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Autoradiographic localization of angiotensin II receptors in rat brain

(peptide neurotransmitter/renin/circumventricular organs/hypothalamus/blood pressure)

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ABSTRACT **The ^{125}I-labeled agonist analog [1-sarcosine]-angiotensin II ([Sar^1]AII) bound with high specificity and affinity ($K_a = 2 \times 10^9$ M^{-1}) to a single class of receptor sites in rat brain. This ligand was used to analyze the distribution of AII receptors in rat brain by *in vitro* autoradiography followed by computerized densitometry and color coding. A very high density of AII receptors was found in the subfornical organ, paraventricular and periventricular nuclei of the hypothalamus, nucleus of the tractus solitarius, and area postrema. A high concentration of receptors was found in the suprachiasmatic nucleus of the hypothalamus, lateral olfactory tracts, nuclei of the accessory and lateral olfactory tracts, triangular septal nucleus, subthalamic nucleus, locus coeruleus, and inferior olivary nuclei. Moderate receptor concentrations were found in the organum vasculosum of the lamina terminalis, median preoptic nucleus, medial habenular nucleus, lateral septum, ventroposterior thalamic nucleus, median eminence, medial geniculate nucleus, superior colliculus, subiculum, pre- and parasubiculum, and spinal trigeminal tract. Low concentrations of sites were seen in caudate-putamen, nucleus accumbens, amygdala, and gray matter of the spinal cord. These studies have demonstrated that AII receptors are distributed in a highly characteristic anatomical pattern in the brain. The high concentrations of AII receptors at numerous physiologically relevant sites are consistent with the emerging evidence for multiple roles of AII as a neuropeptide in the central nervous system.**

In addition to its well-defined peripheral actions in smooth muscle and the adrenal gland, angiotensin II (AII) has important actions in the central nervous system. These include stimulation of drinking, increased salt appetite, elevation of blood pressure (1–3), and release of several pituitary hormones, including vasopressin, corticotropin (3–5), and prolactin (6). All components of the renin-angiotensin system (3), including renin (7, 8), angiotensin-converting enzyme (9–13), and the polypeptide precursor angiotensinogen (14), have been identified in the brain. Renin has been localized in neurones of the medulla oblongata, cerebellar nuclei, and hypothalamus (15) and in cells of the anterior pituitary (16). AII-containing nerve terminals and neurones have been localized in brain by immunohistochemistry (17–22). High densities were found in the substantia gelatinosa of the spinal cord and spinal trigeminal nucleus, sympathetic lateral column and medial external layer of the median eminence (17), and hypothalamus (20, 22).

In addition, receptors for AII have been clearly identified in membrane fractions of brain (23–28). In calf brain, AII binding was highest in the cerebellum (23), whereas in the rat it was found mainly in the thalamus, hypothalamus, midbrain, septum, and medulla (23, 25), with highest concentrations in the lateral septum, superior colliculus, and area postrema (26, 28). Binding sites for blood-borne AII have been localized in the circumventricular organs, particularly the subfornical organ (29), and, after AII injection into the cerebral ventricles, in the organum vasculosum of the lamina terminalis (30). In such studies, labeling of the respective sites depends on access of AII to the brain after intravenous or intracerebroventricular injection as well as the presence of specific binding sites. Because the detailed anatomical distribution of AII receptors in the brain has not been described, we have localized AII receptors in rat brain by an *in vitro* autoradiographic technique (31, 32).

MATERIALS AND METHODS

^{125}I-labeled [1-sarcosine]AII (^{125}I-[Sar^1]AII) was prepared by modified Chloramine-T radioiodination (33) of the potent AII agonist [Sar^1]AII, which was generously provided by M. Khosla (Cleveland Clinic, Ohio). The product was purified by HPLC on a C-18 column in acetonitrile/0.1 M ammonium bicarbonate buffer, pH 8.0 (15:85) and had a specific activity of 800 μCi/μg (1 Ci = 37 GBq) as determined by self-displacement in a radioligand–receptor assay using rat adrenal homogenate (34).

Characterization of the brain binding sites was performed on a block of tissue including the hypothalamus, thalamus, septum, and midbrain, because this area is known to contain a relatively high concentration of AII receptors (25). The brain tissue was homogenized in 10 vol of ice-cold 20 mM $NaHCO_3$ and the 1,000–30,000 $\times$ *g* fraction was suspended in 10 mM sodium phosphate, pH 7.4/120 mM NaCl/5 mM Na_2EDTA/0.1 mM bacitracin/0.2% bovine serum albumin (buffer A) containing 0.1 mM phenylmethylsulfonyl fluoride and 100 kallikrein units of aprotinin per ml as protease inhibitors. Approximately 800 μg of "particulate" protein was incubated in 0.5 ml of buffer A with ^{125}I-[Sar^1]AII (120 pM) for 1 hr at 20°C. Competing ligands were [Sar^1]AII, AII, des-Asp^1-AII, and angiotensin I. Nonspecific binding was determined in the presence of 1 μM AII. After incubation, free and bound tracer was separated by filtration through glass fiber discs and bound radioactivity was measured in a Beckman γ spectrometer. The AII binding data were analyzed by a nonlinear model-fitting computer program (35).

For autoradiography, male Sprague-Dawley rats of ≈300 g were killed by decapitation and the brains were rapidly removed, frozen in 2-methylbutane at −40°C, mounted on

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Abbreviations: AII, angiotensin II; Sar, sarcosine.

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chucks, and cut into 25-μm-thick sections in a cryostat at −14°C. The sections were thaw-mounted on gelatin-coated slides, dried in a desiccator at 4°C for 2 hr, and stored at −80°C (31). The slide-mounted sections were preincubated in 5 ml of buffer (10 mM sodium phosphate, pH 7.4/120 mM NaCl/5 mM Na_2EDTA/0.1 mM bacitracin/0.2% bovine serum albumin) for 15 min at 20°C and then incubated with ^{125}I-[Sar^1]AII (≈160 pM) in 5 ml of fresh buffer for 1 hr at 20°C. Nonspecific binding was determined in the presence of 1 μM AII. The slides were then transferred through four 60-sec successive changes of ice-cold 50 mM Tris·HCl buffer (pH 7.4) to remove nonspecifically bound ligand. At the end of the rinsing period, the slides were rapidly dried under a stream of cold air and placed in cassettes (Wolf X-Ray, West Hempstead, NY) for exposure to LKB Ultrofilm for 4 days at room temperature. The films were then processed and grain density was quantitated by computerized densitometry with color-coded image analysis (36).

RESULTS

Characteristics of [Sar^1]AII Binding Sites. In brain membranes prepared from the hypothalamus-thalamus-septum-midbrain area, [Sar^1]AII bound to a single class of high-affinity sites with a K_a of $2.0 \pm 0.4 \times 10^9$ M^{-1} and concentration of 13 ± 2 fmol/mg of protein. The relative potencies of angiotensin analogs in displacing ^{125}I-[Sar^1]AII were as follows: [Sar^1]AII, 1.00; AII, 0.17; des-Asp^1-AII, 0.12; angiotensin I, 0.0017 (Fig. 1).

Autoradiographic Studies. Angiotensin receptors were found in several discrete brain regions, which were classified after computerized densitometry into the following groups.

Very high levels of AII receptors were found in the subfornical organ (Fig. 2 *c*, *d*, and *j*), paraventricular and periventricular hypothalamic nuclei (Fig. 2 *c*, *d*, *j*, and *k*), nucleus of the tractus solitarius (Fig. 2 *g* and *h*), and area postrema (Fig. 2*h*). In the paraventricular nucleus, very high levels of AII binding sites were seen in both the magnocellular and parvocellular regions.

High densities of AII receptors were present in the lateral olfactory tract (Fig. 2*a*), nucleus of the lateral olfactory tract (Fig. 2*j*), nucleus of the accessory olfactory tract (Fig. 2*j*), olfactory tubercule (Fig. 2*a*), suprachiasmatic nucleus of the hypothalamus (Fig. 2*j*), triangular septal nucleus (Fig. 2*b*), subthalamic nucleus (Fig. 2*l*), locus coeruleus (Fig. 2*f*), and inferior olivary nuclei (Fig. 2 *g* and *h*). Moderate densities of binding sites were seen in the organum vasculosum of the lamina terminalis (not shown), median preoptic nucleus (nucleus medianus), median eminence (not shown), medial habenular nucleus (Fig. 2*k*), superior colliculus (Fig. 2*e*), ventroposterior thalamic nucleus (not shown), hippocampus (Fig. 2*e*), mammillary nuclei (Fig. 2*l*), subiculum (not shown), pre- and parasubiculum (Fig. 2*e*), lateral septum (not shown), medial geniculate nucleus (Fig. 2*e*), and spinal trigeminal tract (not shown). Low concentrations of sites were seen in the caudate-putamen (Fig. 2 *b* and *j*), nucleus accumbens (Fig. 2*a*), amygdala (not shown), and gray matter of the spinal cord (Fig. 2*i*). Very low receptor levels were found in most cortical areas (Fig. 2 *a–e*, *j*, and *l*), globus pallidus (not shown), hippocampus (Fig. 2*e*), cerebellum (Fig. 2 *f–h*), and white matter such as corpus callosum (Fig. 2 *a–c*).

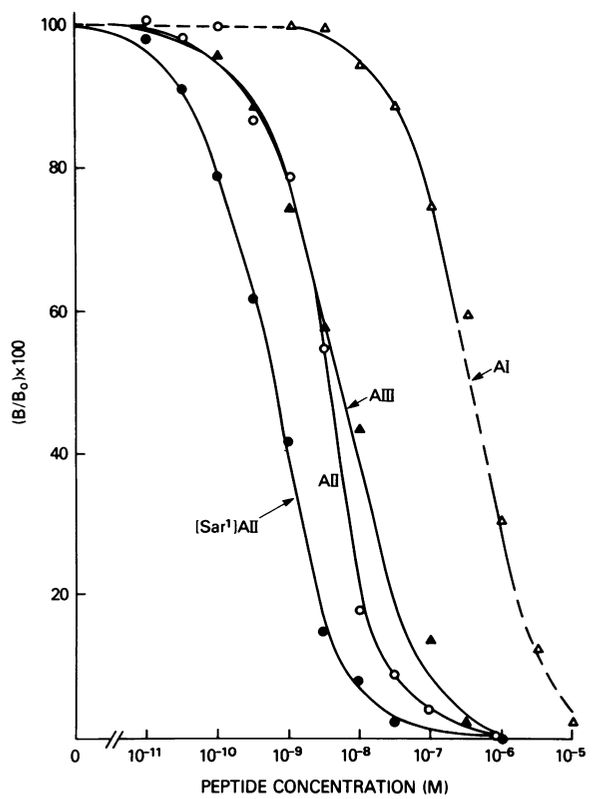

FIG. 1. Binding–inhibition activity of AII analogs in the brain radioligand receptor assay, with ^{125}I-[Sar^1]AII as tracer. The experiment was replicated three times with between-assay variation of <15%. AI, angiotensin I; AIII, des-Asp^1-AII.

DISCUSSION

The potent angiotensin radioligand ^{125}I-[Sar^1]AII bound to a single class of high-affinity, saturable sites in brain tissue with ligand specificity very similar to that observed by others using ^{125}I-labeled AII (23, 25) or [^{3}H]AII (37). The affinity constant and ligand specificity of the brain AII receptors closely resembled those derived for ^{125}I-labeled AII binding sites of the adrenal zona glomerulosa zone (34). The recognized higher affinity of the [Sar^1]AII analog for AII receptors (38, 39; Fig. 1) made it a suitable ligand for these studies. Both AII and [Sar^1]AII showed complete cross-displacement in hypothalamic membrane binding experiments, indicating that both ligands interact with the same receptor as has been reported for the vascular AII receptor (38). In addition, autoradiographic analysis of the bound radioactivity to brain slices using ^{125}I-labeled AII as the labeled ligand, showed identical distribution of the radioactivity to that found using ^{125}I-[Sar^1]AII (data not shown).

The autoradiographic distribution of AII receptors in rat brain was found to be unique and quite distinct from any other receptor distribution previously visualized (40–50). Further light-microscopic autoradiography is necessary to identify the cell type associated with these receptors. Our current autoradiographic findings both confirm and extend earlier descriptions of the distribution of AII receptors in rat brain, based on dissection and binding to membrane fractions. AII binding was reported to be highest in the thalamus-hypothalamus, midbrain, and septum (23, 25, 27, 28), which compares with our finding of a high concentration of receptors at discrete sites within these regions. Previous reports of a high concentration of binding sites in the subfornical organ (51), hypothalamus (25), lateral septum (25), and superior colliculus (26) are also confirmed by the current study. Similarly, specific binding was low or absent in the cerebral cortex, hippocampus, and striatum in our autoradiographic analysis and previous dissection studies (23, 25). In contrast to the calf, rat cerebellum has a low concentration of AII receptors (23, 25), as confirmed in the current study.

Electrophysiological studies have identified neurones specifically receptive to locally applied AII in the cat subfornical organ (52), rat lateral and medial septum (53), rat para-

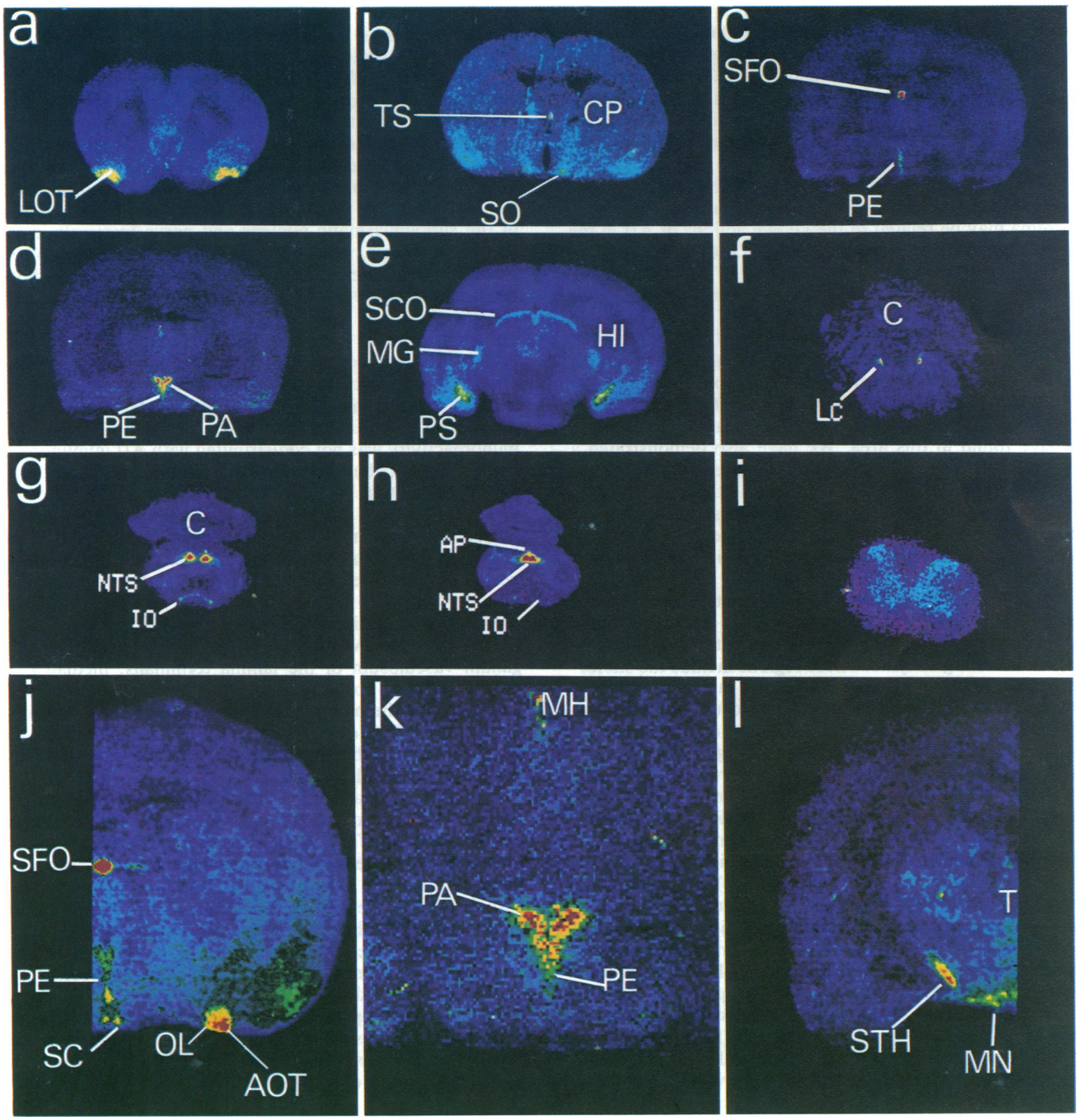

FIG. 2. Pseudocolor reconstructions of ^{125}I-[Sar^1]AII autoradiographs of rat brain sections by computerized densitometry and color-coding (36). Each picture was obtained by using the color code: red, very high density; yellow, high density; green, moderate density; light blue, low density; dark blue and purple, very low density. Densities of ^{125}I-[Sar^1]AII binding sites are 50–60 times higher in the most enriched areas vs. poor areas. AOT, nucleus of the accessory olfactory tract; AP, area postrema; C, cerebellum; CP, caudate-putamen; HI, hippocampus; IO, inferior olivary nuclei; LC, locus coeruleus; LOT, lateral olfactory tract; MN, mammillary nuclei; MH, medial habenular nucleus; MG, medial geniculate nucleus; NTS, nucleus tractus solitarius; OL, nucleus of the lateral olfactory tract; PA, paraventricular nucleus of the hypothalamus; PE, periventricular nucleus of the hypothalamus; PS, pre- and parasubiculum; SC, suprachiasmatic nucleus; SO, medial preoptic area, adjacent to the supraoptic nucleus; SCO, superior colliculus; SFO, subfornical organ; STH, subthalamic nucleus; T, thalamic nuclei; and TS, triangular septal nucleus. (*a–i* ×1.2; *j* and *l*, ×3.0; and *k*, ×6.0.)

ventricular (54) and supraoptic nuclei (54–56), and rat medial preoptic area (57). Moderate to high concentrations of AII receptors were identified in most of these areas. Moreover, the AII receptors localized in this study correspond with many of the sites at which the peptide is known to exert behavioral, endocrine, or physiological actions. For example, sites involved in the drinking response to AII, including the subfornical organ (58), organum vasculosum of the lamina terminalis (59), medial preoptic area (60), and median-preoptic nucleus (61), all contained moderate to high densities of angiotensin receptors. A neural pathway for angiotensin-mediated drinking has been defined by injections into subfornical organ of tritiated amino acids (62, 63) or horseradish-peroxidase (64) and central lesion experiments (65). These studies have revealed anatomical and functional connections between the subfornical organ and the following structures: median preoptic nucleus, organum vasculosum of the lamina terminalis, and supraoptic nucleus. The current finding that

several of these sites contain a high density of AII receptors supports and extends the suggestion that these different projections of the subfornical organ are angiotensinergic (65). In addition, the effects of AII on vasopressin secretion (66) correlate well with the current finding of AII receptors in the paraventricular and suprachiasmatic nuclei, because neurones in these areas contain vasopressin and release the peptide on exposure to AII (67).

The presence of AII receptors in the nucleus tractus solitarius, locus coeruleus, median preoptic nucleus, and area postrema may be related to the known actions of AII in the central regulation of blood pressure (1, 3, 61).

The current finding of a high density of AII receptors in the lateral olfactory tract, primary olfactory cortex, and olfactory bulb supports previous findings of AII receptors in membrane fractions from olfactory bulb (28, 68). In addition, chronic infusion of AII into the olfactory bulb elicits drinking that follows a temporal pattern distinct from that when the peptide is applied to the subfornical organ (69); this action of AII might be related to the high concentration of receptors we have found in this area. We have also demonstrated a high concentration of AII receptors in sites where AII has not been known to have actions (i.e., subthalamic nucleus, inferior olivary nucleus, superior colliculus, and nucleus of the spinal trigeminal tract). These regions represent interesting areas for further investigation of the actions of AII within the central nervous system.

Many of the sites at which high concentration of AII receptors were found in the present study have previously been shown to contain the highest concentrations of angiotensin-converting enzyme. These regions include the subfornical organ, medial habenular nucleus, median eminence, paraventricular nucleus, organum vasculosum of the lamina terminalis, area postrema, locus coeruleus, and nucleus tractus solitarius (11–13). However, in other regions of high converting enzyme activity, only very low levels of AII receptors were detected; these include the choroid plexus, caudate nucleus, and globus pallidus (12, 13). Recently, binding of the tritiated converting enzyme inhibitor [^{3}H]captopril to brain membranes has been shown to be highly localized to the choroid plexus and corpus striatum (70), suggesting that the enzyme might perform different functions in these areas.

It is of interest to compare the distribution of AII receptors with that of the peptide itself in the brain. Immunohistochemical studies of the distribution of AII in the nervous system are conflicting. Fuxe *et al.* (17) reported numerous AII-containing nerve terminals in the substantia gelatinosa of the spinal cord and spinal nucleus of the fifth nerve, sympathetic lateral column, and medial external layer of the median eminence. A moderate density of nerve terminals was found in the dorsomedial hypothalamic nucleus, ventral hypothalamus, locus coeruleus, and nucleus amygdaloideus centralis. Single nerve terminals were identified in most areas of the brain (17). In contrast, Changaris *et al.* (19) reported AII immunoreactivity in neurones of the deep cerebellar nuclei, spinal trigeminal tract, and zone of Lissauer. Fibers within the lateral olfactory tract, pyrifom cortex, nucleus accumbens, hippocampus, and various efferent hippocampal projections contained AII immunoreactivity. Many nonneuronal cells were positive for AII immunoreactivity, including pericapillary pinealocytes, cells of the posterior pituitary, and tanocytes surrounding the third ventricle. Neurones of the paraventricular and supraoptic nuclei were unstained (19). In a different study, immunoreactive AII was located in cell bodies of magnocellular neurones in the supraoptic and paraventricular nuclei and in parvocellular neurones of the suprachiasmatic nucleus, all of which also contained vasopressin (20). Weyhenmeyer and Phillips (21) found AII immunoreactivity in cell bodies of the supraoptic and paraventricular nuclei of the hypothalamus, hippocampus and cortex and fibers in the anterior and middle hypothalamus, basal ganglia, thalamus, locus coeruleus, nucleus of the solitary tract, limbic structures, and reticular formation.

Brownfield *et al.* (71) have attempted to reconcile these conflicting immunohistochemical findings in experiments in which 12 different AII antisera were used. Staining was found with only 3 of the antisera and this AII immunoreactivity was confined to neural elements of the rat brain, including neuronal perikarya in the paraventricular, supraoptic, and accessory magnocellular nuclei and the medial part of the suprachiasmatic nucleus and nerve terminals in the median eminence, neurohypophysis, central nucleus of the amygdala, bed nucleus of the stria terminals, intermediolateral column, and substantia gelatinosa of the spinal cord, and the trigeminal spinal nucleus. Fibers were also seen in the periventricular and lateral hypothalamus and lesser numbers in the caudate nucleus, lateral septum, hippocampus, cingulate and frontal cortex, substantia nigra, medullary reticular formation, motor nucleus of the vagus, and nucleus of the solitary tract. In the same study, converting enzyme immunoreactivity was not codistributed with AII immunoreactivity. This distribution of immunoreactive AII shows only a partial correlation with the distribution of AII receptors in the current study. Such a lack of correlation between the presence of neurotransmitters or neuropeptides and their receptors is not uncommon in the brain and has also been reported for neurotensin, substance P, and catecholamines (46).

High concentrations of angiotensin receptors were localized in the circumventricular organs, subfornical organ, organum vasculosum of the lamina terminalis, median eminence, and area postrema, all highly vascular structures located outside the blood–brain barrier (72) and accessible to circulating AII. However, for most of the other receptor sites, it is likely that AII endogenously formed within the brain is the natural ligand.

It is likely that a major portion of the central regulatory actions of AII is exerted through modulation of sympathetic activity. Several of the structures that contain AII receptors, including the nucleus of the tractus solitarius, locus coeruleus, and peri- and paraventricular hypothalamic nuclei, are associated with the central adrenergic system. AII is known to act on peripheral noradrenergic neurones to stimulate norepinephrine release (73) and may also be involved in the central regulation of norepinephrine and dopamine release in brain regions including the preoptic area (74). It is also possible that the octapeptide could influence the release of other regulatory peptides in the brain, such as vasopressin and corticotropin-releasing factor. Such an interaction is particularly likely in view of the association of both of these peptide neurones of the paraventricular nucleus of the hypothalamus (75) and our current finding of high density of AII receptors in this nucleus.

In summary, we have demonstrated that AII receptors are highly concentrated in relatively few sites, in contrast to the widespread distribution of other central nervous system receptors (40–50). This finding suggests that AII could perform an important modulatory role on a restricted number of selective and precise functions within the central nervous system.

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