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# Characterization and autoradiographical localization of non-adrenoceptor idazoxan binding sites in the rat brain

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**1** In rat whole brain homogenates, saturation analysis revealed that both [$^3$H]-idazoxan and [$^3$H]-RX821002, a selective $\alpha_2$-adrenoceptor ligand, bound with high affinity to an apparent single population of sites. However, the $B_{max}$ for [$^3$H]-idazoxan was significantly ($P<0.01$) greater than that for [$^3$H]-RX821002.

**2** In competition studies, (−)-adrenaline displaced 3 nM [$^3$H]-idazoxan binding with an affinity consistent with [$^3$H]-idazoxan labelling $\alpha_2$-adrenoceptors. However, this displacement was incomplete since 23.68 ± 1.11% of specific [$^3$H]-idazoxan binding remained in the presence of an excess concentration (100 μM) of (−)-adrenaline. In contrast, unlabelled idazoxan promoted a complete displacement of [$^3$H]-idazoxan binding with a Hill slope close to unity and an affinity comparable with its $K_D$ determined in saturation studies.

**3** Displacement of [$^3$H]-idazoxan binding by the $\alpha_2$-adrenoceptor antagonists yohimbine, RX821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline) and RX811059 (2-(2-ethoxy-1,4-benzodioxan-2-yl)-2-imidazoline) was more complex, with Hill slopes considerably less than unity, and best described by a two-site model of interaction comprising a high and low affinity component. The proportion of sites with high affinity for each antagonist was similar (60–80%).

**4** The rank order of antagonist potency for the high affinity component in each displacement curve (RX821002 > RX811059 > yohimbine) is similar to that determined against the binding of [$^3$H]-RX821002 to rat brain, suggesting that these components reflect the inhibition of [$^3$H]-idazoxan binding to $\alpha_2$-adrenoceptors. The remaining component in each displacement curve exhibiting low affinity towards these antagonists is attributable to the displacement of [$^3$H]-idazoxin from a non-adrenoceptor idazoxan binding site (NAIBS) since a comparable amount of [$^3$H]-idazoxan binding was not displaced by an excess concentration of (−)-adrenaline.

**5** The displacement of [$^3$H]-idazoxan binding by RX801023 (6-fluoro-(2-(1,4-benzodioxan-2-yl)-2-imidazoline) was also best described by a model assuming a two site interaction with 20.07 ± 3.11% of the sites labelled displaying high affinity for RX801023. The $K_i$ of RX801023 for the remainder of the sites labelled was similar to its $K_i$ versus [$^3$H]-RX821002, indicating that this drug displays improved affinity and NAIBS/$\alpha_2$-adrenoceptor selectivity compared with idazoxan.

**6** In autoradiographical studies, the distribution of 5 nM [$^3$H]-idazoxan binding to sections of rat whole brain was consistent with that reported from previous studies and resembled the distribution of $\alpha_2$-adrenoceptors. However, when sections of brain were coincubated with concentrations of $\alpha_2$-adrenoceptor agonists or antagonists predicted to saturate $\alpha_2$-adrenoceptors, there remained distinct areas of binding corresponding to discrete brain nuclei. This remaining binding was however displaced by unlabelled idazoxan (3 μM) or RX801023 (3 μM) indicative of the labelling of NAIBS.

**7** Quantitative autoradiography of NAIBS revealed several brain nuclei which contained higher densities of these sites than $\alpha_2$-adrenoceptors, notably the area postrema, interpeduncular nucleus, arcuate nucleus, ependyma and pineal gland.

**Keywords:** $\alpha_2$-Adrenoceptors; non-adrenoceptor idazoxan binding sites; radioligand binding; autoradiography; rat brain; idazoxan; interpeduncular nucleus; area postrema; arcuate nucleus

## Introduction

During recent years, the tritiated form of the $\alpha_2$-adrenoceptor antagonist, idazoxan, has been widely employed for the characterization and identification of such receptors in tissue homogenates from a variety of species since it displays enhanced affinity and $\alpha_2/\alpha_1$ selectivity compared with other antagonists such as rauwolscine (Doxey *et al.*, 1983; 1984). In autoradiographical studies [$^3$H]-idazoxan has also proved useful at the macroscopic level to localize central $\alpha_2$-adrenoceptors (Boyajian *et al.*, 1987; Bruning *et al.*, 1987). However, recent research has shown that both [$^3$H]-idazoxan and [$^3$H]-rauwolscine also bind with high affinity to non-adrenoceptor sites and that careful definition of the specific component of their binding is essential when using these radioligands in receptor binding and autoradiographical investigations of $\alpha_2$-adrenoceptors (see Lehmann *et al.*, 1989; Michel & Insel, 1989). Thus, [$^3$H]-rauwolscine has been reported to bind to 5-$HT_{1A}$ receptors as well as to $\alpha_2$-adrenoceptors in rat (Broadhurst & Wyllie, 1986), rabbit and human frontal cortex membranes (Convents *et al.*, 1989) and in autoradiographic studies its primary distribution is associated with dopaminergic terminals (Boyajian *et al.*, 1987).

Likewise, [$^3$H]-idazoxan binds with equal affinity to $\alpha_2$-adrenoceptors and phentolamine displaceable non-stereoselective sites in rat, rabbit, guinea-pig and human cortex membranes (Convents *et al.*, 1989; Wikberg, 1989; Brown *et al.*, 1990; Wikberg & Uhlen, 1990; Hamilton *et al.*, 1991).

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Similar non-adrenoceptor idazoxan binding sites or NAIBS (Michel & Insel, 1989) have also been observed in membrane preparations from other tissues such as adipocytes (Langin & Lafontan, 1989; MacKinnon *et al.*, 1989; Langin *et al.*, 1990a,b), kidney (Coupry *et al.*, 1989; Michel *et al.*, 1989; Vigne *et al.*, 1989; Hamilton *et al.*, 1991), smooth muscle (Wikberg *et al.*, 1991; Yablonsky & Dausse, 1991) and liver, where binding studies with subcellular fractions suggest a major localization of NAIBS in mitochondria (Tesson *et al.*, 1991). These non-adrenoceptor idazoxan binding sites show only low affinity for compounds such as cimetidine and imidazole-4-acetic acid indicating they are distinct from the non-adrenoceptor imidazoline binding sites labelled by [$^3$H]-clonidine or [$^3$H]-*p*-aminoclonidine in the ventrolateral medulla (Ernsberger *et al.*, 1987; 1988a,b) which may be responsible for the antihypertensive actions of clonidine and related analogues such as rilmenidine (Bricca *et al.*, 1989; Ersberger *et al.*, 1990; Feldman *et al.*, 1990; Gomez *et al.*, 1991). In contrast, no proven function(s) or endogenous neurotransmitter have yet been described for NAIBS.

Interestingly, whilst the 2-alkoxy substituted analogues of idazoxan, RX821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline) and RX811059 (2-(2-ethoxy-1,4-benzodioxan-2-yl)-2-imidazoline) maintain high affinity and selectivity for $\alpha_2$-adrenoceptors (Stillings *et al.*, 1985; Welbourn *et al.*, 1986), their affinity for NAIBS is at least 1000 fold lower than that of idazoxan (Langin *et al.*, 1989; Galitzky *et al.*, 1990; Vauquelin *et al.*, 1990; Hudson *et al.*, 1992). Conversely, preliminary investigations revealed that the 6-fluoro derivative of idazoxan, RX801023 (6-fluoro-(2-(1,4-benzodioxan-2-yl)-2-imidazoline), displays reduced affinity for $\alpha_2$-adrenoceptors (Chapleo *et al.*, 1983) whilst that for NAIBS is maintained (Mallard *et al.*, 1991). These observations suggest that the interaction of idazoxan with NAIBS occurs in a structurally specific fashion and, against this background, we have therefore investigated the radioligand binding profile and autoradiographical distribution of NAIBS in the rat brain. Preliminary accounts of some of this work have been given (Hudson & Nutt, 1990; Hudson *et al.*, 1991a,b).

## Methods

### *Membrane preparation*

Male Wistar rats (200–300 g) were killed by stunning followed by decapitation. Whole brains were rapidly dissected over ice and homogenized in 10 vol of buffered sucrose (0.32 M in 50 mM Tris HCl, pH 7.4 at 4°C) using a motor driven Teflon-glass homogenizer. The homogenate was then centrifuged at 1000 *g* for 10 min at 4°C. The resultant supernatants were pooled and recentrifuged at 31,000 *g* for 20 min at 4°C. The supernatants were then discarded and each pellet resuspended in 10 vol of assay buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4 at 4°C). The pellets were then washed twice by repeated centrifugation at 31,000 *g* for 20 min at 4°C. The final pellets were stored at − 70°C until use.

### *Saturation binding studies*

Membrane aliquots (300–450 μg protein) and nine concentrations of [$^3$H]-idazoxan or [$^3$H]-RX821002, over the range 0.1–60 nM, were incubated, in triplicate, to equilibrium (30 min) in a final volume of 1 ml at 25°C. Bound and free radioligand were then separated by rapid filtration through pre-soaked (0.5% polyethyleneimine) Whatman GF/B filters using a Brandel M-24 cell harvester. Filters were washed twice with 5 ml of ice-cold assay buffer and the radioactivity remaining on the filters determined by liquid scintillation counting. Non-specific binding at each free ligand concentration was determined with either 10 μM or 1 mM phentolamine for [$^3$H]-RX821002 or [$^3$H]-idazoxan respectively.

### *Competition binding studies*

Competition studies were carried out in identical conditions to saturation experiments with either 3–4 nM [$^3$H]-idazoxan or 2–3 nM [$^3$H]-RX821002 and various drugs over the concentration range 0.1 nM–1 mM in a final volume of 1 ml. Non-specific binding was again determined by use of 10 μM or 1 mM phentolamine for [$^3$H]-RX821002 or [$^3$H]-idazoxan respectively. Ascorbic acid (0.05%) was included in the assay buffer for competition studies with (−)-adrenaline.

### *Analysis of binding data*

Saturation and competition binding data were analysed by iterative non-linear regression procedures capable of fitting single and two site models using GraphPad Inplot (ISI Software, 1990). $K_i$ values for competing drugs were calculated from the Cheng & Prusoff (1973) equation: $K_i = IC_{50}/(1 + L/K_D)$. All displacement curves were initially analysed assuming a one site model of radioligand binding. Displacement curves with Hill coefficients significantly less than unity were reanalysed assuming a two site model of radioligand binding and the results compared to the one site model of fit using the differential $F$ value defined by the equation:

$$F = \frac{(SS_1 - SS_2)/(d.f._1 - d.f._2)}{SS_2/(d.f._2)}$$

where $SS_1$ is the sum of the squares error for the single site fit, $SS_2$ is the sum of the squares error for the two site fit, $d.f._1$ is the degrees of freedom for the single site model and $d.f._2$ the degrees of freedom for the two site model (Munson & Rodbard, 1980; Petrash & Bylund, 1986). A two site fit was assumed to describe a displacement curve better than a one site fit if the determined $F$ value had a $P<0.05$.

### *Autoradiography*

Male Wistar rats (280–320 g) were anaesthetized with sodium pentobarbitone (40 mg kg$^{-1}$) and perfused (intracardiac) with 280 ml of ice-cold phosphate (0.01 M) buffered saline (pH 7.4). Brains were rapidly removed, frozen in isopentane at − 40°C and 10 μm thick sections cut on a cryostat at − 15°C. Sections were thaw mounted onto gelatin subbed glass slides, stored at − 70°C for up to three months or used immediately for autoradiography. Additional sections were cut and stained with cresyl-fast violet for histological confirmation of brain structures.

Prior to binding, slide mounted sections were prewashed in buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4) for 30 min at 22°C. Dried sections were then incubated in fresh buffer containing 5 nM [$^3$H]-idazoxan, with or without unlabelled drugs, for 20 min at 22°C. Ascorbate (0.05%) was included in the assay buffer for studies with noradrenaline. Assays were terminated by two 20 s rinses in ice-cold buffer followed by one dip in distilled water (4°C) and rapid drying in a stream of cool air. The radioactivity bound to some sections was then determined by liquid scintillation counting. Other sections were apposed to $^3$H-sensitive film (Hyperfilm, Amersham) with known $^3$H standards for 6–8 weeks before photographic development and computerised densitometry (Quantimet 970, Cambridge Instruments, UK). Binding densities determined in each brain region were corroborated by quantitative analysis of triplicate experiments derived from a minimum of 3 animals. Brains were prepared, sectioned and analysed, to facilitate structural recognition with a Paxinos & Watson (1986) stereotaxic atlas. Resulting autoradiograms may reveal other brain structures where sections were only close to parasagittal or transverse planes relative to the atlas.

### *Protein determinations*

Protein concentrations were determined with Coomassie blue and bovine serum albumin as the standard (Bradford, 1976).

### *Drugs and chemicals*

[$^3$H]-idazoxan (specific activity 40–41 Ci mmol$^{-1}$) and [$^3$H]-RX821002 (specific activity 47.5 Ci mmol$^{-1}$) were produced for Reckitt and Colman by Amersham International.

The following drugs were obtained from external sources; phentolamine mesylate (Research Biochemicals Inc.); rauwolscine hydrochloride (Research Biochemicals Inc.); clonidine hydrochloride (Sigma); (−)-adrenaline (Sigma); noradrenaline (Sigma); yohimbine hydrochloride (Sigma).

Idazoxan, RX811059, RX821002 and RX801023 were synthesized by the Department of Medicinal Chemistry, Reckitt and Colman Products, Hull, UK. All other reagents used were of the highest analytical grade.

## Results

### *Saturation binding studies*

Over the concentration range 0.1–60 nM the binding of both [$^3$H]-idazoxan and [$^3$H]-RX821002 to membranes prepared from rat whole brain was saturable and of high affinity (Figure 1a). Iterative non-linear regression (Figure 1a) and Scatchard analysis (Figure 1b) of the binding isotherms suggested that each ligand was labelling a single population of non-interacting sites. However, the maximal number of binding sites ($B_{max}$) labelled by [$^3$H]-idazoxan was significantly ($P<0.01$) greater than the maximal number of sites labelled by [$^3$H]-RX821002 (Table 1) whereas the equilibrium dissociation constant ($K_D$) for [$^3$H]-RX821002 was significantly ($P<0.05$) less than that for [$^3$H]-idazoxan (Table 1). Nevertheless, at concentrations approximating to their respective $K_D$ values, greater than 90% specific binding was achieved for both ligands.

**Table 1** Specific binding of [$^3$H]-idazoxan and [$^3$H]-RX821002 to rat whole brain membranes

| | *[$^3$H]-idazoxan* | *[$^3$H]-RX821002* |
|---|---|---|
| $B_{max}$ | 328.95 ± 4.31** | 212.46 ± 12.90 |
| $K_D$ | 7.14 ± 1.00 | 1.22 ± 0.25* |

Saturation experiments were performed as described in the methods section. Non-specific binding of [$^3$H]-RX821002 and [$^3$H]-idazoxan was defined in the presence of 10 μM and 1 mM phentolamine respectively. Each value represents the mean ± s.e.mean of observations from five separate determinations performed in triplicate. Data were analysed by iterative non-linear regression analysis (GraphPad Inplot) assuming a one-site model of fit.
**$P<0.01$ compared to the $B_{max}$ for [$^3$H]-RX821002; *$P<0.05$ compared to the $K_D$ for [$^3$H]-idazoxan.

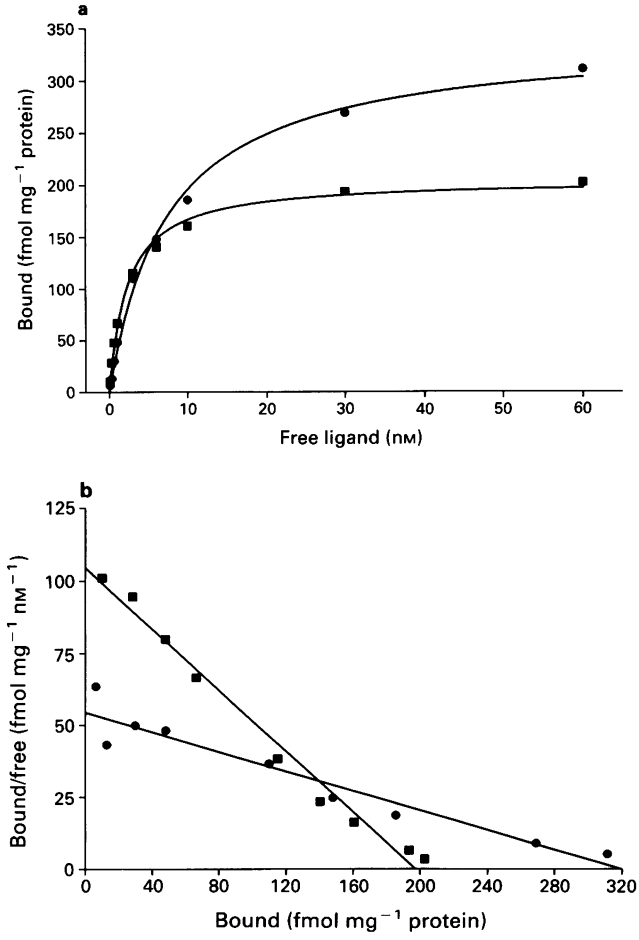


**Figure 1** [$^3$H]-idazoxan (●) and [$^3$H]-RX821002 (■) saturation binding to rat whole brain membranes. (b) Scatchard transformation of the data shown in (a). Incubations were performed for 30 min at 25°C in a Tris HCl buffer (50 mM Tris HCl, 1 mM $MgCl_2$; pH 7.4). Non specific binding at each free ligand concentration was determined with either 10 μM or 1 mM phentolamine for [$^3$H]-RX821002 or [$^3$H]-idazoxan respectively. Both curves describe triplicate results from a single experiment with each ligand performed in parallel. A similar profile was obtained in a further five experiments.

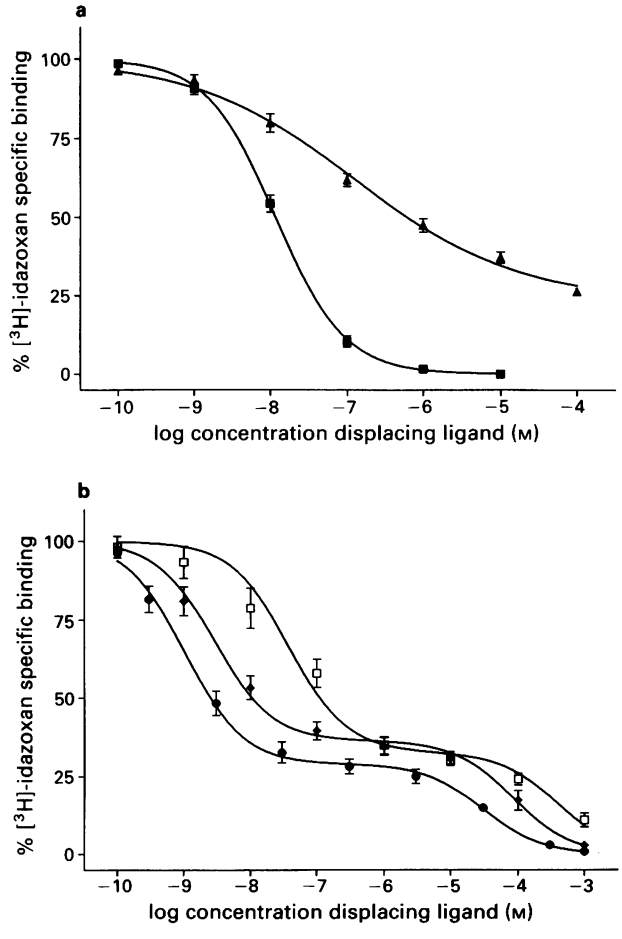


**Figure 2** Concentration-dependent displacement of specific [$^3$H]-idazoxan binding to rat whole brain membranes by α-adrenoceptor agonists and antagonists. (a) Idazoxan (■) and (−)-adrenaline (▲); (b) RX821002 (●), RX811059 (◆) and yohimbine (□). Incubations were performed for 30 min at 25°C in a Tris HCl buffer (50 mM Tris HCl, 1 mM $MgCl_2$; pH 7.4) containing 3–4 nM [$^3$H]-idazoxan. Non specific binding was determined with 1 mM phentolamine. Each point represents the mean (± s.e.mean, vertical bars) of observations from 4 or 5 determinations performed in triplicate.

### Competition binding studies

In competition experiments, a number of α-adrenoceptor agonists and antagonists produced a concentration-dependent inhibition of specific [$^3$H]-idazoxan binding to rat whole brain membranes (Figure 2a,b). The endogenous adrenoceptor agonist (−)-adrenaline inhibited the binding of [$^3$H]-idazoxan in a monophasic fashion (Figure 2a) with a Hill slope substantially less than unity and affinity ($K_i$) consistent with [$^3$H]-idazoxan labelling $\alpha_2$-adrenoceptors (Table 2). Interestingly, however, this displacement was not complete since 23.68 ± 1.11% ($n = 5$) of specific [$^3$H]-idazoxan binding remained in the presence of an excess concentration (100 μM) of (−)-adrenaline (Figure 2a). In contrast, unlabelled idazoxan promoted complete displacement of [$^3$H]-idazoxan binding in a monophasic fashion with a $K_i$ value comparable to its $K_D$ determined in saturation experiments (Table 1) and a Hill slope close to unity (Figure 2a, Table 2).

The displacement of [$^3$H]-idazoxan binding to rat whole brain membranes by the $\alpha_2$-adrenoceptor antagonists, yohimbine, RX821002 and RX811059 was more complex with Hill slopes considerably less than unity (Figure 2b). Computer assisted analysis of the displacement curves indicated that the inhibition of [$^3$H]-idazoxan binding by each antagonist was best described by a two-site model of interaction comprising both a high and low affinity component (Figure 2b, Table 2). Moreover, the proportion of high affinity sites revealed by each antagonist was similar (Figure 2b) and in the range 60–80% (Table 2). The antagonist rank order of potency of RX821002> RX811059> yohimbine derived from the high affinity component in each displacement curve is similar to that determined against the binding of the selective $\alpha_2$-adrenoceptor ligand [$^3$H]-RX821002 in this tissue (Hudson *et al.*, 1992), suggesting that this represents the inhibition of [$^3$H]-idazoxan binding to $\alpha_2$-adrenoceptors. The remaining component (20–40%) in each displacement curve exhibiting only low affinity towards the $\alpha_2$-adrenoceptor antagonists (Figure 2b, Table 2) is then attributable to the displacement of [$^3$H]-idazoxan binding to a non-adrenoceptor site (NAIBS) since a comparable amount of binding was not displaced by an excess concentration of (−)-adrenaline (Figure 2a). A comparison of the antagonist affinities for these two sites reveals that all three are more than 5000 fold selective for $\alpha_2$-adrenoceptors (Table 2).

The displacement of [$^3$H]-idazoxan by its 6-fluoro derivative RX801023 was also best described by a model assuming a two site interaction (Figure 3). Unlike the competition curves obtained with the other $\alpha_2$-adrenoceptor antagonists however, only 20.07 ± 3.11% ($n = 7$) of the sites labelled displayed high affinity for RX801023 ($K_i = 4.79 \pm 0.71$ nM; Figure 3). The remaining 79.93 ± 3.11% of the labelled sites exhibited approximately 54 fold lower affinity for RX801023 ($K_i = 260.71 \pm 25.72$ nM; Figure 3). In concurrent experiments, the displacement of the selective $\alpha_2$-adrenoceptor ligand [$^3$H]-RX821002 by RX801023 was best described a single site model of interaction with a Hill slope close to unity (1.02 ± 0.03, $n = 4$) and a $K_i$ value of 113.46 ± 19.83 nM (Figure 3). The similarity of this latter $K_i$ value to the $K_i$ value determined for the low affinity component in the biphasic displacement of [$^3$H]-idazoxan by RX801023 is again consistent with the suggestion that 60–80% of [$^3$H]-idazoxan binding to this membrane preparation is to $\alpha_2$-adrenoceptors. Moreover, compared to unlabelled idazoxan, RX801023 exhibits enhanced affinity and improved selectivity for the NAIBS component of [$^3$H]-idazoxan binding relative to the $\alpha_2$-adrenoceptor component.

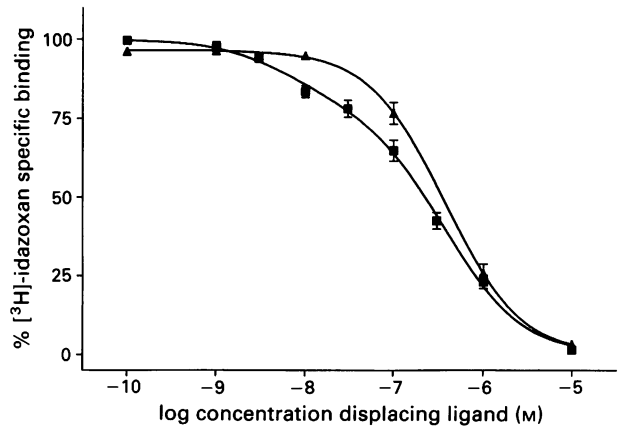


**Figure 3** Concentration-dependent displacement of [$^3$H]-idazoxan (■) or [$^3$H]-RX821002 (▲) binding to rat whole brain membranes by RX801023 (6-fluoro-idazoxan). Incubations were performed for 30 min at 25°C in a Tris HCl buffer (50 mM Tris HCl, 1 mM $MgCl_2$; pH 7.4) containing 3–4 nM [$^3$H]-idazoxan or 2–3 nM [$^3$H]-RX821002. Non specific binding was determined with either 10 μM or 1 mM phentolamine for [$^3$H]-RX821002 and [$^3$H]-idazoxan respectively. Each point represents the mean (± s.e.mean, vertical bars) of observations from 4 or 5 determinations performed in triplicate.

### Autoradiography of [$^3$H]-idazoxan

In preliminary experiments, sections incubated with 5 nM [$^3$H]-idazoxan alone or in the presence of yohimbine (5 μM) or idazoxan (3 μM) were removed from the glass slides and bound radioactivity estimated by liquid scintillation counting (data not shown). In this manner, the washing procedure employed was determined to be optimal for high specific [$^3$H]-idazoxan binding to both $\alpha_2$-adrenoceptors and NAIBS. For other labelled sections, a photographic exposure time of 6 to 8 weeks was chosen as this allowed the detection of NAIBS while the images reflecting $\alpha_2$-adrenoceptor distribution remained within the linear detection phase of the tritium-sensitive film.

Examples of total [$^3$H]-idazoxan (5 nM) binding to sections of brain are shown in Figures 4a, 5 (left hemisections), 6a

**Table 2** Affinity constants ($K_i$) and Hill slopes for the displacement of specific [$^3$H]-idazoxan binding to rat whole brain membranes by α-adrenoceptor agonists and antagonists

| *Ligand* | $K_i$ (nM) | | *nH* | *α2/NAIBS* | n |
|---|---|---|---|---|---|
| Idazoxan | 8.53 ± 1.28 | | 0.97 ± 0.02 | 1 | 5 |
| (−)-Adrenaline | 106.20 ± 29.94 | | 0.42 ± 0.01 | ND | 5 |
| RX821002 | 0.80 ± 0.15 | 21.76 ± 0.97 μM | (71:29)* | 27,200 | 4 |
| RX811059 | 2.32 ± 0.48 | 66.41 ± 1.89 μM | (64:36)* | 28,625 | 4 |
| Yohimbine | 32.39 ± 17.24 | 306.92 ± 129.82 μM | (66:34)* | 9.475 | 5 |

Affinity values were obtained for the displacement of specific 3 nM [$^3$H]-idazoxan binding to rat whole brain membranes prepared as described under methods. RX821002, RX811059, and yohimbine displaced [$^3$H]-idazoxan binding to rat whole brain membranes with Hill slopes (nH) considerably less than unity (*) which could be resolved by computer-assisted curve fitting into high ($\alpha_2$-adrenoceptor) and low (NAIBS) affinity components. For each of these ligands, the proportion of high and low affinity sites differentiated is shown in parentheses. Comparison of $K_i$ values for the high and low affinity [$^3$H]-idazoxan labelled sites reveals each antagonist to be more than 5000 fold selective for $\alpha_2$-adrenoceptors. Each value represents the mean ± s.e.mean of observations from the number of experiments ($n$) performed in triplicate.

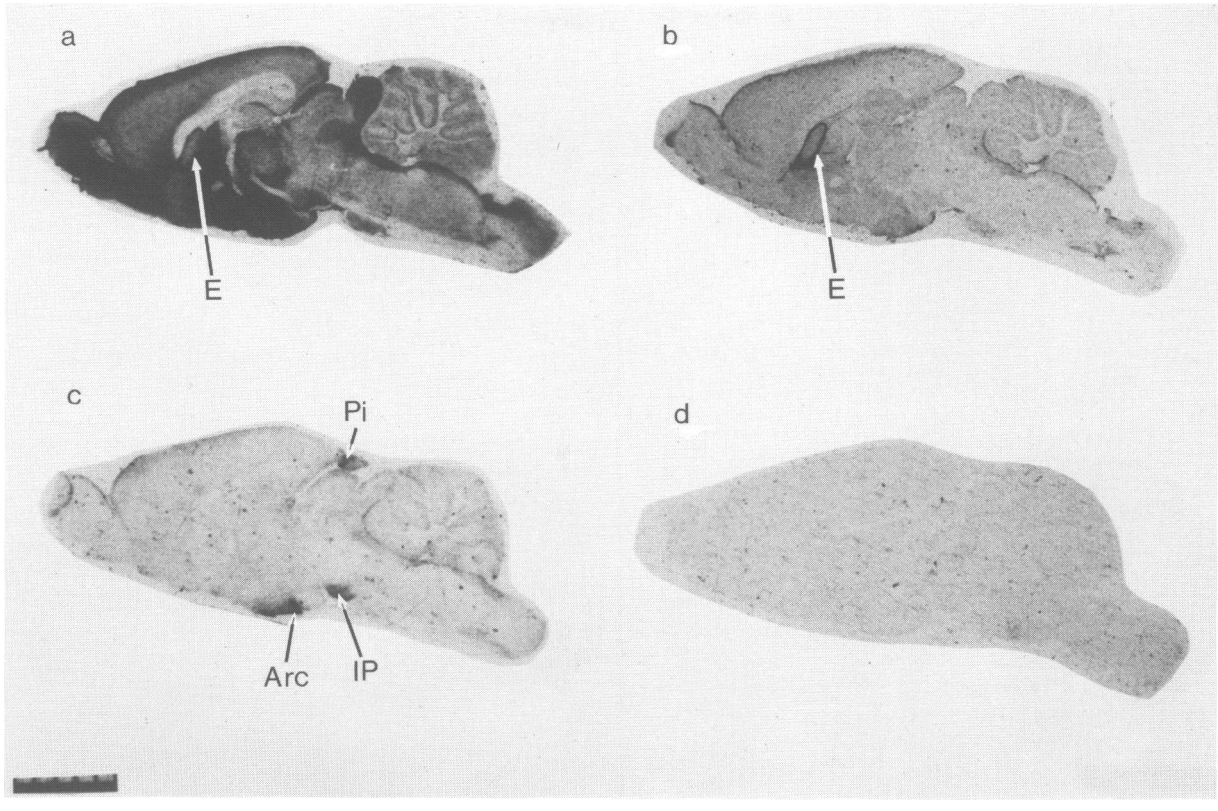


**Figure 4** Autoradiograms of [$^3$H]-idazoxan (5 nM) binding to parasagittal sections of rat brain. Sections (a) and (b) lie approximately 1.2 mm from the midline, whilst (c) and (d) were cut 0.5 mm from the midline. Section (a) demonstrates total binding, (b) is that binding remaining in the presence of rauwolscine (5 μM), (c) is that remaining in the presence of RX821002 (3 μM), whilst (d) represents non-specific binding defined in the presence of unlabelled idazoxan (3 μM). See Table 3 for key to abbreviations. Dark areas depict higher levels of bound ligand. Scale bar = 5 mm.

and 6b. These figures are positive prints of the original autoradiograms, so that dense areas of binding are represented as grey to black areas of silver grains. Non-specific binding as defined by unlabelled idazoxan (3 μM) is shown in Figure 4d and is only slightly darker than normal background fogging.

### *Autoradiographic distribution of NAIBS*

Throughout most regions of the rat brain, rauwolscine (5 μM), yohimbine (5 μM), RX821002 (3 μM), clonidine (3 μM) and noradrenaline (10 μM) each displaced much of the bound [$^3$H]-idazoxan. The remaining uneven images indicating [$^3$H]-idazoxan binding to NAIBS were further reduced in density by coincubation with unlabelled idazoxan (3 μM) or RX-801023 (3 μM). This is demonstrated qualitatively by Figures 4, 5 and 6. Whereas Figure 4a shows total 5 nM [$^3$H]-idazoxan binding to a parasagittal section of rat brain approximately 1.2 mm lateral from the midline, Figure 4b shows a similar section from the same animal incubated with [$^3$H]-idazoxan in the presence of rauwolscine (5 μM) to preclude $\alpha_2$-adrenoceptor binding. Although [$^3$H]-idazoxan binding throughout the section was greatly reduced, a dense area of binding remained over the ependymal layer (E) surrounding the lateral ventricle (Figure 4b). Figure 4c illustrates a section closer to the midline which reveals structures containing [$^3$H]-idazoxan binding insensitive to displacement by RX-821002 (3 μM), namely arcuate nucleus, interpeduncular nucleus and pineal gland. Again, however, this binding to NAIBS was displaced by unlabelled idazoxan (3 μM, Figure 4d), leaving a near undetectable level of non specific binding.

Figure 5 demonstrates [$^3$H]-idazoxan binding to transverse sections of rat brain alone (left hemisections) or in the presence of various $\alpha_2$-adrenoceptor ligands (right hemisections). In rostral areas of brain (Figure 5a) there remained low but detectable amounts of [$^3$H]-idazoxan binding (in the presence of 5 μM yohimbine) to areas of frontal cortex, cingulate cortex and anterior olfactory nucleus. Sections cut through the level of the caudate putamen (Figure 5b, approx bregma − 0.26 mm) show that whilst clonidine (3 μM) displaced much of the bound [$^3$H]-idazoxan (right hemisection) there again remained a residual image. Additionally, dense binding remained around the surface of the 3rd and lateral ventricles corresponding to the ependymal layer and choroid plexus respectively (Figure 5b). Brain sections through bregma − 3.3 mm (Figure 5c) revealed that total [$^3$H]-idazoxan binding was high in the arcuate nucleus although, unlike binding to surrounding brain structures, this was not attenuated in the presence of noradrenaline (10 μM). Likewise, the medial habenular nucleus and ventral medial thalamus also retained a degree of labelling (Figure 5c). Figure 5d illustrates the intense labelling of the interpeduncular nucleus which, as the right hemisection shows, remained in the presence of RX811059 (3 μM), as did a portion of the [$^3$H]-idazoxan bound in the central gray, especially that adjacent to the aqueduct. A more caudal section (bregma − 13.7 mm; Figure 6b,d and f) revealed binding to be high in the area postrema and the nucleus of the solitary tract (Figure 6b) although only the binding in this latter nucleus was prevented by rauwolscine (5 μM; Figure 6d). Figure 6 also demonstrates that the NAIBS component of [$^3$H]-idazoxan binding to brain structures such as the interpeduncular nucleus and area postrema was displaceable by RX801023 (3 μM), consistent with the NAIBS-selectivity of this compound revealed in radioligand binding studies. Thus, total [$^3$H]-idazoxan binding (Figure 6a,b) was preferentially reduced in the presence of RX801023 (3 μM) in the interpeduncular nucleus (Figure 6e) and area postrema

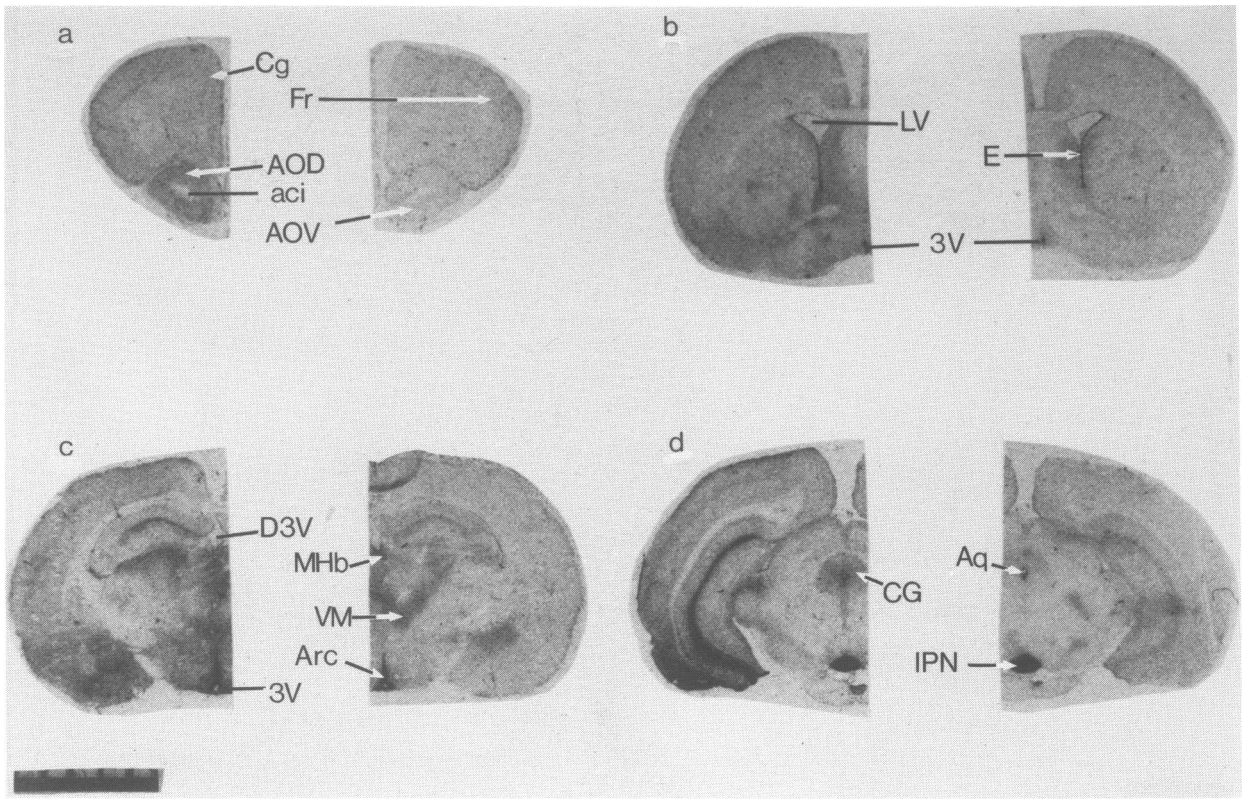


**Figure 5** Autoradiographic distribution of [$^3$H]-idazoxan binding in transverse sections of rat brain. Sections are shown in a rostral to caudal manner taken at (a) bregma 4.2 mm; (b) − 0.26 mm; (c) bregma − 3.3 mm; and (d) bregma − 6.0 mm. At each level the left hemisection demonstrates total [$^3$H]-idazoxan (5 nM) binding, whilst the right hemisections have been co-incubated with (a) clonidine (3 μM); (b) yohimbine (5 μM); (c) noradrenaline (10 μM); and (d) RX811059 (3 μM). Key to abbreviations shown in Table 3. Scale bar = 5 mm.

(Figure 6f), structures in which the binding of [$^3$H]-idazoxan was resistant to displacement by 5 μM rauwolscine (Figure 6c,d). In all autoradiographic studies, [$^3$H]-idazoxan binding which remained in the presence of the various $\alpha_2$-adrenoceptor ligands was reduced to background levels by the addition of RX801023 (3 μM) in an equivalent manner to that found by the addition of unlabelled idazoxan (3 μM).

### *Quantitative autoradiography of [$^3$H]-idazoxan binding*

Table 3 summarises the results of a quantitative analysis of [$^3$H]-idazoxan autoradiography in rat brain. [$^3$H]-idazoxan binding to $\alpha_2$-adrenoceptors was determined as that component displaced by either rauwolscine (5 μM) or RX821002 (3 μM). That bound to NAIBS was identified as [$^3$H]-idazoxan binding remaining in the presence of rauwolscine or RX821002 which was displaceable by either RX801023 (3 μM) or idazoxan (3 μM). The highest density of [$^3$H]-idazoxan binding not displaced by $\alpha_2$-adrenoceptor ligands was in the area postrema (Table 3). The ependyma, interpeduncular and arcuate nuclei also contained high levels of [$^3$H]-idazoxan binding, more of which was insensitive to displacement by rauwolscine or RX821002 than by RX801023 or idazoxan (Table 3). The central gray contained moderate levels of both $\alpha_2$-adrenoceptor and NAIBS binding. Nuclei such as the ventral medial thalamus, hippocampus and entorhinal cortex contained moderate levels of NAIBS binding but were relatively low when compared with the large amount of [$^3$H]-idazoxan binding displaceable by $\alpha_2$-adrenoceptor agonists and antagonists in these regions (Table 3). Most brain nuclei apart from those shown in Table 3 contained low levels of NAIBS binding ($<20$ fmol mg$^{-1}$ wet tissue) and have not therefore been listed. Specific [$^3$H]-idazoxan binding in sections of rat spinal cord was completely displaced by rauwolscine (5 μM).

## Discussion

The present data demonstrate several clear findings. First it confirms recent observations (Brown *et al.*, 1990) that [$^3$H]-idazoxan labels two distinct binding sites in rat brain, namely $\alpha_2$-adrenoceptors and additional non-adrenoceptor binding sites or NAIBS. Secondly, autoradiography reveals that there is marked regional variation in the distribution of these two sites. Thirdly, the close structural analogues of idazoxan, RX821002 and RX811059 are high affinity $\alpha_2$-adrenoceptor antagonists with $\alpha_2$-adrenoceptor/NAIBS selectivity of more than 5000 fold. Conversely, RX801023 is a moderately selective ligand (54 fold) for the NAIBS site with an affinity around 4–5 nM.

On the basis of observations from both the present and previous investigations (Brown *et al.*, 1990) it appears that in rat whole brain homogenates, 70–80% of specific [$^3$H]-idazoxan binding is to $\alpha_2$-adrenoceptors whilst 20–30% is to NAIBS. The technique of autoradiography has provided visual evidence that this ratio of the two sites is not constant throughout the rat brain and that the distribution of NAIBS shows a marked degree of heterogeneity. For example, whilst $\alpha_2$-adrenoceptors were clearly delineated in limbic structures, cortex and olfactory cortex, NAIBS were found to be much less dense. However, NAIBS were clearly visualized in discrete nuclei such as the arcuate and interpeduncular nucleus and pineal gland. A particularly clear demonstration of this differential distribution of $\alpha_2$-adrenoceptors and NAIBS is given in the autoradiograms of the brain stem (Figure 6d,f) where $\alpha_2$-adrenoceptor binding is most dense in the nucleus tractus solitarius and almost absent in the area postrema. Conversely, NAIBS binding shows almost opposite localization, with the area postrema exhibiting the highest density of any brain region. What remains unclear from these studies is whether NAIBS are localized to neuronal and/or glial

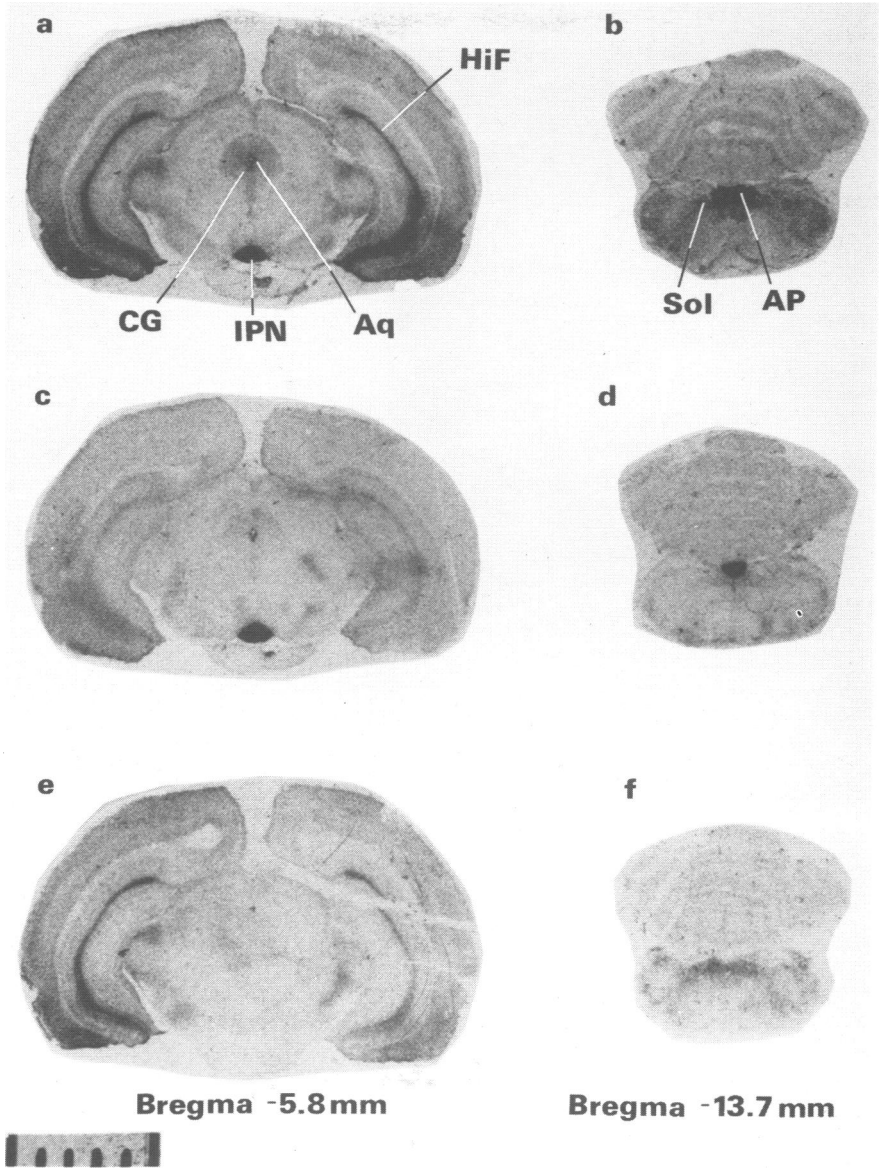


**Figure 6** Autoradiographs of [$^3$H]-idazoxan binding in tranverse sections of rat brain at bregma − 3.3 mm (a,c and e) and at bregma − 13.7 mm (b,d and f). Sections (a) and (b) show total [$^3$H]-idazoxan (5 nM) binding. Sections (c) and (d) demonstrate that binding displaced by rauwolscine (5 μM) whilst sections (e) and (f) show the preferential displacement of binding in those brain nuclei containing NAIBS by RX801023. For key to abbreviations see Table 3. Scale bar = 5 mm.

elements or some other cell type. Possibly the high density of NAIBS in the pineal gland, on or close to ventricle surfaces, for instance around the 3rd ventricle, the aqueduct and associated with the ependyma lining the lateral ventricle, indicate a non-neuronal cell type. Interestingly, the distribution of NAIBS shows a striking resemblance to that of peripheral type benzodiazepine sites in rat brain revealed in autoradiographic studies with [$^3$H]-PK11195 (Benavides *et al.*, 1983). However, whilst it is widely accepted that central [$^3$H]-PK11195 binding is primarily associated with glial and non-neuronal elements, assigning NAIBS to a non-neuronal location based on autoradiographical comparisons alone would be premature. Further studies will be needed to clarify their exact cellular location in the brain, possibly utilizing discrete chemical and electrolytic lesions. Interestingly, however, localization of NAIBS to mitochondria has been postulated on the basis of binding studies with subcellular fractions of peripheral tissues (Lachaud-Pettiti *et al.*, 1991; Tesson *et al.*, 1991).

The differential distribution of [$^3$H]-idazoxan binding to $\alpha_2$-adrenoceptors and NAIBS may help explain many of the actions of this drug. It markedly increases noradrenaline turnover in rat brain (Walter *et al.*, 1984) in regions which possess a high density of $\alpha_2$-adrenoceptors. It seems likely that the antidepressant actions of idazoxan are mediated through an action at $\alpha_2$-adrenoceptors (Osman *et al.*, 1989; see Dickinson, 1991) although confirmation of this will depend on demonstrating clinical efficacy with drugs such as RX811059 which show low affinity for NAIBS. There are effects of idazoxan that are not mimicked by the selective $\alpha_2$-adrenoceptor antagonists RX821002 and RX811059 which may suggest they are mediated by NAIBS. For instance, idazoxan stimulation of eating in rats (Jackson *et al.*, 1991b). This is consistent with the high density of NAIBS in both the area postrema and arcuate nucleus, two regions intimately involved in the control of food intake (see Blundell, 1991). Similarly, idazoxan does not share the typical proconvulsant profile of other $\alpha_2$-adrenoceptor antagonists and can even lower seizure threshold in mice (Jackson *et al.*, 1991a). This could perhaps be mediated in the hippocampus where NAIBS were located to the surface of hippocampal fissure between the molecular layer of the dentate gyrus and the lacunosum moleculare layer (Figure 6c). The finding that RX801023 is relatively a selective ligand for NAIBS will allow the evaluation of these possibilities.

The distribution of [$^3$H]-idazoxan binding in rat brain

**Table 3** Quantitative autoradiographic analysis of [$^3$H]-idazoxan binding to discrete areas of rat brain

| *Brain region* | *Figure symbol* | *$\alpha_2$-adrenoceptors* (fmol mg$^{-1}$ wet tissue) | *NAIBS* (fmol mg$^{-1}$ wet tissue) |
|---|---|---|---|
| Anterior olfactory nucleus | | | |
| dorsal | AOD | 235.1 ± 6.4 | 17.7 ± 0.9 |
| ventral | AOV | 206.5 ± 22.2 | 34.9 ± 1.7 |
| anterior commissure, intrabulbar | aci | | |
| Frontal cortex | Fr | 106.5 ± 6.4 | 24.9 ± 0.7 |
| Cingulate cortex | Cg | 54.3 ± 3.2 | 29.8 ± 1.6 |
| Entorhinal cortex | | 95.1 ± 12.2 | 18.6 ± 4.5 |
| Corpus callosum | | 23.3 ± 5.9 | 4.6 ± 2.0 |
| Caudate putamen | | 56.3 ± 3.2 | 29.7 ± 2.1 |
| Choroid plexus | CP | 12.7 ± 6.0 | 62.3 ± 5.6 |
| Ependymal layer | E | 47.2 ± 31.5 | 101.9 ± 8.1 |
| Hippocampal fissure | HiF | 110.6 ± 30.2 | 61.6 ± 8.2 |
| Arcuate nucleus | Arc | 14.5 ± 1.1 | 102.2 ± 3.3 |
| Lateral hypothalamic area | | 74.1 ± 2.5 | 20.0 ± 2.4 |
| Ventromedial hypothalamic nu | VM | 56.7 ± 6.5 | 40.3 ± 6.4 |
| Central gray | CG | 51.2 ± 1.4 | 27.5 ± 2.8 |
| Superior colliculus | | 73.1 ± 12.7 | 47.9 ± 11.2 |
| Inferior colliculus | | 101.5 ± 9.8 | 16.6 ± 0.7 |
| Interpeduncular nucleus | IPN | 55.1 ± 7.1 | 123.4 ± 12.7 |
| Medial habenular nucleus | MHb | 72.9 ± 5.1 | 45.4 ± 5.9 |
| Lateral habenular nucleus | | 35.8 ± 8.6 | 27.6 ± 1.4 |
| Nuc solitary tract | Sol | 113.6 ± 5.0 | 22.5 ± 3.7 |
| Area postrema | AP | 18.9 ± 18.4 | 208.2 ± 18.7 |
| Aqueduct | Aq | | |
| surface of the aqueduct | | 27.2 ± 7.3 | 134.8 ± 18.2 |
| Dorsal third ventricle | D3V | | |
| Lateral ventricle | LV | | |

[$^3$H]-idazoxan binding to $\alpha_2$-adrenoceptors was taken to be that displaced by rauwolscine (5 $\mu$M) or RX821002 (3 $\mu$M) whereas that bound to NAIBS was that remaining but displaceable by RX801023 (3 $\mu$M) or idazoxan (3 $\mu$M). Data represent the mean ± s.e.mean of 3–5 animals, with triplicate determinations in three sections from each rat brain.

shows considerable overlap with that found in the human brain (De Vos *et al.*, 1991). However, there are several intriguing differences. First, the human brain has a relatively higher ratio of NAIBS to $\alpha_2$-adrenoceptors, with about an equal density of each. In addition, the regions exhibiting the highest NAIBS density in human brain are the dopamine-rich areas such as substantia nigra and caudate nucleus which may explain the therapeutic benefit of idazoxan in the neurodegenerative extrapyramidal condition of progressive supranuclear palsy (Ghika *et al.*, 1991). However, the rat contains sufficient NAIBS binding to allow the opportunity for evaluating the possible role of these binding sites in brain function.

In conclusion, the present results provide further evidence that [$^3$H]-idazoxan can be employed to characterize NAIBS in the rat brain. Moreover, by use of selective $\alpha_2$-adrenoceptor ligands to preclude [$^3$H]-idazoxan binding to $\alpha_2$-adrenoceptors, NAIBS can be visualized in discrete nuclei using *in vitro* autoradiography. In combination with the use of a selective ligand such as RX801023, this localization may provide an opportunity to explore possible functions for these binding sites.

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