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Differential distribution of AT_1 and AT_2 angiotensin II receptor subtypes in the rat brain during development

(autoradiography/circumventricular organs/thalamus)

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ABSTRACT **Angiotensin II (AII) receptor subtypes were analyzed in the brains of adult and 2-week-old rats by *in vitro* autoradiography with ^{125}I-labeled [Sar^1,Ile^8]AII and competition studies with three AII antagonists: the nonpeptide antagonist, DuP 753, which is specific for AT_1 receptors that mediate the calcium–inositol phospholipid signaling actions of AII; and nonpeptide (PD 123177) and peptide (CGP 42112A) antagonists that are selective for AT_2 receptors of yet unknown function. In the adult rat brain, DuP 753 inhibited radioligand binding to the circumventricular organs and paraventricular nucleus but not to the lateral septum, subthalamic nucleus, and inferior olive. However, binding of ^{125}I-labeled [Sar^1,Ile^8]AII in the latter regions was inhibited by the AT_2 receptor antagonists PD 123177 and CGP 42112A. These areas showed similar displacement by the AT_2 receptor subtype-specific antagonists in 2-week-old rats. In addition, radioligand binding at multiple sites of transient expression of AII receptors in 2-week-old rats, including several thalamic nuclei, the nuclei of the 3rd and 12th cranial nerves, geniculate bodies, cerebellum, and cingulate cortex, was displaced by the AT_2 antagonists but not by DuP 753. These studies have demonstrated the presence of two AII receptor subtypes in the brain, one (AT_1) in areas related to regulation of blood pressure, water intake, and pituitary hormone secretion, and one (AT_2) whose function is not yet defined. The abundance and location of brain AT_2 receptors in young animals, and the age-related changes in relative expression of the receptor subtypes, suggest that AII exerts specific actions according to the developmental stage of the central nervous system.**

Angiotensin II (AII) elicits a variety of physiological effects through specific AII receptors in numerous tissues including the adrenal gland, brain, liver, kidney, and vascular system (1–3). These effects of AII are mediated by receptor coupling to intracellular messenger systems, including inositol phospholipid turnover and cytoplasmic calcium mobilization (4), opening of calcium channels (5), and inhibition of adenylate cyclase activity (6). The diverse effects of AII could be mediated by one type of receptor coupled to different signal transducers or by different receptor subtypes that are coupled to the individual second messenger systems (7).

Recently, radioligand binding studies with newly developed nonpeptide and peptide AII receptor antagonists have demonstrated the presence of two AII receptor subtypes in the rat and human adrenal gland and the rat uterus (8, 9). Formerly designated as subtypes AII-1 or B, and AII-2 or A, these are now referred to as AT_1 and AT_2 subtypes of the AII receptor (10). Several tissues contain both receptor subtypes, but one subtype is predominant in certain tissues including the uterus and adrenal medulla (AT_2) and vascular smooth muscle cells and adrenal cortex (AT_1). Unlike the AT_1 receptor, the AT_2 subtype is not inactivated by sulfhydryl reducing agents and does not appear to be coupled to a guanine nucleotide regulatory protein (11). All of the known biochemical and cellular responses to AII have been found to be mediated by the AT_1 receptor, and the function of the putative receptors corresponding to the AT_2 binding sites has not yet been defined. However, recent studies have shown that a major proportion of the AII receptors transiently expressed in the rodent fetus are of the AT_2 subtype (33), suggesting that AT_2 sites could mediate a growth factor-like action of AII during development.

The nature of the AII receptor subtypes is of particular relevance in the brain, since studies in cultured neurons have shown that, in contrast to glial cells and other systems, neuronal AII receptors do not appear to be coupled to calcium–inositol phospholipid signaling pathways (12). These findings, in addition to the demonstration of new areas of AII binding in the brains of young animals (13), have suggested that brain AII receptors may be heterogeneous. The present study was performed to analyze the location of AII receptor subtypes by *in vitro* autoradiography in the rat brain and to evaluate receptor subtype distribution in immature and adult animals.

MATERIALS AND METHODS

AII and [Sar^1,Ile^8]AII were obtained from Peninsula Laboratories, and ^{125}I-labeled [Sar^1,Ile^8]AII (^{125}I-[Sar^1,Ile^8]AII) (2200 Ci/mmol; 1 Ci = 37 GBq) was from NEN. The nonpeptide antagonists DuP 753 [2-*n*-butyl-4-chloro-5-(hydroxymethyl)-1-[2′-(1*H*-tetrazole-5-yl biphenyl-4-yl)methyl]imidazole, potassium salt) and PD 123177, the HCl salt of EXP 655 {1-(4-amino-3-methylphenyl)methyl-5-diphenyl acetyl-4,5,6,7-tetrahydro-1*H*-imidazol[4,5-*c*]pyridine-6-carboxylic acid}, specific for AT_1 and AT_2 subtypes, respectively, were provided by Pancras C. Wong (DuPont). The AT_2 peptide antagonist CGP 42112A [nicotinyl-Tyr-(N^{α}-benzylcarbonyl-Arg)Lys-His-Pro-Ile-OH] was a gift of Marc de Gasparo (CIBA–Geigy).

Sprague–Dawley rats (six rats at 2 weeks of age and three adults) were obtained from Zivic–Miller. The brains were removed after decapitation, cut into blocks, and frozen in powdered dry ice. The frozen tissue was stored at −70°C for up to 2 months prior to sectioning in a cryostat and autoradiographic analysis as described (14). Nonspecific binding was determined in the presence of 1 μM AII. The nonpeptide antagonist DuP 753 was used at 10 μM, the nonpeptide antagonist PD 123177 was used at 1 μM, and the peptide antagonist CGP 42112A was used at 1 μM, concentrations that inhibited radioligand binding to brain membranes by

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Abbreviation: AII, angiotensin II.

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>90%. Tissue sections and films were processed under identical experimental conditions, and semiquantitative estimates of the proportions of the AII receptor subtypes were made in specific brain areas. The binding of ^{125}I-[Sar^1,Ile^8]AII to each region of the brain in the presence of the AT_1 and AT_2 antagonists was assigned a value of +++ to − relative to the control level (+++) at that site.

The binding-inhibition activities of the AII antagonists at the AT_2 receptor subtype were determined in membrane-rich fractions of thalamic nuclei dissected out from brain slices of 2-week-old animals (15). Thalamic tissue was collected in ice-cold phosphate-buffered saline, homogenized in 10 vol of ice-cold 20 mM sodium bicarbonate, and agitated on ice for 20 min. The 1000–30,000 × *g* fraction was resuspended in 10 mM sodium phosphate, pH 7.4/120 mM NaCl/5 mM EGTA/0.1 mM bacitracin/0.2% bovine serum albumin (BSA)/0.1 mM phenylmethylsulfonyl fluoride/100 kallikrein units of aprotinin. For the assay, aliquots equivalent to 50 μg of

Table 1. Distribution of AII receptor subtypes in rat brain

	Immature brain		Adult brain	
	DuP 753 (10^{-5} M)	CGP 42112A (10^{-6} M)	DuP 753 (10^{-5} M)	PD 123177 (10^{-6} M)
Telencephalon				
Piriform cortex (pi)	−	++	−	+++
Cingulate cortex (cc)	−	+	−	++
Hippocampus (HI)	−	+		
Subiculum (sub)	−	+		
Anterior olfactory n external layer (aoe)	−	++		
Organum vasculosum (OVLT)	−	++	−	+++
Lateral septum (sl)	+++	−	+++	−
Lateral amygdaloid n (al)	−	++	−	+++
Bed n-stria terminalis (nist-v)			−	+
Diencephalon				
n preopticus medianus (pome)	−	+++	−	+++
Subfornical organ (SFO)	−	+++	−	+
n of olfactory tract (ol)	−	+++	−	+++
Preoptic periventricular n (pop)	−	+++		
Paraventricular n of hypothalamus (PVN)	−	+++	−	+++
Periventricular n of hypothalamus (hpv)	−	++		
Suprachiasmatic n (scn)	−	+	−	+++
Median eminence (ME)	−	+++	−	+++
Arcuate n (a)	−	+		
Centrolateral thalamic n (cl)	+++	−		
Centromedial n of thalamus (cm)	+++	−		
Dorsomedial n (dm)	−	+	−	+++
Medial thalamic n (tm)	+++	−		
Ventral thalamic n (tv)	−	−		
Median mamillary n (nmm)	+++	−		
Dorsal supraoptic commissure (csdv)	+++	−		
Dorsolateral geniculate n (dcgl)	+++	−		
Medial geniculate body (mgb)	+++	+		
Ventrolateral geniculate n (vcgl)	++	+		
n subthalamicus (sut)	+++	−	+++	+
n reuniens (re)	+++	−		
Anterior ventral preoptic n (avpo)			−	+
Medial forebrain bundle (MFB)			−	+++
Choroid plexus (cp)			−	+++
Hindbrain				
Superior colliculus (SC)	+++	−		
Inferior colliculus (IC)	++	−		
Cerebellum (molecular layer) (c)	+	+		
Cerebellar nuclei (cn)	+++	−		
Inferior olive n (io)	++	−	+	+
n of accessory olive (iom)	+++	−	++	+
Locus coeruleus (lc)	+++	−		
Dorsotegmental n (ntd)	+++	−		
Area postrema (ap)	−	+++	−	+++
n of tractus solitarius (nts)	−	++	−	+++
Lateral lemniscus (ll)	+++	−		
n of oculomotor nerve (nIIIn)	+++	−		
Motor n vagus (nXn)			−	+++
n of hypoglossal nerve (nXIIn)	+++	−		
Ventral bundle (vb)	+	+		
Ventral cochlear n (vc)	+++	−		
Substantia gelatinosa (sg)	+++	−	−	+++

Controls are +++; n, nucleus.

membrane protein were incubated in a vol of 0.2 ml with 120 pM ^{125}I-[Sar^1,Ile^8]AII and the various antagonists. After incubation for 1 hr at 20°C, bound tracer was separated by filtration through GF/C glass fiber filters (Brandel, Gaithersburg, MD) presoaked in 0.1% BSA, and radioactivity was measured in a γ spectrometer. Nonspecific binding was determined in the presence of 1 μM AII.

RESULTS

Inhibition of ^{125}I-[Sar^1,Ile^8]AII Binding in Thalamic Membrane-Rich Fractions. The two AT_2 receptor subtype antagonists CGP 42112A and PD 123177 caused dose-dependent inhibition of ^{125}I-[Sar^1,Ile^8]AII binding to thalamic membranes from 2-week-old animals. The peptide antagonist CGP 42112A inhibited binding by 86% at a concentration of 10 nM and by 93% at 10 μM, whereas PD 123177 was less potent with 31% inhibition at 10 nM and 88% inhibition at 10 μM. In contrast, the nonpeptide AT_1 receptor antagonist DuP 753 caused only minor inhibition of radioligand binding, with maximum displacement of 10% at 10 μM. Furthermore, radioligand binding increased from 2.9% to 5.1% of the added radioactivity in the presence of 2 mM dithiothreitol. The AT_2 receptor antagonists CGP 42112A (1 μM) and PD 123177 (100 μM) caused similar degrees of binding inhibition in slide-mounted frozen sections of lateral septum, thalamic nuclei, subthalamic nucleus, and geniculate bodies during autoradiographic studies with ^{125}I-[Sar^1,Ile^8]AII at both ages (data not shown).

Autoradiographic Analysis of AII Receptor Subtypes. The topographical distribution of AII receptor subtypes was determined by the abilities of the three AII receptor antagonists to inhibit the binding of ^{125}I-[Sar^1,Ile^8]AII to specific areas in brain sections of adult (Table 1, Fig. 1) and 2-week-old rats (Figs. 2 and 3, Table 1). The AT_2 receptor antagonists PD 123177 and CGP 42112A inhibited radioligand binding to AII receptors at both ages in the lateral septum (Figs. 1 *F, G, K,* and *L* and 2 *G* and *L*), nucleus subthalamicus (Figs. 1 *I* and *N* and 3 *G* and *L*), inferior olive and inferior accessory olive (Figs. 1 *J* and *O* and 3 *J* and *O*), superior colliculus (Fig. 3 *H* and *M*), locus coeruleus (Fig. 3 *I* and *N*), and inferior colliculus and substantia gelatinosa (both not shown).

In 2-week-old animals, CGP 42112A inhibited binding in several areas where AII receptors are exclusively or predominantly present only at this period of development. These include thalamic and hindbrain structures such as the centrolateral (Fig. 3 *F* and *K*), medial, ventral, and centromedial (Fig. 2 *J* and *O*) nuclei of the thalamus; dorsal supraoptic commissure (Fig. 2 *J* and *O*); dorsolateral (Fig. 3 *F, G, K,* and *L*), ventrolateral (Fig. 3 *G* and *L*), and medial (Fig. 3 *G, H, L,* and *M*) geniculate body; nucleus of the oculomotor nerve (Fig. 3 *G, H, L,* and *M*); nucleus of the hypoglossal nerve (Fig. 3 *J* and *O*); ventral cochlear nucleus and dorsotegmental nucleus (Fig. 3 *I* and *N*); median mamillary nucleus; lateral lemniscus; and cerebellar nuclei (not shown).

In both adult and 2-week-old animals, DuP 753 abolished binding in the circumventricular organs—i.e., the organum vasculosum of the lamina terminalis (Figs. 1 *A* and *F* and 2 *B* and *G*), subfornical organ (Figs. 1 *C* and *H* and 2 *C* and *H*), and area postrema (Fig. 1 *E* and *J*), as well as in the median eminence (Fig. 1 *D* and *I*). The AT_1 antagonist also abolished binding in the pyriform and cingulate cortices in adult (Fig. 1 *A–C* and *F–H*) and young animals (Figs. 2 *B–E* and *G–J* and 3 *A–C* and *F–H*), as well as in the hippocampus (Figs. 2 *D, E, I,* and *J* and 3 *A–C* and *F–H*) and the subiculum (data not shown). The AT_2 antagonists did not affect, or only slightly decreased, binding in these areas. Binding to the choroid plexus was also completely inhibited by DuP 753 (Figs. 1 *B, D, G,* and *I* and 2 *B, C, E, G, H,* and *J*).

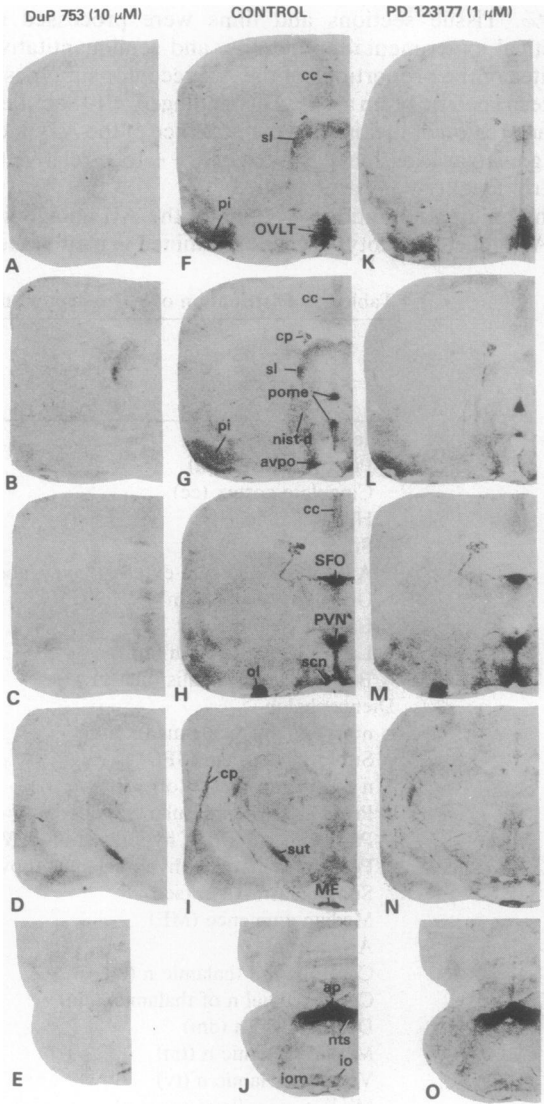

FIG. 1. Autoradiographic analysis of AII receptor subtypes in 20-μm coronal sections of the adult rat brain. Frozen sections were incubated with ^{125}I-[Sar^1,Ile^8]AII (400,000 cpm/ml) for 60 min in the absence of competing ligands (controls; *F–J*) and in the presence of the AT_1 receptor antagonist DuP 753 (10 μM) (*A–E*) or the AT_2 receptor antagonist PD 123177 (1 μM) (*K–O*). Abbreviations are defined in Table 1.

In the diencephalon, DuP 753 inhibited binding at both ages in the nucleus preopticus medianus (Figs. 1 *B* and *G* and 2 *C* and *H*); paraventricular nucleus of the hypothalamus, nucleus of the lateral olfactory tract; suprachiasmatic nucleus (Figs. 1 *C* and *H* and 2 *D* and *I*); lateral amygdaloid nucleus (Figs. 2 *D, E, I* and *J* and 3 *A* and *F*); anterior ventral preoptic nucleus (shown in the adult; Fig. 1 *B* and *G*); anterior olfactory nucleus (external layer) (Fig. 2 *A* and *F*); preoptic periventricular nucleus (Fig. 2 *C* and *H*) and periventricular nucleus of the hypothalamus (shown in 2-week-old animals; Fig. 2 *D* and *I*); dorsomedial nucleus (Fig. 3 *A* and *F*); medial forebrain bundle and the arcuate nucleus (Fig. 3 *B* and *G*).

In the hindbrain, the AT_1 antagonist suppressed binding to AII receptors in the nucleus of the tractus solitarius (Figs. 1 *E* and *J* and 3 *E* and *J*), vagus nerve, and substantia gelatinosa (data not shown). Two areas in the young animal—the ventral bundle (data not shown) and the molecular layer of the cerebellum (Fig. 3 *I* and *J*)—showed a decreased density of binding with both types of antagonists, DuP 753 (Fig. 3 *D* and *E*) and CGP 42112A (Fig. 3 *N* and *O*).

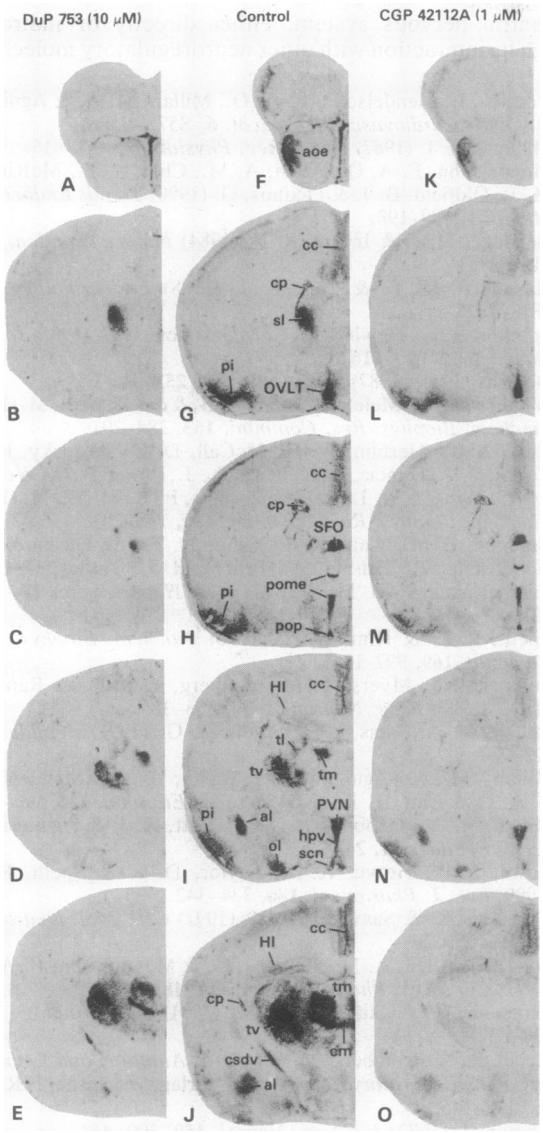

FIG. 2. Autoradiographic analysis of AII receptor subtypes in 20-μm sections of the anterior part of the 2-week-old rat brain. Frozen sections were incubated with ^{125}I-[Sar^1,Ile^8]AII for 60 min in the absence of competing ligands (controls; *F–J*) and in the presence of the AT_1 receptor antagonist DuP 753 (10 μM) (*A–E*) or the AT_2 receptor antagonist CGP 42112A (1 μM) (*K–O*). Abbreviations are defined in Table 1.

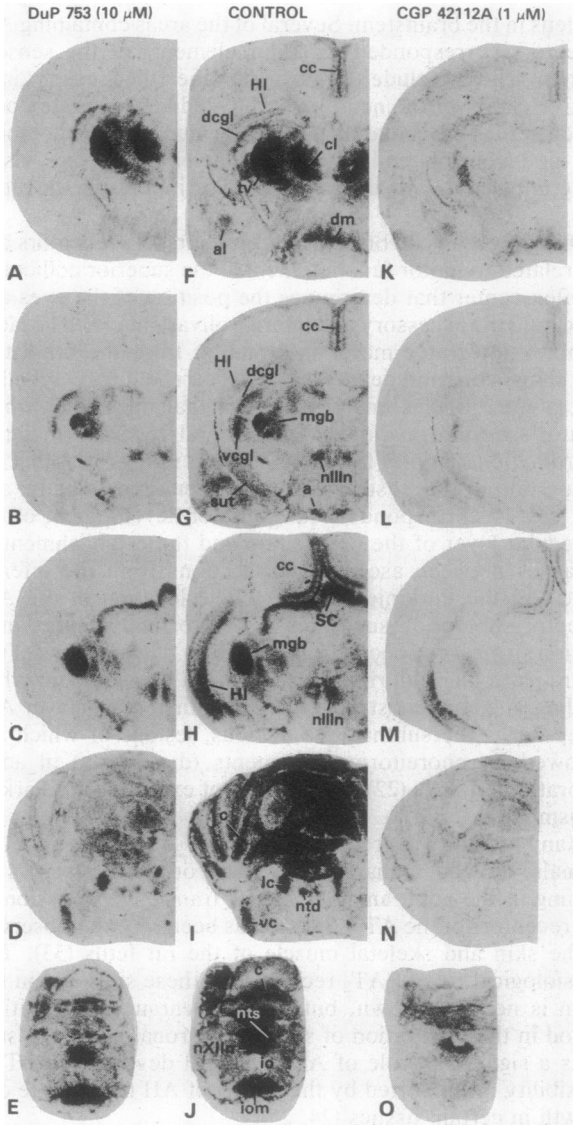

FIG. 3. Autoradiographic analysis of AII receptor subtypes in the posterior part of the 2-week-old rat brain. (*F–J*) Controls. (*A–E*) DuP 753 (10 μM). (*K–O*) CGP 42112A (1 μM). Abbreviations are defined in Table 1.

DISCUSSION

These autoradiographic studies with nonpeptide and peptide AII antagonists have demonstrated the presence and differential distribution of two types of AII receptors in the central nervous system of the rat, as well as changes in their abundance during development. The location of the AT_1 receptor subtype (i.e., the areas of AII receptor binding that were inhibited by DuP 753) in adult and young animals includes the areas in the brain in which AII exerts its well-defined effects on blood pressure regulation, drinking responses, and vasopressin release. The distribution of DuP 753-sensitive AII binding sites in the adult rat brain is in general agreement with recent observations (16) that correlated these sites with areas in which ^{125}I-[Sar^1,Ile^8]AII binding was inhibited by sulfhydryl reducing agents. In our study, binding to areas in which DuP 753-resistant sites were present was shown to be displaced by the AT_2-selective antagonists PD 123177 and CGP 42112A at both ages.

Several areas of AII binding sites in the brains of adult and young animals, at which AII has no known actions, were of the AT_2 receptor subtype as recognized by the ability of the antagonists PD 123177 and CGP 42112A to displace the binding of ^{125}I-[Sar^1,Ile^8]AII. Recent studies have demonstrated AII binding in several brain nuclei of younger animals, which contain few or no AII receptors in the adult (13). The present findings have shown that most of these areas where transient expression of AII binding occurs in immature rats contain receptors of the AT_2 subtype. This is consistent with recent reports on the abundance of AT_2 receptors at several locations in the brains of immature rats based either on differential sensitivity to dithiothreitol (16, 17) or on inhibition of radioligand binding by AT_2 antagonists (18, 19). Some of these locations do not contain AII receptors in the adult brain (e.g., the hypoglossal nucleus, cerebellar cortex, and certain thalamic nuclei) and others contain a much higher abundance of AT_2 receptors than in the adult (e.g., the inferior olive). Additional sites of AT_2 receptors in areas not previously described include the centrolateral thalamic nucleus and the magnocellular division of the ventrolateral geniculate nucleus in the thalamus, and the ventral cochlear

nucleus in the brainstem. Several of the areas containing AT_2 receptors corresponded to major elements of the sensory system. These include the thalamus, the chief sensory integrative center of the neuroaxis; the medial geniculate body, which serves as an auditory relay nucleus; and the dorsolateral and ventrolateral geniculate nuclei, which are visual integrating areas with direct information input from the retina (20).

Other areas of the brain that are rich in AT_2 receptors and are related to motor function include the superior colliculus, a reflex center that determines the position of the eyes and head, and the accessory and inferior olivary nuclei. The latter complex integrates motor information from the brainstem and spinal cord and delivers it to the cerebellum, cerebellar, red, lateral reticular, and ventrolateral thalamic nuclei, which in turn send information to the cerebral cortex to maintain coordination of motor function (20). It is noteworthy that the maturation of this system in the rat occurs postnatally, and that day 14 corresponds to the time of development of the molecular layer of the cerebellum and the establishment of synapses between ascending projections from the inferior olive and the Purkinje cells (21). The location of the AT_2 receptor subtype in such areas of motor and sensory integration suggests a specific role for AII in these regions of the brain, particularly during early postnatal development when binding sites are most abundant. Another site rich in AT_2 receptors is the subthalamic nucleus, lesions of which are followed by choreiform movements (dyskinesia) in adult laboratory animals (22) and reversal of experimental Parkinsonism (23).

Many of the brain areas containing AT_2 receptors in young animals showed a marked decrease or complete loss of binding in the adult animal. Similar transient expression of AII receptors of the AT_2 subtype has been recently observed in the skin and skeletal muscle of the rat fetus (33). The physiological role of AT_2 receptors at these sites and in the brain is not yet known, but their appearance at a critical period in the maturation of specific neuronal pathways suggests a significant role of AII in neural development. This possibility is supported by the ability of AII to promote cell growth in certain tissues (24, 25).

Although the renin–angiotensin system of the rat brain has not been well characterized during development, it is likely that brain AII binding sites are accessible to the peptide at all ages. Angiotensinogen (26, 27) and renin-like activity (28, 29) have been reported in the rat brain as early as the 18th day of fetal life. Angiotensin-converting enzyme has been detected only in the choroid plexus before birth and appears in the brain at 2 weeks of age (30). Also, immunoreactive AII has been detected in brain cells cultured from the 20-day rat fetus (31) and is released from neuronal cultures by adrenergic receptor-mediated mechanisms (32).

In summary, this study has demonstrated the presence and developmental changes of two newly defined AII receptor subtypes during brain maturation. At all ages, the presence of AT_1 receptors in areas related to blood pressure regulation and water intake is consistent with the central actions of AII in cardiovascular homeostasis. Although the function of the AT_2 angiotensin receptor remains to be elucidated, the presence of this receptor subtype in several motor nuclei indicates a possible role for AII in the regulation of motor activity. The abundance and transient expression of brain AT_2 receptors in young animals suggest that AII may have an important function in the development or maturation of the central nervous system, either directly or indirectly through its interaction with other neuroregulatory molecules.

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