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Characterization and distribution of angiotensin II receptor subtypes in the mouse brain

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Abstract

We localized and characterized angiotensin II receptor subtypes (AT_1 and AT_2) in the mouse brain, with the use of autoradiography after incubation with [^{125}I][Sar^1]-angiotensin II or [^{125}I]CGP 42112 and displacement with selective angiotensin AT_1 (losartan and candesartan) or angiotensin AT_2 (CGP 42112(1) and PD 123319(2)) receptor ligands. In the mouse, the receptor subtype affinity for the different ligands was similar to that of the rat. The receptor subtype distribution was also similar to that in the rat, with some notable exceptions, such as the presence of angiotensin AT_1 but not AT_2 receptors in the locus coeruleus, and the expression of angiotensin AT_1 receptors in the caudate putamen. These results confirm that careful consideration of the specific distribution of receptor subtypes in different species, even those closely related such as the mouse and the rat, should be conducted before meaningful comparisons could be proposed. Our data also form the basis for future studies of mouse models such as those with angiotensin receptor gene deficiencies. Published by Elsevier Science B.V.

Keywords: Angiotensin AT_1 receptors; Angiotensin AT_2 receptors; Locus coeruleus; Caudate putamen; Candesartan; Losartan; CGP 42112; PD 123319

1. Introduction

Angiotensin II receptors can be discriminated in two main subtypes (AT_1 and AT_2), characterized pharmacologically on the basis of their affinities for different ligands (Timmermans et al., 1993). While angiotensin AT_1 receptors bind specifically compounds such as losartan or candesartan, angiotensin AT_2 receptors show a high affinity only for compounds such as PD 123319, PD 123177 or CGP 42112 (Timmermans et al., 1993). Nevertheless, there are exceptions known, and angiotensin AT_1 receptors for some mammalian species, such as the dog (Burns et al., 1994) and the gerbil (De Oliveira et al., 1995) show a very reduced affinity for classical angiotensin AT_1 receptor antagonists such as losartan.

Angiotensin AT_1 and AT_2 receptors from humans (Furuta et al., 1992; Tsuzuki et al., 1994) and several animal species (Iwai and Inagami, 1992; Sasamura et al., 1992; Mukoyama et al., 1993; Nakajima et al., 1993) have been cloned, and they are characterized by seven transmembrane helices. Angiotensin AT_1 receptors mediate all of the known physiological effects of angiotensin II in the central nervous system and the periphery (Saavedra, 1992; Timmermans et al., 1993). Only speculations exist about the function of the angiotensin AT_2 receptors.

The brain distribution of angiotensin AT_1 and AT_2 receptors follows a similar pattern across mammalian species, including humans, with several important differences (McKinley et al., 1986; Tsutsumi and Saavedra, 1991; Saylor et al., 1992; Aldred et al., 1993; MacGregor et al., 1995; Jöhren et al., 1997). However, the localization, the pharmacological characteristics, and the expression of the different subtypes of angiotensin II receptors have not been intensively studied in the mouse brain (Harding et al., 1981; Jöhren et al., 1997). A basic knowledge of the distribution and characterization of the mouse angiotensin II receptor subtypes is important, because of the increasing relevance of the gene-deleted mouse models, and in particular the angiotensin AT_{1A}, AT_{1B} and AT_2 receptor, and angiotensin converting enzyme gene deleted strains (Ichiki et al., 1995; Ito et al., 1995; Krege et al., 1995; Chen et al., 1997). Therefore, we characterized pharmacologically angiotensin II receptors in the mouse brain and analyzed their distribution, a study intended as

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the basis for further investigations in different gene-deficient mice-models.

2. Materials and methods

2.1. Animals

We used 8-week-old adult male mice of the C57BL/6J strain, which we obtained from Jackson Laboratory (Bar Harbor, ME). The animals were kept under controlled conditions, according to protocols approved by the NIMH Animal Care and Use Committee, and they had free access to water and food.

Animals were killed between 10:00 a.m. and 11:00 a.m. by decapitation. The brains were immediately removed, and frozen at −30°C by immersion in isopentane, kept on dry ice. Frozen brains were stored at −80°C. For binding studies, 16 μm thick coronal sections were cut in a cryostat at −20°C, thaw-mounted on gelatine-coated slides, and dried overnight in a desiccator at 4°C. Sections were stored at −80°C until binding experiments were performed. Consecutive sections were used for angiotensin II receptor binding studies. Brain regions were designated using a mouse brain atlas (Franklin and Paxinos, 1997).

2.2. Materials

Angiotensin II and [^{125}I][Sar^1]angiotensin II were purchased from Peninsula Laboratories (Belmont, CA). CGP 42112 [nicotinic acid-Tyr-*N*-benzyloxycarbonyl-Arg-Lys-His-Pro-Ile-OH] was purchased from Neosystems Laboratory (Strasbourg, France). [^{125}I][Sar^1]-angiotensin II and CGP 42112 were iodinated by New England Nuclear (Boston, MA) to a specific activity of 2200 Ci/mmol. Candesartan (2-ethoxy-1-[[2′(1*H*-tetrazol-5-yl)biphenyl-4yl]methyl]-1*h*-benzimidazole-7-carboxylic acid), losartan (DuP 753; 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[2′-(1*H*-tetrazol-5-yl)biphenyl-methyl]imidazole), and PD 123319 (1-[[4-(dimethylamino)-3-methylphenyl]-methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-c]pyridine-6-carboxylic acid) were gifts from ASTRA (Hamburg, Germany), DuPont-Merck (Wilmington, DE, USA) and Parke-Davis (Ann Arbor, MI, USA), respectively. Bacitracin and protease-free bovine serum albumin were purchased from Sigma Chemical (St. Louis, MO). Hyperfilm-^{3}H and [^{125}I] micro scales were from Amersham (Arlington Heights, IL), D-19 developer and rapid fixer from Eastman Kodak (Rochester, NY).

2.3. Analysis of ligand metabolism

The degradation of [^{125}I][Sar^1]-angiotensin II during incubation was analyzed by reversed phase high performance liquid chromatography as described by De Oliveira et al. (1995). An acetonitrile gradient in 0.1% trifluoroacetic acid was used on a C-18 column (250 × 4.6 mm, 5 μm) at a constant flow of 2 ml/min. The acetonitrile gradient (15–40% in 0.1% trifluoroacetic acid over 12.5 min) started at 4 min after a wash of 3 min with 0.1% trifluoroacetic acid and ramping up in 1 min to 15% trifluoroacetic acid. In this system, cold Sar^1-angiotensin II eluted at 12.5 min. To determine a possible metabolism of the radiolabeled ligand [^{125}I][Sar^1]-angiotensin II, aliquots of the incubation buffer before and after incubation were analyzed by injecting aliquots of 50 μl in the high performance liquid chromatography system. Fractions of the eluate were collected every 30 s, and radioactivity was measured using a γ-counter. The radioactive peak of the non-metabolized ligand appeared in the eluate after 13.5 min.

2.4. Binding studies

Competition studies were conducted by incubating consecutive brain sections with the radiolabeled angiotensin II receptor agonist [^{125}I][Sar^1]-angiotensin II and increasing concentrations of angiotensin II or selective ligands for the angiotensin AT_1 receptor subtype (losartan and candesartan) or the angiotensin AT_2 receptor subtype (CGP 42112 and PD 123319) as described earlier (Tsutsumi and Saavedra, 1991).

Adjacent brain sections were preincubated for 15 min at 22°C in 10 mM sodium phosphate buffer, pH 7.4, containing 120 mM NaCl, 5 mM EDTA, 0.005% bacitracin and 0.2% protease free bovine serum albumin. Sections were then transferred to fresh buffer, prepared as described above, containing in addition 0.5 nM [^{125}I][Sar^1]-angiotensin II and increasing concentrations of angiotensin II (10^{-12}–10^{-5} M), losartan (10^{-11}–10^{-4} M), candesartan (10^{-12}–10^{-5} M), CGP 42112 (10^{-12}–10^{-5} M), and PD 123319 (10^{-12}–10^{-5} M). Up to five slides were incubated in a chamber containing 10 ml of the buffer. After an incubation for 120 min at 22°C, the sections were washed four times for 1 min each in ice-cold 50 mM Tris–HCl buffer (pH 7.4), followed by a 30 s wash in ice-cold water, and dried under a stream of cold air.

In other experiments, the proportion of angiotensin AT_1 and AT_2 receptors in several brain areas was calculated by incubating the sections with 0.5 nM [^{125}I][Sar^1]-angiotensin II followed by displacement in consecutive sections with single concentrations of losartan (3.3 μM) or CGP 42112 (0.1 μM) that was found to selectively displace angiotensin AT_1 and AT_2 receptors, respectively. In these experiments, non-specific binding was determined by incubating the sections with angiotensin II (1 μM). Binding to angiotensin AT_1 or AT_2 receptors was calculated as the difference of binding of [^{125}I][Sar^1]-angiotensin II (total binding) and binding in the presence of losartan or CGP 42112, respectively.

In addition, [^{125}I]CGP 42112 binding was performed in another set of adjacent sections to confirm the presence of

angiotensin AT_2 receptors (Heemskerk and Saavedra, 1995). Buffers used in this assay had the same composition as those used for the binding with [^{125}I][Sar^1]-angiotensin II. Tissue sections were preincubated for 15 min in incubation buffer followed by an incubation for 120 min in fresh buffer containing 0.2 nM [^{125}I]CGP 42112. To determine non-specific binding and specific binding to angiotensin AT_2 receptors consecutive sections were incubated in the presence of 0.2 nM [^{125}I]CGP 42112 together with 1 μM CGP 42112 or 5 μM angiotensin II, respectively. After incubation, slides were rinsed four times for 1 min each in ice-cold 50 mM Tris-buffer (pH 7.4) and for 30 s in ice-cold water, and dried in a cold air flow.

2.5. Quantitative autoradiography

Dry sections were exposed to Hyperfilm-^{3}H along with 16 μm sections of autoradiographic [^{125}I] micro scales at 4°C. Films were developed in ice-cold D-19 developer for 4 min, fixed in rapid fixer for 4 min at 22°C, and rinsed in water for 15 min. Optical densities were measured in the autoradiograms by computerized micro densitometry using the NIH Image 1.6 analysis system (NIMH, Bethesda, MD). The optical densities were related to the concentration of radioactivity present in the sections by comparison with the [^{125}I] micro scales, and transformed to corresponding values of fmol/mg protein as described earlier (Nazarali et al., 1989).

2.6. Statistical analysis

Results in tables and graphs were expressed as means ± standard error of the mean (S.E.M.). They were calculated and analyzed using GraphPad Prism (version 2.00) and Microsoft Excel (version 7.0a). Statistical analysis for values obtained from the displacement studies using single concentrations of the displacers was performed using an one-way analysis of variance (ANOVA) followed by post hoc analysis with the Newman–Keuls multiple comparison test. A $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Analysis of ligand metabolism

When mouse brain sections were incubated in the presence of 0.5 nM [^{125}I][Sar^1]-angiotensin II, no formation of radioactive breakdown products of the radiolabeled ligand was observed as analyzed by reversed phase high performance liquid chromatography (Fig. 1).

3.2. Localization of [^{125}I][Sar^1]-angiotensin II binding

After incubation with a single concentration of [^{125}I][Sar^1]-angiotensin II (0.5 nM), autoradiographic images revealed that angiotensin II receptors were discreetly distributed in the mouse brain. We found the highest receptor concentrations in the circumventricular organs, the posterodorsal part of the medial nucleus of the amygdala, the paraventricular, periventricular, and suprachiasmatic hypothalamic nucleus, the median eminence, the dorsomedial part of the interpeduncular nucleus, the locus coeruleus, the inferior olive, the dorsal motor nucleus of the vagus, and the nucleus of the solitary tract (Table 1).

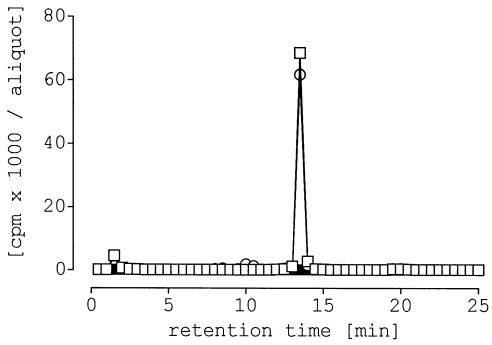

Fig. 1. Stability of [^{125}I][Sar^1]-angiotensin II. Lines show the radioactivity measured in eluted high performance liquid chromatography fractions after injecting aliquots of 50 μl. Radioactivity in the fractions was measured using a γ-counter. Aliquots of incubation buffer without radiolabeled ligand (■), and aliquots of buffer containing 0.5 nM [^{125}I][Sar^1]-angiotensin II before (□) and after a 2 h incubation (○) were analyzed. Results are expressed as mean values ± S.E.M. ($n = 4$).

3.3. Pharmacological characterization of angiotensin II receptors and their subtypes (AT_1 and AT_2)

Competition curves were constructed in selected brain areas (Figs. 2 and 3). [^{125}I][Sar^1]-angiotensin II (0.5 nM) was displaced by using increasing concentrations of angiotensin II (10^{-12} to 10^{-5} M), losartan (10^{-11} to 10^{-4} M), candesartan (10^{-12} to 10^{-5} M), CGP 42112 (10^{-12} to 10^{-5} M) and PD 123319 (10^{-12} to 10^{-5} M).

Angiotensin II displaced [^{125}I][Sar^1]-angiotensin II binding in all areas (Figs. 2 and 3) with an IC_{50} from 6.7×10^{-9} M up to 1.7×10^{-8} M (Table 2).

Displacement with selective angiotensin AT_1 and AT_2 receptor ligands, however, was different for each of the areas examined. In the caudate putamen (Fig. 2A), ventral lateral septum (Fig. 3A), and nucleus of the solitary tract (Fig. 2C) the angiotensin AT_1 receptor selective blocker losartan displaced [^{125}I][Sar^1]-angiotensin II binding with an IC_{50} from 1.5×10^{-8} up to 1.1×10^{-7} M, however to a different extent in each area. Losartan was able to almost completely displace binding in the caudate putamen, but could only partially displace binding in the ventral lateral septum and the nucleus of the solitary tract. Conversely, losartan could not displace binding in the hypoglossal nucleus (Table 2; curve not shown) and the inferior olive (Fig. 2E). The displacement produced by the angiotensin

AT_1 receptor antagonist candesartan was similar to that of losartan. Candesartan displaced binding to a different extent in the areas examined: completely in the caudate putamen (Fig. 2A), and partially in the dorsal lateral septum (Fig. 3C) and in the nucleus of the solitary tract (Fig. 2C). For candesartan, the IC_{50} values were lower (3–15-fold) compared to those obtained with losartan (Table 2). As it was the case with losartan, candesartan was unable to displace [^{125}I][Sar^1]-angiotensin II in the hypoglossal nucleus (Table 2; curve not shown) and inferior olive (Fig. 2E).

Table 1
Specific binding of [^{125}I][Sar^1]-angiotensin II and [^{125}I]CGP 42112 to angiotensin II receptors and angiotensin AT_2 receptors, respectively, in different brain areas of 8-week-old mice

Brain areas	Mean ± S.E.M.	
	[^{125}I][Sar^1] -Ang II	[^{125}I]CGP 42112
I. Circumventricular organs		
Area postrema	28.5 ± 2.1	7.9 ± 0.4
Median eminence	25.1 ± 1.2	nd
Organum vasculosum of the lamina terminalis	28.2 ± 7.1	nd
Subfornical organ	22.0 ± 3.0	nd
II. Telencephalon		
Anterior olfactory nucleus	9.0 ± 1.7	nd
Amygdala		
Basolateral nucleus	16.4 ± 0.6	3.6 ± 0.8
Central nucleus	13.4 ± 1.8	6.6 ± 1.8
Medial nucleus		
anterior part	13.7 ± 1.8	5.9 ± 0.2
posterodorsal part	22.3 ± 2.4	15.1 ± 1.8
Basal ganglia		
Caudate putamen	9.1 ± 0.6	nd
Bed nucleus of the stria terminalis	29.9	11.1 ± 4.4
Lateral septum		
Ventral part	10.5 ± 1.1	4.3 ± 0.6
Dorsal part	18.0 ± 2.4	4.6 ± 0.7
III. Diencephalon		
Hypothalamus		
Arcuate nucleus	14.7 ± 3.0	nd
Dorsomedial nucleus	18.4 ± 2.4	nd
Median preoptic nucleus	22.5 ± 4.9	nd
Paraventricular nucleus	48.2 ± 2.2	nd
Periventricular nucleus	23.5 ± 3.3	nd
Suprachiasmatic nucleus	21.1 ± 6.2	nd
IV. Midbrain		
Interpeduncular nucleus		
Central part	17.5 ± 0.7	nd
Dorsomedial part	33.0 ± 4.8	nd
Lateral part	17.2 ± 2.5	7.4 ± 2.9
Rostral interstitial nucleus of the medial longitudinal fasciculus	15.6 ± 3.1	6.9 ± 1.2
Superficial grey layer of the superior colliculus	14.3 ± 1.2	8.9 ± 0.9
V. Medulla		
Dorsal motor nucleus of the vagus	24.3 ± 2.7	9.0 ± 1.9
Hypoglossal nucleus	14.4 ± 1.7	7.8 ± 0.7
Inferior olive	26.7 ± 4.6	15.3 ± 1.2
Locus coeruleus	21.6 ± 3.3	nd
Nucleus of the solitary tract	24.1 ± 3.1	9.3 ± 1.2
Spinal trigeminal tract	14.5 ± 2.3	3.4 ± 0.2

Values are means ± S.E.M. in fmol/mg protein ($n = 4$–5; $n = 1$ for [^{125}I][Sar^1]-angiotensin II binding in the bed nucleus of the stria terminalis).
Binding of [^{125}I][Sar^1]-angiotensin II to angiotensin II receptors was calculated as the difference in binding after incubating consecutive sections in 0.5 nM [^{125}I][Sar^1]-angiotensin II (total binding) and in the presence of 1 μM angiotensin II (non-specific binding).
Specific binding of [^{125}I]CGP 42112 to angiotensin AT_2 receptors was calculated as the difference in binding of 0.2 nM [^{125}I]CGP 42112 (total binding) and binding of 0.2 nM [^{125}I]CGP 42112 in the presence of 5 μM angiotensin II.
There was no difference, when binding of [^{125}I]CGP 42112 in the presence of angiotensin II was compared to binding in the presence of CGP 42112 (1 μM; = non-specific binding).
nd = binding of [^{125}I]CGP 42112 was not significantly different when compared with binding of [^{125}I]CGP 42112 in the presence of CGP 42112 or angiotensin II.

The angiotensin AT_2 receptor selective ligands displaced [Sar^1]-angiotensin II binding with a different profile than angiotensin AT_1 receptor selective blockers. In the hypoglossal nucleus and the inferior olive, CGP 42112 displaced completely the [^{125}I][Sar^1]-angiotensin II binding, with an IC_{50} of 9.2×10^{-10} M and 2.1×10^{-9} M, respectively (Table 2). In the ventral lateral septum (Fig. 3B) and the nucleus of the solitary tract (Fig. 2D), displacement with CGP 42112 resulted in a bisigmoidal competition curve showing high and low affinity sites. The IC_{50} values for the high affinity site ranged from 4.6×10^{-10} M up to 1.2×10^{-9} M (Table 2), in the same range as the IC_{50} values calculated from the CGP 42112 competition curves in the inferior olive and hypoglossal nucleus, where no losartan-sensitive binding was present. Conversely, the calculated IC_{50} values for the low affinity site to CGP 42112 were higher than 10^{-6} M (Table 2).

CGP 42112 displaced [Sar^1]-angiotensin II binding in the caudate putamen; although the calculated IC_{50} for displacement in this area was low, the relative amount of total binding displaced by CGP 42112 was small (10%; Fig. 2B). Displacement with the angiotensin AT_2 receptor ligand PD 123319 was similar to that obtained with CGP 42112, with complete displacement in the hypoglossal nucleus (curve not shown) and the inferior olive (Fig. 2F), partial displacement in the ventral lateral septum (Fig. 3B) and the nucleus of the solitary tract (Fig. 2D), and very little displacement in the caudate putamen (10% of total

binding; Fig. 2B). The displacement curves were monophasic, and their IC_{50} values were higher (19- to 27-fold in all areas except in the caudate putamen, where IC_{50} was about 500-fold higher) when compared to the IC_{50} values of the high affinity site of the CGP 42112 competitions curves (Table 2).

3.4. Localization of angiotensin II receptor subtypes (AT_1 and AT_2)

To determine the relative expression of angiotensin II receptor subtypes, adjacent sections were incubated with [^{125}I][Sar^1]-angiotensin II (0.5 nM) in the presence of

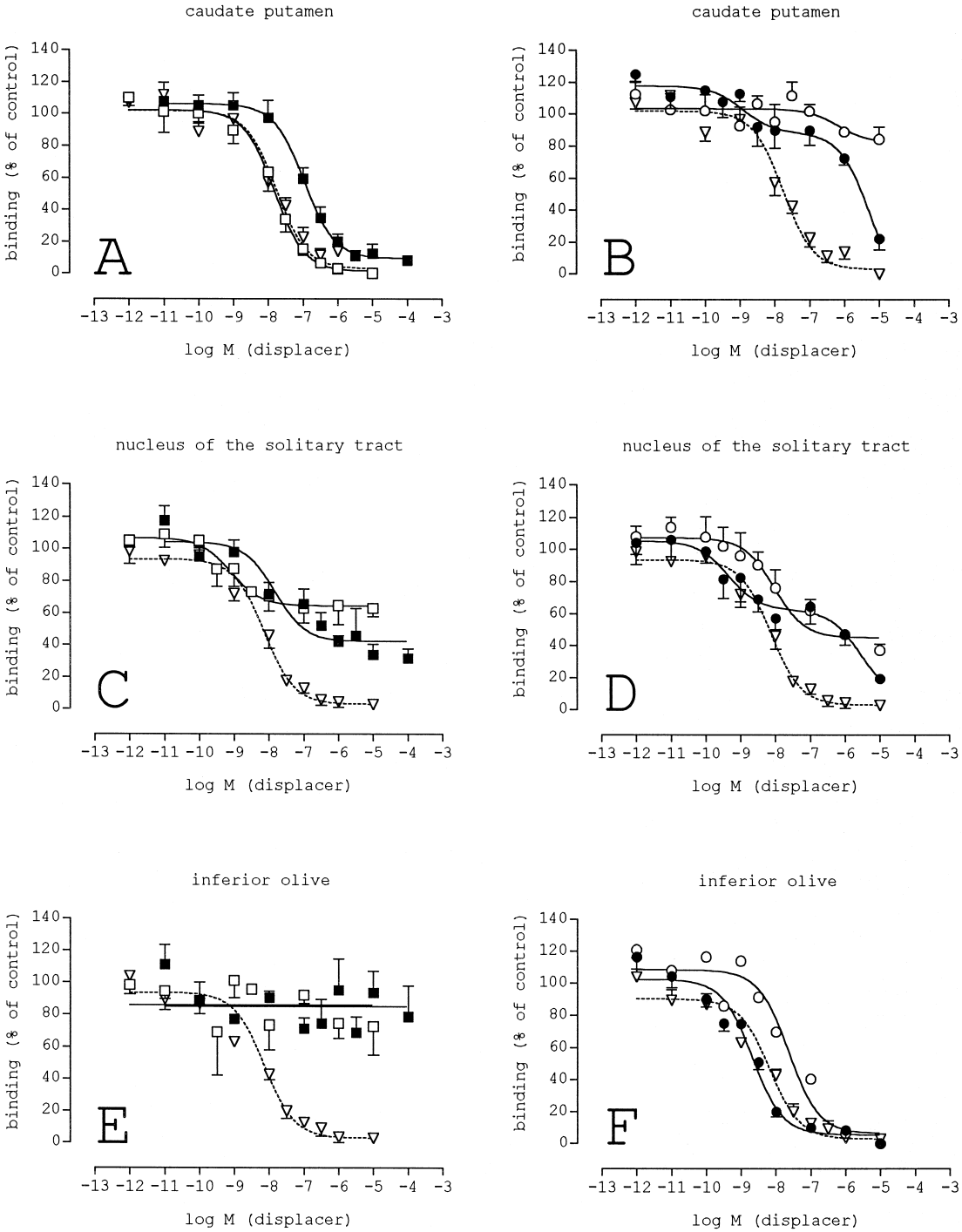

Fig. 2. Characterization of angiotensin II receptors and their subtypes (AT_1 and AT_2) in selected mouse brain areas (caudate putamen [A + B], nucleus of the solitary tract [C + D], and inferior olive [E + F]) by quantitative autoradiography. The figures show competition curves obtained from consecutive brain sections incubated in the presence of 0.5 nM [^{125}I][Sar^1]-angiotensin II and increasing concentrations of angiotensin II (∇) [A–F], of the angiotensin AT_1 receptor antagonists losartan (■) and candesartan (□) [A, C, E], and of the angiotensin AT_2 receptor ligands CGP 42112 (●) and PD 123319 (○)[B, D, F]. Results are expressed as mean values ± S.E.M. (n = 3–4).

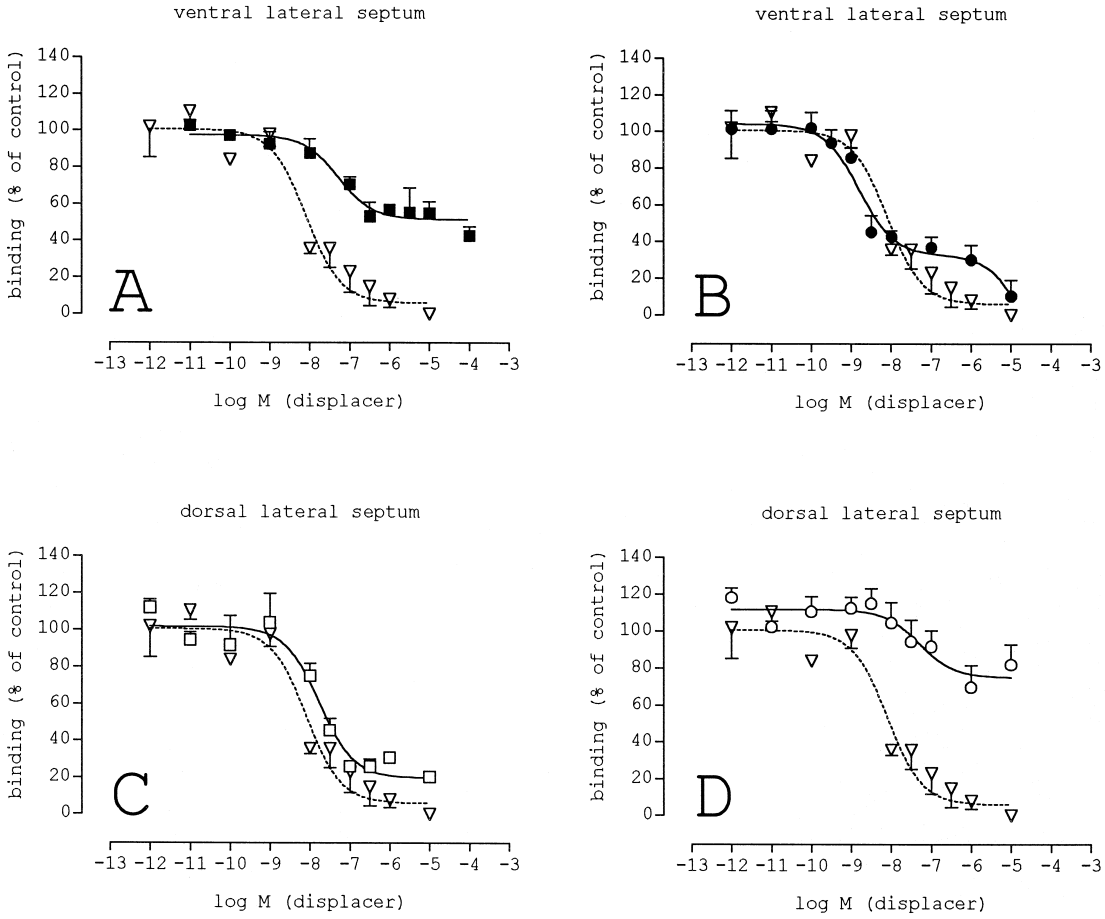

Fig. 3. Characterization of angiotensin II receptors and their subtypes (AT_1 and AT_2) in the ventral and dorsal lateral septum by quantitative autoradiography. The figures show competition curves obtained from consecutive brain sections incubated in the presence of 0.5 nM [^{125}I][Sar^1]–angiotensin II and increasing concentrations of angiotensin II (∇) [A–D], of the angiotensin AT_1 receptor antagonists losartan (■) [A] and candesartan (□) [C], and of the angiotensin AT_2 receptor ligands CGP 42112 (●) [B] and PD 123319 (○)[D]. Results are expressed as mean values ± S.E.M. (n = 3–4).

losartan or CGP 42112 (Figs. 4 and 5). Non-specific binding of [^{125}I][Sar^1]-angiotensin II was determined by incubating adjacent sections in the presence of 1 μM angiotensin II (Figs. 4 and 5). The concentrations for this single-concentration displacement assay were derived from the competition curves (Figs. 2 and 3). We selected the lowest concentration of losartan (3.3 μM) and CGP 42112 (0.1 μM) which produced a maximum specific displacement of angiotensin AT_1 or AT_2 receptors, as determined earlier for the rat brain (Tsutsumi and Saavedra, 1991).

As it was the case for our displacement study, we found differences among the areas in terms of the amount of binding displaced by selective angiotensin AT_1 and AT_2 receptor ligands. In some areas, binding was completely displaced by angiotensin AT_1 receptor selective ligands, and was insensitive to displacement by angiotensin AT_2

Table 2
Kinetics of displacement of [^{125}I][Sar^1]-angiotensin II (0.5 nM) by angiotensin II, losartan, candesartan, CGP 42112, and PD 123319 in selected brain areas

Brain region	IC_{50} (M)				
	Ang II	Losartan	Candesartan	CGP 42112	PD 123319
Caudate putamen	1.7×10^{-8}	1.1×10^{-7}	1.5×10^{-8}	1.2×10^{-9} (5.6×10^{-6})	6.6×10^{-7}
Lateral septum	6.7×10^{-9}	5.2×10^{-8}	1.8×10^{-8}	1.6×10^{-9} (2.7×10^{-5})	2.4×10^{-8}
Nucleus of the solitary tract	7.3×10^{-9}	1.5×10^{-8}	8.2×10^{-10}	4.6×10^{-10} (2.7×10^{-6})	9.4×10^{-9}
Hypoglossal nucleus	6.7×10^{-9}	ND	ND	9.2×10^{-10}	Ø
Inferior olive	6.3×10^{-9}	ND	ND	2.1×10^{-9}	2.3×10^{-8}

Values are means of those concentrations of the competitors needed for half-maximal displacement (IC_{50}) of [^{125}I][Sar^1]-angiotensin II (n = 3–4).
Values in parenthesis show the calculated IC_{50} values for the low affinity site of CGP 42112 A.
ND = no displacement detected at a concentration of 10^{-4} M losartan and 10^{-5} M candesartan, respectively.
Ø = Displacement curve using PD 123319 was not constructed in this brain region.

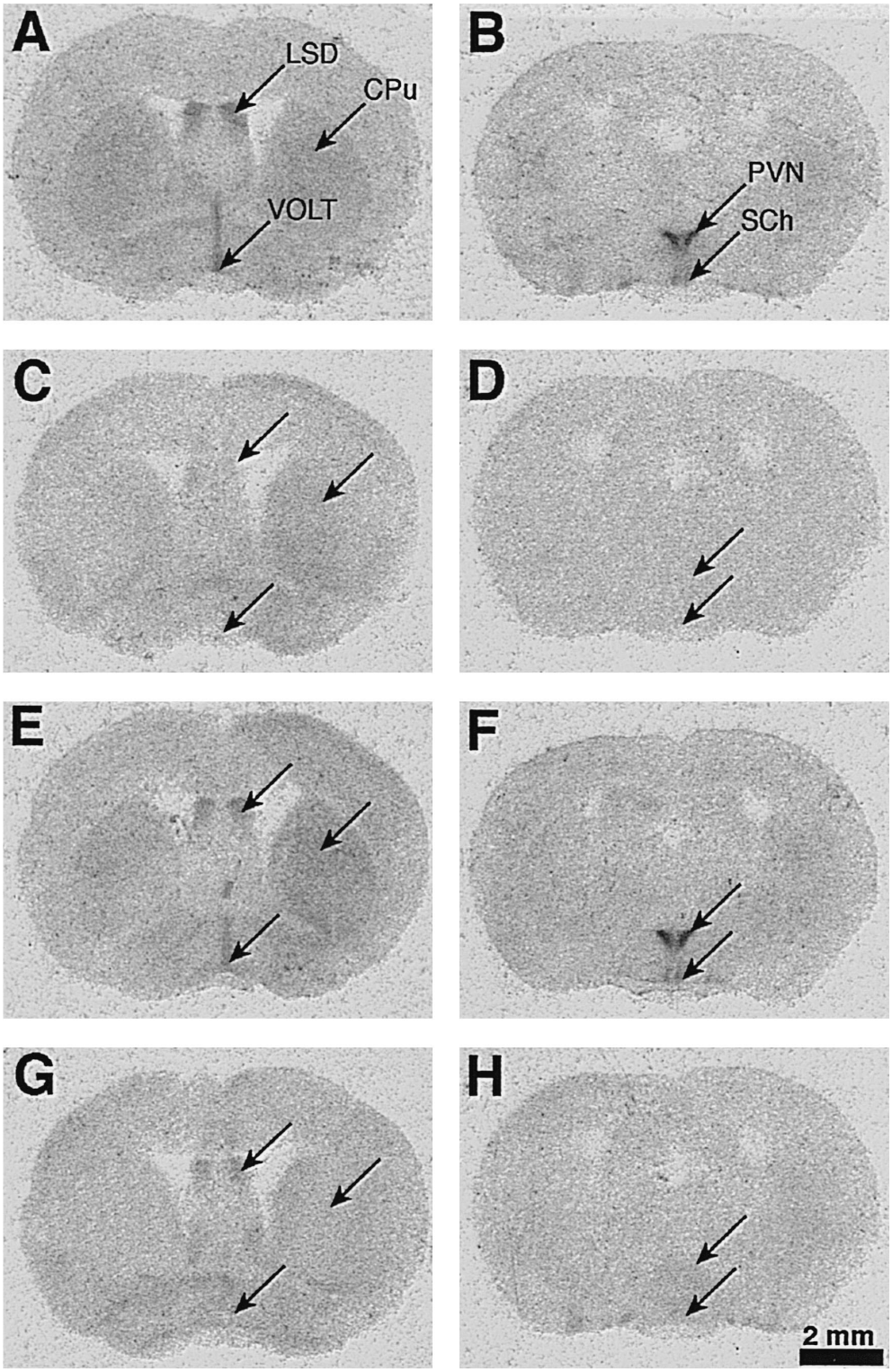

Fig. 4. Autoradiograms show angiotensin II receptors and their subtypes in selected nuclei in the forebrain of the mouse. Sections were incubated in the presence of 0.5 nM [^{125}I][Sar^{1}]-angiotensin II for total binding (A + B). For non-specific binding, adjacent sections were incubated in the presence of angiotensin II (1 μM; C + D). To illustrate angiotensin AT_1 receptors, binding of [^{125}I][Sar^{1}]-angiotensin II to angiotensin AT_2 receptors was displaced by the angiotensin AT_2 receptor ligand CGP 42112 (0.1 μM; E + F). To illustrate angiotensin AT_2 receptors, binding of [^{125}I][Sar^{1}]-angiotensin II to angiotensin AT_1 receptors was displaced by the angiotensin AT_1 receptor ligand losartan (3.3 μM; G + H). In A, binding to the VOLT appears low when compared to values in Table 1, because this represents a caudal section of the VOLT, where expression of binding is lower than in frontal sections. Abbreviations: CPu, caudate putamen; LSD, lateral septum, dorsal part; VOLT, vascular organ of the lamina terminalis; PVN, paraventricular nucleus; SCh, suprachiasmatic nucleus.

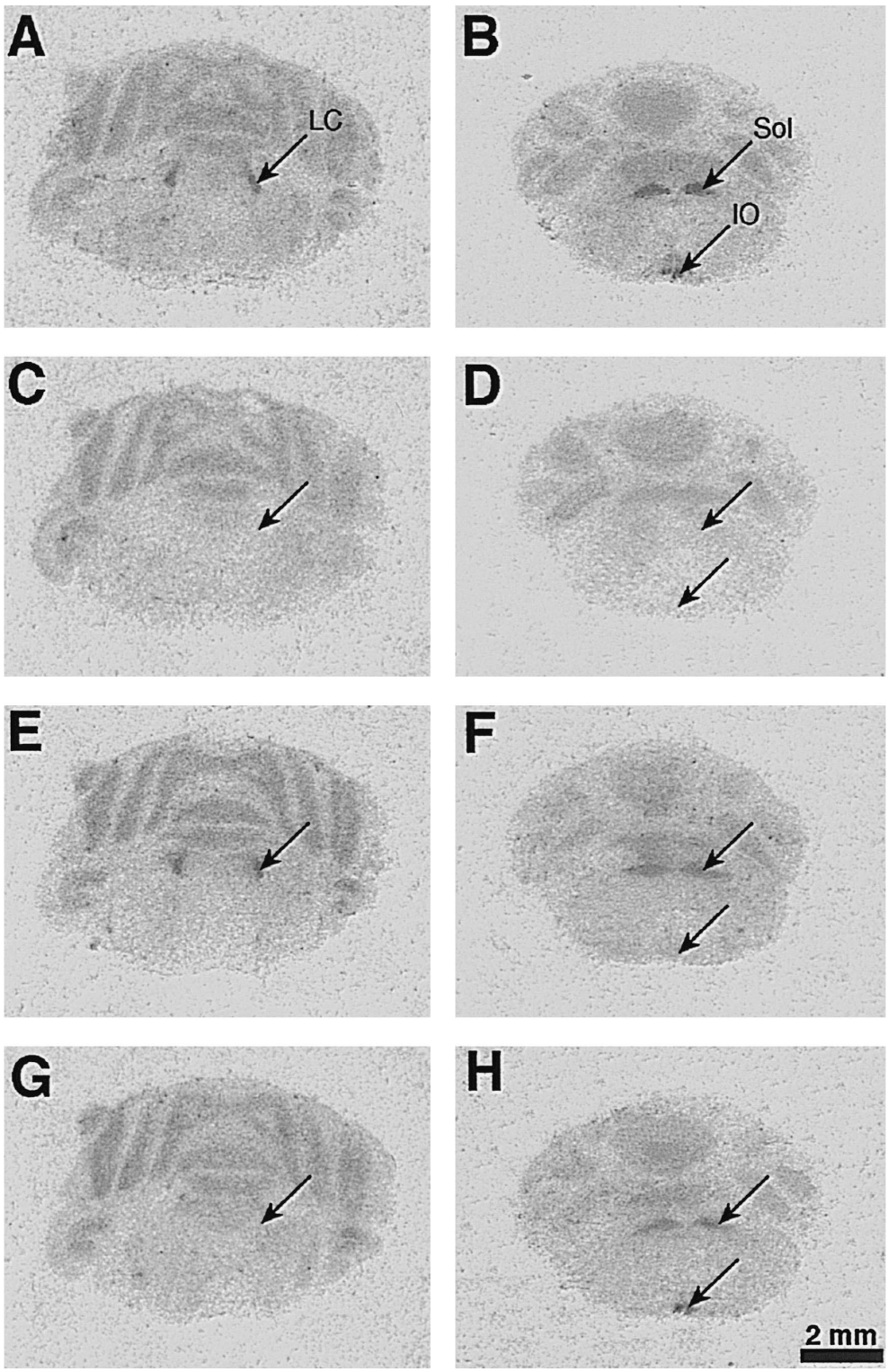

Fig. 5. Autoradiograms show angiotensin II receptors and their subtypes in selected nuclei in the hindbrain of the mouse. Sections were incubated in the presence of 0.5 nM [^{125}I][Sar^{1}]-angiotensin II for total binding (A + B). For non-specific binding, adjacent sections were incubated in the presence of angiotensin II (1 μM; C + D). To illustrate angiotensin AT_1 receptors, binding of [^{125}I][Sar^{1}]-angiotensin II to angiotensin AT_2 receptors was displaced by the angiotensin AT_2 receptor ligand CGP 42112 (0.1 μM; E + F). To illustrate angiotensin AT_2 receptors, binding of [^{125}I][Sar^{1}]-angiotensin II to angiotensin AT_1 receptors was displaced by the angiotensin AT_1 receptor ligand losartan (3.3 μM; G + H). Abbreviations: IO, inferior olive; LC, locus coeruleus; Sol, nucleus of the solitary tract.

receptor selective ligands. These areas were the subfornical organ, arcuate nucleus, median eminence, median preoptic nucleus, suprachiasmatic nucleus, interpeduncular nucleus (dorsomedial and central part), anterior olfactory nucleus, caudate putamen, locus coeruleus, paraventricular and periventricular nucleus and the organum vasculosum of the lamina terminalis (Table 3).

Conversely, only angiotensin AT_2 receptor selective, but not angiotensin AT_1 receptor selective, ligands displaced binding in the hypoglossal nucleus, inferior olive,

Table 3
Distribution of angiotensin II AT_1 and AT_2 receptor subtypes in the mouse brain

	Mean ± S.E.M.	
	AT_1 receptors (% of specific binding displaced by losartan)	AT_2 receptors (% of specific binding displaced by CGP 42112)
1. Areas containing angiotensin AT_1 receptors		
Median preoptic nucleus	84 ± 4	0
Organum vasculosum of the lamina terminalis	87 ± 8	0
Subfornical organ	109 ± 4	0
Anterior olfactory nucleus	81 ± 12	0
Caudate putamen	81 ± 7	0
Arcuate nucleus	84 ± 9	0
Median eminence	96 ± 5	0
Paraventricular nucleus	97 ± 2	0
Periventricular nucleus	87 ± 9	0
Suprachiasmatic nucleus	94 ± 2	0
Interpeduncular nucleus		
central part	82 ± 10	0
dorsomedial part	88 ± 18	0
Locus coeruleus	88 ± 9	0
2. Areas containing angiotensin AT_1 and AT_2 receptors		
Area postrema	61 ± 4	47 ± 5
Amygdala: basolateral nucleus	66 ± 9	31 ± 7
Bed nucleus of the stria terminalis	39	50
Lateral septum		
ventral part	35 ± 16	74 ± 11
dorsal part	67 ± 11	37 ± 6
Dorsomedial nucleus of the hypothalamus	55 ± 15	46 ± 17
Interpeduncular nucleus: lateral part	30 ± 5	75 ± 12
Dorsal motor nucleus of the vagus	47 ± 12	49 ± 20
Nucleus of the solitary tract	52 ± 3	59 ± 5
Spinal trigeminal tract	71 ± 8	46 ± 10
3. Areas containing angiotensin AT_2 receptors		
Amygdala		
central nucleus	0	92 ± 10
medial nucleus		
anterior part	0	91 ± 8
posterodorsal part	0	106 ± 14
Rostral interstitial nucleus of the medial longitudinal fasciculus	0	96 ± 7
Superficial grey layer of the superior colliculus	0	93 ± 4
Hypoglossal nucleus	0	98 ± 5
Inferior olive	0	92 ± 3

Values are means ± S.E.M. in percent of the specific binding of [^{125}I][Sar^1]-angiotensin II (0.5 nM) to angiotensin II receptors displaced by losartan (3.3 μM) or CGP 42112 (0.1 μM; $n = 3–5$; $n = 1$ for the bed nucleus of the stria terminalis).
Displacement with angiotensin II (1 μM) corresponds to a displacement of 100%.
In group 1, binding of [^{125}I][Sar^1]-angiotensin II was significantly displaced by losartan, but not by CGP 42112.
In group 2, binding of [^{125}I][Sar^1]-angiotensin II was significantly displaced by both CGP 42112 and losartan.
In group 3, binding of [^{125}I][Sar^1]-angiotensin II was significantly displaced by CGP 42112, but not by losartan.
0 = binding of [^{125}I][Sar^1]-angiotensin II was not significantly displaced by CGP 42112 (group 1) or losartan (group 3).

central and medial (anterior and posterodorsal part) nucleus of the amygdala, rostral interstitial nucleus of the medial longitudinal fasciculus, and in the superficial grey layer of the superior colliculus (Table 3).

In a third group of brain areas (area postrema, basolateral nucleus of the amygdala, ventral and dorsal parts of the lateral septum, dorsomedial hypothalamic, and interpeduncular nuclei, dorsal motor nucleus of the vagus, nucleus of the solitary tract, and spinal trigeminal tract) binding was sensitive to both angiotensin AT_1 and AT_2 receptor ligands (Table 3).

3.5. Localization of angiotensin AT_2 receptors

To further verify the presence of angiotensin AT_2 receptors in the mouse brain, another set of consecutive sections was incubated with [^{125}I]CGP 42112 (0.2 nM) in the presence of angiotensin II (5 μM) or CGP 42112 (1 μM) (Fig. 6). As expected, most areas in which unlabeled CGP 42112 significantly displaced [^{125}I][Sar^1]-angiotensin II binding (Table 3) did show specific binding of [^{125}I]CGP 42112 (Table 1). There was a positive correlation between the quantification of angiotensin AT_2 receptors using [^{125}I]CGP 42112 with displacement by angiotensin II and [^{125}I][Sar^1]-angiotensin II with displacement by unlabeled CGP 42112, respectively (slope = 0.6112; $r^2 = 0.9838$; Fig. 7). Only in the dorsomedial nucleus of the hypothalamus there was a mismatch between the findings obtained with [^{125}I]CGP 42112 binding and those found after displacement of [^{125}I][Sar^1]-angiotensin II with unlabeled CGP 42112. While the assay using [^{125}I]CGP 42112 did not reveal any binding, a significant amount of [^{125}I][Sar^1]-

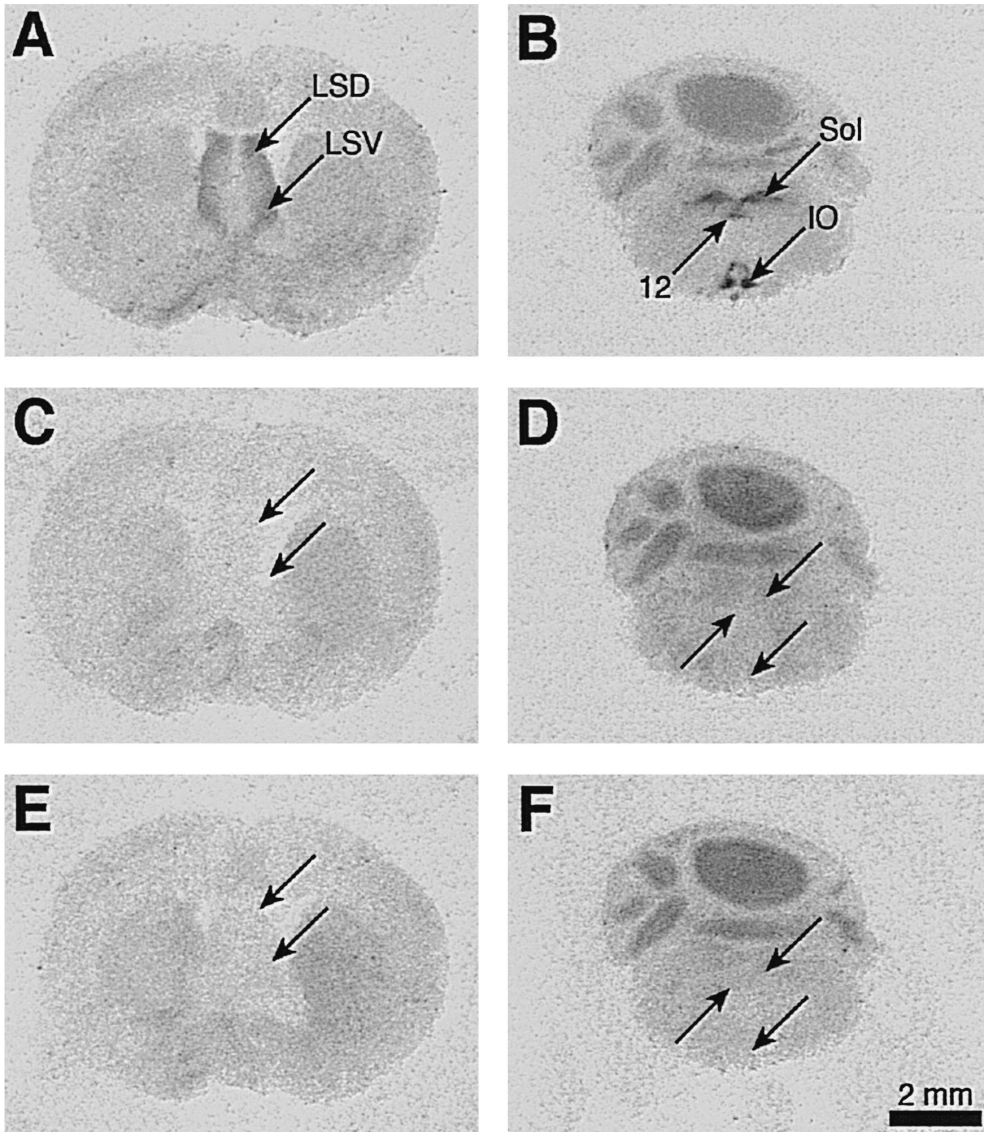

Fig. 6. Displacement of [^{125}I]CGP 42112 from angiotensin AT_2 receptors by angiotensin II and CGP 42112. Sections were incubated in the presence of 0.2 nM [^{125}I]CGP 42112 for total binding (A + B). For non-specific binding, adjacent sections were incubated in the presence of CGP 42112 (1 μM; C + D), and in the presence of angiotensin II (5 μM; E + F) to determine specific binding to angiotensin AT_2 receptors. Abbreviations: 12, hypoglossal nucleus; IO, inferior olive; LSD, lateral septum, dorsal part; LSV, lateral septum, ventral part; Sol, nucleus of the solitary tract.

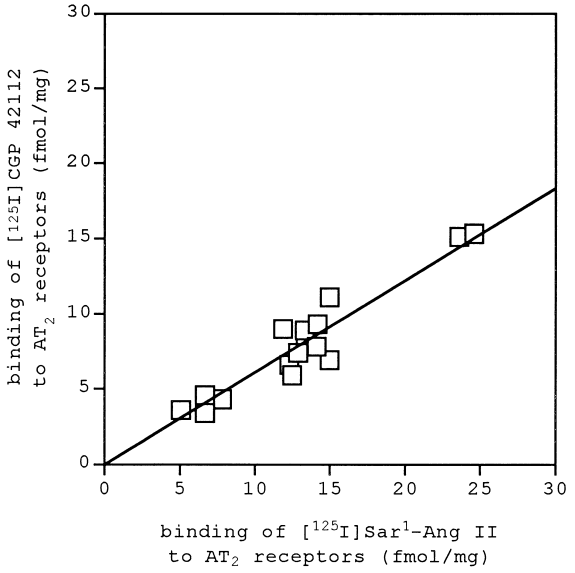

Fig. 7. Correlation between specific binding of [^{125}I][Sar^1]-angiotensin II and [^{125}I]CGP 42112 to angiotensin AT_2 receptors (slope = 0.6112; $r^2 = 0.9838$).

angiotensin II binding was displaced by unlabeled CGP 42112 (Table 3).

4. Discussion

Angiotensin II and derivatives are metabolized very differently under incubation conditions for autoradiography, in different rodent species (De Oliveira et al., 1995). We report, however, that in the mouse brain, binding of [^{125}I][Sar^1]-angiotensin II represents that of angiotensin II and not one of its breakdown products, since we were not able to detect any radioactive metabolites of [^{125}I][Sar^1]-angiotensin II by analyzing aliquots of the incubation buffer after 2 h of incubation.

We used a non-saturating [^{125}I][Sar^1]-angiotensin II concentration, necessary to reliably determine angiotensin II receptor subtypes with displacement by specific angiotensin AT_1 or AT_2 receptor ligands (Tsutsumi and Saavedra, 1991). For this reason, the binding densities measured do not reflect the total receptor number, and cannot be quantitatively compared with our previous studies in the rat brain, in which a saturating concentration of [^{125}I][Sar^1]-angiotensin II (3 nM) was used (Tsutsumi and Saavedra, 1991). The reported concentrations of binding between areas are not more than five-fold different, and it is likely that the use of the ligand at saturating concentrations will reveal a much larger difference in receptor numbers between different areas.

Binding of [^{125}I][Sar^1]-angiotensin II was selectively localized and was specific, being displaced by non-labeled angiotensin II in most areas by more than 90%. The exception was the cerebellar cortex, which exhibited an unusual degree of non-specific binding under the conditions of our experiments.

The pattern of angiotensin II binding expression in the adult mouse brain is in general agreement with that in other mammalian species (McKinley et al., 1986; Tsutsumi and Saavedra, 1991; Saylor et al., 1992; Aldred et al., 1993; MacGregor et al., 1995; Jöhren et al., 1997). Binding is highly expressed in the circumventricular organs, selected hypothalamic nuclei, selected areas in the limbic system, and brain stem areas involved in cardiovascular regulation.

Some notable differences are present when compared to the angiotensin II binding expressed in the rat. For example, there is an expression of angiotensin II binding in the caudate putamen, interpeduncular and rostral interstitial nuclei of the mouse, where none is detected in the rat (Tsutsumi and Saavedra, 1991). Conversely, in the medial geniculate nucleus, where angiotensin II receptors are expressed in the rat (Tsutsumi and Saavedra, 1991) we found no binding in the mouse. There are some additional developmental differences: for example, angiotensin II binding is expressed in the hypoglossal nucleus of the adult mouse, but only occurs in the immature, not the adult, rat brain.

The relative expression of specific angiotensin AT_1 or AT_2 receptor subtypes was determined by displacing [^{125}I][Sar^1]-angiotensin II binding with angiotensin AT_1 or AT_2 receptor selective ligands. Our estimates are meaningful because we chose the angiotensin AT_1 and AT_2 receptor displacers at concentrations which maintain the selectivity for the corresponding receptor subtype (see below). As a note of caution, we should point out that [^{125}I][Sar^1]-angiotensin II has higher affinity for the angiotensin AT_1 receptor than for the angiotensin AT_2 receptor (Rowe et al., 1992; Tsutsumi and Saavedra, 1992) and it is possible that we are underestimating the absolute angiotensin AT_2 receptor concentration in our [^{125}I][Sar^1]-angiotensin II binding experiments. To fully clarify this issue, it would be essential to use saturating concentrations of totally specific angiotensin AT_1 and AT_2 receptor radiolabeled ligands, which are not presently available.

The selectivity of different ligands for each of the angiotensin II receptor subtypes is not absolute, but relative. That is, there are optimum ligand concentrations which selectively displace binding from one receptor subtype, and not the other. With higher ligand concentrations, the specificity can be lost, and binding is displaced from both subtypes (Tsutsumi and Saavedra, 1991). To determine the precise ligand concentrations which selectively displace a specific receptor subtype, we first established the affinity of the angiotensin AT_1 and AT_2 receptors for selective angiotensin AT_1 and AT_2 receptor ligands in some specific brain areas. Because of their small size, not all displacement curves could be performed in all areas of interest.

We found that the affinity of mouse angiotensin II receptors for angiotensin AT_1 or AT_2 receptor ligands, is

similar to that in the rat and human (Tsutsumi and Saavedra, 1991; Sasamura et al., 1992; Nakajima et al., 1993), and that in the mouse brain, like in the brain of all other mammalian species studied (McKinley et al., 1986; Tsutsumi and Saavedra, 1991; Saylor et al., 1992; Aldred et al., 1993; MacGregor et al., 1995; Jöhren et al., 1997), there is a discrete localization of both angiotensin AT_1 and AT_2 receptors. Similarly, the ligand affinities for both angiotensin AT_1 and AT_2 receptors in the mouse brain do not differ among the nuclei studied and are similar to those in similar areas of the rat (Tsutsumi and Saavedra, 1991, 1992; Rowe et al., 1992; Jöhren et al., 1997). Since we used candesartan for the first time as a selective angiotensin AT_1 receptor displacer (Noda et al., 1993) for competition curves in autoradiographic studies, there are no available data to compare with values in the rat. The binding affinity for candesartan observed in our study is one order of magnitude higher than that of losartan, and is consistent with data obtained from membrane binding studies (Noda et al., 1993; Shibouta et al., 1993; Flesch et al., 1995).

A number of areas in the mouse brain expressed angiotensin AT_1 receptors only, as determined by total displacement of [^{125}I][Sar^1]-angiotensin II by losartan, and lack of significant displacement by CGP 42112. These areas were concentrated in the forebrain, including hypothalamic nuclei and circumventricular organs. In other brain areas, only CGP 42112 completely displaced angiotensin II binding, while losartan was ineffective. We conclude that these areas express angiotensin AT_2 receptors only.

In addition, the presence of mixed populations of angiotensin AT_1 and AT_2 receptors is a widespread occurrence in the mouse brain. We conclude that angiotensin AT_1 and AT_2 receptor subtypes are colocalized in specific brain areas when both angiotensin AT_1 receptor antagonists and angiotensin AT_2 receptor ligands partially but significantly displace [^{125}I][Sar^1]-angiotensin II binding. In areas with a mixture of angiotensin AT_1 and AT_2 receptors, competition with CGP 42112 revealed two-sites, one with high and another with low affinity. In these cases, the site with a high affinity for CGP 42112 corresponds to a angiotensin AT_2 receptor site, and the site with a low affinity for CGP 42112 to the angiotensin AT_1 receptor site, since the angiotensin AT_1 receptors can be displaced by very high CGP 42112 concentrations (Tsutsumi and Saavedra, 1991, 1992).

Thus, with the use of several angiotensin AT_1 and AT_2 receptor ligands, we can determine the presence of each of the angiotensin II receptor subtypes with a reasonable degree of accuracy, even in areas where the accurate determination of receptor subtypes appeared problematic. For example, in the caudate putamen, (Fig. 2A and B) displacement of a small fraction of the [Sar^1]-angiotensin II binding by CGP 42112 and PD 123319 might have suggested the presence of small numbers of angiotensin AT_2 receptors. However, our complete displacement profile does not support this conclusion. First, although losartan displaces only about 90% of the angiotensin II binding, the high affinity angiotensin AT_1 receptor antagonist candesartan displaces 100% of binding. In addition, incubation with [^{125}I]CGP 42112 does not reveal any significant binding in the caudate putamen. Furthermore, although the selective angiotensin AT_2 receptor ligand PD 123319 was able to displace about 10% of the binding, the calculated EC_{50} value (6.6×10^{-7} M) is very high, and therefore this effect cannot be considered a specific displacement from angiotensin AT_2 receptors. Unlabeled CGP 42112 appears to displace some [Sar^1]-angiotensin II binding as well (Fig. 2B). However, a close examination of the displacement curve revealed that very low CGP 42112 concentrations actually increased [Sar^1]-angiotensin II binding above 100%, a phenomenon which does not occur with higher CGP 42112 concentrations (Fig. 1B). The reason for the [Sar^1]-angiotensin II binding increase in the presence of low CGP 42112 concentrations is unknown.

Both angiotensin AT_1 and AT_2 receptors are expressed in the lateral septum, and their relative proportion varies with the area studied. In the dorsal part of the lateral septum, the majority of the binding sites are of the angiotensin AT_1 receptor subtype. In the ventral part, the angiotensin AT_2 receptor subtype is the predominant receptor.

The use of a specific angiotensin AT_2 receptor ligand, [^{125}I]CGP 42112, (Heemskerk and Saavedra, 1995) further confirmed the presence of angiotensin AT_2 receptors in selected areas of the mouse brain. For the most part, an excellent correlation was found between [^{125}I]CGP 42112 binding and that portion of the [^{125}I][Sar^1]-angiotensin II binding displaced by 0.1 μM CGP 42112. The only exception was the dorsomedial nucleus of the hypothalamus. We could not detect any [^{125}I]CGP 42112 binding in this area, although unlabeled CGP 42112 displaced a significant portion of the [^{25}I][Sar^1]-angiotensin II binding.

We found that binding values obtained with [^{125}I][Sar^1]-angiotensin II were higher than those obtained with [^{125}I]CGP 42112. The reason for this discrepancy is not clear. Since the binding assay has been optimized for [^{125}I][Sar^1]-angiotensin II, it is possible that binding conditions are not optimal for [^{125}I]CGP 42112 binding. However, the correlation between values for each radiolabeled ligand in many brain areas, was excellent, and this validates our data.

The present results are partially in agreement with a recent semi-quantitative study of angiotensin II receptors in the mouse brain (Jenkins et al., 1997). The discrepancies between both studies can be understood by the use of very high concentrations of angiotensin AT_1 and AT_2 receptor displacers by Jenkins et al. (1997). With large concentrations, ligand selectivity is lost, and this may result in erroneous estimation of angiotensin II receptor subtypes (Tsutsumi and Saavedra, 1991). The selection of displacer

concentrations is of crucial importance for the precise quantification of angiotensin II receptor subtypes.

It was interesting to compare mouse brain angiotensin II receptors with previous findings in the rat brain, since most of previous studies on brain angiotensin II receptors have been carried out in the rat (Saavedra, 1992). Besides the differences in total angiotensin II binding mentioned above, there are differences between rat and mouse in terms of the receptor subtype expressed. For example, in the locus coeruleus the rat expresses angiotensin AT_2 receptors, and the mouse angiotensin AT_1 receptors. While in the rat, the nucleus of the solitary tract, the area postrema and the basolateral nucleus of the amygdala express angiotensin AT_1 receptors, the mouse expresses both receptor subtypes in these nuclei.

Our results clarify the presence of angiotensin AT_1 and AT_2 receptor subtypes in selected brain areas of the adult mouse, and will serve as a basis for future studies in genetic and gene deleted mouse models. Careful determination of the type of receptor subtype is necessary for each species under study, even for related rodent species such as the rat, the mouse and the gerbil (De Oliveira et al., 1995). The questions of cellular localization of receptor subtypes, of whether a single neuron can express both subtypes, and the reasons for differential expression of receptor subtypes in some areas such as the locus coeruleus, remain to be clarified in future studies.

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