

# Autoradiographic Comparison of [<sup>3</sup>H]-Clonidine Binding to Non-Adrenergic Sites and $\alpha_2$ -Adrenergic Receptors in Human Brain

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Clonidine is a partial agonist at brain  $\alpha_2$ -adrenoceptors ( $\alpha_2$ AR), but also has high affinity ( $K_D = 51$  nM) in homogenate binding assays for non-adrenergic imidazoline-binding sites (I-sites; imidazoline receptors). Herein, an autoradiographic comparison of [<sup>3</sup>H]-clonidine binding to I-sites and  $\alpha_2$ AR in sections of human brain is reported. For I-sites, the adrenergic component of 50 nM [<sup>3</sup>H]-clonidine binding was masked with either 60  $\mu$ M norepinephrine (NE;  $\alpha_2$ AR agonist) or 12.5  $\mu$ M methoxy-idazoxan (MIDX; selective  $\alpha_2$ AR antagonist), whereas the remaining non-adrenergic sites were studied by displacement with 20  $\mu$ M cirazoline. Levels of [<sup>3</sup>H]-clonidine binding to  $\alpha_2$ AR and I-sites, determined in adjacent tissue sections, were positively correlated across 27 brain regions ( $p = 0.0003$ ;  $r^2 = 0.385$ ). The principal olivary nucleus and the rostral portion of the ventrolateral medulla had highest ratios of I-sites:  $\alpha_2$ AR (>4:1). Quantitative transepts drawn across hippocampal images revealed  $\alpha_2$ AR enrichments in the CA-1 and inner molecular layers of the dentate gyrus—areas

not enriched in I-sites. Competition curves were generated for I-sites in caudate sections using 10 ligands known to distinguish between  $I_1$  and  $I_2$  subtypes. The rank-order of affinities were cirazoline > harmane > BDF6143 > idazoxan = tizanidine (affinities of agmatine, efaroxan, moxonidine, NE, and oxymetazoline were too low to be reliable). Only the endogenous I-site ligand, harmane, had a monophasic displacement curve at the non-adrenergic sites ( $K_i = 521 \pm 12$  nM). In conclusion: 1) the distribution of non-adrenergic [<sup>3</sup>H]-clonidine binding sites in human brain sections was correlated with, but distinct from  $\alpha_2$ AR; and 2) the affinities of these sites was distinct from  $\alpha_1$ AR,  $\alpha_2$ AR,  $I_1$  or  $I_2$  sites as previously defined in membrane binding assays. The properties of this non-adrenergic [<sup>3</sup>H]-clonidine binding site are consistent with I-sites previously labeled by [<sup>3</sup>H]-cirazoline in rat brain.

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Clonidine is the prototypic imidazoline compound that can elicit a diversity of CNS effects (Buccafusco 1992). Originally thought to be a selective agonist for  $\alpha_2$ -adrenoceptors ( $\alpha_2$ AR), clonidine is now realized to possess only slightly lower affinity for non-adrenergic imidazoline receptors (IR) compared to  $\alpha_2$ AR (Bousquet et al. 1984; Ernsberger et al. 1987).

The first subtype of IR (IR<sub>1</sub>) was discovered by comparing the hypotensive effects of clonidine to other non-imidazoline  $\alpha_2$ AR agonists, which were far less hypotensive agents than clonidine when micro-injected

into the rostral ventrolateral medulla (RVLM) of anesthetized rats (Bousquet et al. 1984). In those studies, the pharmacological control of blood pressure was found to best correlate with agents classified as imidazoline compounds (Ernsberger and Haxhiu 1997). In fact, the recommended therapeutic doses for clonidine and other imidazoline compounds to treat patients with hypertension have been positively correlated with their affinities for non-adrenergic imidazoline binding sites (of the  $I_1$  subtype)—whereas no such correlation exists with their affinities for  $\alpha_2$ AR (Buccafusco et al. 1995).

In psychiatry, novel imidazoline compounds are currently being explored for possible neuro-protective properties (Regunathan et al. 1999), for treating drug withdrawal symptoms (Vandergriff et al. 1999), and as possible antidepressants (Raddatz and Lanier 1997). Second-generation imidazoline anti-hypertensives are also now available, which include rilmenidine and moxonidine (Molderings 1997). Rilmenidine and moxonidine possess approximately 5-fold and 100-fold selective affinities, respectively, for  $I_1$  over  $\alpha_2$ AR sites (Ernsberger et al. 1995a; Piletz et al. 1996), and this affinity difference probably explains their diminished sedative side-effects relative to clonidine (van Zwieten 1997).

Like most receptor families, there are several IR subtypes identified using radioligand binding techniques.  $I_1$  sites have high affinity and  $I_2$  sites have low affinity for clonidine (Michel and Ernsberger 1992). By contrast, planar imidazoline compounds, like idazoxan, exhibit preferential affinity for  $I_2$  sites, of which there are two subclasses:  $I_{2A}$  and  $I_{2B}$  (Eglen et al. 1998). There are at least two other I-site subtypes defined by various other imidazolines: a cirazoline-sensitive I-site in kidney and brain (Angel et al. 1995) and a novel efaroxan-sensitive I-site in pancreas (Monks et al. 1999). In addition, the search for endogenous ligands has identified two putative IR neurotransmitters: a decarboxylated metabolite of arginine, agmatine (Reis and Regunathan 1999), and a  $\beta$ -carboline, harmaline (Hudson et al. 1999).

Only one previous autoradiographic study exists of [ $^3$ H]-clonidine binding to brain  $I_1$  sites (De Vos et al. 1994). While improving on that autoradiographic method, we have now provided a more detailed visualization of those sites in human brain. A comparison is made of the distribution of non-adrenergic versus adrenergic sites labeled by [ $^3$ H]-clonidine across 27 brain regions. De Vos et al. (1994) did not previously distinguish non-adrenergic versus adrenergic sites labeled by [ $^3$ H]-clonidine. Special attention is given to the hippocampus, where previous studies of depressed suicide victims have previously revealed a decrease in IR immunoreactivity by western blotting (Piletz et al. 2000b). A pharmacological rank-ordering of affinities is also presented for I-sites labeled by [ $^3$ H]-clonidine using competitive ligands known to distinguish  $I_1$ ,  $I_2$ , and  $\alpha_2$ AR sites (taken from previous homogenate binding studies).

In summary, we are describing a non- $\alpha_2$ AR binding site for [ $^3$ H]-clonidine which has a distribution distinct from [ $^3$ H]-clonidine-labeled  $\alpha_2$ AR in human brain, and the pharmacology of this site resembles an imidazoline binding site previously thought by De Vos and coworkers (1994) to be an  $I_1$  site.

## MATERIALS AND METHODS

### Human Tissue Collection

Discarded brain tissues from five subjects (three males and two females) who died of sudden natural or accidental causes were collected at the time of autopsy at the Cuyahoga County Coroner's Office, Ohio, in accordance with an approved Institutional Review Board protocol. Tissues were coded to protect the identity of subjects. Deaths occurred within four hours of injury. Causes of death were determined by the county medical examiner. Four of the subjects died of cardiovascular failure and one died of asphyxiation secondary to smoke inhalation. The average age of the subjects was  $52 \pm 10$  yr (age range = 24 to 80 yr). All cadavers were refrigerated prior to autopsy, immediately upon arrival at the coroner's facility. The average postmortem interval (from death to freezing of brain tissue) was  $16 \pm 3$  h (range 6 to 21 h). The coroner performed toxicological screens for 50 compounds in the blood, bile, urine, and/or vitreous fluids of cadavers to identify numerous psychoactive and other compounds. Four of the subjects had no detectable drugs in the toxicological screen. One subject had evidence of lidocaine in the blood. Postmortem autopsies revealed no record of neurological or psychiatric histories for any of the subjects.

At autopsy, blocks of brain tissue were dissected. The techniques for dissection of blocks of tissue have been published for frontal cortex (Brodman's area 10) and hippocampus (Klimek et al. 1999), and for locus coeruleus (Klimek et al. 1997). The tissue block containing the hypothalamus was dissected just anterior to the substantia nigra and was sectioned in the coronal plane. A block of tissue containing the medulla was obtained by a transverse cut just caudal to the caudal end of the locus coeruleus, and this block of tissue was cut in the transverse plane, perpendicular to the rostro-caudal axis of the medulla. Tissue blocks were dipped in 2-methylbutane cooled on dry ice ( $-50^\circ\text{C}$ ), placed on powdered dry ice for 10 min, and then stored in an ultra-cold freezer ( $-82^\circ\text{C}$ ) until sectioned. Frozen blocks of brain tissue were mounted on a specimen chuck of a cryostat microtome (Cryocut 1800, Leica, Reichert-Jung, Deerfield, IL) and tissue sections (20  $\mu\text{m}$  thick) were cut at  $-16^\circ\text{C}$  and thaw-mounted onto gelatin-coated microscope slides. Sections were dried under refrigeration and stored at  $-82^\circ\text{C}$  until assayed.

### Quantitative Autoradiography

Quantitative autoradiography of [ $^3\text{H}$ ]-clonidine binding was performed using a modification of the method of De Vos et al. (1994). [ $^3\text{H}$ ]-clonidine (50 nM) was used to label both  $\alpha_2\text{AR}$  and I-sites in adjacent tissue sections. Sections were removed from the freezer and dried on the counter top at room temperature (22°C). Subsequently, sections were incubated with [ $^3\text{H}$ ]-clonidine, in the presence and absence of competitors as designated below, in a reaction buffer containing 50 mM Tris-HCl, 2 mM  $\text{MgCl}_2$ , and 252  $\mu\text{M}$  ascorbic acid (pH 7.4). This was performed by placing a small drop of reaction buffer containing the radioligand, directly on each slide-mounted section, and then incubating 1 h in a humidity chamber at 22°C. Unbound radioligand was removed by three consecutive washes (1 min each) of the slides in ice-cold reaction buffer, then dipped for 1 sec in ice cold water to remove buffer salts. Slide-mounted sections were dried immediately afterwards under a stream of air at room temperature overnight. Slides were apposed, along with brain-mash calibrated  $^3\text{H}$  standards (American Radiolabeled Chemicals Inc., St. Louis, Mo.), to Hyperfilm- $^3\text{H}$  (Amersham, Arlington Hts, IL) in x-ray cassettes and stored at room temperature for four weeks. Films were processed with GBX developer and fixer (Eastman Kodak Co., Rochester, NY). The same sections were then stained with cresyl violet to identify anatomical structures. The resulting autoradiograms were quantified using the M2 Microcomputer Imaging Device (Imaging Research, St. Catharines, Ontario, Canada). Hippocampal fields (Klimek et al. 1999) were outlined based on morphological criteria (Duvernoy 1988). Brainstem and medullary regions were identified with the aid of an atlas (Paxinos and Huang 1995).

Total binding was determined by incubating sections with 50 nM [ $^3\text{H}$ ]-clonidine without any competitors. In adjacent sections, binding to  $\alpha_2\text{AR}$  was determined by subtracting the binding of [ $^3\text{H}$ ]-clonidine in the presence of 60  $\mu\text{M}$  norepinephrine (NE) from the total binding of [ $^3\text{H}$ ]-clonidine. Binding to I-sites was determined by subtracting the binding of [ $^3\text{H}$ ]-clonidine in the presence of 60  $\mu\text{M}$  NE plus 20  $\mu\text{M}$  cirazoline from the binding of [ $^3\text{H}$ ]-clonidine in the presence of 60  $\mu\text{M}$  NE. NE exhibited negligible binding to I-sites under the conditions of this assay (Figure 5). On the other hand, cirazoline is known to displace [ $^{125}\text{I}$ ]p-iodoclonidine from human  $\text{I}_1$  sites ( $K_i = 99$  nM) (Piletz et al. 1996). The percentage of total binding that was specific binding (including I-sites and  $\alpha_2\text{AR}$ ) was  $72 \pm 1\%$  (means  $\pm$  S.E.M. of all brain regions from all subjects). Of this specific binding,  $54 \pm 1\%$  was binding to I-sites and  $46 \pm 1\%$  was binding to  $\alpha_2\text{AR}$ .

Competition curves were generated from slide wipes using ligand concentrations from  $10^{-10}$  to  $10^{-4}$  M (11 points in triplicate for each of 3–4 subjects). The mea-

surements were made on serial adjacent caudate sections (2–4 sections per slide, wiped together). After incubation for 1 h and a rapid cold water dip, the caudate sections were wiped from the slides onto S&S #32 glass fiber filters (Schleicher and Schuell, Keene, NH). Radioactivity was measured by liquid scintillation counting. In a typical experiment, total radioactivity remaining in the presence of 60  $\mu\text{M}$  NE was  $1503 \pm 222$  cpm, and that remaining in the presence of 20  $\mu\text{M}$  cirazoline plus 60  $\mu\text{M}$  NE was  $433 \pm 28$  cpm. Competition curves were analyzed by both LIGAND (McPherson 1985) and Prism (GraphPad, San Diego, CA), producing similar results. Data were modeled to one-site and two-site fits (McPherson 1985) and the preferred model was chosen by an F test.  $\text{IC}_{50}$  values were computed from 2–3 experiments and were expressed as mean  $\pm$  SEM.

### Drugs

[ $^3\text{H}$ ]-clonidine (66.5 Ci/mmol) was purchased from NEN<sup>TM</sup> (Life Science Products, Inc., Boston, MA) and stored at  $-20^\circ\text{C}$  before reconstitution in reaction buffer (defined below). Moxonidine and BDF6143 were gifts of Lilly Forschung GmbH Company (Hamburg, Germany). Tizanidine was a gift of Sandoz Pharmaceuticals (Tokyo, Japan). Efaroxan was a gift from Pierre Fabre Pharmacie (Castres Cedex, France). Cirazoline and RX821002 (MIDX; methoxy-idazoxan) were purchased from Research Biochemicals International (Natick, MA). Agmatine, harmane, NE, oxymetazoline, and idazoxan (IDX) were from Sigma Chemical Company (St. Louis, MO). All chemicals were of the highest grade available. Drug solutions were made fresh daily before each experiment. Drug stock solutions were prepared (0.01 M) in either distilled water or 10 mM acetic acid depending on their solubilities and were serially diluted in the reaction buffer.

### Technical Note

Several differences are noteworthy comparing our method to the autoradiographic method of De Vos and coworkers (1994): 1) The concentration of [ $^3\text{H}$ ]-clonidine in our study (50 nM) was nearer its  $K_D$  concentration for I-sites (57 nM), rather than 8 nM used by De Vos and coworkers; 2) MIDX (12.5  $\mu\text{M}$ , instead of 2  $\mu\text{M}$  used by De Vos and coworkers) and NE (60  $\mu\text{M}$ ) were compared as displacing agents to demonstrate that the adrenergic binding of [ $^3\text{H}$ ]-clonidine was of the  $\alpha_2\text{AR}$  type, not  $\alpha_1\text{AR}$ , as well as to ensure complete masking of  $\alpha_2\text{AR}$  in the case of I-sites; 3) In our study, non-specific binding was assessed with 20  $\mu\text{M}$  cirazoline rather than with 10  $\mu\text{M}$  cirazoline, because we found 20  $\mu\text{M}$  cirazoline resulted in more complete inhibition of [ $^3\text{H}$ ]-clonidine binding (Figure 5); 4) Binding reactions were conducted for 60 min, as compared to 20 min by De Vos and colleagues (1994). This was

based on earlier binding studies (Piletz et al. 1991); and 5) De Vos and colleagues used the antagonist, [ $^3\text{H}$ ]-MIDX, to assess  $\alpha_2\text{AR}$  binding. By contrast, we used the agonist [ $^3\text{H}$ ]-clonidine to assess I-sites and  $\alpha_2\text{AR}$  binding.

## RESULTS

The specific binding of [ $^3\text{H}$ ]-clonidine to  $\alpha_2\text{AR}$  and I-sites was quantified by autoradiography in 27 human brain regions, including specific areas of prefrontal cortex, hippocampus, caudate, hypothalamus, pons, and the medulla oblongata (Table 1). Comparison of [ $^3\text{H}$ ]-clonidine binding to  $\alpha_2\text{AR}$  and I-sites reveals overlapping, yet distinctly different distributions. For example, at the level of the medulla, there was considerable similarity between the distribution of I-sites and  $\alpha_2\text{AR}$  (Figure 1). In fact, there was a significant linear correlation between the amounts of [ $^3\text{H}$ ]-clonidine binding to  $\alpha_2\text{AR}$  and to I-sites when considering all brain regions in a scatter plot ( $p = .0003$ ,  $r^2 = 0.385$ ; Figure 2).

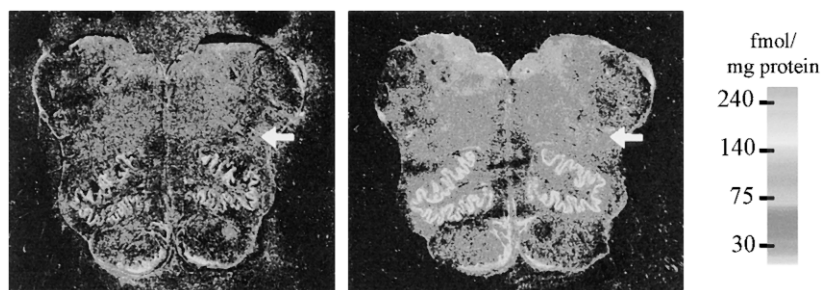
Despite the overall correlation between the densities of  $\alpha_2\text{AR}$  and I-sites (Figure 2), there were distinct sub-regional differences in the distributions of  $\alpha_2\text{AR}$  and I-sites in the brain. Highest binding to I-sites was in the principle nucleus of the inferior olive ( $130 \pm 18$  fmol/mg protein) wherein the amount of binding was 10-fold higher than that in the white matter of the prefrontal cortex (Table 1, Figure 2). Other areas of high density for [ $^3\text{H}$ ]-clonidine binding to I-sites included the polymorphic layer of the hippocampus, dorsal and median raphe, dentate gyrus, hypothalamus, locus coeruleus, central gray, and the hypoglossal nucleus (Table 1). Lowest [ $^3\text{H}$ ]-clonidine binding to I-sites was in the white matter of the prefrontal cortex, the striosomes of the caudate, and the pyramidal tract of the medulla. The distribution of I-sites contrasted the distribution of [ $^3\text{H}$ ]-clonidine binding to  $\alpha_2\text{AR}$ . The highest binding to  $\alpha_2\text{AR}$  occurred in the molecular layer of CA1 of the hippocampus and in the hypothalamus. Moderate amounts of binding were observed in the locus coeruleus.

To further illustrate the distinction between  $\alpha_2\text{AR}$

**Table 1.** Binding of [ $^3\text{H}$ ]-Clonidine to I-Sites and  $\alpha_2$ -Adrenoceptors in Human Brain

Brain Region	Imidazoline Sites (fmol/mg protein)	$\alpha_2$ -Adrenoceptors (fmol/mg protein)
Prefrontal Cortex		
Layer 1	$36.3 \pm 9.6$	$47.9 \pm 12.7$
Layers 2–3–4	$41.1 \pm 9.5$	$41.7 \pm 12.6$
Layers 5–6	$34.9 \pm 7.8$	$42.1 \pm 6.7$
White matter	$13.8 \pm 6.5$	$7.9 \pm 2.3$
Hippocampus		
CA4	$76.8 \pm 8.5$	$67.3 \pm 7.3$
CA3	$55.5 \pm 3.1$	$43.4 \pm 5.0$
CA2	$49.2 \pm 5.4$	$21.5 \pm 8.8$
CA1 stratum molecular	$56.0 \pm 11.1$	$91.6 \pm 18.8$
CA1 stratum radiatum	$48.2 \pm 13.9$	$34.1 \pm 7.8$
CA1 stratum lacunosum	$45.4 \pm 15.0$	$48.8 \pm 15.6$
CA1 stratum pyramidale	$46.7 \pm 12.4$	$30.5 \pm 3.1$
Polymorphic layer	$97.0 \pm 3.6$	$65.5 \pm 16.5$
Dentate gyrus		
Granular layer	$86.2 \pm 4.5$	$52.4 \pm 13.4$
Molecular layer	$76.2 \pm 6.0$	$58.0 \pm 12.2$
Head of Caudate		
Medial caudate	$32.9 \pm 9.6$	$24.5 \pm 10.9$
Lateral caudate	$27.4 \pm 5.0$	$23.3 \pm 11.1$
Striasomes	$19.6 \pm 5.4$	$12.4 \pm 3.7$
Hypothalamus	$88.9 \pm 12.7$	$69.6 \pm 6.2$
Pons		
Locus coeruleus	$77.7 \pm 10.8$	$45.1 \pm 9.4$
Dorsal raphe (caudal)	$96.7 \pm 14.8$	$59.8 \pm 9.8$
Medial raphe	$90.3 \pm 10.8$	$38.5 \pm 15.5$
Central gray	$70.5 \pm 14.0$	$56.8 \pm 7.3$
Medulla		
Inferior olive, principle nucleus	$130.3 \pm 17.6$	$43.4 \pm 16.3$
Inferior olive, dorsal nucleus	$68.0 \pm 18.2$	$32.8 \pm 18.8$
Hypoglossal nucleus	$81.4 \pm 11.2$	$34.3 \pm 4.4$
Rostral ventrolateral region	$51.3 \pm 23.0$	$11.6 \pm 2.0$
Pyramidal tract	$21.8 \pm 6.3$	$9.0 \pm 4.0$

Each value is the mean  $\pm$  SEM data from five subjects.



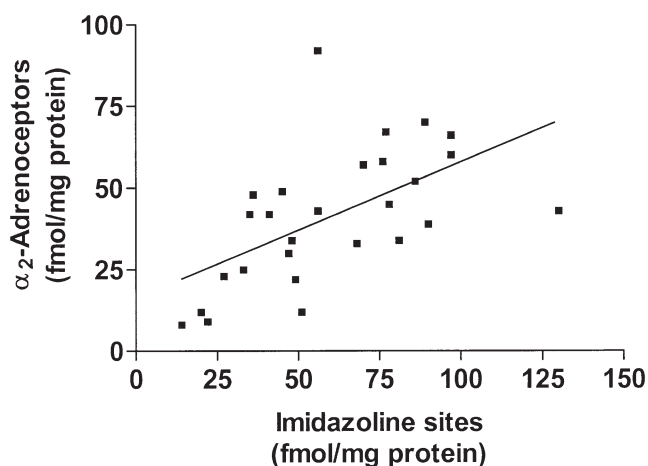
**Figure 1.** Autoradiograms of the binding of 50 nM [ $^3$ H]-clonidine to I-sites (left) and to  $\alpha_2$ -adrenoceptors (right) in human medulla. *Left panel:* the specific binding of [ $^3$ H]-clonidine to I-sites generated by digital subtraction of an autoradiogram of [ $^3$ H]-clonidine binding in the presence of NE (60  $\mu$ M) plus cirazoline (20  $\mu$ M) from an autoradiogram of the binding of [ $^3$ H]-clonidine in the presence of NE alone. *Right panel:* the specific binding of [ $^3$ H]-clonidine to  $\alpha_2$ -adrenoceptors generated by digital subtraction of the autoradiogram of the binding of [ $^3$ H]-clonidine in the presence of NE (60  $\mu$ M) from the autoradiogram of the binding of [ $^3$ H]-clonidine alone. For both panels, the two autoradiograms used to produce the image were generated from adjacent tissue sections. The calibration bar illustrates the relationship between the color spectrum and radioligand binding density. The arrows indicate the rostral ventrolateral region, based on human brain atlas maps.

and I-sites, quantitative transepts were drawn through specific binding images of hippocampi using an image analysis system (Figure 3). Amounts of binding across a transept through a subregion may differ from the levels outlined in the entire subregion (i.e., data in Table 1). In the outlining method (Table 1), uneven binding densities within subregions are averaged by the computer program. In the transept method (Figure 3), pixel by pixel densities are recorded across the subregions. Therefore, quantitative transepts should allow a closer examination of complex regions, like the hippocampus, where uneven binding distributions exist. Histograms of the transept densities of [ $^3$ H]-clonidine binding to I-sites and  $\alpha_2$ AR are shown in Figures 3 and 4. The dif-

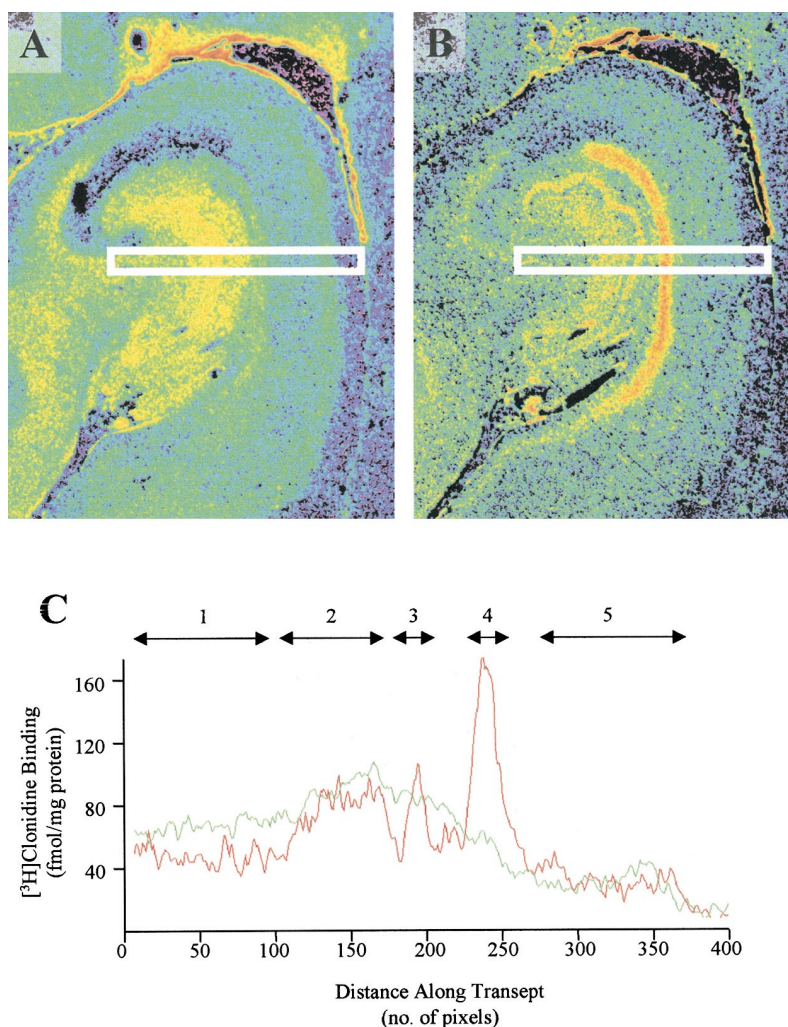
ference between these sites was most obvious in the molecular layer of CA1. There, binding to  $\alpha_2$ AR clearly exceeded that of I-sites by 4:1, whereas in other subregions of the hippocampus the ratios of  $\alpha_2$ AR:I sites were closer to 1:1.

To rule out the possibility that the agonist-induced state of  $\alpha_2$ AR might in some way account for brain I-sites (e.g., an artifact of using the agonists, [ $^3$ H]-clonidine and NE), we defined  $\alpha_2$ AR with either NE (60  $\mu$ M) or the  $\alpha_2$ AR antagonist, MIDX (12.5  $\mu$ M). MIDX is a selective antagonist for  $\alpha_2$ AR with very low affinity for the human brain I-sites ( $IC_{50}$  at I-sites  $\geq 60$   $\mu$ M) (De Vos et al. 1994; Flamez et al. 1997). We observed that specific binding to  $\alpha_2$ AR was only slightly greater using MIDX, and the specific binding to I-sites was only slightly lower using MIDX compared to the results with NE. Furthermore, the relative distributions of binding to  $\alpha_2$ AR and I-sites were identical using either of these competitors (Figure 4, Panel C). The near identity of these results with either displacing agent makes it highly unlikely that an  $\alpha_2$ AR affinity state could account for I-sites.

Competition curves were next generated for [ $^3$ H]-clonidine binding (under a mask of 12.5  $\mu$ M MIDX) at caudate I-sites (Figure 5). [ $^3$ H]-Clonidine was found to have a  $K_D = 57 \pm 3$  nM for I-sites, in keeping with the results of De Vos and colleagues (1994). The head of the caudate was chosen for these studies because of the availability of tissue. Five compounds were capable of completely displacing [ $^3$ H]-clonidine from caudate I-sites, but these mostly had shallow competition curves (Figure 5). The only exception was the  $\beta$ -carboline, harmaline, which exhibited a monophasic competition curve (Figure 5). The rank order of  $IC_{50}$  values for I-sites (generated using a single site model) was as follows: cirazoline > harmaline > BDF6143 > idazoxan = tizanidine. Agmatine, efaroxan, moxonidine, NE, and



**Figure 2.** Correlation between the binding of [ $^3$ H]-clonidine to I-sites and to  $\alpha_2$ -adrenoceptors in 27 brain regions from human prefrontal cortex, hippocampus, caudate, hypothalamus, pontine region, and the medulla oblongata ( $p = .0003$ ,  $r^2 = 0.385$ ). Each point represents the mean value of binding obtained for each region (five subjects/region).



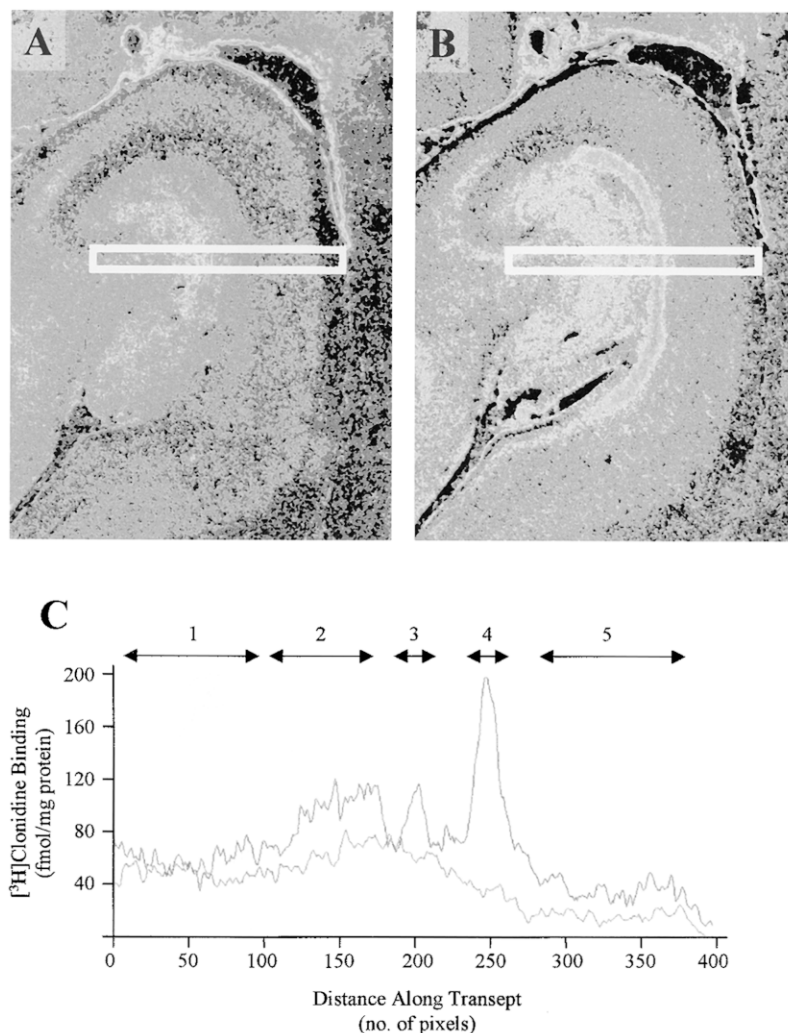
**Figure 3.** Comparison of the distribution of the binding of  $[^3\text{H}]\text{-clonidine}$  to I-sites (**A**) and to  $\alpha_2$ -adrenoceptors (**B**) in the human hippocampus using norepinephrine (NE) to identify  $\alpha_2$ -adrenoceptors. (**A**): The specific binding of  $[^3\text{H}]\text{-clonidine}$  to I-sites was generated by digital subtraction of an autoradiogram of  $[^3\text{H}]\text{-clonidine}$  binding in the presence of NE (60  $\mu\text{M}$ ) plus cirazoline (20  $\mu\text{M}$ ) from an autoradiogram of the binding of  $[^3\text{H}]\text{-clonidine}$  in the presence of NE alone. (**B**): The specific binding of  $[^3\text{H}]\text{-clonidine}$  to  $\alpha_2$ -adrenoceptors generated by digital subtraction of the autoradiogram of the binding of  $[^3\text{H}]\text{-clonidine}$  in the presence of NE (60  $\mu\text{M}$ ) from the autoradiogram of the binding of  $[^3\text{H}]\text{-clonidine}$  alone. For both images, the two autoradiograms used to produce the image shown were generated from adjacent tissue sections. White outline bars overlaid upon the images of A and B indicate where a quantitative transept bar was placed. (**C**): The quantitative distributions of  $[^3\text{H}]\text{-clonidine}$  binding to I-sites (lower line) and to  $\alpha_2$ -adrenoceptors (upper line in 4), expressed pixel by pixel along the horizontal axis. Representative regions along these transepts are indicated by numbers: 1 = CA3; 2 = CA4; 3 = polymorphic area; 4 = Molecular layer of CA1; 5 = transitional area between CA1 and subiculum.

oxymetazoline did not inhibit  $[^3\text{H}]\text{-clonidine}$  binding sufficiently to determine  $\text{IC}_{50}$  values. Actually, agmatine failed to displace any I-sites labeled by  $[^3\text{H}]\text{-clonidine}$  in caudate (Figure 5, Table 2).

For comparative purposes, Table 2 shows our  $K_i$  values beside those previously determined by De Vos and colleagues (1994), who also used the head of the human caudate for competition binding studies. The rank-order for human  $I_1$  sites in homogenate binding assays is known to be moxonidine  $\geq$  oxymetazoline  $>$  tizanidine  $\geq$  agmatine  $\geq$  efaroxan = clonidine  $>$  cirazoline  $>$  BDF6143  $>>$  idazoxan  $>>$  NE (Piletz et al. 1996). The

rank-order for human  $I_2$  sites in homogenate binding assays is cirazoline  $>$  idazoxan  $>>$  oxymetazoline  $>$  moxonidine  $\geq$  efaroxan  $>$  clonidine (Piletz and Sletten 1993). Thus, the I-site(s) under study has(have) a unique pharmacological profile.

Moxonidine, a compound with preferential  $I_1$  affinity, was found to have low affinity in caudate sections for I-sites labeled by  $[^3\text{H}]\text{-clonidine}$  (Figure 5, Table 2). To examine this further, moxonidine was used in parallel with cirazoline to define non-adrenergic binding of  $[^3\text{H}]\text{-clonidine}$  in a number of regions of the medulla from three subjects. The RVLM is often cited as mediating the non-



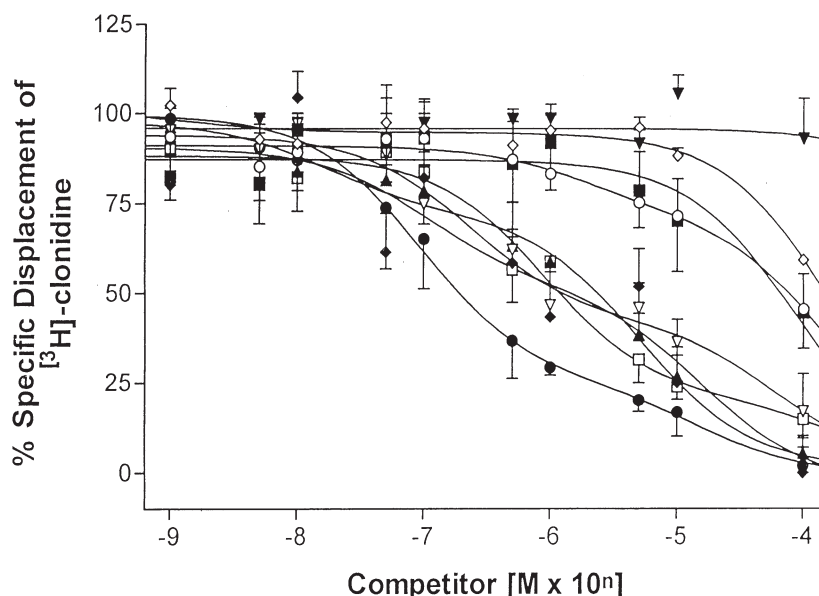
**Figure 4.** Comparison of the distribution of the binding of  $[^3\text{H}]$ -clonidine to I-sites (**A**) and to  $\alpha_2$ -adrenoceptors (**B**) in the human hippocampus using methoxy-iodazoxan (MIDX) to identify  $\alpha_2$ -adrenoceptors. (**A**): The specific binding of  $[^3\text{H}]$ -clonidine to I-sites was generated by digital subtraction of an autoradiogram of  $[^3\text{H}]$ -clonidine binding in the presence of MIDX (12.5  $\mu\text{M}$ ) and cirazoline (20  $\mu\text{M}$ ) from an autoradiogram of the binding of  $[^3\text{H}]$ -clonidine in the presence of MIDX alone. (**B**): The specific binding of  $[^3\text{H}]$ -clonidine to  $\alpha_2$ -adrenoceptors generated by digital subtraction of the autoradiogram of the binding of  $[^3\text{H}]$ -clonidine in the presence of MIDX (12.5  $\mu\text{M}$ ) from the autoradiogram of the binding of  $[^3\text{H}]$ -clonidine alone. For both images, the two autoradiograms used to produce the image shown were generated from adjacent tissue sections. White outline bars overlaid upon the images of A and B indicate where a quantitative transept bar was placed. (**C**): The quantitative distributions of  $[^3\text{H}]$ -clonidine binding to I-sites (lower line) and to  $\alpha_2$ -adrenoceptors (upper line), expressed pixel by pixel along the horizontal axis. Representative regions along these transepts are indicated by numbers: 1 = CA3; 2 = CA4; 3 = polymorphic layer; 4 = Molecular layer of CA1; 5 = transitional area between CA1 and subiculum.

adrenergic hypotensive effects of clonidine, moxonidine, and rilmenidine (Chan et al. 1996). Table 3 shows four conditions of  $[^3\text{H}]$ -clonidine binding: (a) without any competitors (total binding); (b) in the presence of 60  $\mu\text{M}$  NE (displacing  $\alpha_2\text{AR}$ ); (c) in the presence of 60  $\mu\text{M}$  NE plus 20  $\mu\text{M}$  cirazoline (displacing  $\alpha_2\text{AR}$  and I-sites); and (d) in the presence of 60  $\mu\text{M}$  NE plus 5  $\mu\text{M}$  moxonidine (displacing  $\alpha_2\text{AR}$  and only a small additional amount of I-sites). The amount of  $[^3\text{H}]$ -clonidine binding in any region remaining in the presence of NE was not significantly different from the amount remaining in the pres-

ence of NE plus moxonidine, but was significantly greater than the amount of binding remaining in the presence of NE plus cirazoline (Table 3). Even in the RVLM (Figure 1, arrow),  $[^3\text{H}]$ -clonidine binding was not substantially more displaced by moxonidine than NE (Table 3).

## DISCUSSION

In the present study, the distributions of  $[^3\text{H}]$ -clonidine binding to I-sites and  $\alpha_2\text{AR}$  were distinct (Figures 1, 3,



**Figure 5.** Inhibition of the binding of [ $^3\text{H}$ ]-clonidine (50 nM) to I-sites in consecutive human caudate section wipes by imidazoline and non-imidazoline compounds. In all cases, the binding of [ $^3\text{H}$ ]-clonidine to  $\alpha_2$ -adrenoceptors was prevented by adding 12.5  $\mu\text{M}$  methoxy-idazoxan to the binding reaction mixture. Competing ligands were (▼) agmatine, (◇) NE, (■) moxonidine, (○) oxymetazoline, (▲) BDF6143, (▽) idazoxan, (◆) tizanidine, (□) harmane, and (●) cirazoline. Each point represents the mean  $\pm$  S.E.M. of values generated from three separate experiments.

and 4) but correlated across 27 brain regions (Figure 2). As expected, the RVLM (Figure 1, arrow) had a high ratio of I-sites:  $\alpha_2\text{AR}$  (nearly 5:1) compared to other brain regions (Table 1). This is noteworthy because the RVLM is the site of the anti-hypertensive action ascribed to  $\text{IR}_1$  stimulation when imidazolines are micro-injected into the brainstem (Bousquet et al. 1984). However, a number of brain regions were more abundantly enriched with I-sites than the RVLM, notably the inferior olive, pontine regions, and hypothalamus (Table 1). Furthermore, our competition binding curves (Figure 5) make it doubtful

that the non-adrenergic site labeled by [ $^3\text{H}$ ]-clonidine in the human RVLM is of the  $\text{I}_1$  subtype (see below).

A clear distinction in the regional distribution of [ $^3\text{H}$ ]-clonidine labeling to  $\alpha_2\text{AR}$  and I-sites was obvious in the hippocampus (see Figures 3 and 4). The distribution of I-sites and  $\alpha_2\text{AR}$  was virtually identical whether defined by masking [ $^3\text{H}$ ]-clonidine binding with an  $\alpha_2\text{AR}$  agonist (NE) or an  $\alpha_2\text{AR}$  antagonist (MIDX; Figures 3 and 4). Approximately 50% of the [ $^3\text{H}$ ]-clonidine binding in the human hippocampal CA1 molecular layer was to  $\alpha_2\text{AR}$ . This compared to  $\approx 35\%$  binding to I-sites and  $\approx 15\%$  non-specific binding in the same CA1 region.

It should be noted that  $\alpha_2\text{AR}$  would be nearly saturated at a concentration of 50 nM [ $^3\text{H}$ ]-clonidine, since clonidine has a high affinity at each of the human  $\alpha_2\text{AR}$  ( $\alpha_{2A}\text{AR}$ ,  $K_D = 1.5$  nM;  $\alpha_{2B}\text{AR}$ ,  $K_D = 7.8$  nM;  $\alpha_{2C}\text{AR}$ ,  $K_D = 1.3$  nM for [ $^{125}\text{I}$ ]p-iodoclonidine) (Piletz et al. 1996). On the other hand, I-sites would only be half-saturated ( $K_D = 55$  nM for [ $^{125}\text{I}$ ]p-iodoclonidine). Therefore, the proportions of these two sites in, for instance, the CA1 molecular layer might actually be re-estimated to be 6:5 for I-sites: $\alpha_2\text{AR}$ . Using similar calculations, the CA4 region of the hippocampus might be estimated to possess a 9:3 ratio of I-sites: $\alpha_2\text{AR}$  as labeled by [ $^3\text{H}$ ]-clonidine. Thus, I-sites and  $\alpha_2\text{AR}$  appeared unevenly distributed in the hippocampus with each site having a unique distribution.

A variety of previous autoradiographic studies of I-sites, mostly in animals, exist in the literature (De Vos et al. 1994; Ernsberger et al. 1994; King et al. 1995; Lione

**Table 2.**  $K_i$  Values for I-Sites in Caudate Section Wipes Labeled with 50 nM [ $^3\text{H}$ ]-Clonidine.

Ligand	DeVos et al., (1994)	Present Findings
Cirazoline	$8.4 \pm 0.5$ nM	$88.3 \pm 2.1$ nM
Guanoxan	$56 \pm 8$	—
Rilmenidine	$214 \pm 36$	—
Harmane	—	$521 \pm 12$
BDF6143	—	$693 \pm 19$
Idazoxan	$20.7 \pm 1.1$	$1330 \pm 32$
UK14,304	$770 \pm 10$	—
ST587	$950 \pm 13$	—
Tizanidine	—	$1540 \pm 62$
Oxymetazoline	—	$>36,200^a$
Norepinephrine	—	$>50,400^a$
Moxonidine	—	$>55,600^a$
Efaroxan	—	$>74,700^a$
Agmatine	—	$>100,000$

<sup>a</sup> In these cases the non-linear regression fits were forced through  $y = 0$ .

**Table 3.** Binding of [<sup>3</sup>H]-Clonidine in Subregions of the Human Medulla in the Absence and Presence of Non-Imidazoline and Imidazoline Compounds<sup>a</sup>

Brain Region	Total [ <sup>3</sup> H]-clonidine binding	[ <sup>3</sup> H]-Clonidine Binding in the Presence of:		
		Norepinephrine	Norepinephrine and Cirazoline	Norepinephrine and Moxonidine
Inferior olive, principle nucleus	270±12	226±20	96±19	199±17
Inferior olive, dorsal nucleus	180±23	148±4	80±21	140±15
Hypoglossal nucleus	150±11	116±9	35±4	110±1
Rostral ventrolateral region	129±5	117±7	89±41	103±6
Pyramidal tract	50±8	41±10	20±6	44±9

<sup>a</sup>Concentrations of inhibitors were: norepinephrine, 60 μM; cirazoline, 20 μM; moxonidine, 5 μM.

Each value (in fmol/mg protein) is the mean ± SEM of determinations made in three subjects.

et al. 1998; Mallard et al. 1992). Unfortunately, the results of those previous studies have been as diverse as the radioligands used to define them. Besides us, only De Vos and colleagues (1994) have used [<sup>3</sup>H]-clonidine to visualize brain I-sites. Our results compare favorably in a number of ways with theirs (Table 2). On the other hand, Ernsberger and coworkers (1994) reported diffuse [<sup>125</sup>I]p-iodoclonidine binding to "I<sub>1</sub>-like" sites in rat brainstem sections. Their diffuse images contrasted sharply with the highly regionalized images of Figures 1, 3, and 4. For instance, the striking intensity of I-sites in the olivary nuclei (Figure 1) appears to be a "signature" for human I-sites labeled with [<sup>3</sup>H]-clonidine, but this was not reported for [<sup>125</sup>I]p-iodoclonidine autoradiograms (Ernsberger et al. 1994). We believe this discrepancy from the results with [<sup>125</sup>I]p-iodoclonidine reported by Ernsberger and coworkers (1994) may be related to the lipophilicity of [<sup>125</sup>I]p-iodoclonidine (see below). There are also autoradiographic images from rat brain using [<sup>3</sup>H]idazoxan (Mallard et al. 1992), [<sup>3</sup>H]rilmenidine (King et al. 1995), and [<sup>3</sup>H]2-(2-benzofuranyl-2-imidazoline (Lione et al. 1998). The latter three studies have all related their findings to I<sub>2</sub> sites (King et al. 1995; Lione et al. 1998; Mallard et al. 1992).

By convention, I-sites labeled with [<sup>3</sup>H]-clonidine have been designated as I<sub>1</sub> sites (Ernsberger et al. 1987). However, De Vos and colleagues (1994) used cautionary language when making an assignment of their non-adrenergic [<sup>3</sup>H]-clonidine binding site as an I<sub>1</sub> subtype. They reported that idazoxan and guanoxan had higher affinities than expected for an I<sub>1</sub> site (Table 2), but clonidine and rilmenidine had affinities consistent with an I<sub>1</sub> subtype. In that same study, they clearly distinguished their site from the I<sub>2</sub> site labeled by [<sup>3</sup>H]-idazoxan and from the α<sub>2</sub>AR labeled by [<sup>3</sup>H]-MIDX (De Vos et al. 1994). Therefore, De Vos and colleagues concluded: "At first glance, IR appear to correspond to the I<sub>1</sub> imidazoline receptor subtype . . . however, this comparison is subject to some caution." Their cautionary note is echoed by our present results.

Special emphasis may be given to our choice of cirazoline for defining specific binding to I-sites (Figure 5).

NE or MIDX were used to displace [<sup>3</sup>H]-clonidine from adrenergic sites, and cirazoline was used (under NE or MIDX masks) to displace [<sup>3</sup>H]-clonidine from non-adrenergic I-sites. Nearly identical images were obtained with either NE or MIDX as displacers (Figures 3 and 4). Therefore, any hypothesis invoking an agonist-induced state of [<sup>3</sup>H]-clonidine binding to α<sub>2</sub>AR in order to explain the existence of I-sites seems unlikely. Equally important, cirazoline is an imidazoline α<sub>1</sub>AR agonist without high affinity for known human α<sub>2</sub>AR subtypes (K<sub>i</sub> values for α<sub>2</sub>AR subtypes vary from 193 to 657 nM) (Piletz et al. 1996). At 50 nM, [<sup>3</sup>H]-clonidine would bind minimally to α<sub>1</sub>AR because the K<sub>i</sub> value of clonidine for brain α<sub>1</sub>AR is 750 nM (Glossmann et al. 1981).

[<sup>3</sup>H]-cirazoline has previously been shown to label a non-adrenergic/ non-I<sub>1</sub> / non-I<sub>2</sub> site in brain homogenate binding assays (Angel et al. 1995). In characterizing that I-site, Angel et al. (1995) reported the K<sub>i</sub> values of several of the same drugs studied here. They reported a rank ordering of affinities for I-sites of cirazoline (K<sub>i</sub> = 4 nM) > clonidine (K<sub>i</sub> = 50 nM) > idazoxan (K<sub>i</sub> = 158 nM) >> oxymetazoline (22,000 nM) > efaxoxan (219,000 nM) > (-) epinephrine (635,000 nM). This rank-order of K<sub>i</sub> values corresponds closer to our data (Table 2) than any other I-sites reported. Therefore, the same receptor(s) may have been under study by [<sup>3</sup>H]-clonidine displacement with cirazoline (in our study) and by [<sup>3</sup>H]-cirazoline displacement with clonidine (Angel et al. 1995). The identity and function of the [<sup>3</sup>H]-cirazoline-sensitive I-site studied by Angel and coworkers (1995) remains unknown.

De Vos and colleagues (1994) found no correlation between I-sites labeled with [<sup>3</sup>H]-clonidine and α<sub>2</sub>AR sites labeled with [<sup>3</sup>H]-MIDX (r<sup>2</sup> = - 0.068) when comparing the densities of these two sites in human brain regions. This is a surprising difference from the positive correlation we observed (p = .0003, r<sup>2</sup> = 0.385) across 27 brain regions between these sites (Figure 2). However, of the eight brain regions in common between our two studies (cortical layers 1, 2–4, 5–6, hippocampal layer CA1, dentate gyrus, caudate putamen, raphe nuclei,

and olivary nucleus), we also found no correlation between I-sites labeled with [ $^3\text{H}$ ]-clonidine and  $\alpha_2\text{AR}$  sites labeled with [ $^3\text{H}$ ]-clonidine ( $p = .58$ ,  $r^2 = 0.053$ ). Therefore, the discrepancy between our studies could simply be due to different brain regions under study. It should be emphasized that De Vos and coworkers (1994) used the selective  $\alpha_2\text{AR}$ -antagonist, [ $^3\text{H}$ ]-MIDX, to measure  $\alpha_2\text{AR}$  binding, while we used the mixed  $\alpha_2\text{AR}$ /I-site agonist [ $^3\text{H}$ ]-clonidine.

De Vos and coworkers (1994) did not report on  $\alpha_2\text{AR}$  binding with [ $^3\text{H}$ ]-clonidine, although they used it for I-sites. Agonists, like [ $^3\text{H}$ ]-clonidine, preferentially induce a high affinity state of the  $\alpha_2\text{AR}$  (Alburges et al. 1993), whereas antagonists bind equally to low and high affinity states of  $\alpha_2\text{AR}$ . This difference in the states of  $\alpha_2\text{AR}$  labeled by [ $^3\text{H}$ ]-clonidine (high affinity states), versus both low and high affinity states in their study (De Vos et al. 1994) might underlie the different correlations between our studies. For instance, the correlation observed in Figure 2 may in fact relate to G-proteins in common with  $\alpha_2\text{AR}$  and I-sites.

The subtype or subtypes of I-sites labeled by [ $^3\text{H}$ ]-clonidine in human brain remains open to question. In competition studies with human caudate sections, we observed the affinities of I-sites labeled by [ $^3\text{H}$ ]-clonidine were not consistent with either an  $\text{I}_1$  or  $\text{I}_2$  site (Table 1). Three potent  $\text{I}_1$  ligands (efaroxan, oxymetazoline, and moxonidine) did not displace [ $^3\text{H}$ ]-clonidine binding from caudate I-sites, even at relatively high concentrations (Table 1). The possibility that the I-sites labeled by [ $^3\text{H}$ ]-clonidine are actually  $\text{I}_2$  sites also is unlikely because higher than expected affinities were observed for several agents (i.e., BDF6143 and rilmenidine affinities were 4-fold and 54-fold higher, respectively, than expected for human  $\text{I}_2$  sites) (Table 2) (Diamant et al. 1992). Furthermore, [ $^3\text{H}$ ]-clonidine binding was examined in human medullary slices with the goal of identifying a typical  $\text{I}_1$  site (i.e., moxonidine-displaceable). A series of medullary slices that included the human RVLM were studied (Figure 1, arrow). Our autoradiographic technique was the same as in caudate, except, in addition to the presence of 20  $\mu\text{M}$  cirazoline in some slices, 5  $\mu\text{M}$  moxonidine was added to measure the non-specific binding in adjacent sections. Yet, moxonidine competed for only slightly more total sites than NE in the medullary slices (Table 3). Oxymetazoline was also ineffective at displacing [ $^3\text{H}$ ]-clonidine from I-sites in the medulla (data not shown). Moxonidine was also found to be an ineffective displacer of I-sites in the hippocampus and prefrontal cortex (data not shown). Thus, we found no autoradiographic evidence for typical  $\text{I}_1$  sites labeled by [ $^3\text{H}$ ]-clonidine, even in the medulla.

It should also be mentioned that the conditions in our autoradiographic procedure, while routine for many receptors (Klimek et al. 1997), were vastly differ-

ent from those recommended for filtration binding assays of homogenate preparations of  $\text{I}_1$  sites (Ernsberger et al. 1995b). Optimal buffer conditions for homogenate  $\text{I}_1$  sites invoke low osmotic and ionic strengths (Ernsberger et al. 1995b). Furthermore, purified plasma membranes are considered useful to lower the signal-to-noise ratio of  $\text{I}_1$  sites in most filtration binding assays of  $\text{I}_1$  sites (Ernsberger et al. 1995b). Also, a high flow-through vacuum system is considered critical for filtration binding assays of  $\text{I}_1$  sites, due to their fast off-rate of binding (Piletz et al. 1991). Hypo-osmolarity and hypotonic buffer conditions could not be used autoradiographically, because these conditions destroy the anatomical integrity of slide-mounted tissue sections. Hence, comparisons of affinities derived from autoradiographic binding studies and homogenate binding studies may not be legitimate for  $\text{I}_1$  or  $\text{I}_2$  sites. It remains a possibility that the different conditions used in the present study might have significantly altered the affinities of certain ligands, such as moxonidine, for true  $\text{I}_1$ -sites.

Mention should also be made that most previous membrane binding studies of  $\text{I}_1$  and  $\alpha_2\text{AR}$  sites (Piletz and Sletten 1993; Klimek et al. 1999) have used [ $^{125}\text{I}$ ]-p-iodoclonidine as radioligand. In preliminary studies, we found that if [ $^{125}\text{I}$ ]-p-iodoclonidine was substituted for [ $^3\text{H}$ ]-clonidine using the autoradiographic method, a substantial amount of [ $^{125}\text{I}$ ]-p-iodoclonidine binding was localized to white matter (data not shown). Although [ $^3\text{H}$ ]-para-aminoclonidine and [ $^{125}\text{I}$ ]-p-iodoclonidine are known to have similar properties (Alburges et al. 1993), we found little or no specific labeling of white matter with [ $^3\text{H}$ ]-clonidine. Therefore, even closely related imidazoline radioligands, like [ $^3\text{H}$ ]-clonidine and [ $^{125}\text{I}$ ]-p-iodoclonidine, might bind different sites.

The molecular basis of imidazoline receptors is another area of intense research. There is little doubt that the diversity of ligand-defined subtypes will be matched by protein subtypes. A cDNA clone which encodes a peptide with  $\text{I}_1$ -like properties has recently been described (Ivanov et al. 1998; Piletz et al. 2000a) and, surprisingly, the predicted protein has a novel amino acid sequence, different from known receptors. Therefore, it is uncertain if the cloned  $\text{I}_1$  site is actually a true receptor (Piletz et al. 2000a).  $\text{I}_2$  sites are somewhat better understood, and have been physically linked to monoamine oxidases (Tesson et al. 1995). In addition, there are at least two subtypes of  $\text{I}_2$  sites that exhibit biphasic binding curves (Eglen et al. 1998). An  $\text{I}_3$  site has been hypothesized to reside on the ion conducting pore component of the ATP-sensitive potassium channel (Monks et al. 1999). To these sites we now add another I-site labeled similarly by [ $^3\text{H}$ ]-cirazoline (Angel et al. 1995) and [ $^3\text{H}$ ]-clonidine (present study).

Harmane had high affinity ( $K_i = 521 \text{ nM}$ ) for I-sites

labeled by [ $^3\text{H}$ ]-clonidine in the human caudate (Figure 2). This high affinity  $K_i$  value supports the candidacy of harmaline as an endogenous I-site ligand (Hudson 1999). Harmaline has been reported to have high affinity for  $I_1$  sites in filtration binding assays and to be active in modulating blood pressure in the CNS (Hudson 1999). The other endogenous I-site ligand, agmatine, had no affinity whatsoever for I-sites in the present study. Little is known about the regional distribution of harmaline in the brain, and future studies will be required to determine if it co-localizes with putative IR sites.

In summary, the distribution of a novel I-site for [ $^3\text{H}$ ]-clonidine has been described in human brain. The pharmacology of this site is in agreement with a [ $^3\text{H}$ ]-clonidine binding site described by De Vos and co-workers (1994) and a [ $^3\text{H}$ ]-cirazoline site described by Angel and coworkers (1995). It remains to be discovered what the function of this site is, but given the plethora of actions previously ascribed to clonidine (Buccafusco 1992), it should be of interest to psychiatry. Currently, we are attempting to determine whether this is the same I-site that was reported previously to be decreased in the hippocampus of depressed suicide victims (Piletz et al. 2000b) and increased in the frontal cortex of depressed suicide victims (Garcia-Sevilla et al. 1996).

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