This article was downloaded by: *[Blackmore, Robert][informa internal users]*
On: *26 September 2008*
Access details: *Access Details: [subscription number 755239602]*
Publisher *Informa Healthcare*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK

Stress
Publication details, including instructions for authors and subscription information: http://www-intra.informaworld.com/smpp/title~content=t713652982

Angiotensin II AT_1 receptor blockade selectively enhances brain AT_2 receptor expression, and abolishes the cold-restraint stress-induced increase in tyrosine hydroxylase mRNA in the locus coeruleus of spontaneously hypertensive rats

C. Bregonzio [a]; A. Seltzer [a]; I. Armando [a]; J. Pavel [b]; J. M. Saavedra [a]
[a] Department of Pharmacology, Faculty of Chemical Sciences, National University of Cordoba, Cordoba, Argentina [b] Section on Pharmacology, National Institute of Mental Health, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA

First Published on: 13 June 2008

To cite this Article Bregonzio, C., Seltzer, A., Armando, I., Pavel, J. and Saavedra, J. M.(2008)'Angiotensin II AT_1 receptor blockade selectively enhances brain AT_2 receptor expression, and abolishes the cold-restraint stress-induced increase in tyrosine hydroxylase mRNA in the locus coeruleus of spontaneously hypertensive rats',Stress,

To link to this Article: DOI: 10.1080/10253890801892040

URL: http://dx.doi.org/10.1080/10253890801892040

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www-intra.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Stress, 2008; iFirst article, 1–10

informa
healthcare

ORIGINAL RESEARCH PAPER

Angiotensin II AT_1 receptor blockade selectively enhances brain AT_2 receptor expression, and abolishes the cold-restraint stress-induced increase in tyrosine hydroxylase mRNA in the locus coeruleus of spontaneously hypertensive rats

C. BREGONZIO[1], A. SELTZER[1], I. ARMANDO[1], J. PAVEL[2], & J. M. SAAVEDRA

[1]*Department of Pharmacology, Faculty of Chemical Sciences, National University of Cordoba, Cordoba, Argentina, and* [2]*Section on Pharmacology, National Institute of Mental Health, National Institutes of Health, Department of Health and Human Services, 10 Center Drive, MSC 1514, Building 10, Room 2D-57, Bethesda, MD 20892, USA*

(*Received 10 September 2007; revised 4 December 2007; accepted 20 December 2007*)

Downloaded By: [Blackmore, Robert][informa internal users] At: 15:41 26 September 2008

Abstract

Spontaneously hypertensive rats, a stress-sensitive strain, were pretreated orally for 14 days with the AT_1 receptor antagonist candesartan before submission to 2 h of cold-restraint stress. In non-treated rats, stress decreased AT_1 receptor binding in the median eminence and basolateral amygdala, increased AT_2 receptor binding in the medial subnucleus of the inferior olive, decreased AT_2 binding in the ventrolateral thalamic nucleus and increased tyrosine hydroxylase mRNA level in the locus coeruleus. In non-stressed rats, AT_1 receptor blockade reduced AT_1 receptor binding in all areas studied and enhanced AT_2 receptor binding in the medial subnucleus of the inferior olive. Candesartan pretreatment produced a similar decrease in brain AT_1 binding after stress, and prevented the stress-induced AT_2 receptor binding decrease in the ventrolateral thalamic nucleus. In the locus coeruleus and adrenal medulla, AT_1 blockade abolished the stress-induced increase in tyrosine hydroxylase mRNA level. Our results demonstrate that oral administration of candesartan effectively blocked brain AT_1 receptors, selectively increased central AT_2 receptor expression and prevented the stress-induced central stimulation of tyrosine hydroxylase transcription. The present results support a role of brain AT_1 and AT_2 receptors in the regulation of the stress response, and the hypothesis that AT_1 receptor antagonists may be considered as potential therapeutic compounds in stress related disorders in addition to their anti-hypertensive properties.

Keywords: *Angiotensin II receptors, brain, central sympathetic system, locus coeruleus, renin angiotensin system, stress*

Introduction

Brain angiotensin II (Ang II), through AT_1 receptor stimulation, is a multitasking peptide with established important roles in hormone formation and release, the control of central sympathetic system activity and stress (Saavedra 2005). Lines of evidence supporting the hypothesis of a major role of brain Ang II in stress include stress-induced increases in circulating and brain Ang II levels (Yang et al. 1993, 1996), high AT_1 receptor expression in all areas involved in the stimulation of the hypothalamic–pituitary–adrenal axis (HPA) activity, including the hypothalamic paraventricular nucleus (PVN), the median eminence (ME) and the subfornical organ (SFO) (Tsutsumi and Saavedra 1991b), and a stress-induced increase in AT_1 receptor expression in the parvocellular PVN, where cell bodies forming the corticotropin-releasing hormone (CRH) are located (Castrén and Saavedra 1988; Aguilera et al. 1995a). AT_1 receptors from the PVN are transported to the ME through axons co-expressing CRH (Oldfield et al. 2001). Stimulation

Correspondence: J. M. Saavedra, Section on Pharmacology, DIRP, NIMH, NIH, DHHS. 10 Center Drive, Bldg. 10, Room 2D-57, Bethesda, MD 20892, USA. Tel: + 1 301 496 0160. Fax: + 1 301 402 0337. E-mail: Saavedrj@mail.nih.gov

ISSN 1025-3890 print/ISSN 1607-8888 online © 2008 Informa UK Ltd.
DOI: 10.1080/10253890801892040

Downloaded By: [Blackmore, Robert][informa internal users] At: 15:41 26 September 2008

of PVN AT_1 receptors by Ang II increases CRH formation and release followed by enhanced ACTH release (Ganong and Murakami 1989; Aguilera et al. 1995b). In turn, increased levels of adrenal glucocorticoids regulate the expression of Ang II receptors in the PVN (Castrén and Saavedra 1989; Aguilera et al. 1995a) through stimulation of glucocorticoid response elements (GREs) in the AT_1 receptor promoter (Guo and Inagami 1994).

Antagonism of brain AT_1 receptors decreases CRH production and release in the PVN, explaining the blockade of the hormonal response to isolation stress by this class of compounds (Armando et al. 2001, 2007). Sustained inhibition of peripheral and brain AT_1 receptors by peripheral administration of the AT_1 receptor antagonist candesartan prevents not only the hormonal, but also the sympathoadrenal response to isolation stress (Armando et al. 2001). In addition, candesartan pretreatment prevents the activation of the brain sympathetic system during isolation (Saavedra et al. 2006), the isolation-induced cerebrocortical alterations in CRH_1 receptors and the $GABA_A$ complex, reducing anxiety (Saavedra et al. 2006). These results suggest that the effect of AT_1 receptor antagonists may not be limited to their action in the hypothalamus. In addition to hypothalamic areas, AT_1 receptors are expressed in brain areas regulating the response of the limbic system to stress, such as the basolateral amygdaloid nucleus (Tsutsumi and Saavedra 1991a). This, and the anxiolytic and cortical effects of AT_1 receptor blockade, suggest a role for Ang II in the regulation not only of the autonomic and hormonal, but also the behavioral response to stress (Shekhar et al. 2003).

The brain also expresses another Ang II receptor type, the AT_2 receptors (Tsutsumi and Saavedra 1991a) located, in the adult rat brain, in areas related to sensory and motor function and behavior, such as the inferior olivary complex and thalamic nuclei (Tsutsumi and Saavedra 1991a). In the rat, there are high numbers of AT_2 receptors in the locus coeruleus (Tsutsumi and Saavedra 1991a), the major site for catecholamine synthesis projecting to the forebrain (Sawchenko and Swanson 1981). A role for brain AT_2 receptors in the regulation of central catecholamine formation and stress is further supported by the decrease in AT_2 receptor mRNA in the locus coeruleus and inferior olive of rats submitted to chronic cold stress (Peng and Phillips 2001) and by the increased stress response (Watanabe et al. 1999), HPA axis stimulation and AT_1 receptor expression (Armando et al. 2002) in AT_2 gene-disrupted ($AT_2 -/-$) mice.

We have earlier reported that candesartan pretreatment prevented a stress-induced disorder, the development of cold-restraint induced gastric ulcers in spontaneously hypertensive rats (SHRs) (Bregonzio et al. 2003). In SHR, both the brain Ang II and sympathetic systems are hyperactive (Phillips and Kimura 1988; Palmer and Printz 1999). In addition, and perhaps as a consequence of the central Ang II and sympathetic hyperactivity, SHR are hypersensitive to a variety of stressors, including immobilization (McMurtry and Wexler 1981). To further clarify, the central mechanisms involved in the therapeutic effect of candesartan, and the role of Ang II AT_1 and AT_2 receptors in the regulation of the central response to stress, we studied these receptors in brain areas proposed to be involved in the hypothalamic and limbic response to cold-restraint stress in SHR.

Materials and methods

Animals

Adult, 8-week-old male spontaneously hypertensive rats (SHRs) weighing 200–250 g were purchased from Taconic Farms, Germantown, NY, USA, housed at 22°C, under a 12-h dark, 12-h light cycle (lights on at 07:00 h) and given free access to normal rat diet and tap water. The National Institute of Mental Health Animal Care and Use Committee approved all procedures. All efforts were made to minimize the number of animals used and their suffering. Animals were killed by decapitation without anesthesia to prevent biochemical changes as a result of the anesthetic procedures (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 80-23, revised 1996).

Groups of 12 animals received oral candesartan-cilexetil (TCV 116, from ASTRA, Mölndal, Sweden), 10 mg/kg per day for 14 days, or vehicle, dissolved in their drinking water. Candesartan-cilexetil was first dissolved as a 1 mg/ml stock solution in polyethylene glycol (PGE) 400/ethanol/Cremophor EL (Sigma Chemicals)/water (10/5/2/83%) adjusted to pH9 with 0.2 M Na_2CO_3. The stock solution was diluted in water to a final concentration equal to or less than 1/0.5/0.2% PEG/ethanol/Cremophor EL.

Systolic blood pressures were measured by the tail-cuff procedure in control, non-stressed animals on the day before administration of candesartan and after 14 days of administration.

Stress protocol

The rats were fasted overnight with free access to water, on the night before the experiment. Fasting overnight was required in this model to prevent gastric protection by stomach contents (Tanaka et al. 2007). On the day of the experiment, the group of 12 rats treated with candesartan and the group of 12 rats treated with vehicle were randomly divided into two groups of six rats each. Two of the groups, one previously treated with candesartan and one group treated with vehicle, were submitted to stress for 2 h between 9:00 AM and

12:00 noon, by placing them in standard adjustable plastic restraining devices (IITC Inc., Woodland Hills, CA, USA, model 82) maintained at 4°C. Two other groups, one treated with candesartan and the another one treated with vehicle, were not submitted to stress and kept in their home cages. Immediately after the end of the restraining period, the rats were killed by decapitation. The other two groups of rats were treated with vehicle or candesartan and served as controls. They were fasted but not submitted to cold-restraint stress.

Tissue preparation

The brain and adrenal glands were immediately removed, frozen in isopentane at −30°C on dry ice, and stored at −80°C until assayed. Consecutive, 16 μm thick coronal brain sections and adrenal sections were cut at −20°C in a cryostat. For anatomical localization of Ang II receptor binding or TH 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 rat was evaluated independently, and four sections per brain region were studied for each animal and for each procedure.

Quantitative autoradiography of angiotensin II AT_1 and AT_2 receptors

Coronal brain sections, 16 μm-thick, were cut in a cryostat at −20°C, thaw-mounted on poly-1-lysine-coated slides (Labscientific Inc., Livingston, NJ, USA), dried overnight in a desiccator at 4°C, and stored at −80°C until use. Sections were labeled *in vitro* with 0.5 nM of $[^{125}I]$sarcosine^1-Ang II ($[^{125}I]$Sar^1-Ang II, Peninsula Laboratories, Belmont, CA, USA; iodinated by the Peptide Radioiodination Service Center, School of Pharmacy, University of Mississippi, Mississippi, MS, USA, to a specific activity of 2176 Ci/mmol). Sections were pre-incubated for 15 min at 22°C in 10 mM sodium phosphate buffer, pH 7.4, containing 120 mM NaCl, 5 mM Na_2EDTA, 0.005% bacitracin (Sigma Chemical, St. Louis, MO, USA), and 0.2% proteinase-free bovine serum albumin (Sigma Chemical), followed by incubation for 120 min in fresh buffer containing 0.5 nM of $[^{125}I]$Sar^1–Ang II. We determined total binding by incubating the sections as described above (Tsutsumi and Saavedra 1991a). Non-specific 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 AT_1 and AT_2 receptors, we incubated consecutive sections with 0.5 nM of $[^{125}I]$Sar^1-Ang II in the presence of the selective AT_1 receptor antagonist losartan (10 μM; DuPont-Merck, Wilmington, DE, USA) or the selective AT_2 receptor antagonist PD 123319 (Sigma Chemical), respectively, to give maximum specific displacement. The number of AT_1 and AT_2 receptors was defined as the binding displaced by the AT_1 and AT_2 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, dipped in ice-cold distilled water, and dried under air. Sections were exposed to Kodak Biomax MR film (Eastman Kodak Company, Rochester, NY, USA) together with ^{14}C-labeled microscales (American Radiolabeled Chemicals, St. Louis, MO, USA). 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 autoradiograms generated by incubation with the ^{125}I-labeled ligands were quantified by computerized densitometry using the Image 1.6 Program (National Institute of Mental Health, Bethesda, MD, USA) after calibration with ^{14}C-labeled standards as described (Tsutsumi and Saavedra 1991a). The films were exposed for different times to obtain film images within the linear portion of the standard curve and the optical densities were converted to corresponding values of fmol per mg protein (Nazarali et al. 1989). Because, we used single ligand concentrations below saturation, we could not determine whether the changes described represent alterations in receptor number or receptor affinity.

In situ *hybridization of tyrosine hydroxylase mRNA*

For *in situ* hybridization, sections at the level of the locus coeruleus consecutive to those used for autoradiography were thaw-mounted on silanated glass slides (Digene Diagnostics, Beltsville, MD, USA) and stored at −80°C. Sections from the adrenal gland were collected as above.

An antisense oligonucleotide probe corresponding to 48 nucleotides of the rat tyrosine hydroxylase (TH) cDNA sequence (nt 1562–1609) was synthesized by Lofstrand Labs Ltd (Gaithersburg, MD, USA) (Grima et al. 1985). We labeled the probe to a specific activity of 3 to 4×10^8 dpm/μg with a 3′-end labeling kit (Amersham) that used terminal deoxynucleotidyl transferase. Each reaction was performed with 70 pmol of probe in the presence of 70 μCi of $[\alpha\text{-}^{35}S]$ATP (SJ 1334) (Amersham). The labeled probes were separated from unincorporated nucleotides using MicroSpin G-25 columns (Amersham). *In situ* hybridization of rat brain and adrenal sections and post-hybridization washings were performed as described (Wisden and Morris 1994). *In situ* hybridization was performed in consecutive sections, one with the TH antisense (AS) probe and another with

Downloaded By: [Blackmore, Robert][informa internal users] At: 15:41 26 September 2008

Downloaded By: [Blackmore, Robert][informa internal users] At: 15:41 26 September 2008

added excess unlabeled TH-AS probe (157 pmol/ml). After the washing, sections were dehydrated in alcohols containing 0.3 M ammonium acetate, air-dried and exposed to Hyperfilm-^{3}H (Amersham, Arlington Heights, IL, USA) for 14 days. Films were developed in D-19 developer (Eastman Kodak, Rochester, NY, USA) 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 [^{14}C] micro-scales.

Statistical analysis

Data from TH mRNA and Ang II receptor binding were analyzed by one-way ANOVA and Newman–Keuls post tests. Blood pressure data were analyzed by *t*-test. A value of $P < 0.05$ was considered significant.

Results

Effects of candesartan on blood pressure

Oral administration of candesartan reduced the systolic blood pressure in SHR from 190 ± 6 to 114 ± 5 mm Hg ($n = 6$, $P < 0.01$).

Autoradiographic localization of angiotensin II receptor types

We detected AT_1 binding in the SFO, PVN, ME, piriform cortex, basolateral amygdaloid nucleus, median preoptic nucleus, area postrema and the nucleus of the solitary tract (Figures 1–3). AT_2 binding was located in the nucleus of the lateral olfactory tract, the mediodorsal and ventrolateral thalamic nuclei, the inferior olivary complex (dorsal, medial subnucleus A and B, and medial nuclei) and in the locus coeruleus (Figures 4 and 5). In each of the brain areas studied, we could detect only one receptor type, and not the other. This selective expression was maintained in stressed rats treated with vehicle and in control or stressed rats treated with the AT_1 receptor antagonist (Figures 1–5).

Effects of oral administration of an AT_1 receptor antagonist on brain AT_1 and AT_2 receptor expression

Long-term oral treatment with the AT_1 receptor antagonist inhibited binding to AT_1 receptors in all brain areas studied, either outside (SFO, ME, and area postrema) or inside (median preoptic nucleus, PVN, basolateral amygdaloid nucleus, piriform cortex and nucleus of the solitary tract) the blood brain barrier (Figures 1–3).

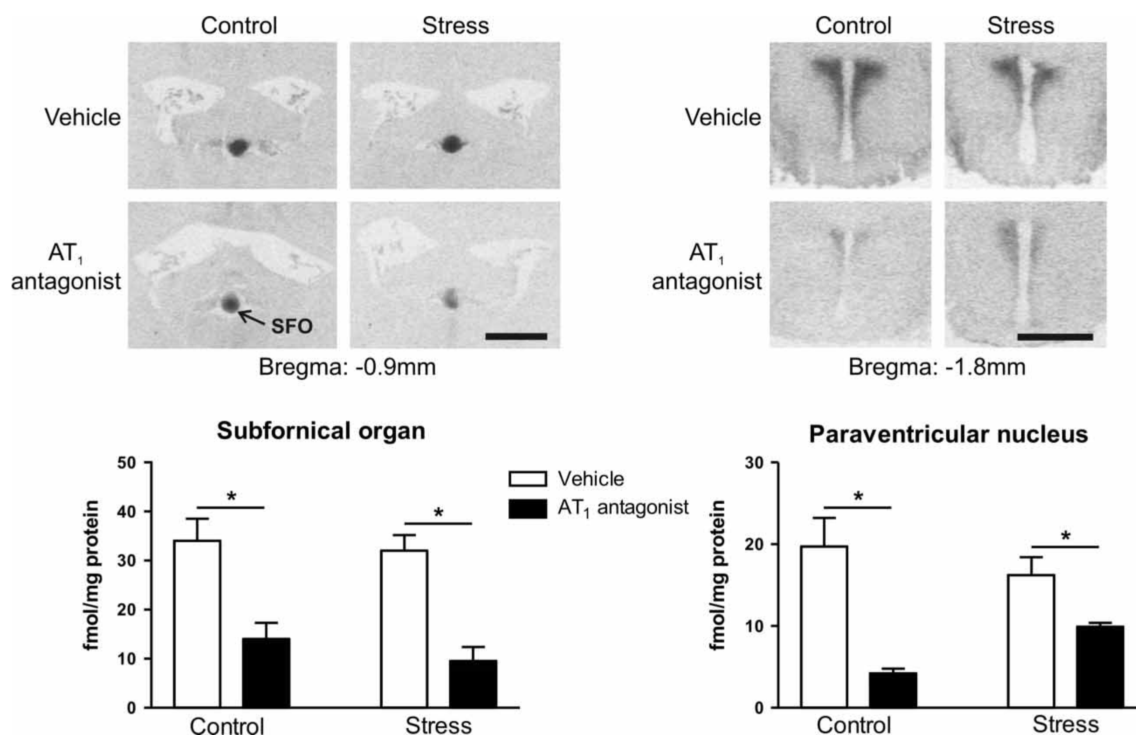

Figure 1. Effects of stress and AT_1 receptor blockade on AT_1 receptor binding in the subfornical organ and paraventricular nucleus. AT_1 receptor binding, as defined in "Materials and methods," is visualized in the autoradiograms of sections incubated with [^{125}I]Sar^1-AngII in the presence of the AT_2 receptor selective antagonist PD123319. The autoradiographs show one representative individual of each group. SFO, subfornical organ and PVN, paraventricular nucleus. Columns are means ± SEM obtained from six rats, measured individually. *$P < 0.05$. Bars are 3 mm.

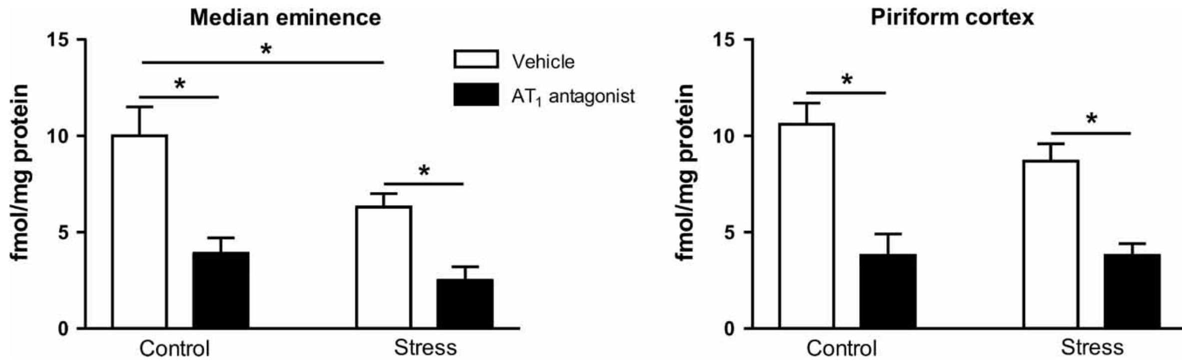

Figure 2. Effects of stress and AT_1 receptor blockade on AT_1 receptor binding in the median eminence and piriform cortex. Columns are means ± SEM obtained from six rats, measured individually. $^*P < 0.05$.

No significant changes in AT_2 binding occurred in the nucleus of the lateral olfactory tract, the mediodorsal or ventrolateral thalamic nuclei, or the locus coeruleus after long-term AT_1 receptor blockade (Figure 4). Conversely, after pretreatment with candesartan, the number of AT_2 receptors was significantly increased in the medial subnucleus A and B of the inferior olivary complex (Figure 5).

Effect of cold-restraint stress on brain AT_1 and AT_2 receptor expression

Cold restraint stress did not alter AT_1 receptor number in the median preoptic nucleus, SFO, PVN, piriform cortex, or nucleus of the solitary tract (Figures 1–3). However, stress produced a significant increase in AT_1 receptor binding in the area postrema, and a decrease in AT_1 binding in the median eminence and the basolateral amygdaloid nucleus (Figures 2 and 3).

After stress, we found significant increases in AT_2 receptor binding only in the medial subnucleus A and B of the inferior olivary complex (Figure 5). Stress did not affect AT_2 receptor binding in the nucleus of the locus coeruleus, the lateral olfactory tract, or the mediodorsal thalamic nucleus (Figure 4). Conversely, stress produced a significant decrease in AT_2 receptor binding localized exclusively to the ventrolateral thalamic nucleus (Figure 4).

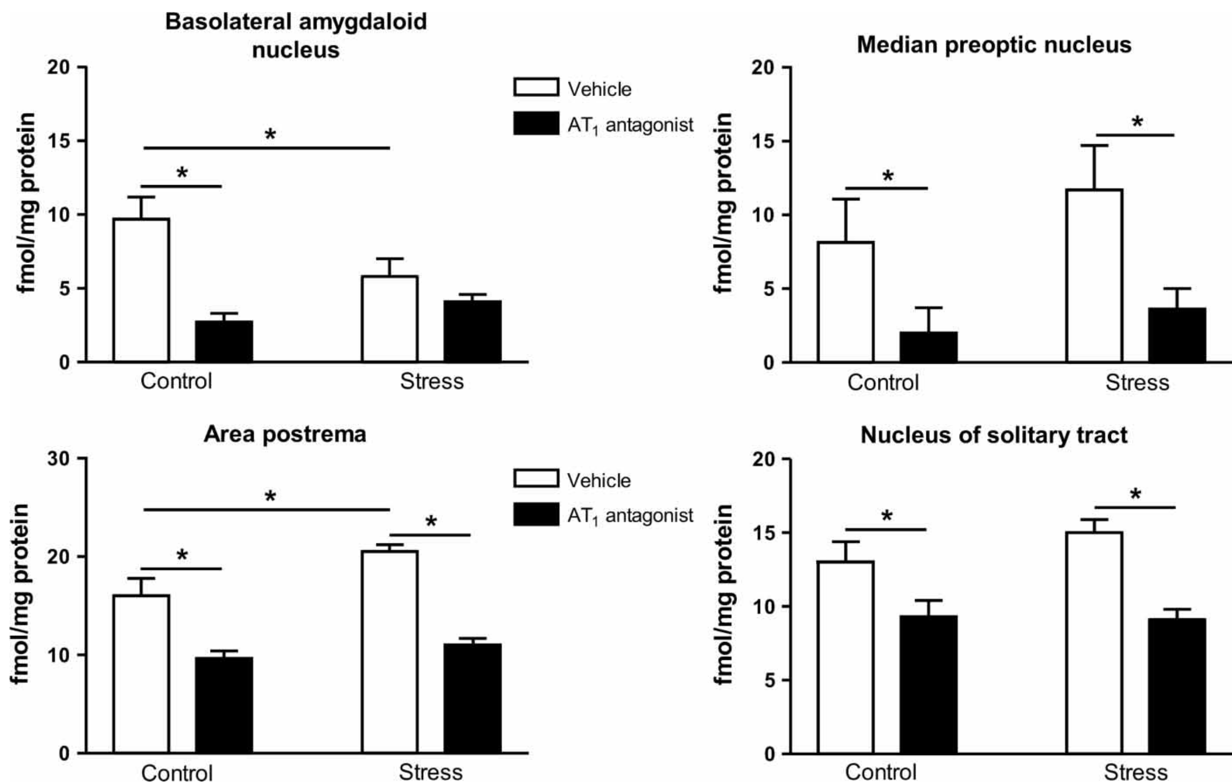

Figure 3. Effects of stress and AT_1 receptor blockade on AT_1 receptor binding in the basolateral amygdaloid nucleus, median preoptic nucleus, area postrema and nucleus of the solitary tract. Columns are means ± SEM obtained from six rats, measured individually. $^*P < 0.05$.

Downloaded By: [Blackmore, Robert][informa internal users] At: 15:41 26 September 2008

Downloaded By: [Blackmore, Robert][informa internal users] At: 15:41 26 September 2008

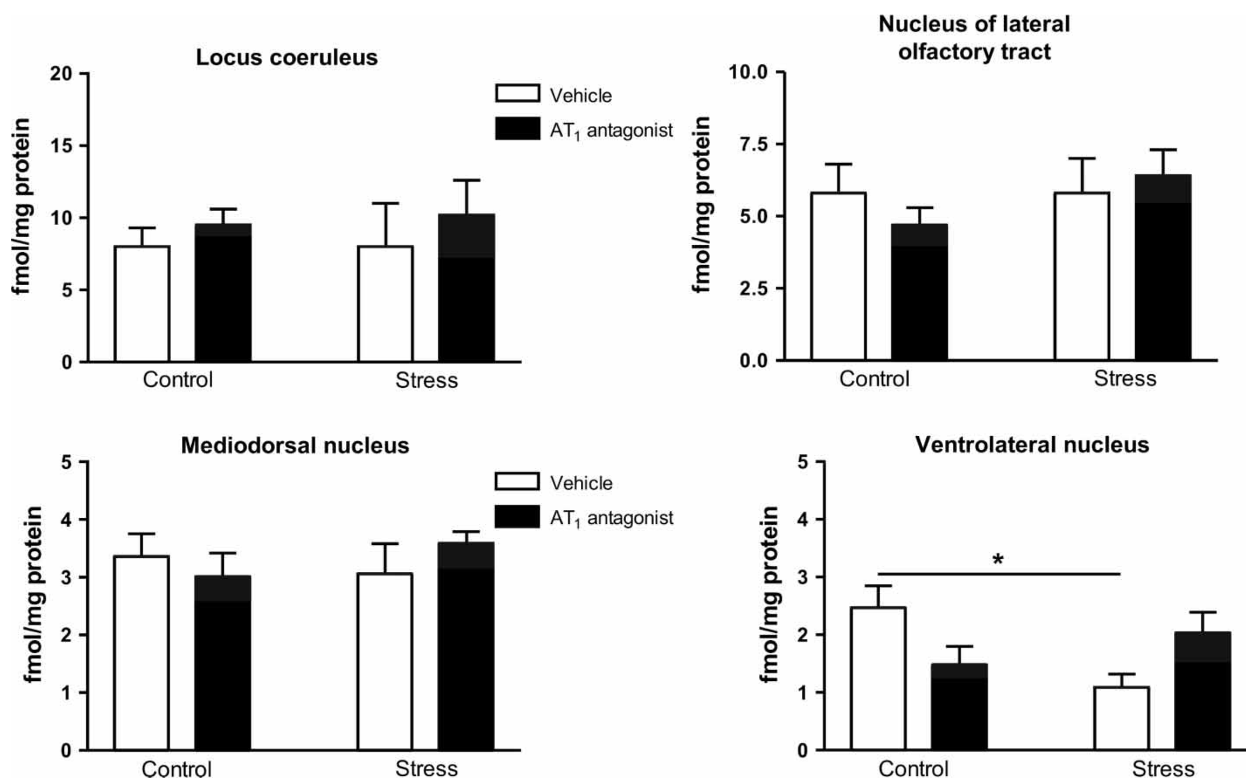

Figure 4. Effects of stress and AT_1 receptor blockade on AT_2 receptor binding in the locus coeruleus, the nucleus of the lateral olfactory tract, and in the mediodorsal and ventrolateral nuclei of the thalamus. Columns are means ± SEM obtained from six rats, measured individually. $^*P < 0.05$.

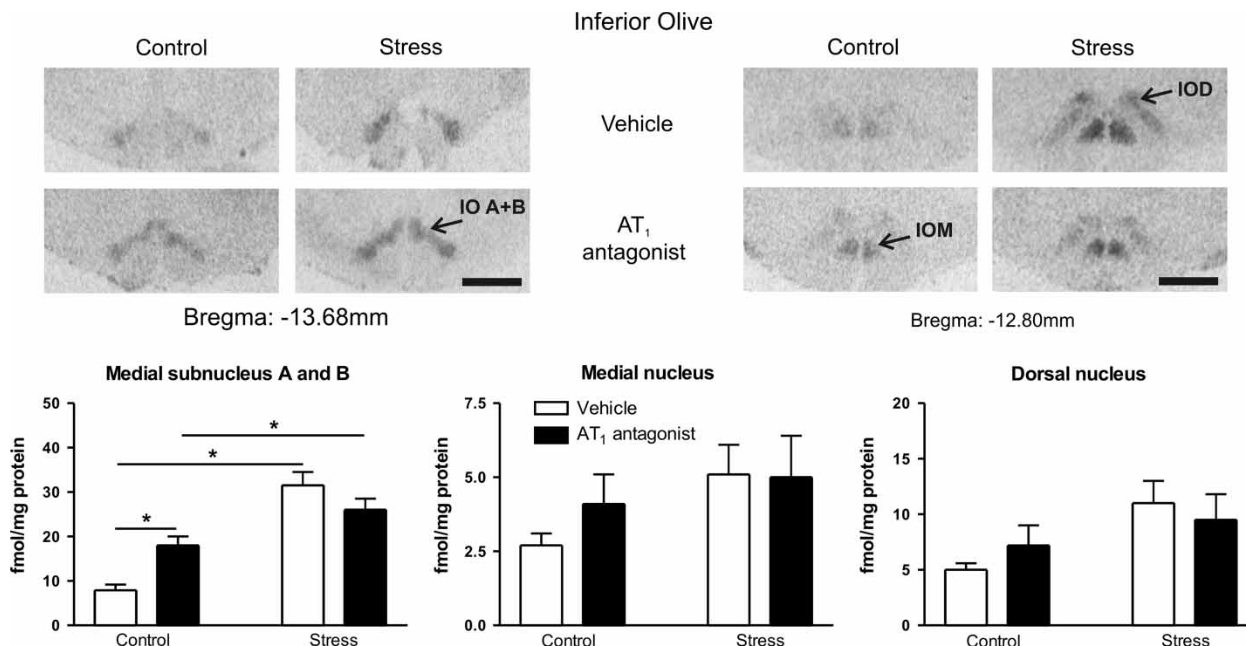

Figure 5. Effects of stress and AT_1 receptor blockade on AT_2 receptor binding in the inferior olive. IO A + B, inferior olive, medial subnucleus A and B; IOM, inferior olive, medial nucleus; and IOD, inferior olive, dorsal nucleus. AT_2 receptor binding, as defined in Materials and methods is visualized in the autoradiograms of sections incubated with $[^{125}I]Sar^1$-Ang II and displaced with the AT_1 receptor-selective ligand losartan. Autoradiographs show one representative individual of each group. Columns are means ± SEM obtained from six rats, measured individually. $^*P < 0.05$. Bar is 1 mm.

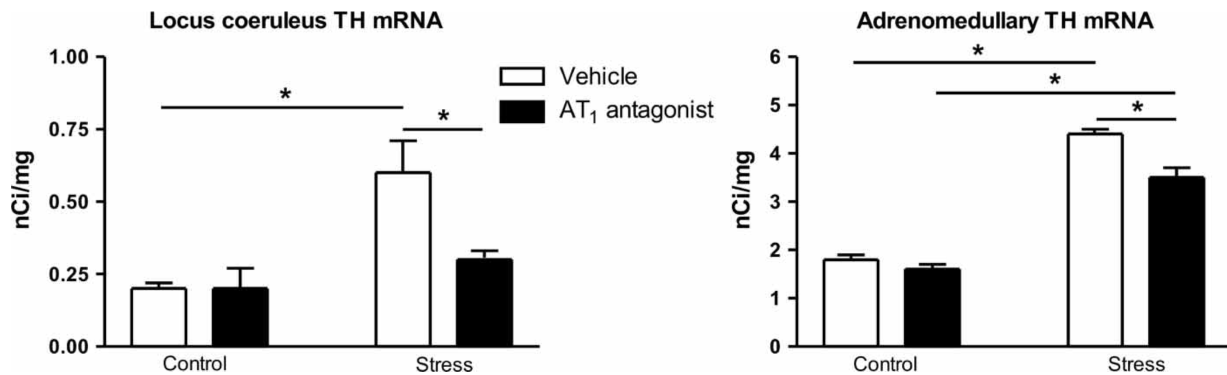

Figure 6. Effect of stress and AT_1 receptor blockade on tyrosine hydroxylase mRNA in the locus coeruleus and in the adrenal medulla. Tyrosine hydroxylase was measured by quantitative *in situ* hybridization as described in Materials and methods. Candesartan treatment prevents the stress-induced increase in tyrosine hydroxylase mRNA expression in locus coeruleus, but not in the adrenal medulla. Columns are means ± SEM obtained from six rats, measured individually. $*P < 0.05$.

Downloaded By: [Blackmore, Robert][informa internal users] At: 15:41 26 September 2008

Effect of pretreatment with the AT_1 receptor antagonist on AT_1 and AT_2 receptor expression after cold-restraint

Pretreatment with the AT_1 receptor antagonist inhibited AT_1 receptor binding after cold-restraint in all brain areas studied to the same extent as the inhibition observed in non-stressed rats (Figures 1–3).

AT_2 receptor binding in the medial subnucleus A and B of the inferior olive was still significantly greater after AT_1 blockade followed by cold-restraint (Figure 5). Similarly, AT_1 receptor antagonism did not modify the AT_2 receptor expression in the locus coeruleus, the nucleus of the lateral olfactory tract, and the mediodorsal thalamic nucleus after stress (Figure 4). On the other hand, the significant stress-induced reduction in AT_2 receptor binding in the ventrolateral thalamic nuclei was no longer present (Figure 4).

Effect of cold restraint and AT_1 receptor blockade on tyrosine hydroxylase mRNA expression in the locus coeruleus

Cold restraint produced a significant increase in TH mRNA expression in the locus coeruleus. Oral pretreatment with the AT_1 receptor antagonist did not influence the expression of TH mRNA in control rats, but completely abolished the increased TH mRNA expression in the locus coeruleus that was produced by cold-restraint (Figure 6)

Effect of cold restraint and AT_1 receptor blockade on adrenal tyrosine hydroxylase mRNA

After cold-restraint there was a significant increase in adrenomedullary TH mRNA. Oral pretreatment with the AT_1 receptor antagonist did not modify the expression of TH mRNA in non-stressed rats, but significantly reduced the stress-induced increase in the expression of adrenomedullary TH mRNA (Figure 6).

Discussion

Effects of candesartan treatment and cold-restraint stress on Ang II AT_1 receptors

Orally administered candesartan, at doses that decreased systolic blood pressure in SHR to normotensive levels, significantly blocked brain AT_1 receptors situated outside or inside the blood brain barrier and effectively prevented the effects of centrally administered Ang II in SHR (Seltzer et al. 2004). The present results confirm our previous observations, demonstrating that orally administered candesartan may be used to study the activity and the role of brain Ang II in a manner similar to that of candesartan administered subcutaneously at an equivalent dose (Nishimura et al. 2000).

Acute cold-restraint stress produced profound and selective alterations in AT_1 receptor expression. Only repeated, but not acute immobilization produces alterations in AT_1 receptor expression (Leong et al. 2002). The decrease in AT_1 receptor binding in the ME after acute cold-restraint, a more complex stress than immobilization alone, may be due to fast receptor internalization following enhanced Ang II binding as a consequence of the stress-induced increase in circulating and hypothalamic levels of the peptide (Yang et al. 1993). Receptor binding, however, was enhanced in another circumventricular organ, the area postrema. These changes are not likely to result from alterations in receptor synthesis, because of the short time of exposure to stress in our model.

Alterations in AT_1 receptor binding during stress are not limited to hypothalamic structures. Of particular interest is the decrease in AT_1 receptor binding in the basolateral amygdaloid nucleus. The basolateral amygdala has been implicated in the emotional response to stress (McIntyre et al. 2003; Sah et al. 2003). The presence of AT_1 receptors in the basolateral amygdala has been previously recognized

Downloaded By: [Blackmore, Robert][informa internal users] At: 15:41 26 September 2008

(Tsutsumi and Saavedra 1991a). Our results indicate that AT_1 receptors in the basolateral amygdala are involved in the acute response to a major stress, and are in agreement with the recent report (Shekhar et al. 2003) of an Ang II-mediated activation of the basolateral amygdala during stress. We postulate that enhanced stimulation by Ang II may increase receptor internalization, resulting in the observed decrease in receptor binding.

In most brain areas, the decrease in AT_1 receptor binding produced by candesartan pretreatment was not changed when candesartan-treated rats were submitted to cold-restraint. These observations indicate that brain AT_1 receptor blockade was effective during the stress procedure.

Effects of candesartan treatment and cold-restraint stress on Ang II AT_2 receptors

The AT_1 antagonist increased AT_2 binding in the inferior olivary complex and especially in the medial subnuclei A and B in non-stressed rats, confirming previous observations (Seltzer et al. 2004). In our study, candesartan did not increase AT_2 receptor binding in the locus coeruleus or in the thalamic nuclei. We had previously observed increased AT_2 binding in the locus coeruleus after four weeks of candesartan administration to non-fasted SHR (Seltzer et al. 2004). Differences in treatment conditions could best explain the discrepancy between the two studies.

We conclude that brain AT_1 receptor antagonism has selective and profound influences on brain AT_2 receptor expression, and that AT_1 receptor activity regulates AT_2 receptor number, at least in some selected brain areas. In turn, AT_2 receptor activity regulates AT_1 receptor number, as demonstrated by the increased AT_1 receptor expression in the PVN of $AT_2-/-$ mice (Armando et al. 2002). The reciprocal interaction between AT_1 and AT_2 receptor expression supports the hypothesis of AT_1/AT_2 receptor interaction, or intracellular "cross-talk" (Gelband et al. 1997). However, we have not detected co-localization of AT_1 and AT_2 receptors in any brain area studied. If same cell AT_1/AT_2 co-localization exists in the brain, it is the exception rather than the norm (Tsutsumi and Saavedra 1991a). The cross-talk between brain AT_1 and AT_2 receptors is therefore due to intercellular rather than intracellular interactions, and its precise mechanisms remain to be elucidated.

We report major increases in AT_2 receptor expression in the medial subnucleus A and B of the inferior olive after stress. Changes in AT_2 receptor binding are probably not the result of increased receptor synthesis, because of the short period of stress, but they may represent increased receptor affinity or decreased receptor agonist occupancy. These results indicate that AT_2 receptors in the inferior olive may be important for the regulation of the stress reaction by brain Ang II. Ang II, by stimulation of AT_2 receptors, increases neuronal firing in the inferior olive (Ambuhl et al. 1992). The role of the inferior olive during the stress reaction has not been clarified, although it is well-known that this system controls sensory information and responds readily to sensory inputs that are not anticipated (Devor 2002).

In the ventrolateral thalamic nucleus, AT_2 receptor expression substantially decreased after stress. Thalamic structures are among the few areas of the rat brain expressing substantial numbers of AT_2 receptors in adult animals and this may signal a role in processing of sensory information in adults (Tsutsumi and Saavedra 1991a). The present results indicate an active role of AT_2 receptors in the ventrolateral thalamic nucleus in the processing of information during stress. The mechanism of the fast decrease in binding may represent increased receptor agonist occupancy, because the AT_2 receptors do not internalize after agonist binding (De Gasparo and Siragy 1999), and the timing of the changes is too short to be the result of alterations in receptor turnover.

Alterations in AT_2 receptor binding in the inferior olivary complex did not change when candesartan-treated rats were submitted to cold-restraint. Conversely, the decrease in AT_2 receptor binding in the ventrolateral thalamic nucleus observed during stress was abolished in rats pretreated with candesartan. The mechanism and significance of this finding remains an open question.

Effects of candesartan treatment and cold-restraint stress on tyrosine hydroxylase mRNA in the locus coeruleus

Candesartan completely prevented the stress-induced increase in TH mRNA expression in the locus coeruleus, a hallmark of the central response to stress and an indication of increased catecholamine synthesis and central sympathetic activity (Rusnak et al. 1998). This indicates that AT_1 receptor antagonism abolishes the increased TH transcription not only during the relatively mild stress of isolation (Saavedra et al. 2006), but also during a major challenge such as cold-restraint as reported here. These findings indicate that the control of central catecholamine formation by brain Ang II (Saavedra and Benicky 2007) has important physiological correlates.

While the locus coeruleus of the rat expresses only AT_2 receptors (Tsutsumi and Saavedra 1991a), the locus coeruleus of the mouse expresses only AT_1 receptors, indicating that receptor type expression in this area is species-dependent (Häuser et al. 1998). There is evidence for a localization of Ang II (Fuxe et al. 1988) and Ang II receptors (Rowe et al. 1990) in catecholaminergic neurons in the locus coeruleus.

However, Ang II does not affect norepinephrine release from this region (Huang et al. 1987). Instead, Ang II depresses the depolarizing effect of glutamate and excitatory postsynaptic potentials in the locus coeruleus through AT_2 receptor stimulation (Xiong and Marshall 1994). We did not detect alterations in AT_2 receptor expression in the locus coeruleus during stress or after AT_1 receptor blockade. Others have reported increased AT_2 receptor mRNA in the locus coeruleus of Wistar rats after acute or chronic immobilization or air-jet stress (Dumont et al. 1999) and decreased AT_2 receptor mRNA expression after chronic cold stress (Peng and Phillips 2001). We conclude that the effect of AT_1 receptor antagonists in the expression of TH mRNA in the locus coeruleus may not be direct. Indirect effects may include inhibition of brain stem AT_1 receptors located in the nucleus of the solitary tract and the area postrema, as reported here, along with the blood pressure decrease and changes in the baroreflex that follow AT_1 receptor antagonism (Hasser et al. 2000) and inhibition of the CRH mediation of the stress-induced stimulation of neuronal activity in this area (Porter 2000; Makino et al. 2002; Zeng et al. 2003).

Decreased TH transcription in the locus coeruleus may be causally related to the increase in AT_2 receptor expression in the inferior olive, since inferior olivary neurons containing AT_2 receptors (Tsutsumi and Saavedra 1991a) receive norepinephrine afferents from the locus coeruleus (Kobayashi et al. 1974). The regulation of AT_2 receptor expression by alterations in central catecholamine formation is a novel hypothesis in need of further support.

In addition to the central alterations described above, oral pretreatment with the AT_1 receptor blocker partially reduces the well-known stress-induced increase in adrenomedullary TH transcription (Kvetnansky et al. 1996). This supports our previous observations in rats submitted to isolation or cold-restraint stress and pretreated with candesartan administered subcutaneously (Armando et al. 2001; Bregonzio et al. 2003). These results are in agreement with recent findings of reduction in central and peripheral sympathetic nerve activity in neurogenic hypertensive rats by the AT_1 receptor antagonist losartan (Ye et al. 2002).

In conclusion, our observations support a role for hypothalamic and limbic system AT_1 receptors and brain stem AT_2 receptors in the control of the central response to stress. The present findings demonstrate that AT_1 receptor antagonists with dual peripheral and central effects inhibit the stress-induced increase in central and peripheral sympathetic activity. The anti-stress properties of candesartan are shared by other AT_1 receptor antagonists such as losartan (Vinícius et al. 2007). These observations indicate that AT_1 receptor blockers may be considered as effective anti-stress agents.

Acknowledgements

This study was supported by the Division of Intramural Research Programs, National Institute of Mental Health, National Institutes of Health, Department of Health and Human Services, USA.

References

Aguilera G, Kiss A, Luo X. 1995a. Increased expression of type 1 angiotensin II receptors in the hypothalamic paraventricular nucleus following stress and glucocorticoid administration. J Neuroendocrinol 7:775–783.

Aguilera G, Young WS, Kiss A, Bathia A. 1995b. Direct regulation of hypothalamic corticotrophin-releasing-hormone neurons by angiotensin II. Neuroendocrinology 61:437–444.

Ambuhl P, Felix D, Imboden H, Khosla MC, Ferrario CM. 1992. Effects of angiotensin II and its selective antagonists on inferior olivary neurons. Regul Pept 41:19–26.

Armando I, Carranza A, Nishimura Y, Hoe KL, Barontini M, Terrón JA, Falcón-Neri A, Ito T, Juorio AV, Saavedra JM. 2001. Peripheral administration of an angiotensin II AT_1 receptor antagonist decreases the hypothalamic–pituitary–adrenal response to isolation stress. Endocrinology 142:3880–3889.

Armando I, Terrón JA, Falcón-Neri A, Ito T, Häuser W, Inagami T, Saavedra JM. 2002. Increased angiotensin II AT_1 receptor expression in paraventricular nucleus and hypothalamic–pituitary–adrenal axis stimulation in AT_2 receptor gene disrupted mice. Neuroendocrinology 76:137–147.

Armando I, Volpi S, Aguilera G, Saavedra JM. 2007. Angiotensin II AT_1 receptor blockade prevents the hypothalamic corticotropin-releasing factor response to isolation stress. Brain Res 1142:92–99.

Bregonzio C, Armando I, Ando H, Jezova M, Baiardi G, Saavedra JM. 2003. Anti-inflammatory effects of angiotensin II AT_1 receptor antagonism prevent stress-induced gastric injury. Am J Physiol 285:G414–G423.

Castrén E, Saavedra JM. 1988. Repeated stress increases the density of angiotensin II binding sites in the rat paraventricular nucleus and subfornical organ. Endocrinology 122:370–372.

Castrén E, Saavedra JM. 1989. Angiotensin II receptors in paraventricular nucleus, subfornical organ, and pituitary gland of hypophysectomized, adrenalectomized, and vasopressin-deficient rats. Proc Natl Acad Sci USA 86:725–729.

De Gasparo M, Siragy HM. 1999. The AT_2 receptor: Fact, fancy and fantasy. Regul Pept 81:11–24.

Devor A. 2002. The great gate: Control of sensory information flow to the cerebellum. Cerebellum 1:27–34.

Dumont EC, Rafrafi S, Laforest S, Drolet G. 1999. Involvement of central angiotensin receptors in stress adaptation. Neuroscience 93:877–884.

Fuxe K, Bunnemann B, Aronsson M, Tinner B, Cintra A, von Euler G, Agnati LF, Nakanishi S, Ohkubo H, Ganten D. 1988. Pre- and postsynaptic features of the central angiotensin system: Indications for a role of angiotensin peptides in volume transmission and for interactions with central monoamine neurons. Clin Exp Hypertens A 10(Suppl 1):143–168.

Ganong WF, Murakami K. 1987. The role of angiotensin II in the regulation of ACTH secretion. Ann NY Acad Sci 512:176–186.

Gelband CH, Zhu M, Lu D, Reagan LP, Fluharty SJ, Posner P, Raizada MK, Sumners C. 1997. Functional interaction between neuronal AT_1 and AT_2 receptors. Endocrinology 138:2195–2198.

Grima B, Lamouroux A, Blanot F, Biguet NF, Mallet J. 1985. Complete coding sequence of rat tyrosine hydroxylase mRNA. Proc Natl Acad Sci USA 82:617–621.

Guo DF, Inagami T. 1994. The genomic organization of the rat angiotensin II receptor AT_{1B}. Biochim Biophys Acta 1218:91–94.

Downloaded By: [Blackmore, Robert][informa internal users] At: 15:41 26 September 2008

Hasser EM, Cunningham JT, Sullivan MJ, Curtis KS, Blaine EH, Hay A. 2000. Area postrema and sympathetic nervous system effects of vasopressin and angiotensin II. Clin Exp Pharmacol Physiol 27:432–436.

Häuser W, Jöhren O, Saavedra JM. 1998. Characterization and distribution of angiotensin II receptor subtypes in the mouse brain. Eur J Pharmacol 348:101–114.

Huang Y, Rogers J, Henderson G. 1987. Effects of angiotensin II on [3H]noradrenaline release and phosphatidylinositol hydrolysis in the parietal cortex and locus coeruleus of the rat. J Neurochem 49:1541–1549.

Kobayashi RM, Palkovits M, Kopin IJ, Jacobowitz DM. 1974. Biochemical mapping of noradrenergic nerves arising from the rat locus coeruleus. Brain Res 77:269–279.

Kvetnansky R, Nankova B, Hiremagalur B, Viskupic E, Vietor I, Rusnak M, McMahon A, Kopin IJ, Sabban EL. 1996. Induction of adrenal tyrosine hydroxylase mRNA by single immobilization stress occurs even after splanchnic transection and in the presence of cholinergic antagonists. J Neurochem 66:138–146.

Leong DS, Terrón JA, Falcón-Neri A, Armando I, Ito T, Jöhren O, Tonelli LH, Hoe KL, Saavedra JM. 2002. Restraint stress modulates brain, pituitary and adrenal expression of angiotensin II AT_{1A}, AT_{1B} and AT_2 receptors. Neuroendocrinology 75:227–240.

Makino S, Smith MA, Gold PW. 2002. Regulatory role of glucocorticoids and glucocorticoid receptor mRNA levels on tyrosine hydroxylase gene expression in the locus coeruleus during repeated immobilization stress. Brain Res 943:216–223.

McIntyre CK, Power AE, Roozendaal B, McGaugh JL. 2003. Role of the basolateral amygdala in memory consolidation. Ann NY Acad Sci 985:273–293.

McMurtry JP, Wexler BC. 1981. Hypersensitivity of spontaneously hypertensive rats (SHR) to heat, ether, and immobilization. Endocrinology 108:1730–1736.

Nazarali AJ, Gutkind JS, Saavedra JM. 1989. Calibration of ^{125}I-polymer standards with ^{125}I-brain paste standards for use in quantitative receptor autoradiography. J Neurosci Methods 30:247–253.

Nishimura Y, Ito T, Hoe K, Saavedra JM. 2000. Chronic peripheral administration of the angiotensin II AT_1 receptor antagonist candesartan blocks brain AT_1 receptors. Brain Res 871:29–38.

Oldfield BJ, Davern PJ, Giles ME, Allen AM, Badoer E, McKinley MJ. 2001. Efferent neural projections of angiotensin receptor (AT_1) expressing neurons in the hypothalamic paraventricular nucleus of the rat. J Neuroendocrinol 13:139–146.

Palmer AA, Printz MP. 1999. Strain differences in Fos expression following airpuff startle in spontaneously hypertensive and Wistar kyoto rats. Neuroscience 89:965–978.

Paxinos G, Watson C. 1986. The rat brain in stereotaxic coordinates. NY: Academic Press.

Peng JF, Phillips MI. 2001. Opposite regulation of brain angiotensin type 1 and type 2 receptors in cold-induced hypertension. Regul Pept 97:91–109.

Phillips MI, Kimura B. 1988. Brain angiotensin in the developing spontaneously hypertensive rat. J Hypertens 6:607–612.

Porter JP. 2000. Contribution of central ANG II to acute stress-induced changes in baroreflex function in young rats. Am J Physiol 279:R1386–R1391.

Rowe BP, Kalivas PW, Speth RC. 1990. Autoradiographic localization of angiotensin II receptor binding sites on noradrenergic neurons of the locus coeruleus of the rat. J Neurochem 55:533–540.

Rusnak M, Zorad S, Buckendahl P, Sabban E, Kvetnansky R. 1998. Tyrosine hydroxylase mRNA levels in locus coeruleus of rats during adaptation to long-term immobilization stress exposure. Mol Chem Neuropathol 33:249–258.

Saavedra JM. 2005. Brain angiotensin II: New developments, unanswered questions and therapeutic opportunities. Cell Mol Neurobiol 25:485–512.

Saavedra JM, Benicky J. 2007. Brain and peripheral angiotensin II play a major role in stress. Stress 10:185–193.

Saavedra JM, Armando I, Bregonzio C, Juorio A, Macova M, Pavel J, Sánchez-Lemus E. 2006. A centrally acting, anxiolytic angiotensin II AT_1 receptor antagonist prevents the isolation stress-induced decrease in cortical CRF_1 receptor and benzodiazepine binding. Neuropsychopharmology 31:1123–1134.

Sah P, Faber ES, Lopez De Armentia M, Power J. 2003. The amygdaloid complex: Anatomy and physiology. Physiol Rev 83:803–834.

Sawchenko P, Swanson L. 1981. Central noradrenergic pathways for the integration of hypothalamic neuroendocrine and autonomic responses. Science 214:683–687.

Seltzer A, Bregonzio C, Armando I, Baiardi G, Saavedra JM. 2004. Oral administration of an AT_1 receptor antagonist prevents the central effects of angiotensin II in spontaneously hypertensive rats. Brain Res 1028:9–18.

Shekhar A, Sajdyk TJ, Gehlert DR, Rainnie DG. 2003. The amygdala, panic disorder, and cardiovascular responses. Ann NY Acad Sci 85:308–325.

Tanaka A, Hatazawa R, Takahira Y, Izumi N, Filaretova L, Takeuchi K. 2007. Preconditioning stress prevents cold restraint stress-induced gastric lesions in rats: Roles of COX-1, COX-2, and PLA2. Dig Dis Sci 52:478–487.

Tsutsumi K, Saavedra JM. 1991a. Characterization and development of angiotensin II receptor subtypes (AT_1 and AT_2) in rat brain. Am J Physiol 261:R209–R216.

Tsutsumi K, Saavedra JM. 1991b. Angiotensin II receptor subtypes in median eminence and basal forebrain areas involved in regulation of pituitary function. Endocrinology 129:3001–3008.

Vinícius F, Donadio M, Kunrath A, Corezola KL, Franci CR, Anselmo-Franci JA, Lucion AB, Sanvitto GL. 2007. Effects of acute stress on the day of proestrus on sexual behavior and ovulation in female rats: Participation of the angiotensinergic system. Physiol Behav 92:591–600.

Watanabe T, Hashimoto M, Okuyama S, Inagami T, Nakamura S. 1999. Effects of targeted disruption of the mouse angiotensin II type 2 receptor gene on stress-induced hypothermia. J Physiol 515:881–885.

Wisden W, Morris BJ. 1994. *In situ* hybridization with synthetic oligonucleotide probes. In: Wisden W, Morris BJ, editors. *In situ* hybridization protocols for the brain. San Diego: Academic Press. p 9–34.

Xiong H, Marshall KC. 1994. Angiotensin II depresses glutamate depolarizations and excitatory postsynaptic potentials in locus coeruleus through angiotensin II subtype 2 receptors. Neuroscience 62:163–175.

Yang G, Xi ZX, Wan Y, Wang H, Bi G. 1993. Changes in circulating and tissue angiotensin II during acute and chronic stress. Biol Signals 2:166–172.

Yang G, Wan Y, Zhu Y. 1996. Angiotensin II – An important stress hormone. Biol Signals 5:1–8.

Ye S, Zhong H, Duong VN, Campese VM. 2002. Losartan reduces central and peripheral sympathetic nerve activity in a rat model of neurogenic hypertension. Hypertension 39:1101–1106.

Zeng J, Kitayama I, Yoshizato H, Zhang K, Okazaki Y. 2003. Increased expression of corticotropin-releasing factor receptor mRNA in the locus coeruleus of stress-induced rat model of depression. Life Sci 73:1131–1139.

Downloaded By: [Blackmore, Robert][informa internal users] At: 15:41 26 September 2008