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A peripherally administered, centrally acting angiotensin II AT₂ antagonist selectively increases brain AT₁ receptors and decreases brain tyrosine hydroxylase transcription, pituitary vasopressin and ACTH

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Abstract

The physiological actions of brain Angiotensin II AT₂ receptors and their relationship to Angiotensin II AT₁ receptors remain controversial. To further clarify their role, we determined to what extent systemic administration of an AT₂ receptor antagonist affected AT₂ receptor binding within the brain and the expression of AT₁ receptors. For this purpose, we subcutaneously administered the AT₂ receptor antagonist PD123319 (1 mg/kg/day) to adult male rats for two weeks via osmotic minipumps. We also studied the content of pituitary adrenocorticotrophic hormone and vasopressin, representative of hypothalamic–pituitary–adrenal axis activation, and the tyrosine hydroxylase gene expression in the locus coeruleus as a measure of central norepinephrine function. We found significant decreases in AT₂ receptor binding in brain areas inside the blood brain barrier, the inferior olive and the locus coeruleus. AT₂ receptor blockade increased AT₁ receptor binding and mRNA expression not only in the subfornical organ and the median eminence, situated outside the blood brain barrier, but also in the hypothalamic paraventricular nucleus, located inside the blood brain barrier. These changes paralleled decreased expression of tyrosine hydroxylase mRNA in the locus coeruleus and decreased pituitary adrenocorticotrophic and vasopressin content. Our results demonstrate that sustained peripheral administration of an AT₂ antagonist decreases binding to brain AT₂ receptors, indicating that this drug is a useful tool for the study of their central role. AT₂ receptor activity inhibition up-regulates AT₁ receptor expression in specific brain areas. Blockade of brain AT₂ receptors is compatible with enhanced hypothalamic–pituitary–adrenal axis and decreased central sympathetic system activity.

Keywords

Brain Angiotensin II receptor; Central sympathetic system; Pituitary hormone; Locus coeruleus; Paraventricular nucleus; Inferior olive

1. Introduction

Brain Angiotensin II (Ang II) is a multitasking peptide controlling central sympathetic system activity, pituitary hormone formation and release, stress and cerebrovascular flow (Saavedra, 2005). There are two Ang II receptor types, the AT₁ and AT₂ receptors (de Gasparo et al.,

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2000). AT₁ receptors predominate in the adult rodent brain, and their expression is high in areas involved in autonomic, hormone, cerebrovascular, and behavior regulation (Tsutsumi and Saavedra, 1991a; Rowe et al., 1992; Lenkei et al., 1998). In particular, AT₁ receptors are the only Ang II receptor type expressed in brain areas regulating pituitary hormone formation and release (Tsutsumi and Saavedra, 1991b). For this reason, and because the effects of centrally-administered Ang II were prevented by peripherally administered AT₁ receptor blockers (Toney and Porter, 1993; Seltzer et al., 2004), the effects of Ang II in the brain are considered as a consequence of AT₁ receptor stimulation (McKinley et al., 1996; de Gasparo et al., 2000).

On the other hand, the function of brain AT₂ receptors remains elusive. AT₂ receptors are very highly expressed in the immature rodent brain and dramatically decrease in number during the first two weeks of postnatal life, an indication of a still undetermined role in brain development (Tsutsumi and Saavedra, 1991a; Millan et al., 1991). In the adult rat brain, AT₂ receptors remain significantly expressed in sensory and motor areas (Tsutsumi and Saavedra, 1991a; Lenkei et al., 1996). The high number of AT₂ receptors in the locus coeruleus of the rat suggests a role in the regulation of central norepinephrine activity (Tsutsumi and Saavedra, 1991a; Lenkei et al., 1996). It has also been reported that the AT₂ receptor modulates neuronal differentiation and nerve degeneration, protecting against neuronal injury during ischemia, and regulates water intake and vasopressin (AVP) release (Hogarty et al., 1992; Li et al., 2003, 2005). These results, however, are still controversial (de Gasparo and Siragy 1999; Jung et al., 2007).

The overall effect of Ang II was originally proposed to be the result of a balance or crosstalk between AT₁ and AT₂ receptor function (Gelband et al., 1997). The original “cross talk” hypothesis was based on results obtained with cell culture studies. The hypothesis suggested an intracellular interaction between AT₁ and AT₂ receptors regulating opposing signal transduction mechanisms, and that the balance between AT₁ and AT₂ activity was important to ensure intracellular homeostasis upon stimulation by Ang II (Gelband et al., 1997; Inagami et al. 1999). AT₁ and AT₂ receptors were also considered to stimulate distinct, but converging intracellular pathways with some responses simultaneously mediated by both receptor types (Bleuel et al. 1995).

In the brain, however, most if not all AT₁ and AT₂ receptors are expressed in different brain nuclei and in different neurons (Tsutsumi and Saavedra 1991a; Jöhren et al. 1995; Lenkei et al., 1996, 1998). The selective neuronal localization of brain AT₁ and AT₂ receptors makes the universality of the intracellular “cross-talk” hypothesis highly unlikely. Nevertheless, it appears that the expression of AT₁ and AT₂ receptors in the brain is reciprocally regulated, because AT₁ receptor blockade enhances AT₂ receptor expression in selected areas (Seltzer et al., 2004; Zhou et al., 2006; Bregonzio et al., 2008). In addition, AT₂ receptor $-/-$ mice express higher numbers of AT₁ receptors in the hypothalamic paraventricular nucleus (PVN) and subfornical organ (SFO), leading to hypothalamic–pituitary–adrenal (HPA) axis hyperactivity (Armando et al., 2002).

Functional studies on brain AT₂ receptors have been hampered by the limited number of selective ligands (de Gasparo et al., 2000) and by questionable characterization and specificity of AT₂ receptor antibodies (Reagan et al., 1994; Yiu et al., 1997). Experimental studies on the effects of AT₂ receptor agonists and antagonists directly injected into the brain yielded controversial results (Toney and Porter, 1993; Li et al., 2003; Saad et al., 2005; Kerr et al., 2005). There are few reports of central effects of peripherally administered AT₂ agonists or antagonists (Li et al., 2005), and there is no direct evidence that these compounds actually enter the brain, decreasing binding to central AT₂ receptors. For this purpose, we decided to determine to what extent the non-peptidic AT₂ receptor antagonist PD123319, administered peripherally, can cross the blood brain barrier to inhibit central AT₂ receptors. Because of the

proposed mutual influence in brain binding expression between AT₁ and AT₂ receptors, we studied the effects of PD123319 on AT₁ receptor expression in selected areas expressing AT₁ receptors. The locus coeruleus (LC) is a very important site for expression and transcription of the catecholamine rate-limiting enzyme tyrosine hydroxylase (TH), and TH transcription in this area regulates the production of forebrain norepinephrine (Sabban and Serova, 2007). In the rat, the LC expresses large numbers of AT₂ receptors (Tsutsumi and Saavedra, 1991a,b), indicating that this receptor type may be involved in central norepinephrine function. For this reason we studied TH mRNA expression in the locus coeruleus after PD123319 administration. AT₂^{-/-} mice showed increased PVN AT₁ receptor expression and signs of enhanced HPA axis activation. Consequently we studied levels of pituitary AVP and adrenocorticotrophic hormone (ACTH), hormones partially regulated by AT₁ receptor activity (Armando et al., 2001).

2. Results

2.1. Peripheral administration of an AT₂ receptor antagonist decreases AT₂ binding in the brain

We detected AT₂ binding in the LC, the inferior olivary complex and the lateral septal nucleus of vehicle-treated rats. No AT₂ binding was expressed in the SFO, PVN, median eminence (ME), piriform cortex, basolateral amygdaloid nucleus or in the nucleus of the solitary tract and area postrema. Peripheral administration of PD123319 significantly decreased AT₂ receptor binding in the LC by about 40% (Fig. 1) and in the olivary complex (dorsal and medial nuclei and subnucleus A and B of the medial nucleus) between 50 and 60% (Fig. 2). No significant changes in AT₂ binding were noted in the lateral septal nucleus (0.7 ± 0.2 and 0.6 ± 0.3 fmol/mg protein for vehicle-treated and PD123319-treated, respectively, $P > 0.05$ for groups of six to eight animals).

2.2. Peripheral administration of an AT₂ receptor antagonist increases AT₁ binding and AT_{1A} mRNA expression in specific brain nuclei

We found AT₁ binding in the SFO, PVN, ME, piriform cortex, basolateral amygdaloid nucleus, the nucleus of the solitary tract and the area postrema. No AT₁ binding was found in the lateral septal nucleus, the LC or the inferior olivary complex. Peripheral administration of PD123319 significantly increased the AT₁ receptor expression by 40 to 60% in the SFO, the PVN and the ME (Fig. 3). In addition, PD123319 increased AT_{1A} mRNA expression in both the SFO and the PVN (Fig. 4).

Conversely, no significant changes in AT₁ receptor expression after PD123319 administration were noted in the piriform cortex, the basolateral amygdaloid nucleus, the nucleus of the solitary tract or the area postrema. Values were 8.2 ± 2.1 and 9.1 ± 2.3 for the piriform cortex, 2.4 ± 0.7 and 1.8 ± 0.2 for the basolateral amygdaloid nucleus, 11.0 ± 3.0 and 16.4 ± 3.5 for the nucleus of the solitary tract, and 12.9 ± 2.4 and 13.8 ± 2.4 for the area postrema, fmol/mg protein for vehicle-treated and PD123319-treated, respectively, $P > 0.05$, for groups of 6 to 8 animals.

2.3. Peripheral administration of an AT₂ receptor antagonist decreased tyrosine hydroxylase mRNA expression in the locus coeruleus

PD123319 administration produced a significant decrease in TH mRNA expression in the locus coeruleus (Fig. 5).

2.4. Peripheral administration of an AT₂ receptor antagonist decreases pituitary ACTH and vasopressin content

Treatment with PD123319 resulted in a pronounced decrease in the content of pituitary ACTH and AVP (Fig. 6).

3. Discussion

In an effort to further clarify the role of brain Ang II AT₂ receptors, we determined to what extent sustained administration of an AT₂ receptor antagonist blocked central AT₂ receptor binding. Ligand selectivity for AT₁ and AT₂ receptors is relative, because very high concentration of AT₁ or AT₂ receptor antagonists can displace AT₂ or AT₁ receptor binding, respectively. We chose PD123319, an effective and widely used AT₂ receptor antagonist, administered peripherally at a dose of 1 mg/kg/day, significantly decreasing AT₂ receptor binding in peripheral organs without interference with AT₁ receptor binding (Jezova et al., 2003). Similar doses of PD123319 have been used by other investigators to test the role of AT₂ receptors *in vivo* (Nagai et al., 2007; Lukkarinen et al., 2005). There are no literature reports suggesting that PD123319 exerts important effects in addition to its AT₂ receptor-blocking effects. Peripheral administration was selected to avoid artifacts resulting from infusion of large doses of PD123319 into the brain. For our initial experiments, we prolonged PD123319 treatment for two weeks to determine the effects of sustained receptor blockade beyond the initial acute response. We also studied the effect of the AT₂ receptor antagonist on AT₁ receptor transcription and expression, because absence of AT₂ receptor function in AT₂^{-/-} mice is accompanied by enhanced AT₁ receptor expression in selected brain areas (Armando et al., 2002).

We selected brain areas with a well-characterized receptor presence, AT₁ receptors in hypothalamic and brain stem nuclei, and AT₂ receptors in the inferior olive, LC and septal nuclei (Tsutsumi and Saavedra, 1991a). We found significant AT₂ receptor blockade in all areas of the inferior olivary complex and in the LC, of a magnitude similar to that observed for peripheral AT₂ receptors (Jezova et al., 2003), indicating that this compound crosses the blood brain barrier and may be used as a tool for the study of the regulation and role of brain AT₂ receptors. Binding to AT₂ receptors was not significantly decreased only in the lateral septal nucleus, suggesting that some brain areas where AT₂ binding is low may be more resistant to PD123319 blockade. Since PD123319 does not bind tightly to AT₂ receptors some of the compound may be washed out during the incubation procedure. For this reason, the reported degree of reduction in receptor binding may underestimate the actual proportion of receptors occupied *in vivo*. Additionally, chronic drug treatment can decrease receptor binding without actually blocking the receptor, by affecting receptor transcription and translation. However, we have found that a similar treatment with the AT₂ receptor antagonist did not modify the expression of AT₂ receptor protein in the adrenal gland (Jezova et al., 2003). Nevertheless, we considered that receptor inhibition sustained for two weeks might be sufficient to determine the consequences of this partial, but significant AT₂ receptor blockade.

In the SFO and PVN, AT₁ receptors are of the AT_{1A} receptor subtype (Leong et al., 2002). A partial inhibition of AT₂ receptor binding, interpreted as a possible decrease in receptor activation, significantly up-regulated AT_{1A} receptor transcription and AT₁ receptor expression in the SFO and PVN, and AT₁ receptor expression in the ME. There were no significant changes in AT₁ receptor expression in brain stem areas indicated that the influence of AT₂ receptor activation was anatomically selective. Our observations are in line with previous reports of enhanced AT₁ receptor expression in the PVN, ME, kidney and adrenal gland of animals with life-long absence of AT₂ receptor function, the AT₂^{-/-} mice (Armando et al., 2002; Saavedra et al., 2001a,b). We found up-regulation of AT₁ receptor expression in two different rodent models, the AT₂^{-/-} mice where the receptor activity does not develop (Armando et al.,

2002, Saavedra et al., 2001a,b), and adult rats with pharmacological AT₂ receptor blockade (present results). In addition, in normotensive adult rats, knock down of AT₂ receptors in peripheral tissues and in brain increases blood pressure, indirectly suggesting enhanced AT₁ receptor activation (Wang et al., 2004).

The conclusion is that AT₂ receptor activity appears to limit AT₁ receptor expression, since either decreased or absent AT₂ receptor function increases the number of AT₁ receptors in some brain areas and peripheral tissues. Conversely, in normal rats, overexpression of AT₂ receptors in the rostral ventrolateral medulla decreased blood pressure and urinary norepinephrine secretion (Gao et al., 2008), a finding compatible with decreased AT₁ receptor function. Additionally, there is indirect evidence that over expressing AT₂ receptors in the heart decreases AT₁ receptor activity (Masaki et al., 1998).

The enhanced AT₁ receptor number has consequences expressed in the mouse AT₂^{-/-} phenotype and in the response of adult rats to pharmacological AT₂ receptor blockade. AT₂^{-/-} mice exhibit an exaggerated HPA axis activity with increased ACTH and corticosterone release (Armando et al., 2002), adrenomedullary activity (Armando et al., 2002), AVP secretion (Li et al., 2003), and increased locomotion and anxiety (Ichiki et al., 1995; Okuyama et al., 1999). This phenotype may be best interpreted as an indirect consequence of AT₁ receptor up-regulation. Similarly, we may interpret our current findings of decreased ACTH and AVP pituitary stores after AT₂ receptor blockade not as a direct result of decreased AT₂ receptor function, because AT₂ receptors are not expressed in brain areas directly controlling the HPA axis or in the pituitary gland (Tsutsumi and Saavedra, 1991b). In addition, AT₁, but not AT₂, receptors are a major factor controlling pituitary ACTH and AVP formation and release (Becú-Villalobos et al., 1994; Hogarty et al., 1992). For these reasons the most logical explanation is that increased hormone secretion and utilization is an indirect consequence of increased AT₁ receptor function in the SFO, PVN and ME, leading to enhanced HPA axis activity. AT₂^{-/-} mice exhibit enhanced blood pressure increase as a result of peripheral or central administration of Ang II (Li et al., 2003), indication of increased AT₁ receptor activation. However, administration of PD123319 to normal animals and to humans does not significantly affect blood pressure (Welch et al., 2007; Chappellaz and Smith, 2007; Brillante et al., 2005; Duke et al., 2005). This suggests a differential influence of AT₂ receptor activity on blood pressure control when comparing the mouse AT₂^{-/-} model and pharmacological blockade of AT₂ receptors in adult subjects.

There is a reciprocal influence between Ang II receptor types and limitations in AT₁ receptor activity enhance AT₂ receptor expression in brain and peripheral tissues. For example, AT₁ receptor blockers increase AT₂ binding in the LC, inferior olive and cerebral microvessels and prevent the isolation-stress AT₂ decrease in expression in LC and inferior olive (Seltzer et al., 2004; Zhou et al., 2006; Bregonzio et al., 2008), adipocytes (Zorad et al., 2006) and in the muscular gastric layer during cold-restraint stress (Bregonzio et al., 2003). Because AT₁ receptor blockade enhances renin production and release, increasing Ang II levels, their use is expected to result in over-stimulation of AT₂ receptors.

Our observations and those of the literature confirm the hypothesis of an interaction between AT₁ and AT₂ receptors. We have found no evidence of same cell AT₁ and AT₂ receptor co-localization in the brain. In addition, the reciprocal influence between AT₁ and AT₂ receptor activity appears to be anatomically selective, with receptor expression modulated in some brain areas but not in others. The conclusion is that the reciprocal AT₁/AT₂ receptor influence may be indirect and dependent on differential regulatory mechanisms, since one receptor changes in one area and the other in an anatomically distinct and distant location.

A good example of such indirect interaction is the role of AT₁ and AT₂ receptors on central norepinephrine formation. TH transcription in the locus coeruleus is a major factor in the regulation of central norepinephrine activity, and increase in TH transcription is an important part of the brain reaction to stress (Sabban and Serova, 2007). It is well-established that brain Ang II contributes to regulate brain norepinephrine activity through AT₁ receptor stimulation (Toney and Porter, 1993). Intracerebral Ang II injection, isolation stress and cold-restraint stress increase TH transcription in the LC, effects prevented by AT₁ receptor blockade (Seltzer et al., 2004; Saavedra et al., 2006; Bregonzio et al., 2008). However, the rat LC expresses only AT₂ receptors (Tsutsumi and Saavedra, 1991a), and we demonstrate here that AT₂ receptor blockade in the LC decreases TH transcription in this area. We may conclude from our results that AT₂ receptor activity is also important to maintain TH transcription in the LC, and therefore to achieve a normal level of central norepinephrine activation. On the other hand, AT₁ receptor blockade also decreases TH transcription while increasing AT₂ receptor expression in the LC (Seltzer et al., 2004), isolation stress enhances TH transcription while decreasing AT₂ receptor expression in the LC (Saavedra et al., 2006), and cold-restraint stress increases TH transcription without modification in AT₂ receptor expression (Bregonzio et al., 2008). Thus, the level of TH transcription does not necessarily correlate with the degree of AT₂ receptor expression. Consideration of indirect mechanisms is necessary to explain this apparent contradiction. TH transcription in the LC is controlled by multiple systems, including direct corticotrophin-releasing hormone (CRH) projections originated in the PVN and distinct from those regulating the HPA axis (Reyes et al., 2005). AT₁ receptor blockade prevents the extra-hypothalamic stimulation of the CRH system during stress (Saavedra et al., 2006). We may speculate that PVN CRH projections to the LC stimulate TH transcription, and that these projections are under AT₁ receptor control. Stress increases PVN AT₁ receptor function, resulting in enhanced TH transcription in the LC, effects prevented by AT₁ receptor blockade. In this case, increased AT₂ receptor expression in the LC resulting from AT₁ receptor blockade may be considered a compensatory mechanism to maintain TH activity. When the AT₂ receptors in LC are blocked, as reported here, TH transcription decreases in spite of enhanced PVN AT₁ receptor expression. We may conclude that TH transcription in the LC is under the indirect influence of the activity of both Ang II receptor types, and that AT₁ and AT₂ receptors may act in synchrony through similar, and not opposing, mechanisms. A similar effect was noted in the adrenal medulla, where TH transcription, previously considered exclusively under AT₁ receptor control (Wong et al., 1990), has been shown to be decreased to a similar extent by either AT₁ or AT₂ receptor antagonists (Jezova et al., 2003).

In conclusion, our results demonstrate that brain AT₂ receptor binding can be decreased by peripheral administration of an AT₂ receptor antagonist, selectively enhancing AT₁ receptor transcription, stimulating the HPA axis, and decreasing central norepinephrine activity. Revised, the AT₁/AT₂ receptor cross-talk hypothesis may be formulated as a series of different inter-cellular relationships, with receptor activities sometimes in opposition and sometimes in synergy, regulated in a complex, selective and indirect series of mechanisms.

4. Experimental procedures

4.1. Animals and tissue preparation

Wistar Hanover male rats (8 wk old) were purchased from Taconic Farms, Inc. (Germantown, NY), were kept at 22 °C under a 12-h dark, 12-h light cycle with lights on at 07.00 h and were given free access to normal rat diet and tap water. The National Institute of Mental Health (NIMH) Animal Care and Use Committee approved all procedures. Rats were anesthetized with pentobarbital (30 mg/kg) and Alzet osmotic minipumps (Alza Scientific Products, Palo Alto, CA) were implanted subcutaneously. Groups of eight animals (treated) received minipumps containing vehicle or the AT₂ receptor antagonist PD123319 (Sigma-Aldrich

Corp., St. Louis, MO; 1 mg/kg/day), respectively. Since the half-life of PD123319 is short (Levy et al., 1996), we chose subcutaneous administration for two weeks to provide continuous receptor blockade (Jezova et al., 2003). After minipump implantation the rats were kept in their cages in groups of three to four under standard conditions with regular rat food and water *ad libitum* for 14 days. At the end of the experiment the animals were killed by decapitation and the brains and pituitary glands were removed, frozen in isopentane at -30°C on dry ice, and stored at -80°C until used.

Consecutive, 16- μm thick coronal brain sections were cut at -20°C in a cryostat. Alternate brain sections were collected for receptor binding and *in situ* hybridization. For receptor autoradiography, sections were thaw-mounted on gelatin-coated glass slides, dried overnight in a desiccator at 4°C , and stored at -80°C . For *in situ* hybridization, sections were thaw-mounted on silanated glass slides (Digene Diagnostics, Beltsville, MD), dried at 50°C for 5 min on a slide warmer and stored at -80°C until use. For anatomical localization of Ang II receptor binding or mRNA, sections were stained with Toluidine Blue, and brain regions were identified and designated according to a rat brain atlas (Paxinos and Watson, 1986). Each animal was quantified independently, and four sections per brain region were studied for each animal and for each procedure.

4.2. Quantitative autoradiography of Angiotensin II AT₁ and AT₂ receptors

Brain coronal sections containing the SFO, the PVN, the ME, the piriform cortex, the basolateral amygdaloid nucleus, LC, the nucleus of the solitary tract, the area postrema and the inferior olivary complex were preincubated for 15 min at 22°C in 10 mM Na phosphate buffer, pH 7.4, containing 120 mM NaCl, 5 mM Na₂EDTA, 0.005% bacitracin (Sigma Chemical, St. Louis, MO), and 0.2% proteinase-free bovine serum albumin (Sigma Chemical), followed by incubation for 120 min in fresh buffer containing 0.5 nM of [¹²⁵I]Sarcosine¹-Ang II ([¹²⁵I]Sar¹-Ang II, Peninsula Laboratories, Belmont, CA; iodinated by the Peptide Radioiodination Service Center, School of Pharmacy, University of Mississippi, to a specific activity of 2176 Ci/mmol). We determined total binding by incubating the sections as described above (Tsutsumi and Saavedra, 1991a). Nonspecific binding was determined in consecutive sections incubated as above in the presence of 1 μM unlabeled Ang II (Peninsula), and was defined as the binding remaining in the presence of excess unlabeled agonist. To determine selective binding to the Ang II AT₁ and AT₂ receptors, we incubated consecutive sections with 0.5 nM of [¹²⁵I]Sar¹-Ang II in the presence of 10 μM of the selective AT₁ receptor antagonist losartan (DuPont-Merck, Wilmington, DE, USA) or 10 μM of the selective AT₂ receptor antagonist PD123319 (Sigma Aldrich), respectively, to give maximum specific displacement. The number of AT₁ and AT₂ receptors was defined as the binding displaced by the AT₁ and AT₂ receptor antagonists, respectively (Tsutsumi and Saavedra, 1991a).

After incubation, slides were rinsed four consecutive times, for 1 min each, in fresh ice-cold 50 mM Tris-(hydroxymethyl) aminomethane HCl buffer, pH 7.6, followed by a dip in ice-cold distilled water, and the sections were dried under air. Sections were exposed to Kodak Biomax MR film (Eastman Kodak Company, Rochester, NY) together with ¹⁴C-labeled microscales (American Radiolabeled Chemicals, St Louis, MO). Films were developed in ice-cold GBX developer (Eastman Kodak) for 4 min, fixed in Kodak GBX fixer for 4 min at 22°C , and rinsed in water for 15 min. Optical densities of auto-radiograms generated by incubation with the ¹²⁵I-labeled ligands were quantified by computerized densitometry using the Image 1.6 Program (National Institute of Mental Health, Bethesda, MD) after calibration with ¹⁴C-labeled standards as described (Miller and Zahniser, 1987). The films were exposed for different times to obtain film images within the linear portion of the standard curve and transformed to corresponding values of fmol per mg protein (Nazarali et al., 1989 and Miller and Zahniser, 1987).

4.3. In situ hybridization histochemistry

To obtain rat AT_{1A} receptor-specific riboprobes, partial fragments of full-length cDNA were subcloned into the polylinker site of the pBluescript KS (+) vector (Stratagene, La Jolla, Calif., USA) as described (Jöhren et al., 1995). The rAT_{1A} cDNA was restricted with HaeIII. The restriction fragment of 368 bp (from nt 1317 through 1684) was prepared and ligated into the EcoRV site of the vector. The fragment corresponded to the 3'-untranslated region (3'-UTR) of the gene with a 35-bp ORF region that did not show significant homology with rAT_{1B} cDNA ORF. The subclone, named rAT_{1A}-S2, was confirmed by DNA sequencing.

For *in vitro* transcription of the ³⁵S-labeled antisense and sense (control) riboprobes, the subclones were linearized with HindIII or EcoRI for the rAT_{1A}-S2 and then they were treated with T7 or T3 RNA polymerase. *In vitro* transcription was performed using the RNA transcription kit (Pharmacia Biotech, Piscataway, NJ) as described (Jöhren et al., 1995). The quality of riboprobes was monitored with liquid scintillation counting, polyacrylamide gel electrophoresis by a preliminary experiment using adrenal gland as a positive control and subsequent autoradiography (Jöhren et al., 1995).

To perform *in situ* hybridization histochemistry, sections were fixed for 10 min in 4% paraformaldehyde, and rinsed twice in phosphate-buffered saline. After acetylation in 0.1 M triethanolamine HCl, pH 8.0, containing 0.25% acetic anhydride for 10 min, sections were dehydrated through graded ethanol and air-dried. *In situ* hybridization was performed as described earlier (Jöhren et al., 1995). Each section was covered with 150 µl hybridization buffer [containing 50% formamide, 0.3 M NaCl, 2 mM EDTA, 20 mM tris(hydroxymethyl) aminomethane (Tris), pH 8.0, 1× Denhardt's solution, 10% dextran sulfate, 100 pg/ml salmon sperm DNA, 250 pg/ml yeast RNA, 250 pg/ml yeast tRNA, 100 mM dithiothreitol, 0.1% sodium dodecyl sulfate, and 2×10⁷ cpm/ml labeled sense or antisense probes] applied to each slide, and sections were incubated for 16–18 h at 54 °C. After hybridization, sections were rinsed several times in 4× standard saline citrate (SSC) buffer to remove coverslips and excess riboprobes (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate). Nonhybridized riboprobes were digested by incubation with 40 pg/ml ribonuclease A (Sigma Chemical, St. Louis, MO) for 30 min at room temperature. After a final high-stringency wash in 0.1× SSC at 65 °C for 60 min, sections were dehydrated in graded ethanol containing 0.3 M ammonium acetate and air-dried. Sections were exposed to Hyperfilm-3H (Amersham, Arlington Heights, IL) for 14 days. Films were developed in D-19 developer (Eastman Kodak, Rochester, NY) for 4 min at 0 °C and fixed in Kodak rapid fixer for 4 min at 22 °C. The intensities of hybridization signals were quantified as nCi/g tissue equivalent (Wisden and Morris, 1994) by measuring optical film densities using the NIH Image 1.61 program after calibration with the [¹⁴C] micro-scales. Sense probes were considered as control to determine nonspecific hybridization.

4.4. In situ hybridization of tyrosine hydroxylase mRNA

One antisense (TH-AS) oligonucleotide of 48-mer for the rat TH cDNA sequence (Grima et al., 1985), localized in nt 1562–1609, synthesized by Lofstrand Labs Ltd (Gaithersburg, Md., USA) was labeled with a 3'-end labeling kit (Amersham) using terminal deoxynucleotidyl transferase to a specific activity of 3–4×10⁸ dpm/µg. Each reaction was performed with 70 pmol of oligonucleotides in the presence of 70 µCi of [³⁵S] ATP (SJ 1334) (Amersham). The labeled oligonucleotides were separated from unincorporated nucleotides using MicroSpin G-25 columns (Amersham). *In situ* hybridization of rat LC sections and post-hybridization washings were performed as described (Wisden and Morris, 1994). *In situ* hybridization was performed in consecutive LC sections, one with the TH-AS antisense oligonucleotide and another with excess unlabelled TH-AS probe (157 pmol/ml). After the washing, sections were dehydrated in alcohol gradient containing 0.3 M ammonium acetate, air-dried and exposed to Hyperfilm-³H (Amersham). The films were developed and quantified as described above by

comparison with ^{14}C standards (American Radiolabeled Chemicals, Inc., St. Louis, Mo., USA).

4.5. Hormone determinations

Pituitary hormones were determined by RIA using commercially available kits [ICN Biomedicals, Inc. (Costa Mesa, CA) for ACTH and Phoenix Pharmaceuticals, Inc. (Belmont, CA), for pituitary vasopressin].

4.6. Statistics

Data are means $\pm$ SEM, for groups of 6 to 8 animals measured individually. For quantitative autoradiography and *in situ* hybridization methods, values for 2 to 3 consecutive sections were averaged for each animal. We used Student's *t* test to assess the significance of differences between groups. $P < 0.05$ was considered statistically significant.

Abbreviations

Ang II, Angiotensin II
 LC, locus coeruleus
 PVN, hypothalamic paraventricular nucleus
 SFO, subfornical organ
 ME, median eminence
 IOM, inferior olive, medial nucleus
 IOD, inferior olive, dorsal nucleus
 IOA, inferior olive, subnucleus A of medial nucleus
 IOB, inferior olive, subnucleus B of medial nucleus
 ACTH, Adrenocorticotrophic hormone
 AVP, vasopressin
 TH, tyrosine hydroxylase
 HPA, axis, hypothalamic–pituitary–adrenal axis
 CRH, corticotropin-releasing hormone

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AT₂ receptor binding

Locus coeruleus

Fig. 1.

PD123319 decreases brain Angiotensin II AT₂ receptor binding in the locus coeruleus. Figures are from images obtained from brain sections including the locus coeruleus after peripheral administration of vehicle or PD123319. Figures represent *in vitro* binding to AT₂ receptors as described under Experimental procedures. Arrows point to the locus coeruleus. Note decreased AT₂ binding in the locus coeruleus after PD123319 administration, when compared with vehicle-treated control. Bars are means $\pm$ S.E.M. of groups of six to eight animals measured individually. * $P < 0.05$ vs. vehicle treated rats.

AT₂ receptor binding

Inferior Olive

Fig. 2.

PD123319 decreases brain Angiotensin II AT₂ receptor binding in the inferior olive. Figures are from images obtained from brain sections including the inferior olive after peripheral administration of vehicle or PD123319. Figures represent *in vitro* binding to AT₂ receptors as described under Experimental procedures. Arrows point to the inferior olive. IOM: inferior olive, medial nucleus. IOD: inferior olive, dorsal nucleus. IOA: inferior olive, subnucleus A of medial nucleus. IOB: inferior olive, subnucleus B of medial nucleus. Note decreased AT₂ binding in the inferior olive, subnucleus A and B, in the medial and in the dorsal inferior olive nuclei after PD123319 administration, when compared with vehicle-treated control. Bars are

means $\pm$ S.E.M. of groups of six to eight animals measured individually. * $P<0.05$ vs. vehicle treated rats.

AT₁ receptor binding

Fig. 3. PD123319 selectively increases brain Angiotensin II AT₁ receptor binding in the subfornical organ, hypothalamic paraventricular nucleus and median eminence. Figures are from images obtained from brain sections including the SFO, PVN and ME after peripheral administration of vehicle or PD123319. Figures represent *in vitro* binding to AT₁ receptors as described under Experimental procedures. Arrows point to the SFO, PVN and ME. Note increased AT₁ binding in the SFO, PVN and ME after PD123319 administration, when compared with vehicle-treated control. Bars are means ± S.E.M. of groups of six to eight animals measured individually. **P* < 0.05 vs. vehicle treated rats.

AT₁ receptor mRNA

Fig. 4.

PD123319 increases Angiotensin II AT_{1A} receptor mRNA expression in the paraventricular nucleus and the subfornical organ. Figures are from images obtained from brain sections including the SFO and PVN after peripheral administration of vehicle or PD123319. Figures represent AT_{1A} receptor mRNA expression as described under Experimental procedures. Arrows point to the SFO and PVN. Note increased AT_{1A} mRNA expression in the SFO and PVN after PD123319 administration, when compared with vehicle-treated control. Bars are means±S.E.M. of groups of six to eight animals, assayed individually. **P*<0.05 vs. vehicle-treated group.

Tyrosine Hydroxylase mRNA

Locus coeruleus

Fig. 5. PD123319 decreases tyrosine hydroxylase mRNA expression in the locus coeruleus. Figures are from images obtained from brain sections including the locus coeruleus after peripheral administration of vehicle or PD123319. Arrows point to the locus coeruleus. Note the significant decrease in tyrosine hydroxylase mRNA after treatment with the AT₂ receptor antagonist. Bars are means±S.E.M. of groups of six to eight animals, assayed individually. * $P<0.05$ vs. vehicle-treated group.

Pituitary gland

Fig. 6. PD123319 decreases pituitary adrenocorticotrophic hormone and vasopressin content. Bars are means $\pm$ S.E.M. of groups of six to eight animals, assayed individually. * P <0.05 vs. vehicle-treated rats.