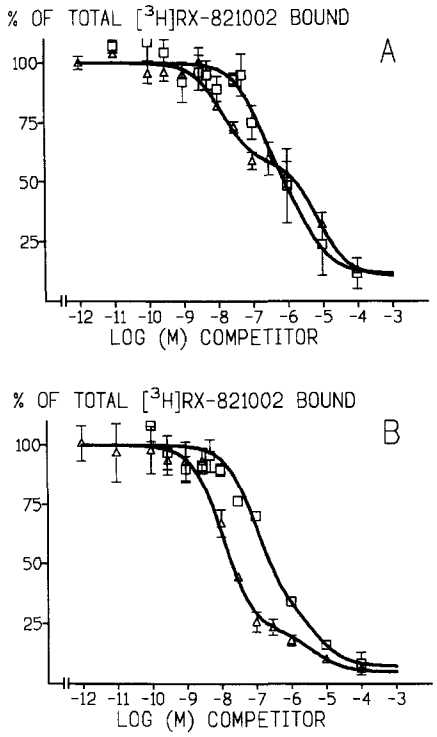

Fig. 6. Inhibition of total [^{3}H]RX-821002 (6 nM) binding by BRL-44408 (□) and ARC-239 (△) in human hippocampus. (A) Lacunosum-moleculare layer of CA_1; (B) pyramidal layer of CA_1. The points are means ± S.E. of 3 experiments. The curved lines represent computer drawn fits obtained from the simultaneous fitting of all data in each experiment to a model that assumed that ligands bound to one or two independent sites according to the law of mass action. The selection between a one-site or two-site model was made statistically by means of an *F*-test.

ing in the cortical layer I (5% of the total binding at 3×10^{-7} M) (Fig. 3).

In tissue sections from human hippocampus, the profiles of competition curves were more complex and dependent on the microscopic area studied. The 5-HT_{1A} receptor agonist 8-OH-DPAT showed high affinity for a certain component of [^{3}H]RX-821002 binding: 0.3 mM 8-OH-DPAT inhibited 13%, 36% and 10% of total [^{3}H]RX-821002 binding in the lacunosum-moleculare layer, the stratum pyramidale and the dentate gyrus, respectively (Fig. 5). The α_{2A}-adrenoceptor antagonist BRL-44408 competed in a biphasic manner in all areas studied, the high affinity component being always predominant (Table 3 and Figs. 5 and 6). The competition curves corresponding to ARC-239 were also biphasic, but the high affinity component was only predominant in the stratum pyramidale (Table 3 and Figs. 6 and 7).

Competition experiments with BRL-44408 and ARC-239 in sections from human cerebellum were monophasic. While BRL-44408 exhibited a high affinity for [^{3}H]RX-821002 binding, the affinity of ARC-239 was very low (Table 3).

Finally, in tissue sections from the human basal ganglia, 100 nM BRL-44408 did not significantly affect [^{3}H]RX-821002 binding in the putamen. In contrast, 0.3 mM ARC-239 inhibited about 60% of the specific binding of this radioligand in this structure (Fig. 8).

4. Discussion

[^{3}H]RX-821002 has been proposed as a very appropriate ligand to label the total population of α_2-adrenoceptors, due to its high affinity for the three receptor subtypes (O'Rourke et al., 1994a; Devedjian et al., 1994). Therefore, competition experiments with subtype-selective drugs versus this ligand are an adequate approach to delineate the relative presence of α_2-adrenoceptor subtypes in the different regions of the human brain. BRL-44408 (highly α_{2A}-adrenoceptor-selective) and ARC-239 (highly α_{2B} and α_{2C}-adrenoceptor-selective) are now considered as the best available antagonists to discriminate these receptor subtypes (Bylund et al., 1988, 1994; Young et al., 1989; Devedjian et al., 1994; Sastre and García-Sevilla, 1994). For this reason, these drugs were chosen for the competition autoradiographic experiments.

The present work confirms and extends previous data indicating the presence of different α_2-adrenoceptors in the human brain, although with a degree of predominance of the α_{2A} subtype (Meana et al., 1989; De Vos et al., 1992; Ordway et al., 1993; Sastre and García-Sevilla, 1994). In addition, our results provide the direct visualization of the anatomical distribution of these sites at the microscopic level. On the other hand, our data indicate that [^{3}H]RX-821002 recognizes in the human brain, in addition to α_2-adrenoceptors, two additional populations of binding sites.

It has been previously reported that [^{3}H]RX-821002, as well as other α_2-adrenoceptor related compounds, such as rauwolscine and yohimbine, bind with high affinity to 5-HT_{1A} receptors (Convents et al., 1989; Vauquelin et al., 1990; De Vos et al., 1991, Winter and Rabin, 1992). Taking into account the strong homology in amino acid sequence between α_2-adrenoceptors and 5-HT_{1A} receptors, such affinities could indicate a certain degree of resemblance between the binding domains of both receptors (Kobilka et al., 1987; Fargin et al., 1988). We have dissected out the 5-HT_{1A} component of [^{3}H]RX-821002 binding in the different regions of the human brain. The specific compound 8-OH-DPAT (Hoyer et al., 1985) does not show high affinity for any portion of [^{3}H]RX-821002 binding in striatum and cerebellum, two regions very poor in 5-HT_{1A} receptors (Pazos et al., 1987). In frontal cortex membranes, 8-OH-DPAT recognizes with high affinity a very small component (around 3%) of the specific binding of [^{3}H]RX-821002. Autoradiographic data reveal that such component is localized over layer I (Pazos et al., 1987). These results indicate that the 'contaminant' 5-HT_{1A} binding of this radioligand in the human frontal cortex is not of special relevance. In contrast, our autoradiographic results

in the hippocampus demonstrate that a relevant component of [^{3}H]RX-821002 binding in this structure corresponds to 5-HT_{1A} receptors. Autoradiographic competitions with 8-OH-DPAT show that about 40% of the labelling over the stratum pyramidale of the CA_1 field and about 15% in other strata is of serotonergic (5-HT_{1A}) nature. In this regard, it is noteworthy that the hippocampus presents the highest densities of 5-HT_{1A} receptors in the human brain (Pazos et al., 1987). Furthermore, our results explain the striking discrepancy between the pattern of autoradiographic identification of [^{3}H]UK-14304 and [^{3}H]RX-821002 in the human hippocampus (Pascual et al., 1992; De Vos et al., 1994): labelling of [^{3}H]UK-14304 is mainly restricted to the lacunosum-moleculare layer, as this ligand is devoid of affinity for 5-HT_{1A} receptors. Taken together, our results support the notion of adding a masking concentration of 5-HT to the incubation buffer when labelling α_2-adrenoceptors in the brain with [^{3}H]RX-821002 (see De Vos et al., 1992; Sastre and García-Sevilla, 1994).

The ability of RX-821002 to recognize 5-HT_{1A} receptors in the human brain is of special interest, as we have recently found that BRL-44408 and ARC-239 displace with relevant affinity (pK_i 6.6 and pK_i 7.3, respectively) the binding of [^{3}H]8-OH-DPAT to human brain membranes (Meana et al., submitted). Such ability to bind to 5-HT_{1A} receptors explains the fact that the high affinity component of [^{3}H]RX-821002 binding for both drugs represents 75–80% of the total amount of sites in the stratum pyramidale of the hippocampus. Therefore, it can be concluded that the 5-HT_{1A} receptors labelling has to be taken into consideration when using BRL-44408 and ARC-239 in the definition of α_2-adrenoceptor subtype populations.

Our results show that, in the frontal cortex, [^{3}H]RX-821002 labels a further population of binding sites, for which several drugs including 5-HT, BRL-44408, yohimbine and ARC-239 show nanomolar affinity. Competition autoradiographic data demonstrate that this population is restricted to the external layers, mainly over layers II and III. This site does not correspond to any of the α_2-adrenoceptors so far identified. Furthermore, although 5-HT ex-

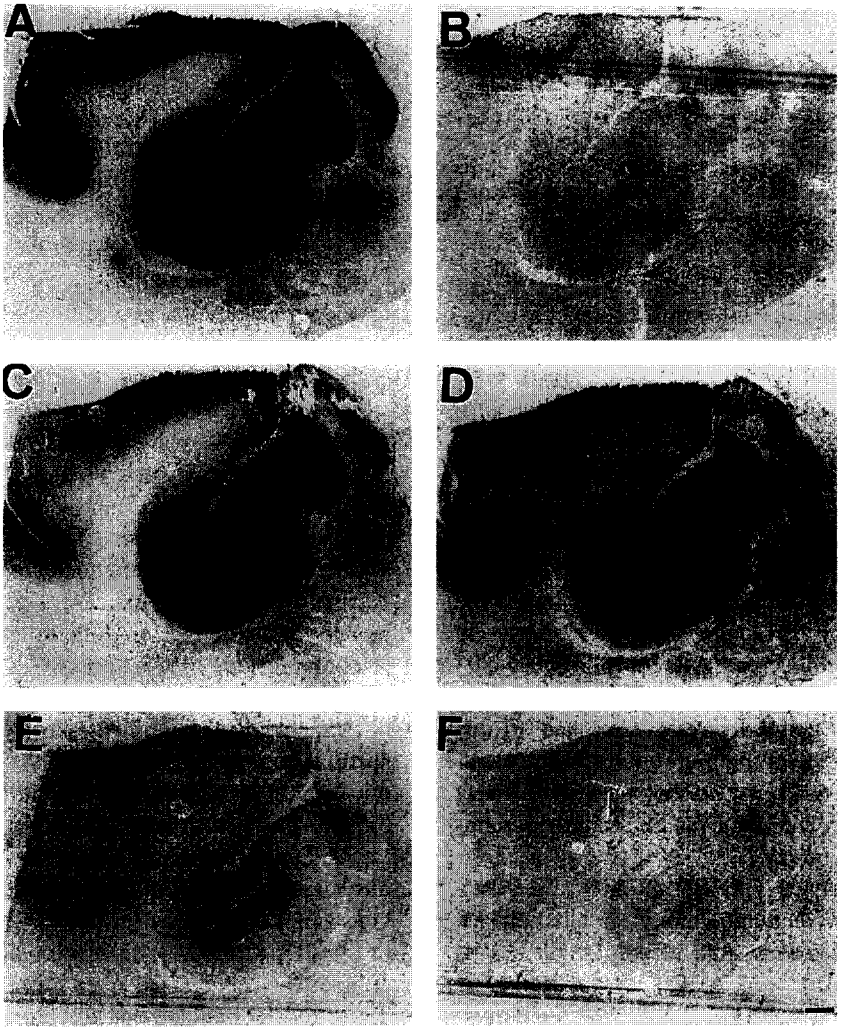

Fig. 7. Autoradiography of [^{3}H]RX-821002 (6 nM) binding in human hippocampus. (A) Total binding; (B) binding in the presence of 10 μM (−)-adrenaline 10 mM; (C, D, E and F) binding in the presence of 10 nM, 100 nM, 10 μM and 100 μM ARC-239, respectively. Bar = 2 mm.

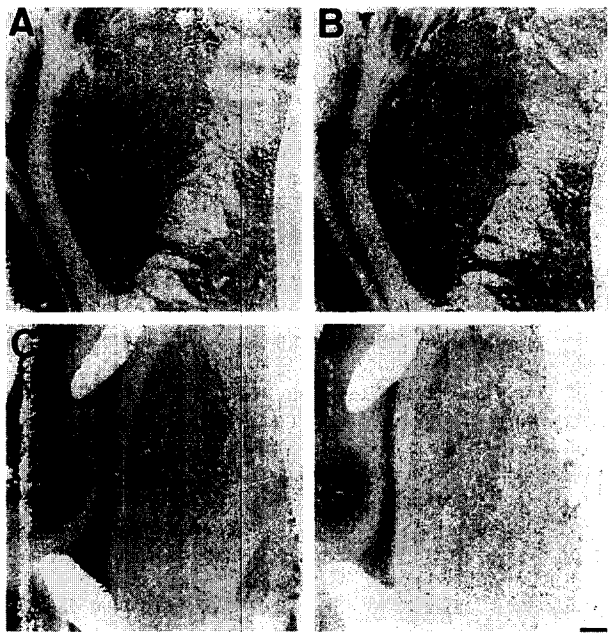

Fig. 8. Autoradiography of [^{3}H]RX-821002 (6 nM) binding in human basal ganglia. (A and C) Total binding; (B) binding in the presence of 100 nM BRL-44408; (D) binding in the presence of 0.3 μM ARC-239. P = putamen. Bar = 2 mm.

hibited high affinity for the site, this binding site does not correspond to the 5-HT_{1D} subtype (abundant in the human neocortex) as the 5-HT_{1D} receptor-selective agonist sumatriptan (see Hartig et al., 1992) shows very low affinity for this site. Further work is needed to clarify the characteristics of this component of [^{3}H]RX-821002 binding. The fact that both BRL-44408 and ARC-239 bind with nanomolar affinity to this unidentified site gives a special complexity to the analysis of competition curves of these compounds.

In the frontal cortex and cerebellum, the predominant α_2-adrenoceptor subtype is α_{2A}. This conclusion is based on the profile of affinities versus [^{3}H]RX-821002 binding in both membranes and sections. In membranes, BRL-44408 (between 42- and 85-fold α_{2A}-selective relative to α_{2B}, Young et al., 1989; Devedjian et al., 1994), oxymetazoline (between 108- and 230-fold α_{2A}-selective relative to α_{2B}, Petrash and Bylund, 1986; Devedjian et al., 1994) and guanfacine (60-fold α_{2A}-selective to α_{2B}, Uhlén and Wikberg, 1991) display high affinity when inhibiting [^{3}H]RX-821002 binding. In contrast, prazosin (13–79 fold α_{2B}-selective; Petrash and Bylund, 1986; Devedjian et al., 1994) exhibits a low value of pK_i, quite similar to that expected for the α_{2A} subtype. In addition, the potencies of ratios of pairs of drugs corresponding to the inhibition of the predominant component of [^{3}H]RX-821002 binding in the frontal cortex are quite similar to those previously found for α_{2A} -adrenoceptors in different tissues and cell lines (Uhlén and Wikberg, 1991; Bylund et al., 1992; Devedjian et al., 1994; Renouard et al., 1994). In this sense, BRL-44408 also shows high affinity for [^{3}H]RX-821002 binding in competition autoradiographic experiments in frontal cortex and cerebellar layers, although the existence of a minority low affinity component in layer III probably indicates the existence of a small population of α_{2B}- and α_{2C}-adrenoceptors in this cortical layer. In the frontal cortex, the α_{2B}- and α_{2C}-adrenoceptor-selective antagonist ARC-239 displaced [^{3}H]RX-821002 binding to cortical membranes in a biphasic mode, the low affinity component (α_{2A}) being predominant. In addition, the combined analysis of the competition autoradiographic curves demonstrates that most of the high affinity component of ARC-239 inhibition does not correspond to α_{2B}-adrenoceptor, but to the unidentified [^{3}H]RX-821002 binding site mentioned above.

This is the first direct visualization of a detailed distribution of α_2-adrenoceptor subtypes in the human frontal cortex and cerebellum. The very predominant presence of α_{2A}-adrenoceptors in these regions is in good agreement with previously reported results obtained in membranes (De Vos et al., 1992; Ordway et al., 1993; Sastre and García-Sevilla, 1994), as well as with data of mRNA expression (Perälä et al., 1992; Berkowitz et al., 1994).

The population of α_2-adrenoceptors in the human basal ganglia is mixed, but with a predominance of the α_{2B}- and α_{2C}-adrenoceptor subtype. This is illustrated by the existence of high and low affinity components of the competition curves of subtype-selective drugs in caudate membranes, as well as the higher affinity shown by ARC-239, in comparison to BRL-44408, in putamen sections. The potency ratios of pairs of drugs in competition binding assays also appear to agree with such predominance. Other groups have also reported the existence of a mixed population of α_2-adrenoceptors in the basal ganglia, although the information on the predominant subtype is contradictory (De Vos et al., 1992; Ordway et al., 1993; Sastre and García-Sevilla, 1994).

When the amount of 5-HT_{1A} labelling of [^{3}H]RX-821002 in the hippocampus is taken into account, autoradiographic data reveal the presence of a mixed population of α_2-adrenoceptors in this area. The α_{2A}-adrenoceptor subtype is predominant over the dentate gyrus and the stratum pyramidale, while both α_{2A}- and non-α_{2A}- (α_{2B}- and α_{2C}) adrenoceptor subtypes appear to exist in a similar proportion in the lacunosum-molecular layer of the CA_1 field. Previous studies carried out in membranes have detected a single population of α_2-adrenoceptors in the hippocampus (α_{2A}) (De Vos et al., 1992; Sastre and García-Sevilla, 1994). This fact illustrates the limited resolution of binding assays in homogenates when they are performed in tissues with a heterogeneous anatomical constitution: the existence of a high density of receptors in a very discrete area, i.e. the lacunosum-molecular layer of the hippocampus, can be masked by the 'dilution' into a larger amount of tissue poor in such receptor. Our results are in contrast with those reported by Ordway et al. (1993), who have found, also by autoradiography, a homo-

geneous population of α_{2A}-adrenoceptors in the hippocampus. However, these authors did not carry out full competition experiments in the sections; they only defined the proportion of receptor subtypes by the addition of a critical concentration of oxymetazoline (170 nM). Besides the fact that such concentration may in fact affect a significant proportion of α_{2C}-adrenoceptors (Devedjian et al., 1994), it is noteworthy that oxymetazoline, although it is the best drug for differentiating the three α_2-adrenoceptors in terms of rank order of affinities (Bylund et al., 1992), is not very suitable for discriminating subtypes in competition assays, because of its agonistic properties (see also De Vos et al., 1992).

Because ARC-239 and prazosin are very poorly α_{2B}-adrenoceptor-selective, relative to the α_{2C}-adrenoceptor subtype (Lawhead et al., 1992; Devedjian et al., 1994), it is difficult, to ascribe to one of these subtypes, the high affinity component found for both antagonists in the different regions examined (mainly in the basal ganglia and the hippocampus). In this sense, it has been shown that the α_2-C4 mRNA (α_{2C}-adrenoceptor subtype) is mostly expressed in the caudate nucleus, while the presence of the α_{2B}-subtype mRNA has not been reported in the human brain (Perälä et al., 1992). In addition, in situ hybridization studies in the rat brain also show that the subtype expressed in the striatum is the α_{2C} (Nicholas et al., 1993; Scheinin et al., 1994). All these data appear to indicate that non-α_{2A}-adrenoceptors throughout the human brain belong to the α_{2C}-adrenoceptor subtype. However, our K_i ratios for different pairs of compounds in human caudate membranes are closer to those values for tissues and cells expressing α_{2B}-adrenoceptors.

In conclusion, [^{3}H]RX-821002 binding in human brain mainly corresponds to α_{2A}-adrenoceptors. However, α_{2B}- or α_{2C}-adrenoceptors were also found, specially in the neostriatum and hippocampus. To some extend, [^{3}H]RX-821002 was also found to bind to 5-HT_{1A} receptors, mainly in the hippocampus and to an unidentified binding site in the outer layers of the frontal cortex.

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