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Characterisation and localisation of [^{3}H]2-(2-benzofuranyl)-2-imidazoline binding in rat brain: a selective ligand for imidazoline I_2 receptors

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Abstract

In rat whole brain homogenates, saturation binding analysis revealed that both [^{3}H]2-BFI (2-(2-benzofuranyl)-2-imidazoline) and [^{3}H]idazoxan (in the presence of 5 μM rauwolscine) bound with high affinity to an apparent single population of sites. However, the K_d for [^{3}H]2-BFI (1.74 ± 0.14 nM) was significantly ($P < 0.01$) less than that for [^{3}H]idazoxan (10.4 ± 2.68 nM). In competition studies idazoxan, 2-BFI, BU224 (2-(4,5-dihydroimidaz-2-yl)-quinoline), amiloride and guanabenz displayed high affinity (K_i values = 7.32, 1.71, 2.08, 21.80 and 14.90 nM, respectively) for 70–80% of sites, and low μM affinity for the remaining 20–30% of sites labelled by [^{3}H]2-BFI. In contrast, several α_2-adrenoceptor, imidazoline I_1 receptor and histamine receptor ligands exhibited only micromolar affinity for the [^{3}H]2-BFI labelled site. Quantitative receptor autoradiography revealed high binding by [^{3}H]2-BFI to discrete brain nuclei, notably the area postrema, interpeduncular nucleus, arcuate nucleus, mammillary peduncle, ependyma and pineal gland. These data indicate that [^{3}H]2-BFI recognises imidazoline I_2 receptors in rat brain with higher affinity and selectivity than [^{3}H]idazoxan and thus represents a superior radioligand to [^{3}H]idazoxan for the study of imidazoline I_2 receptors.

Keywords: Imidazoline I_2 receptor; [^{3}H]2-BFI; Autoradiography; Brain, rat; Idazoxan; Arcuate nucleus

1. Introduction

Extensive examination of the binding characteristics and distribution of the imidazoline radioligands [^{3}H]clonidine, [^{3}H]p-aminoclonidine and [^{3}H]idazoxan, as well as the biochemical and functional effects of imidazolines and related compounds has led to a widely accepted view that at least two principle classes of imidazoline (I) receptors now exist, designated I_1 and I_2 (for review see Parini et al., 1996). Imidazoline I_1 receptors are recognised as those labelled by [^{3}H]clonidine and [^{3}H]p-aminoclonidine and imidazoline I_2 receptors are recognised as those labelled by [^{3}H]idazoxan (Ernsberger et al., 1987; Kamisaki et al., 1990; Mallard et al., 1992). Both imidazoline I_1 and I_2 receptors show a high nanomolar affinity for cirazoline and idazoxan, but are distinguished from each other by the low affinity (> micromolar) of clonidine, rilmenidine, moxonidine, efaroxan, imidazole-4-acetic acid and cimetidine for the imidazoline I_2 receptor and the low affinity (μM) of the guanadides, guanabenz and amiloride, for the imidazoline I_1 receptor. Moreover, Ernsberger further subdivided the imidazoline I_2 receptor into I_{2A} and I_{2B} according to differential affinity for amiloride in different species and tissues (see Parini et al., 1996). Biochemical studies have purified various imidazoline binding proteins of 27–85 kDa, from several tissues and species, which clearly indicate the existence of a heterogeneity of imidazoline binding proteins, which is in agreement with previous pharmacological studies (Wang et al., 1992; Limon et al., 1992; Lanier et al., 1993; Escribá et al., 1994, 1995; Greney et al., 1994). Interestingly, some of these groups have more recently disclosed an association of imidazoline I_2 receptors and imidazoline receptor binding proteins with monoamine oxidase (for review see Parini et al., 1996). Furthermore binding and functional studies have revealed the existence of additional imidazoline receptors which cannot be grouped into I_1, I_{2A} or I_{2B} subtypes (Chan et al., 1994; Olmos et al., 1994a; King et al., 1992, 1995).

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For many years [^{3}H]idazoxan has proved invaluable as the radioligand for the study of both α_2-adrenoceptors and imidazoline I_2 receptors in rat brain (Boyajian et al., 1987; Bruning et al., 1987; Mallard et al., 1992; Olmos et al., 1996). This dual identity of idazoxan has limited the characterisation of imidazoline I_2 receptors, as the inclusion of α_2-adrenoceptor antagonists is required to study imidazoline I_2 receptors in isolation (Mallard et al., 1992). More recently new selective radioligands for imidazoline I_2 receptors have been developed. The azido derivative of cirazoline, AZIPI (2,[3-amino-4-{^{125}I}iodophenoxy]methyl imidazoline), has been used to purify three different putative imidazoline binding proteins of three different apparent molecular weights, two of which correspond with the molecular weights of monoamine oxidase-A and monoamine oxidase-B (Lanier et al., 1993) and whose distribution mirrors that of monoamine oxidase-B in rat brain (Saura et al., 1992). Also [^{3}H]RS-45041-190 (4-chloro-2-(imidazolin-2-yl)-isoindolene) has been characterised and identified as a radioligand that has a higher affinity and selectivity for imidazoline I_2 receptors in rat kidney, compared with non-adrenoceptor [^{3}H]idazoxan binding in rat kidney and brain (Mackinnon et al., 1995). Furthermore, these workers have revealed a correlation of > 0.95 between the affinities of several competitors for [^{3}H]RS-45041-190 binding in rat kidney and non-adrenoceptor [^{3}H]idazoxan binding in rat kidney and brain. In addition the distribution of [^{3}H]RS-45041-190 binding in rat brain has been shown to substantially overlap the previously reported distribution of [^{3}H]idazoxan labelling of imidazoline I_2 receptors in rat brain (Mallard et al., 1992).

The 2-benzofuranyl-derivative of idazoxan, 2-BFI (2-(2-benzofuranyl)-2-imidazoline) has been found to possess a high affinity for imidazoline I_2 receptors and has an improved I_2: α_2 selectivity profile in rat brain, such that it is $>$ 1500-fold selective (Hudson et al., 1997). [^{3}H]2-BFI has recently been characterised as a selective ligand for imidazoline I_2 receptors in rabbit brain and kidney, and found to be a superior ligand to [^{3}H]idazoxan for the study of these receptors (Lione et al., 1996; Hosseini et al., 1997; Alemany and García-Sevilla, 1997). We have now characterised the binding of [^{3}H]2-BFI in rat brain, and used quantitative autoradiography to confirm the distribution of central I_2 receptors in this species (Mallard et al., 1992).

2. Materials and methods

2.1. Membrane preparation

Male Wistar rats (240–300 g) were sacrificed by stunning followed by decapitation. Whole brains were immediately removed over ice and homogenised in 10 volumes (w/v) of buffered sucrose (0.32 M in 50 mM Tris–HCl, pH 7.4 at 4°C) using a motor driven Teflon-glass homogeniser. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C. The resultant supernatants were pooled and recentrifuged at $32\,000 \times g$ for 20 min at 4°C. The supernatants were discarded and each pellet resuspended in 10 volumes of assay buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.4 at 4°C) and spun at $32\,000 \times g$ for 20 min at 4°C. The pellets were washed twice by repeated centrifugation at $32\,000 \times g$ for 20 min at 4°C. The final pellets were stored at -70°C until use. Prior to radioligand binding studies, membrane pellets were thawed and washed a further 3–4 times by resuspension in 10 volumes of assay buffer (as above) and repeated centrifugation ($32\,000 \times g$), to remove any possible endogenous inhibitors of binding. The protein content of the membrane preparations was determined utilising Coomassie blue and bovine serum albumin as the standard (Bradford, 1976).

2.2. Kinetic binding studies

Radioligand binding studies used the method of Lione et al. (1996). All binding experiments were performed at 25°C in the aforementioned assay buffer. Association binding studies were performed by incubating membrane aliquots (300–450 μg; 750 μl) with 1 nM [^{3}H]2-BFI, for periods of time ranging from 30 s to 120 min. After equilibrium was reached (40 min) dissociation was initiated by the addition of 10 μM 6-fluoro-idazoxan (RX801023) for periods of time ranging from 30 s to 60 min. In the dissociation experiments non-specific binding represented the binding remaining after 120 min incubation in the presence of 10 μM 6-fluoro-idazoxan. All data points were quadruplicate determinations. Bound and free radioactivity were separated by vacuum assisted rapid filtration through pre-soaked (0.5% polyethyleneimine) glass-fibre filters (Whatman GF/B) using a Brandel M-24 cell harvester. Filters were then washed twice with 5 ml of ice-cold assay buffer and membrane-bound radioactivity remaining on the filters was determined by liquid scintillation counting (Wallac 1409 β-counter).

2.3. Saturation binding studies

Membrane aliquots and 12 concentrations of [^{3}H]2-BFI and [^{3}H]idazoxan (in the presence of 5 μM rauwolscine to preclude binding to α_2-adrenoceptors) over the range 0.01–5 nM and 0.01–50 nM, respectively were incubated, in triplicate, to equilibrium (40 min) in a final volume of 1 ml. Specific binding was defined at each free radioligand concentration with either 10 μM 6-fluoro-idazoxan or BU224 (2-(4,5-dihydroimidaz-2-yl)-quinoline; Mallard et al., 1992; Hudson et al., 1994).

2.4. Competition binding studies

The ability of various drugs to displace [^{3}H]2-BFI (1 nM) binding was assessed by use of at least 10 concentra-

tions ranging between 0.01 nM to 0.1 mM in a final volume of 1 ml. Specific binding was defined with 10 μM 6-fluoro-idazoxan or BU224. Ascorbate (0.05%) was included in the assay buffer for competition studies with (−)-noradrenaline to prevent its degradation. All concentration points were performed in triplicate.

2.5. Analysis of binding studies

Results from the kinetic, saturation and competition binding studies were analysed by iterative non-linear regression curve-fitting procedures (GraphPAD Prism, version 2.01) capable of fitting data to one or two site models of binding. Each experiment was analysed independently. The IC_{50} (concentration of drug displacing 50% specific binding) was converted to the inhibition constant (K_i) by the equation of Cheng and Prussof (1973). All displacement curves were initially analysed assuming a one site model of binding. Displacement curves with Hill coefficients (n_H) significantly less than unity were reanalysed assuming a two site model of high and low affinity binding. The *F*-test was used to statistically analyse whether the more complex two site model was a significantly better ($P < 0.05$) fit than the simpler one site model (Munson and Rodbard, 1980).

2.6. [^{3}H]2-BFI and [^{3}H]idazoxan autoradiography

Male Wistar rats (240–300 g) were anaesthetised with sodium pentobarbitone (60 mg kg^{-1} i.p.) and perfused (intra-cardiac) with ice-cold phosphate (0.01 M) buffered saline (pH 7.4; 1 ml g^{-1}). Brains were removed and rapidly frozen in isopentane cooled on dry ice. Sections of frozen brain were cut (12 μm thick; transverse or parasagittal in the plane of the atlas of Paxinos and Watson, 1986) using a cryostat (2800 Frigocut, Reichert-Jung, Germany) at −16°C to −20°C and thaw-mounted on to gelatin-coated glass microscope slides and stored at −70°C until used.

Conditions for the autoradiography of [^{3}H]2-BFI and [^{3}H]idazoxan were based on the previously published methods of Mallard et al. (1992). Prior to binding, sections were thawed and prewashed for 30 min at room temperature (21–25°C) in a 50 mM Tris–HCl buffer containing 1 mM $MgCl_2$ (pH 7.4) and dried under a stream of cool air. Sections were incubated in 200 μl of assay buffer containing either 0.5 nM [^{3}H]2-BFI or 5 nM [^{3}H]idazoxan (in the presence of 5 μM rauwolscine) for 40 min at room temperature. Specific binding for each ligand was defined with 10 μM 6-fluoro-idazoxan or BU224. Assays were terminated by aspirating off the free ligand and the sections washed by two 20 s rinses in ice-cold assay buffer, followed by a dip rinse in ice-cold distilled water. Sections were then rapidly dried under a stream of cool air. The sections were apposed to [^{3}H]-Hyperfilm (Amersham, UK) with ^{3}H-microscale standards (Amersham), in X-ray cassettes, at room temperature for 6 weeks. The films were developed in Ilford High Contrast developer for 3–4 min, immersed in an acetic acid (0.01%) stop-bath for 20 s, fixed with Ilford Hypam fixer plus Ilford rapid hardener for 6–8 min, washed for 20 min in distilled water with wetting agent to prevent spotting and allowed to air dry. The films were then quantified by computer-assisted densitometry (Quantimet 970, Cambridge Instruments, UK) and values converted to fmol ^{3}H-ligand/mg wet tissue equivalent using calibrated [^{3}H]-microscale standards (Amersham). For each brain region analysed, three densitometric determinations were made from each section.

2.7. Drugs and chemicals

[^{3}H]2-BFI and [^{3}H]idazoxan were synthesised to a specific activity of 58 Ci $mmol^{-1}$ and 45 Ci $mmol^{-1}$ by Amersham International (UK) and stored at 4°C and −20°C, respectively.

The following drugs were used: amiloride, histamine, clorgyline, naphazoline, clonidine, (−)-noradrenaline, urea, putriscene, D-medetomidine, L-medetomidine, UK 14,304 (Sigma); rauwolscine (Research Biochemicals); moxonidine (UCB-Pharma, Belgium); idazoxan, efaroxan, 6-fluoro-idazoxan (RX801023), guanabenz acetate (Reckitt and Colman Products, UK); 2-BFI (2-(2-benzofuranyl)-2-imidazoline), BU224 (2-(4,5-dihydroimidaz-2-yl)-quinoline), BU239 ((2-(4,5-dihydroimidaz-2-yl)-quinoxaline; Prof. J. Lewis, Dept. of Chemistry, University of Bristol); agmatine (Aldrich Chemical, UK); RS-45041-190 (4-chloro-2-(imidazolin-2-yl)-isoindoline; Tocris Cookson, UK).

3. Results

3.1. Characterisation of [^{3}H]2-BFI and [^{3}H]idazoxan binding to rat whole brain membranes

3.1.1. Kinetics binding studies

The association of [^{3}H]2-BFI (1 nM) binding to rat whole brain membranes was rapid, with equilibrium being reached within 20 min and remained at equilibrium for a further 2 h (Fig. 1). Consequently an incubation time of 40 min was chosen for future experiments. On the addition of 10 μM 6-fluoro-idazoxan, the specific binding of [^{3}H]2-BFI was rapidly and fully dissociated (Fig. 1). Four independent studies followed the association and dissociation time course of [^{3}H]2-BFI. Based on the *F*-test, the data from all experiments were better fitted to a single exponential phase of binding (*F*-test; $P < 0.05$). The analysis revealed an apparent association rate constant of $K_{obs} = 0.59 \pm 0.06$ min^{-1} and $t_{1/2} = 1.21 \pm 0.13$ min, and a dissociation rate constant of $K_2 = 0.97 \pm 0.09$ min^{-1} and $t_{1/2} = 0.73 \pm 0.06$ min. The K_d value was calculated based

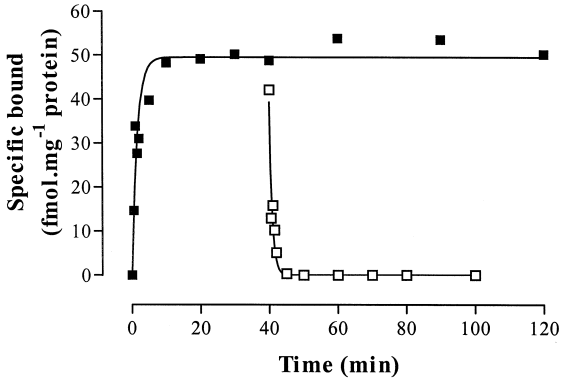

Fig. 1. Kinetic study of the specific binding of [^{3}H]2-BFI (1 nM) to rat whole brain membranes. Time course of the association and dissociation. The data represent a single experiment performed in quadruplicate. Essentially similar data were obtained for a further three experiments (see text for mean values). Dissociation was initiated by the addition of 10 μM 6-fluoro-idazoxan after 40 min. Non-specific binding represented the binding remaining after 120 min in the presence of 10 μM 6-fluoro-idazoxan.

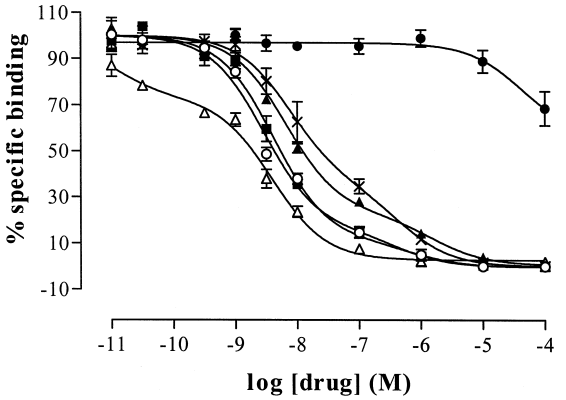

Fig. 3. Concentration-dependent displacement of specific [^{3}H]2-BFI binding to rat whole brain membranes by 2-BFI (○), idazoxan (×), 6-fluoro-idazoxan (▲), BU224 (■), RS-45041-190 (△), and agmatine (●). All data were better fitted to a two-site model of low and high affinity binding, except for agmatine which was better fitted to a one-site model (GraphPAD Prism). Specific binding was defined with either 10 μM 6-fluoro-idazoxan or BU224. The data represent the mean ± S.E.M. (vertical bars) of 3 or 4 experiments each performed in triplicate.

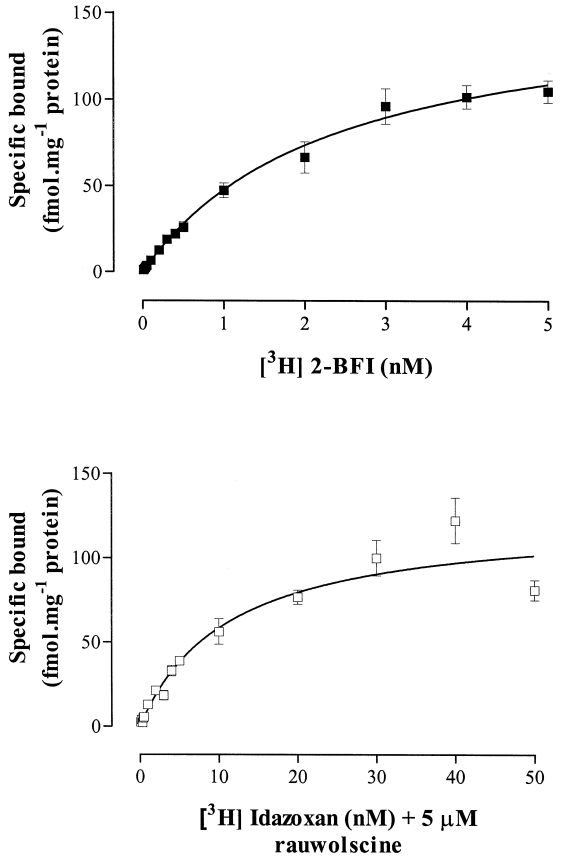

Fig. 2. Saturation of [^{3}H]2-BFI (upper panel) and [^{3}H]idazoxan (lower panel: in the presence of 5 μM rauwolscine) specific binding to rat whole brain membranes. Analyses of the saturation data by non-linear regression (GraphPad Prism, version 2.01) resolved a single high affinity binding site for both radioligands. Specific binding at each free radioligand concentration was defined with either 10 μM BU224 or 6-fluoro-idazoxan for [^{3}H]2-BFI and [^{3}H]idazoxan. Both curves describe triplicate results from 6 experiments (mean ± S.E.M.; vertical bars) with each radioligand performed in parallel.

Table 1
Inhibition constants (K_i, nM) and Hill slopes for compounds against [^{3}H]2-BFI (1 nM) binding to rat whole brain membranes

Compound	K_i (high), nM	K_i (low), nM	% high	n_H
2-BFI	1.71 ± 0.24	242 ± 106	82 ± 3	0.81 ± 0.12[a]
Idazoxan	7.32 ± 1.72	411 ± 74	68 ± 4	0.68 ± 0.04[a]
6-fluoro-idazoxan	3.56 ± 0.43	1571 ± 454	77 ± 3	0.68 ± 0.04[a]
amiloride	21.8 ± 5.80	1231 ± 605	45 ± 15	0.64 ± 0.06[a]
naphazoline	1.68 ± 0.40	849 ± 110	55 ± 6	0.27 ± 0.11[a]
guanabenz	14.9 ± 5.72	768 ± 446	77 ± 3	0.75 ± 0.06[a]
BU224	2.08 ± 0.57	561 ± 274	80 ± 9	0.93 ± 0.16[a]
BU239	4.30 ± 0.68	789 ± 179	59 ± 4	0.48 ± 0.02[a]
RS-45041-190	0.0068 ± 0.0026	2.58 ± 0.53	27 ± 2	0.77 ± 0.03[a]
clonidine	3.16 ± 0.80	3602 ± 1,619	27 ± 4	0.30 ± 0.06[a]
moxonidine	54 273 ± 17,018		–	0.48 ± 0.09
efaroxan	> 100 000		–	0.90 ± 0.02
UK 14,304	35.2 ± 8.90	3157 ± 383	63 ± 6	0.60 ± 0.05[a]
D-medetomidine	66.0 ± 11.0	11 436 ± 4787	46 ± 1	0.45 ± 0.03[a]
L-medetomidine	56.4 ± 32.8	> 100,000	44 ± 18	0.55 ± 0.01[a]
clorgyline	11.7 ± 3.20	6767 ± 1505	46 ± 3	0.35 ± 0.06 [a]
rauwolscine	> 100 000		–	> 1.0
(−)-noradrenaline	60 075 ± 8692		–	0.78 ± 0.11
histamine	> 100 000		–	0.76 ± 0.15
agmatine	> 100 000		–	> 1.0
urea	> 100 000		–	> 1.0
putriscene	> 100 000		–	> 1.0

Inhibition constants, Hill slopes and percentage of high affinity sites were obtained for the displacement of 1 nM [^{3}H]2-BFI binding to rat whole brain membranes by a range of compounds (0.01 nM–0.1 mM) as described under Section 2.

[a]denotes that iterative non-linear regression analyses (GraphPAD Prism software, version 2.01) revealed these data best fitted to a two-site model of high and low affinity binding, as compared to a one-site model using the F-test, $P < 0.05$.

Each value represents the mean ± S.E.M. from 3 or 4 experiments each performed in triplicate.

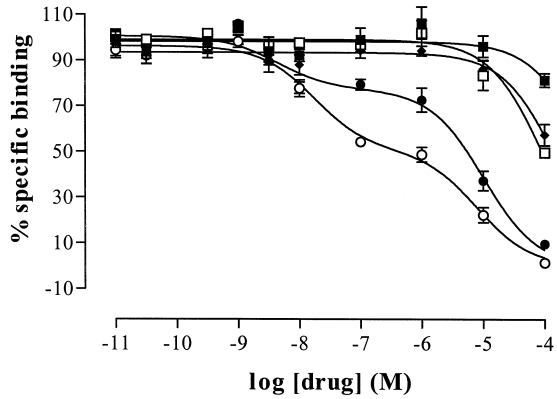

Fig. 4. Concentration-dependent displacement of specific [^{3}H]2-BFI (1 nM) binding to rat whole brain membranes by the α_2-adrenoceptor antagonists, rauwolscine (■) and efaroxan (◆), the α_2-adrenoceptor agonists, (−)-noradrenaline (□) and clonidine (●) and the monoamine oxidase inhibitor, clorgyline (○). For clorgyline and clonidine the data were better fitted to a two-site model of high and low affinity binding (GraphPAD Prism). Non-specific binding was determined with either 10 μM 6-fluoro-idazoxan or BU224. The data represent the mean ± S.E.M. (vertical bars) of 3 or 4 experiments each performed in triplicate.

on the percentage of rapidly associating and dissociating components, thus a K_d value of 1.64 ± 0.28 was obtained.

3.1.2. Saturation binding studies

Over the concentration range 0.01–5 nM and 0.01–50 nM the specific binding of [^{3}H]2-BFI and [^{3}H]idazoxan (in the presence of 5 μM rauwolscine), respectively, was saturable and of high affinity in rat brain membranes (Fig. 2). Iterative non-linear regression analysis of the saturation binding curves yielded B_{max} values of 144 ± 4.8 and 128 ± 12 fmole mg^{-1} protein and K_d values of 1.74 ± 0.14 and 10.4 ± 2.68 nM for specific [^{3}H]2-BFI and [^{3}H]idazoxan binding (in the presence of 5 μM rauwolscine), respectively, revealing that both radioligands bound to a single population of non-interacting sites and a similar maximal number of sites. However, the equilibrium dissociation constant (K_d) for [^{3}H]2-BFI was significantly less ($P < 0.01$) than that for [^{3}H]idazoxan. At concentrations approximating the K_d value, greater than 80% specific binding was achieved for both [^{3}H]2-BFI and [^{3}H]idazoxan, as defined with 10 μM 6-fluoro-idazoxan or BU224.

3.1.3. Competition binding studies

For the competition experiments 1 nM [^{3}H]2-BFI was used, which is close to the K_d of the high affinity site, a concentration predicted to label mainly high affinity binding sites. In competition experiments several imidazoline and guanadino compounds known to have high affinity for imidazoline I_2 receptors, produced a concentration-dependent inhibition of specific [^{3}H]2-BFI binding to rat whole brain homogenates (Fig. 3). For instance, unlabelled 2-BFI

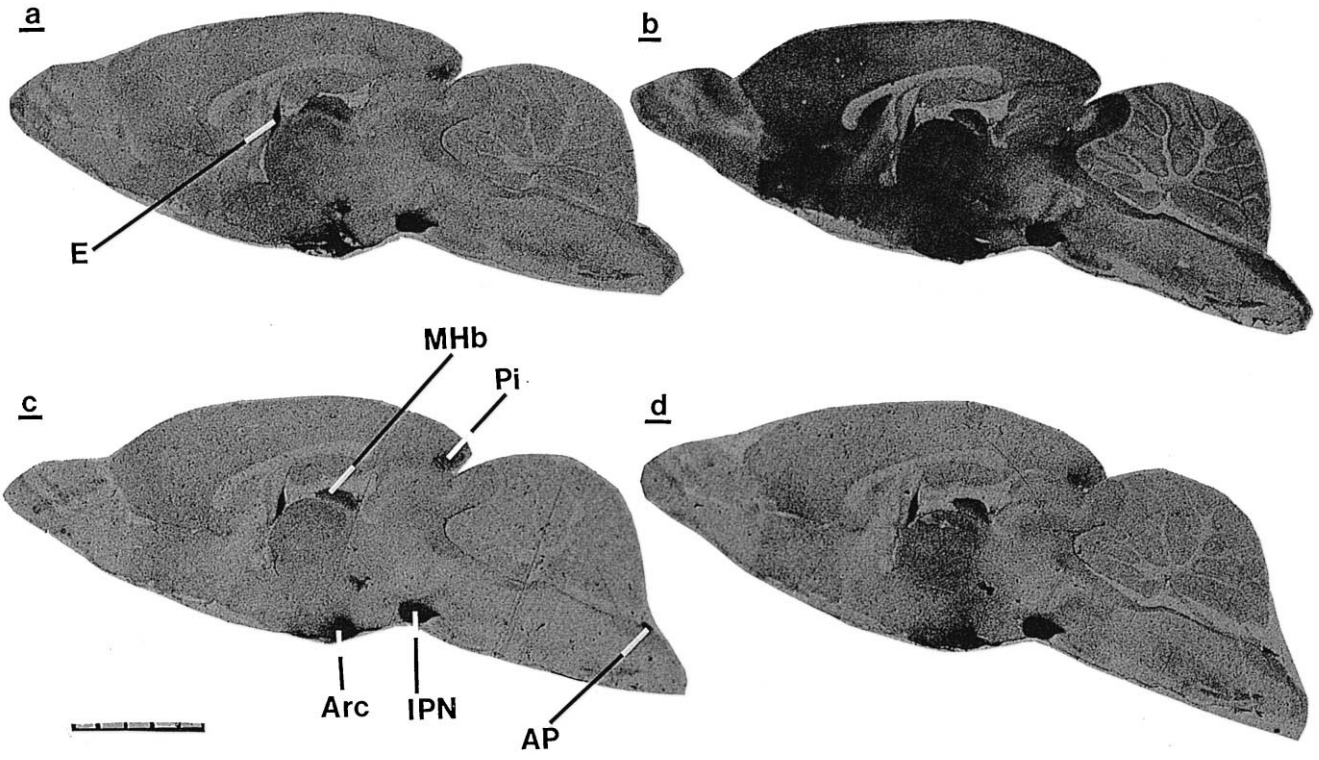

Fig. 5. Autoradiographic distribution of 0.5 nM [^{3}H]-BFI (sections a and c) and 5 nM [^{3}H]idazoxan (sections b and d) binding in parasagittal sections of rat brain. Sections were cut 0.4 mm from the midline. Sections a and b represent total ligand binding and sections c and d represent total ligand binding remaining in the presence of rauwolscine (5 μM). See Table 2 for key to abbreviations. Figures are positive prints of the original autoradiograms, so that dense areas of binding are represented as grey to black areas of silver grains. Scale bar = 5 mm.

showed high affinity ($K_i = 1.71 \pm 0.24$ nM) closely in agreement with the K_d of its labelled form (Fig. 2), and a Hill slope of 0.81 ± 0.12. The imidazoline I_2 receptor compounds: idazoxan, 6-fluoro-idazoxan, BU224, BU239, naphazoline and RS-45041-190 displaced [^{3}H]2-BFI binding with high affinity (Fig. 3; Table 1). Similarly, the

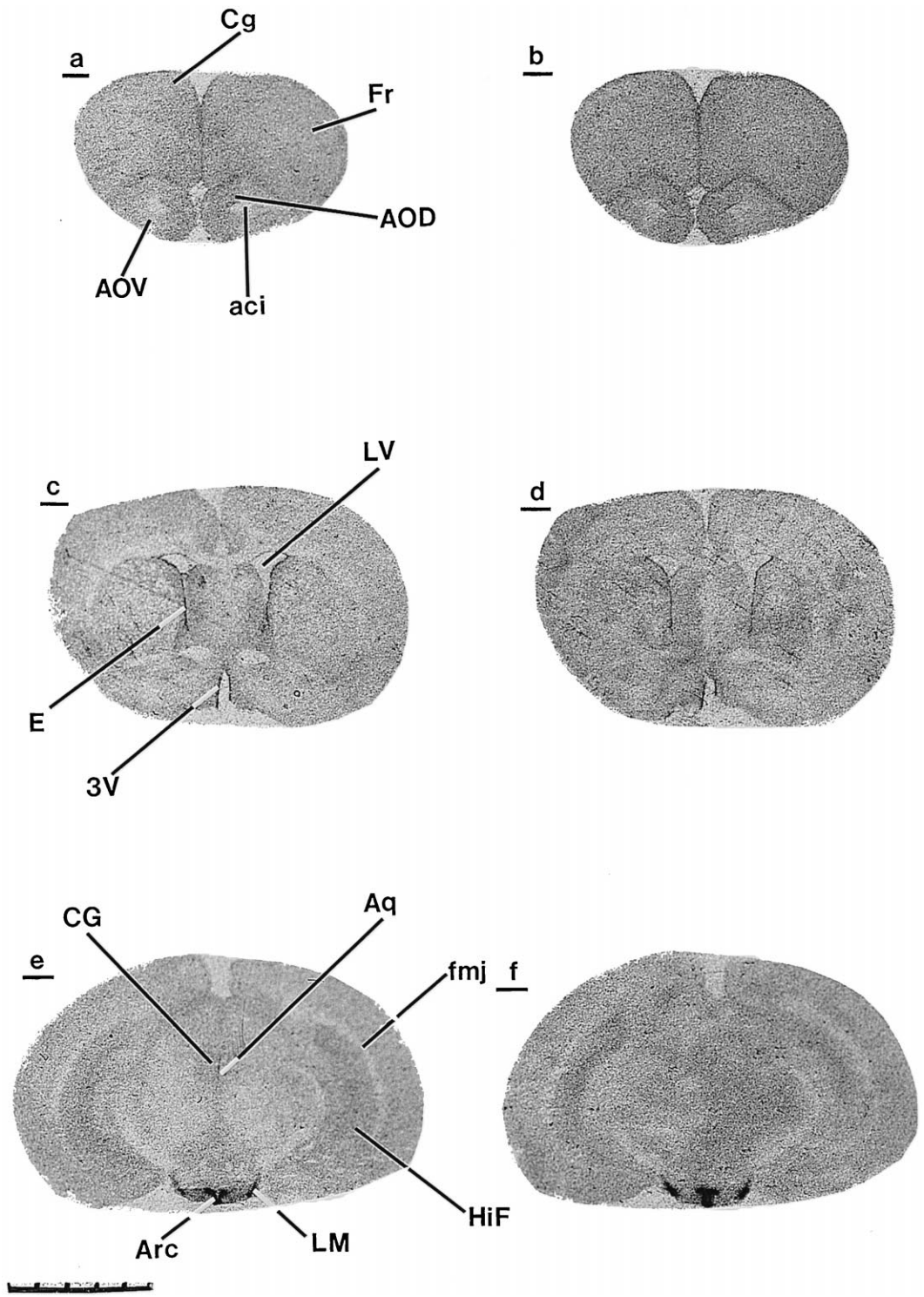

Fig. 6. Autoradiographic distribution of imidazoline I_2 receptors in transverse sections of rat brain. Sections a, c and e represent total [^{3}H]2-BFI (0.5 nM) binding. Imidazoline I_2 receptor distribution is shown in comparable sections taken from the same brain, where [^{3}H]idazoxan (5 nM) was incubated in the presence of 5 μM rauwolscine to preclude binding to α_2-adrenoceptors (sections b, d and f). Sections are shown in a rostral–caudal direction taken at (a,b) bregma 4.2 mm; (c,d) bregma −0.26 mm; (e,f) bregma −3.3 mm. See Table 2 for key to abbreviations. Figures are positive prints of the original autoradiograms, so that dense areas of binding are represented as grey to black areas of silver grains. Scale bar = 5 mm.

guanadino compounds, guanabenz and amiloride, also showed high affinity for [^{3}H]2-BFI binding (Table 1). Analysis of the competition curves revealed all the data were significantly better fit to a two-site model of binding (Fig. 3; Table 1). The high affinity component of binding represented 70–80% of the specifically bound ligand. In contrast, the α_2-adrenoceptor agonist (−)-noradrenaline, the α_2-adrenoceptor antagonists, rauwolscine and efaroxan, and the imidazoline I_1 receptor-selective drug moxonidine, were weak at competing against [^{3}H]2-BFI binding (Fig. 4, Table 1). These data suggest that 70–80% of the sites labelled with [^{3}H]2-BFI show characteristics consistent with imidazoline I_2 receptors. The α_2-adrenoceptor agonist/imidazoline I_1 receptor ligand, clonidine, showed a high nanomolar affinity for a proportion ($27 \pm 4\%$) of [^{3}H]2-BFI binding (Fig. 4; Table 1). The drugs, UK 14,304, L- and D-medetomidine all had moderate affinity for 40–50% of the sites being labelled by [^{3}H]2-BFI (Table 1). The monoamine oxidase-A inhibitor, clorgyline also displaced 40–50% of the binding with high affinity (Fig. 4; Table 1). Other compounds tested, including the putative endogenous ligand for imidazoline I_1/I_2 receptors, agmatine and its metabolites, urea and putrescine, had no affinity against [^{3}H]2-BFI binding even at concentrations up to 100 μM (Fig. 3; Table 1).

3.2. Autoradiography of [^{3}H]2-BFI and [^{3}H]idazoxan binding

Bound radioactivity to sections of rat whole brain incubated with [^{3}H]2-BFI were estimated by liquid scintillation counting. In this manner the washing procedure (based on the method of Mallard et al. (1992) for [^{3}H]idazoxan) was determined to be optimal for [^{3}H]2-BFI yielding high specific, low non-specific binding (< 10%).

3.2.1. Comparative autoradiographical distribution of [^{3}H]2-BFI and [^{3}H]idazoxan binding

The distribution of [^{3}H]2-BFI and [^{3}H]idazoxan labelling of imidazoline I_2 receptors was highly localised to discrete regions of rat brain (Figs. 5–7).

Fig. 5b shows total 5 nM [^{3}H]idazoxan binding to a parasagittal section of rat brain approximately 0.4 mm lateral from the midline, whereas Fig. 5d shows an adja-

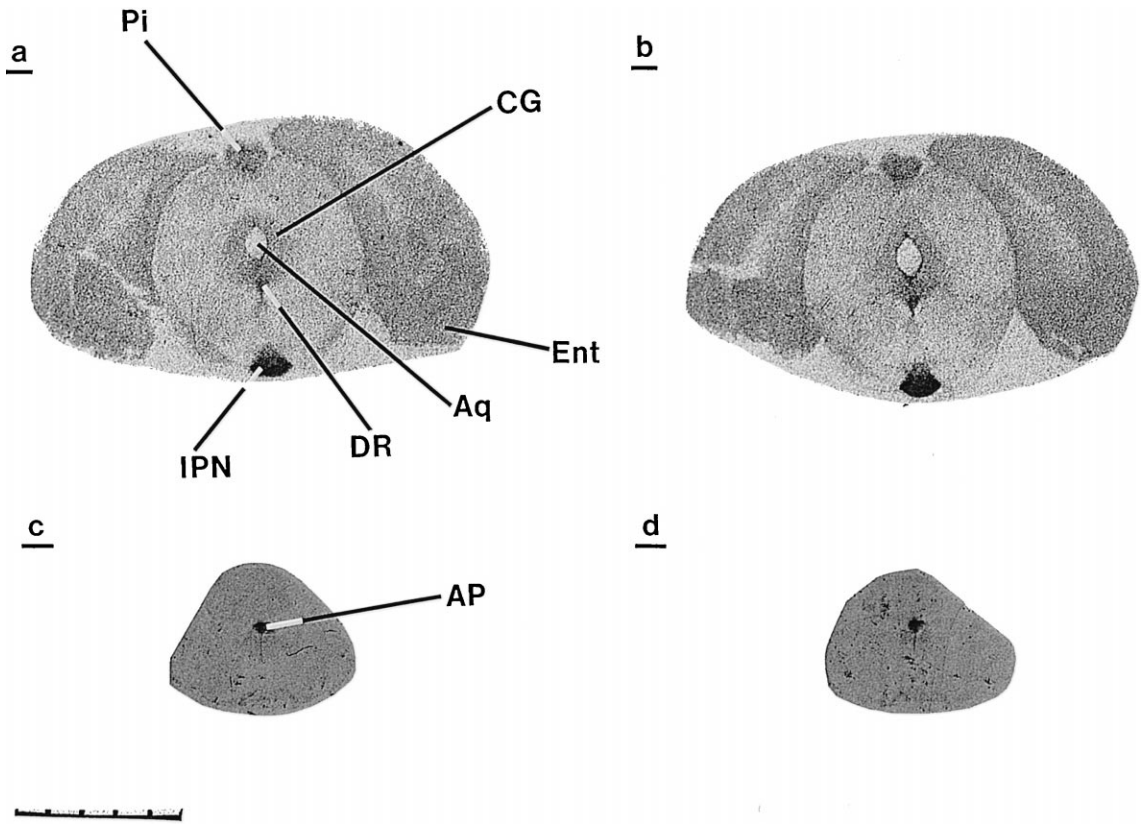

Fig. 7. Autoradiographic distribution of imidazoline I_2 receptors in more caudal transverse sections of rat brain. Sections a and c represent total [^{3}H]2-BFI (0.5 nM) binding. I_2 site distribution is shown in comparable sections taken from the same brain, where [^{3}H]idazoxan (5 nM) was incubated in the presence of 5 μM rauwolscine to preclude binding to α_2-adrenoceptors (sections b and d). Sections are shown in a rostral–caudal direction taken at (a,b) bregma −7.06 mm; (c,d) bregma −13.8 mm. See Table 2 for key to abbreviations. Figures are positive prints of the original autoradiograms, so that dense areas of binding are represented as grey to black areas of silver grains. Scale bar = 5 mm.

Table 2
Comparative distribution of [^{3}H]2-BFI and [^{3}H]idazoxan binding in rat brain

Brain region	Fig. symbol	Specific binding (fmol mg^{-1} tissue) [^{3}H]2-BFI	[^{3}H]idazoxan
Anterior olfactory nucleus			
lateral	AOL	7.30 ± 1.09	10.23 ± 1.38
ventral	AOV	7.51 ± 0.58	13.12 ± 1.71
dorsal	AOD	6.88 ± 0.34	12.74 ± 0.78
lateral ventricle	LV		
dorsal third ventricle	D3V		
surface of dorsal third ventricle		102.15 ± 6.92	141.25 ± 23.30
aqueduct	Aq		
surface of aqueduct		25.54 ± 2.12	30.50 ± 10.30
fourth ventricle	4V		
surface of fourth ventricle		29.94 ± 3.29	48.48 ± 8.02
Cortical regions			
frontal	Fr	6.80 ± 0.69	11.69 ± 2.56
entorhinal	Ent	7.42 ± 1.08	7.73 ± 1.13
occipital	Oc	6.04 ± 1.56	5.31 ± 0.27
temporal	Te	6.06 ± 1.88	5.41 ± 0.58
ventral orbital	VO	7.74 ± 0.95	12.17 ± 1.56
cor/amygdaloid fissure/zone	AF	6.71 ± 0.39	9.18 ± 1.28
Basal ganglia			
caudate putamen	Cpu	8.28 ± 2.06	10.76 ± 2.34
nucleus accumbens	Acb	5.68 ± 0.49	8.01 ± 1.70
globus pallidus	GP	4.39 ± 0.79	7.83 ± 1.55
intrabulbar anterior commissure	aci	4.05 ± 0.53	6.59 ± 0.75
corpus callosum	cc	2.46 ± 0.51	2.68 ± 1.12
ependymal layer	E	34.04 ± 2.36	41.71 ± 6.22
Hippocampal regions			
dentate gyrus	DG	9.11 ± 1.39	10.66 ± 2.03
subiculum	S	7.50 ± 0.96	10.25 ± 3.61
stratum oriens CA1	CA1	8.86 ± 1.18	11.81 ± 2.43
stratum oriens CA2	CA2	8.92 ± 1.17	10.45 ± 2.53
stratum pyramidal	CA3	8.84 ± 0.81	12.29 ± 1.61
hippocampal fissure	HiF	12.56 ± 1.82	12.41 ± 3.37
Thalamic regions			
paraventricular thalamic nucleus	PVP	26.60 ± 4.39	26.74 ± 4.95
medial habenular nucleus	MHb	22.52 ± 1.02	25.25 ± 8.12
lateral habenular nucleus	LHb	8.11 ± 0.71	13.94 ± 4.47
fasciculus retroflexus of Meynart	fr	11.11 ± 1.76	15.80 ± 0.10
posteromedian thalamic nucleus	POMN	17.21 ± 0.67	25.64
Hypothalamic regions			
dorsomedial	DM	10.28 ± 3.05	23.17 ± 9.04
ventromedial	VM	9.41 ± 3.00	21.23 ± 10.21
arcuate hypothalamic nucleus	Arc	112.09 ± 16.93	145.41 ± 12.27
lateral mammillary nucleus	LM	54.33 ± 5.52	82.92 ± 10.23
central grey	CG	9.69 ± 1.16	9.77 ± 2.23
superficial grey layer, superior colliculus	SuG	10.28 ± 1.50	8.18 ± 0.72
superior colliculus	SC	6.36 ± 0.66	9.00 ± 2.30
nucleus Brachium inferior colliculus	BIC	4.61 ± 1.24	5.02 ± 1.20
optic chiasm/supraoptic decuss.	ox/sox	77.71	155.90
pineal gland	PG	63.99 ± 4.97	68.79 ± 10.96
Midbrain			
interpeduncular nucleus	IPN	88.75 ± 6.16	126.07 ± 17.63
substantia nigra	SN	4.53 ± 0.53	5.48 ± 0.92
Pons			
dorsal raphé nucleus	DR	32.66 ± 1.04	33.64 ± 1.61
pontine nucleus	Pn	8.07 ± 0.79	7.65 ± 0.49
dorsal tegmental nucleus	dtg	5.45 ± 1.07	5.46 ± 0.41

Table 2 (continued)

Brain region	Fig. symbol	Specific binding (^{3}H]2-BFI	(fmol mg^{-1} tissue) [^{3}H]idazoxan
Medulla			
area postrema	AP	96.73 ± 9.66	94.06 ± 4.25
pyramidal tract	py	2.22 ± 0.20	4.46 ± 1.13
nucleus solitary tract	Sol	11.29 ± 0.61	19.92 ± 5.72
inferior olive	io	4.62 ± 0.41	19.17 ± 11.15
spinal trigeminal tract	sp5	3.41 ± 0.35	4.54 ± 0.81
cerebellum		6.77 ± 1.04	7.14 ± 0.76

Sections were labelled with 0.5 nM [^{3}H]2-BFI and 5 nM [^{3}H]idazoxan (in the presence of 5 μM rauwolscine), respectively, as described under Section 2. Regional binding densities were determined by optical density measurements in 3 areas of each brain region from at least 3 sections of rat brain. Data represent the mean ± S.E.M. from 4–5 animals.

cent section from the same animal incubated with 5 nM [^{3}H]idazoxan in the presence of rauwolscine (5 μM) to preclude α_2-adrenoceptor binding. Although [^{3}H]idazoxan binding throughout the section was greatly reduced, dense binding remained in the arcuate nucleus, interpeduncular nucleus, area postrema, and pineal gland. A comparable distribution of labelling was shown for total 0.5 nM [^{3}H]2-BFI binding to an adjacent parasagittal section 0.4 mm lateral to the midline (Fig. 5). This distribution of binding was unaltered when a similar section was incubated with [^{3}H]2-BFI in the presence of 5 μM rauwolscine (Fig. 5c). Overall the distribution of [^{3}H]2-BFI binding (Fig. 5a and c) was virtually indistinguishable from the distribution of [^{3}H]idazoxan in the presence of 5 μM rauwolscine (Fig. 5d).

Fig. 6a, c and e demonstrate total [^{3}H]2-BFI binding to transverse sections of rat brain (left panel) and Fig. 6b, d, and f demonstrate total [^{3}H]idazoxan binding in the presence of rauwolscine (5 μM; right panel). In rostral areas of brain (Fig. 6a and b) there remained low but detectable amounts of [^{3}H]2-BFI and [^{3}H]idazoxan binding to frontal cortex, cingulate cortex and anterior olfactory cortex. Sections cut through approximately bregma −0.26 mm showed dense [^{3}H]2-BFI and [^{3}H]idazoxan binding around the surface of the 3rd and lateral ventricles (Fig. 6c and d). Brain sections through bregma −3.3 mm revealed high [^{3}H]2-BFI and [^{3}H]idazoxan binding in the arcuate nucleus, mamilliary peduncle and a moderate degree of labelling in the medial habenular nucleus. Fig. 7 demonstrates total [^{3}H]2-BFI binding (left panel) and total [^{3}H]idazoxan binding in the presence of rauwolscine (5 μM; right panel) to more caudal transverse sections of rat brain. Fig. 7a and b show the intense labelling of the interpeduncular nucleus and the pineal gland by both [^{3}H]2-BFI and [^{3}H]idazoxan. These radioligands also labelled the central grey, especially that adjacent to the aqueduct. More caudally (bregma −13.8 mm; Fig. 7c and d) binding was high in the area postrema. In all autoradiographic studies total [^{3}H]2-BFI and [^{3}H]idazoxan (in the presence of 5 μM rauwolscine) binding was reduced to background levels by the addition of the high affinity specific imidazoline I_2 receptor ligands, 6-fluoro-idazoxan and BU224 (10 μM) (data not shown).

3.2.2. Quantitative autoradiography of [^{3}H]2-BFI and [^{3}H]idazoxan binding

Densitometric analysis of the autoradiograms from brain sections obtained from four separate rats determined levels of radioligand bound in each brain area which are summarised in Table 2 and Fig. 8. The highest density of [^{3}H]2-BFI and [^{3}H]idazoxan binding (> 90 fmol mg^{-1} tissue) was observed in the arcuate nucleus, surface of

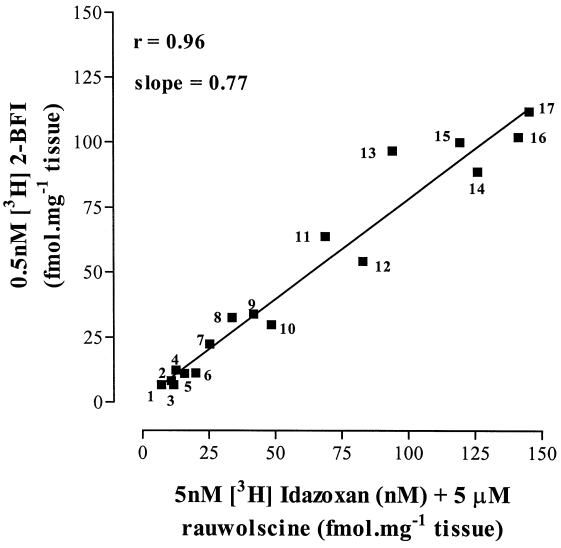

Fig. 8. Correlation of the density of [^{3}H]2-BFI binding vs. the density of [^{3}H]idazoxan binding (in the presence of 5 μM rauwolscine) to 17 rat brain regions (data from Table 2; i.e., data derived from the optical density measurements from the autoradiograms of 4–5 rat brains; r = 0.96, slope = 0.77). Key: 1 (cerebellar C9); 2 (caudate putamen); 3 (frontal cortex); 4 (hippocampal fissure); 5 (fasciculus retroflexus of Meynart); 6 (nucleus solitary tract); 7 (medial habenular nucleus); 8 (dorsal raphé nucleus); 9 (ependymal layer); 10 (surface of the fourth ventricle); 11 (pineal gland); 12 (lateral mammilary nucleus); 13 (area postrema); 14 (interpeduncular nucleus); 15 (mammilary peduncle); 16 (surface of the dorsal third ventricle); 17 (arcuate hypothalamic nucleus).

dorsal third ventricle, mammillary peduncle, interpeduncular nucleus and area postrema, with moderate binding (50–90 fmol mg^{-1} tissue) in the pineal gland, surface of fourth ventricle, ependyma and dorsal raphé. Areas such as the hippocampus, frontal cortex and caudate-putamen contained low but detectable amounts of binding (<20 fmol mg^{-1} tissue). Most brain nuclei, apart from those shown in Table 2, contain negligible [^{3}H]2-BFI and [^{3}H]idazoxan binding and have therefore not been listed. A correlation of 0.96 exists between the density of [^{3}H]2-BFI binding and the density of [^{3}H]idazoxan binding in 17 regions of rat brain (Fig. 8); when the binding of both radioligands was less than or close to background levels, these brain regions were not included in this correlation.

4. Discussion

Until 1995 [^{3}H]idazoxan had been the only radioligand commercially available to study imidazoline I_2 receptors, albeit indirectly, as such studies were complicated by the interaction of [^{3}H]idazoxan with α_2-adrenoceptors. In 1995, 2-BFI was reported to have a higher affinity for imidazoline I_2 receptors and a >1000-fold selectivity for imidazoline I_2 receptors vs. α_2-adrenoceptors in mammalian brain, compared with idazoxan (see Hudson et al., 1997). More recently, we have reported [^{3}H]2-BFI to have a higher affinity, higher specific activity and higher selectivity for imidazoline I_2 receptors in rabbit brain than [^{3}H]idazoxan (Lione et al., 1996). In this present study both [^{3}H]2-BFI and [^{3}H]idazoxan have been compared for their use in radioligand homogenate and autoradiographical binding studies of imidazoline I_2 receptors in rat brain.

These results demonstrate [^{3}H]2-BFI binding to rat whole brain membranes is both saturable and reversible. Similar to [^{3}H]idazoxan binding (Wikberg, 1989) [^{3}H]2-BFI binding attains equilibrium within 20 min. Non-linear regression analysis of the [^{3}H]2-BFI and [^{3}H]idazoxan (in the presence of rauwolscine to isolate the imidazoline I_2 receptor component) saturation binding curves indicates both radioligands recognise a similar maximal number of binding sites with [^{3}H]2-BFI possessing a >6 fold higher affinity, compared with [^{3}H]idazoxan.

Competition studies show the α_2-adrenoceptor agonist, (−)-noradrenaline and the α_2-adrenoceptor antagonists, rauwolscine and efaroxan have negligible affinity for the sites labelled by [^{3}H]2-BFI, showing that this radioligand does not label α_2-adrenoceptors. This is compatible with previous reports using 2-BFI (Lione et al., 1996; Hosseini et al., 1997; Alemany and García-Sevilla, 1997) confirming the selectivity of [^{3}H]2-BFI for imidazoline I_2 receptors over α_2-adrenoceptors. Several compounds with an imidazoline and guanidine moiety, namely idazoxan, 2-BFI, naphazoline, and guanabenz display biphasic displacement curves retaining a high nM affinity for 70–80% of the binding and a low μM affinity for the remaining 20–30%. A comparison of the affinities of these compounds for [^{3}H]2-BFI binding in this study with their affinities for [^{3}H]idazoxan in this tissue (Mallard et al., 1992; Miralles et al., 1993), shows a good correlation. Overall, these results indicate that the pharmacology of 70–80% of [^{3}H]2-BFI binding in rat brain is related to imidazoline I_2 receptors labelled with [^{3}H]idazoxan. Although it was originally proposed that all rat brain imidazoline I_2 receptors were amiloride-insensitive (I_{2B} subtype; Michel and Insel, 1989; Olmos et al., 1996; Alemany and García-Sevilla, 1997), our results demonstrate that rat brain contains imidazoline I_2 receptors labelled with [^{3}H]2-BFI which show high and low affinity for amiloride (see Table 1). An explanation for this disparity may relate to their being a mixture of I_{2A}/I_{2B} receptors in rat whole brain, yet solely I_{2B} receptors in cerebral cortex utilised the preparation in the former studies. The moderate affinity of UK 14,304, L- and D-medetomidine for [^{3}H]2-BFI binding correlate with affinities of these compounds for [^{3}H]idazoxan labelling of imidazoline I_{2B} receptors in rat brain (Olmos et al., 1996; Hosseini et al., 1997), further supporting the possibility for the existence of both imidazoline I_{2A} and I_{2B} receptors in rat brain.

Competition studies with unlabelled 2-BFI and several imidazoline and guanidinium compounds, were biphasic, suggesting [^{3}H]2-BFI recognises two binding sites, perhaps I_{2A} and I_{2B} subtypes. However the complex binding could represent two different affinity conformational states of the same binding site (see Wikberg et al., 1992).

In rat brain imidazoline I_1 receptors are preferentially located in the brainstem reticular formation (Kamisaki et al., 1990; Ernsberger et al., 1995). The lack of displacement of [^{3}H]2-BFI binding by imidazoline I_1 receptor ligands, moxonidine and efaroxan, implies that at 1 nM this radioligand is not labelling imidazoline I_1 receptors in rat brain, which is in agreement with our findings in rabbit brain (Lione et al., 1996). In contrast, the high nM affinity displayed by clonidine for 30% of [^{3}H]2-BFI binding could be explained by [^{3}H]2-BFI labelling an imidazoline I_1 receptor. Moreover 2-BFI has been shown to have an equipotent high nM affinity for imidazoline I_2 receptors and 'novel putative I-receptors' labelled with [^{3}H]clonidine in rabbit kidney cortex (Baines et al., 1995) that are distinct from imidazoline I_1 receptors labelled with [^{3}H]clonidine in bovine brainstem (Ernsberger et al., 1987).

The present study questions recent claims that agmatine may be an endogenous ligand for imidazoline I_2 receptors (Li et al., 1994). Such discrepancies have also been reported in other studies (Piletz et al., 1995; Mackinnon et al., 1995; Lione et al., 1996; Hosseini et al., 1997). The inability of urea and putrescine to displace the binding of [^{3}H]2-BFI also suggests these metabolites of agmatine do not interact with the imidazoline I_2 subtype. It is interesting, however, that agmatine has comparable affinity to idazoxan for imidazoline I_{2A} receptors labelled with [^{3}H]2-BFI in frog brain (Hudson et al., 1996) which may provide

a useful model for understanding the function of agmatine and thus imidazoline I_2 receptors in non-mammalian species.

In this study clorgyline discriminates two [^{3}H]2-BFI binding sites of high and low affinity. The high nM affinity of clorgyline for 50% of the sites labelled by [^{3}H]2-BFI is comparable to its affinity for imidazoline I_{2B} receptors labelled by [^{3}H]idazoxan in rat cerebral cortex (Olmos et al., 1996). These results support recent evidence that identifies two members of the family of imidazoline I_2 binding proteins as monoamine oxidase isoforms (for review see Parini et al., 1996). Whether this will be the case requires further study.

The characteristics of [^{3}H]2-BFI binding make it an ideal ligand for the autoradiographical study of imidazoline I_2 receptors. This is well demonstrated by the definition of the autoradiograms shown in Figs. 5–7. In this study the distribution of [^{3}H]2-BFI and [^{3}H]idazoxan (in the presence of rauwolscine to mask binding to α_2-adrenoceptors) binding in rat brain is indistinguishable and parallels that distribution observed using [^{3}H]idazoxan (Mallard et al., 1992), [^{3}H]RS-45041-190 (Mackinnon et al., 1995) and [^{125}I]AMIPI (Ivkovic et al., 1994). The binding of [^{3}H]2-BFI and [^{3}H]idazoxan to imidazoline I_2 receptors in rat brain shows greatest abundance in discrete nuclei, namely the arcuate and interpeduncular nucleus, mammillary peduncle, area postrema and pineal gland. Furthermore, in this study there was a good correlation between the regional distribution of [^{3}H]2-BFI and [^{3}H]idazoxan binding ($r = 0.96$). These results indicate that the distribution of [^{3}H]2-BFI binding is related to the distribution of [^{3}H]idazoxan in rat brain, which is in agreement with the pharmacological profile obtained for these radioligands from the homogenate radioligand binding studies. [^{3}H]2-BFI displayed significantly lower levels of binding than [^{3}H]RS-45041-190 throughout rat brain as would be expected from the difference in the K_d values obtained for the two radioligands from the homogenate radioligand binding studies. Also the difference may reflect the relative receptor occupancy given that Mackinnon et al. (1995) utilised a concentration of [^{3}H]RS-45041-190 above its K_d value, whilst in this study a concentration of [^{3}H]2-BFI just below its K_d value was utilised, taken from preliminary saturation experiments on rat brain sections (data not shown).

It should be pointed out that from all the autoradiographical studies describing the distribution of imidazoline I_2 receptors as discussed thus far, it still remains unclear whether imidazoline I_2 receptors are neuronal and/or glial. Possibly the high density of imidazoline I_2 receptors in the pineal gland and on ventricle surfaces, particularly around the third ventricle, indicate a non-neuronal cell type, and recent functional studies now support this view. For instance chronic treatment of rats with imidazoline I_2 receptor ligands have been shown to increase glial fibrillary acidic protein (GFAP) immunoreactivity (Alemany et al., 1995; Olmos et al., 1994b). Conversely, imidazoline I_2 receptors have recently been associated with neurones that degenerate in Huntington's disease (Reynolds et al., 1996). Further studies will be required to elucidate the exact cellular localisation of imidazoline I_2 receptors in the brain, possibly by combining the technique of autoradiography with discrete chemical and electrolytic lesions.

The highly localised distribution of [^{3}H]2-BFI and [^{3}H]idazoxan binding to imidazoline I_2 receptors in rat brain may help uncover the possible physiological role of these binding sites in brain function. For instance the high density of imidazoline I_2 receptors in the arcuate nuclei and area postrema is of particular interest relative to the action of idazoxan and those actions of the selective imidazoline I_2 receptor ligands, 2-BFI, LSL 60101 and RS-45041-190 on food intake (Jackson et al., 1991, 1995; Menargues et al., 1994; Brown et al., 1995).

Autoradiography studies in rat brain with the monoamine oxidase-A inhibitor, [^{3}H]-Ro 41-1049, have shown high levels of binding in the medial habenular and interpeduncular nuclei, whereas the distribution of monamine oxidase-B using the selective inhibitor, [^{3}H]-Ro 19-6237, have shown the highest density of binding in the circumventricular organs (which include the area postrema), the pineal gland and the arcuate nucleus (Saura et al., 1992). Thus the distribution of both isoforms of monoamine oxidase have a degree of overlap with the distribution of [^{3}H]2-BFI binding in rat brain, which supports recent evidence relating imidazoline I_2 receptors and monoamine oxidase (Parini et al., 1996). The affinity of the selective monoamine oxidase-A and monoamine oxidase-B inhibitors, Ro 41-1049 and Ro 19-6237, for [^{3}H]2-BFI binding have not been investigated in this study, although these inhibitors have no affinity for imidazoline I_2 receptors defined by the displacement of [^{3}H]2-BFI binding in rabbit brain (Lione et al., 1996). It is tempting to speculate that some [^{3}H]2-BFI binding may represent binding to monoamine oxidase-A given 50% of [^{3}H]2-BFI binding is sensitive to clorgyline and given for some brain areas, its similar distribution to monoamine oxidase-A in rat brain, however the active site of monoamine oxidase-A appears not to be an imidazoline I_2 receptor (Parini et al., 1996).

The results of the present study demonstrate two clear findings. Firstly, it confirms previous observations that [^{3}H]2-BFI represents a radioligand with greater affinity, specific activity and selectivity in mammalian brain, compared with [^{3}H]idazoxan (Nutt et al., 1995; Lione et al., 1996). Secondly, autoradiography reveals the overall distribution for both [^{3}H]2-BFI and [^{3}H]idazoxan (in the presence of 5 μM rauwolscine) in this study is comparable with that previously described for imidazoline I_2 receptors in rat brain labelled with [^{3}H]idazoxan (Mallard et al., 1992), [^{3}H]RS-45041-190 (Mackinnon et al., 1995) and [^{125}I]AMIPI (Ivkovic et al., 1994). Given these characteristics it can be concluded that [^{3}H]2-BFI is preferable to

[^{3}H]idazoxan for future in vitro and ex vivo autoradiography studies of imidazoline I_2 receptors, and should help researchers to determine the exact structure, function, location and therapeutic potential of imidazoline I_2 receptors.

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