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Enhanced water and salt intake in transgenic mice with brain-restricted overexpression of angiotensin (AT_1) receptors

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Lazartigues E, Sinnayah P, Augoyard G, Gharib C, Johnson AK, Davisson RL. Enhanced water and salt intake in transgenic mice with brain-restricted overexpression of angiotensin (AT_1) receptors. *Am J Physiol Regul Integr Comp Physiol* 295: R1539–R1545, 2008. First published August 27, 2008; doi:10.1152/ajpregu.00751.2007.—To address the relative contribution of central and peripheral angiotensin II (ANG II) type 1A receptors (AT_{1A}) to blood pressure and volume homeostasis, we generated a transgenic mouse model [neuron-specific enolase (NSE)-AT_{1A}] with brain-restricted overexpression of AT_{1A} receptors. These mice are normotensive at baseline but have dramatically enhanced pressor and bradycardic responses to intracerebroventricular ANG II or activation of endogenous ANG II production. Here our goal was to examine the water and sodium intake in this model under basal conditions and in response to increased ANG II levels. Baseline water and NaCl (0.3 M) intakes were significantly elevated in NSE-AT_{1A} compared with nontransgenic littermates, and bolus intracerebroventricular injections of ANG II (200 ng in 200 nl) caused further enhanced water intake in NSE-AT_{1A}. Activation of endogenous ANG II production by sodium depletion (10 days low-sodium diet followed by furosemide, 1 mg sc) enhanced NaCl intake in NSE-AT_{1A} mice compared with wild types. Fos immunohistochemistry, used to assess neuronal activation, demonstrated sodium depletion-enhanced activity in the anteroventral third ventricle region of the brain in NSE-AT_{1A} mice compared with control animals. The results show that brain-selective overexpression of AT_{1A} receptors results in enhanced salt appetite and altered water intake. This model provides a new tool for studying the mechanisms of brain AT_{1A}-dependent water and salt consumption.

thirst; sodium appetite; transgenic mice; circumventricular organs; volume homeostasis; anteroventral third ventricle

THERE IS SUBSTANTIAL EVIDENCE that activation of the renin-angiotensin system (RAS) is involved in water and sodium intake. Thirst and sodium depletion are monitored by osmoreceptors and/or sodium receptors located at the periphery and in the brain (5). Although several candidates have been identified (6, 27, 28, 49), the nature and mechanism of action of these receptors remain unclear. However, it is well known that systemically and brain-generated angiotensin II (ANG II) can act directly or indirectly on ANG II receptor type 1 (AT_1) receptors located in the lamina terminalis to produce water and salt intake (20, 36). While the role and independence of centrally vs. peripherally generated ANG II in these responses still remain unclear, the pivotal involvement of central AT_1 receptors was established by studies where intracerebroventricular (ICV) administration of RAS antagonists reduced sodium appetite of sodium-depleted intact rats (41) and adrenalectomized rats (42). Furthermore, the exaggerated salt appetite observed in spontaneously hypertensive rats was reduced by central treatment with angiotensin-converting enzyme inhibitors, suggesting a role for the brain RAS in the exaggerated salt appetite in this model of hypertension (14).

In rodents, water deprivation upregulates the AT_1 receptor in various areas of the brain, including the subfornical organ (SFO) and the anterior pituitary (43), as well as in the periphery (19). In addition, water and sodium intake can be abolished by pretreatment with selective antagonists (3, 11) and antisense oligonucleotides (42) for the AT_1 receptor. A significant role for brain AT_1 receptors in volume regulatory mechanisms has been suggested in both AT_{1A}- and AT_{1B}-receptor knockout mice (13, 32). Interestingly, increased neuronal activation and vasopressin expression were observed in the paraventricular neucleus (PVN) of $AT_{1A}^{-/-}$ mice exposed to dehydration (32), supporting a role for the AT_{1B}-receptor subtype.

With the long-term goal of characterizing the signaling pathways involved in thirst and salt-craving behavior, we generated a new transgenic mouse model with brain-selective overexpression of AT_1 receptors [neuron-specific enolase (NSE)-AT_{1A}] (23). These transgenic mice exhibit widespread overexpression of the AT_1 receptor in the brain, including in areas known to be involved in the regulation of cardiovascular function and body fluid homeostasis. We have shown that, although NSE-AT_{1A} mice have normal basal blood pressure (BP), they are hyperresponsive to both exogenous and endogenous ANG II activation (23, 24).

Here we address the hypothesis that overexpression of AT_1 receptors in the brain of these mice would lead to increased water intake and salt appetite. We investigated whether NSE-AT_{1A} mice have enhanced water and/or salt intake at baseline and in the face of increased ANG II levels, and whether this is accompanied by changes in neuronal activation in brain regions implicated in the control of thirst and salt appetite.

MATERIALS AND METHODS

Generation of transgenic mice and animal husbandry. NSE-AT_{1A} transgenic mice were generated as described previously (23). Briefly, a fusion transgene consisting of 4 kb of the rat NSE 5′ flanking region and a cDNA encoding the full open-reading frame of the rat AT_{1A} receptor was microinjected into fertilized C57BL/6J × SJL/J (B6SJL F2) mouse embryos. The transgenic line selected (6085-2) showed brain-specific expression of the transgene. All mice were fed standard

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mouse chow (LM-485; Teklad Premier Laboratory Diets, containing 0.32% sodium), unless specified, and water ad libitum. All procedures were approved by the University Animal Care and Use Committee at the University of Iowa.

Baseline water intake. NSE-AT$_{1A}$ mice (n = 18) and control littermates (n = 28), from both sexes, were housed separately in plastic cages with free access to food and water. Burettes containing water were made with 25-ml pipettes connected to a glass nozzle. Animals, food, and water were weighed daily around 11:00 AM to determine the amounts ingested.

Chronic ICV losartan administration. NSE-AT$_{1A}$ transgenic (n = 12) and control littermates (n = 12) were surgically instrumented with an ICV cannula connected, via a PE50 catheter, to a mini-osmotic pump implanted subcutaneously (Alzet, model 1003D), for central administration of the AT$_1$-receptor antagonist losartan (10 μg/h) or saline during 3 days. Following recovery from anesthesia, mice were returned to their home cage, and water intake was measured as described above. This dose of losartan was previously shown to block the central pressor response to peripheral ANG II (25) but does not affect water intake when administered at the periphery in our pilot studies (baseline: 2.8 ± 0.3 ml/day, after 3 days sc losartan: 3.1 ± 0.5 ml/day; $P > 0.05$).

Acute ICV ANG II administration. NSE-AT$_{1A}$ transgenic (n = 30) and control littermates (n = 38) were surgically instrumented with ICV cannulas for central administration of ANG II (200 ng) or saline, as described (23). Following 2–3 days recovery from surgery, mice from both sexes were injected, in their home cages, with ANG II or saline, and drinking was recorded for 30 min, as described above. Compounds injected ICV were dissolved in 0.9% saline and delivered in a volume of 200 nl over at least 30 s. Following all experiments, Evans blue dye (200 nl) was injected ICV for histological verification of proper cannula placement.

Vasopressin and electrolytes. Urine from NSE-AT$_{1A}$ transgenic (n = 30) and control littermates (n = 36) was analyzed for vasopressin levels using radioimmunoassay, as described (7). In addition, urines were diluted (1:4), and osmolality was determined using a vapor pressure osmometer (Wescor Instruments). Using the same dilution, electrolyte concentrations were determined with an automatic flame photometer (model 943, Instrumentation Laboratory, Lexington, MA), according to the manufacturer's instructions. Additionally, blood (200 μl) was collected by eye bleed, and plasma was assessed for vasopressin levels using an ELISA kit (Assay Designs, Ann Arbor, MI), according to the manufacturer's instructions.

Salt deprivation. NSE-AT$_{1A}$ transgenic (n = 15) and control littermates (n = 16) were housed separately and maintained on low-salt diet (Harlan TD90228, with a 0.01–0.02% Na^+ content) for 10 days. Meanwhile, animals were allowed free access to water and 0.3 M NaCl solution from the drinking burettes. The position of the burettes was alternated daily. In addition to fluid intake, body weight and food intake were measured daily, as described above. On *day 10*, the salt solution was removed, and mice were injected with furosemide (1 mg sc). After 48-h salt deprivation, the NaCl solution was reintroduced, and parameters recorded every 30 min during 4 h and then daily for 2 additional days. In a separate group, mice (n = 9) were killed 24 h after furosemide injection, and brains processed for c-Fos immunostaining.

Immunohistochemistry. Mice (n = 3 per group) were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffer, as described previously (23). Free-floating sections (30 μm, coronal) from each transgenic and nontransgenic line were incubated in 10% normal goat serum (Sigma, St. Louis, MO) for 1 h and then in 0.5% blocking reagent (NEN Life Science Products) for 30 min at room temperature. Sections were then incubated at 4°C with the primary antibody; rat AT$_{1A}$-receptor antibody (gift of Dr. M. McKinley, Howard Florey Institute, Australia; 1:5,000 dilution, 48 h) or c-Fos antibody (Santa Cruz Biotechnologies; 1:500 dilution, overnight); followed by biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories) and streptavidin-horseradish peroxidase (1:100, NEN Life Science Products) for 1 h each.

For AT$_{1A}$-receptor immunohistochemistry, this was followed by a 10-min amplification of biotinyl tyramide (1:100 NEN Life Science Products) and 1 h incubation in fluorescein-avidin D (1:100, Vector Laboratories). In addition, sections were incubated overnight with a mouse anti-MAP-2 monoclonal antibody (1:500, Sigma), followed by 2 h in rhodamine-conjugated goat anti-mouse antibody (1:200, Sigma).

For c-Fos immunohistochemical staining, visualization of the avidin-biotinylated-horseradish peroxidase complex was performed with a diaminobenzidine/H_2O_2 reaction, as described previously (48).

Sections were mounted with buffered glycerol (Molecular Probes, Eugene, OR). AT$_{1A}$ and MAP-2 immunostainings were analyzed using fluorescence confocal microscopy (Zeiss LSM 510). Sections were scanned using an Argon laser-emitting light at 488 nm for visualization of fluorescein and 570 nm for rhodamine. c-Fos immunostaining was analyzed using light microscopy (Nikon OptiPhot), and dark grains were counted inside a standard area (100 × 100 pixels). Images were obtained using ×10 and ×20 magnifications.

Statistics. Data are expressed as means ± SE. Data were analyzed by Student's t-test, or ANOVA (following Bartlett's test of homogeneity of variance) followed by Newman-Keuls correction for multiple comparisons between means or a Dunnett's multiple-comparison test when appropriate. Statistical comparisons were performed using Prism (version 3.0) software package (GraphPad Software, San Diego, CA).

RESULTS

Water intake. Increased baseline water consumption was observed in NSE-AT$_{1A}$ mice from both sexes compared with nontransgenic littermates (Fig. 1*A*, $P < 0.05$). While a typical enhanced baseline water intake was observed in control females vs. males, no sex differences were noticed in NSE-AT$_{1A}$

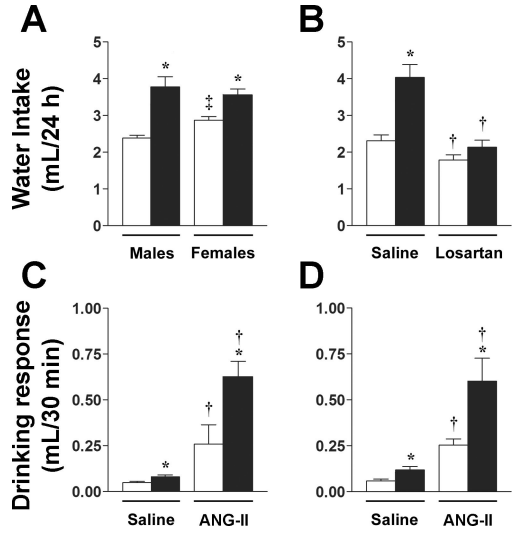

Fig. 1. Enhanced water intake in neuron-specific enolase (NSE)-angiotensin II (ANG II) receptor type 1A (AT$_{1A}$) transgenic mice. Basal water intake was measured for 24 h in nontransgenic (open bars, 15 males and 13 females) and NSE-AT$_{1A}$ transgenic mice (solid bars, 9 males and 9 females) with free access to water (*A*) and following chronic intracerebroventricular infusion with saline or losartan (n = 6 per group) (*B*). Induced drinking was recorded during 30 min following intracerebroventricular administration of saline (200 nl) or ANG II (200 ng) in both males (*C*) and females (*D*) from nontransgenic (open bars, 11 males and 12 females) and NSE-AT$_{1A}$ transgenic mice (solid bars, 10 males and 7 females). Values are means ± SE. Statistical comparisons were made using Student's t-test. $P < 0.05$ vs. *nontransgenic mice, ‡males, and †saline.

mice (Fig. 1*A*). Additional groups of mice receiving chronic infusion of losartan ICV during 3 days show that central AT_1 antagonist administration reduces the daily water intake in male (Fig. 1*B*) and female (1.5 ± 0.3 ml/day) NSE-AT_{1A} mice to a similar level seen in nontransgenic littermates (Fig. 1*B*, $P > 0.05$).

Following ICV saline, a small drinking response was observed in all groups (Fig. 1, *C* and *D*), and this dipsogenic response was significantly increased in transgenic mice vs. controls (Fig. 1, *C* and *D*, $P < 0.05$). In nontransgenics, ANG II induced a typical dipsogenic effect, but the magnitude of this response was dramatically increased in NSE-AT_{1A} mice (Fig. 1, *C* and *D*, $P < 0.05$) without significant differences between males and females ($P > 0.05$). This drinking response was prevented by ICV pretreatment with losartan (data not shown).

Vasopressin and electrolytes. Diuresis, plasma, and urine vasopressin levels were not significantly different among the groups (Table 1, $P > 0.05$). In addition, electrolyte concentrations in urine were similar between the different groups (Table 1), with the exception of the male transgenics exhibiting increased osmolality.

Salt deprivation. Low-salt diet was well accepted by all mice with no significant change in body weight (Table 2). Under low-sodium diet and before any sign of sodium depletion (first week), NaCl solution intake in mice remained lower than water intake (Fig. 2, *A* and *C*, and Table 2). In addition, females ingested significantly more salt and water than males (Fig. 2, *A* and *C*). In NSE-AT_{1A} mice, NaCl intake was significantly greater than in control littermates, representing, after 10 days, ~30% of the total daily fluid intake in male NSE-AT_{1A} mice compared with 15% in nontransgenic mice (Table 3). Water and salt intake under both diets indicates that male NSE-AT_{1A} have changed the source of their fluid intake by reducing the amount of water ingested, preferring the NaCl solution. Female NSE-AT_{1A} maintained the same water intake, in addition to a significant volume of NaCl solution (Table 3). Administration of a single injection of furosemide (1 mg sc) followed by removal of the NaCl solution induced a dramatic diminution in food intake and an increase in water intake (Table 2). After 48 h of sodium depletion, NaCl was reintroduced, resulting in a significant increase in sodium in both transgenic and control mice ($P < 0.05$), compared with predepletion values (Fig. 2, *A* and *C*). Four hours after reintroduction of NaCl, NSE-AT_{1A} mice exhibit an enhanced sodium intake compared with control animals (Fig. 2, *B* and *D*, $P < 0.05$). Again, this salt intake was accompanied by a reduction in water intake in male NSE-AT_{1A} (even below the control mice baseline values), while females maintained their water intake in addition to an increase in NaCl intake (Fig. 2, *B* and *D*).

Immunohistochemistry. AT_{1A}-receptor expression was investigated in the sensory circumventricular organs (CVO) of

Table 1. *Vasopressin and electrolytes concentrations*

	Nontransgenic	NSE-AT_{1A}
Diuresis, ml/24 h		
Males	2.8±0.2 (11)	2.6±0.2 (7)
Females	2.9±0.2 (10)	2.6±0.2 (12)
Vasopressin, pg/ml		
Plasma	97±15 (9)	86±8 (7)
Urine	114±20 (13)	120±19 (12)
Osmolality, mosmol/kgH_2O		
Males	1,461±78 (12)	2,169±167* (11)
Females	1,498±116 (12)	1,523±87 (15)
Sodium, mmol/24 h		
Males	0.43±0.02 (12)	0.52±0.06 (9)
Females	0.41±0.04 (16)	0.39±0.03 (13)
Potassium, mmol/24 h		
Males	0.66±0.3 (12)	0.81±0.06 (9)
Females	0.67±0.07 (16)	0.59±0.04 (13)

Values are expressed as mean ± SE; $n = 36$ nontransgenic and $n = 30$ neuron-specific enolase (NSE)-angiotensin II receptor type 1A (AT_{1A}) mice. Nos. in parentheses are n values. *$P < 0.05$ vs. nontransgenic mice.

Table 2. *Food and water intakes during low-salt diet*

	Nontransgenic		NSE-AT_{1A}	
	Males	Females	Males	Females
n	6	10	7	8
Food intake, g/24 h				
Before low-salt diet	3.0±0.2	3.0±0.1	3.1±0.2	3.3±0.2
Before furosemide	3.6±0.1	3.9±0.1	3.8±0.1	3.6±0.1*
24 h after furosemide	2.6±0.1	3±0.1	3.2±0.3	3.4±0.2
48 h after furosemide	2.7±0.2	3.6±0.2	3.4±0.3	3.9±0.2
Body weight, g				
Before low-salt diet	33.5±2.3	27.8±0.7	34.7±0.6	26.2±0.7
Before furosemide	33.6±2.3	27.8±0.7	34.5±0.7	26.2±0.7
24 h after furosemide	33.4±2.3	27.8±0.7	34.4±0.7	26.1±0.7
48 h after furosemide	33.5±2.3	27.8±0.7	34.4±0.7	26.2±0.7
Water intake, ml/24 h				
Before low-salt diet	2.4±0.1	2.9±0.1	3.8±0.3*	3.6±0.2*
Before furosemide	2.7±0.1	2.7±0.1	2.5±0.1	3.4±0.1*
24 h after furosemide	3.9±0.2	4.9±0.3	4.4±0.3	4.5±0.2
48 h after furosemide	3.1±0.2	4.1±0.3	3.5±0.2	4.5±0.2

Values are means ± SE; n, no. of mice. Weight gain is relative to the weight before introduction to low-salt diet. Statistical significance: *$P < 0.05$ vs. nontransgenic mice.

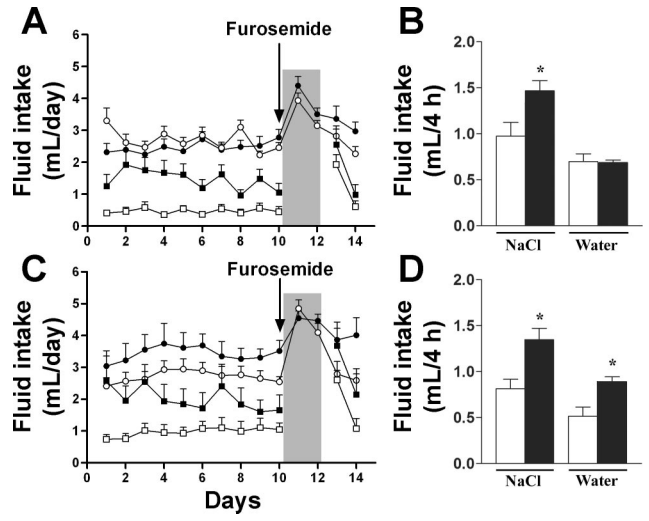

Fig. 2. Enhanced salt intake in NSE-AT_{1A} transgenic mice. Male (*A* and *B*) and female (*C* and *D*) NSE-AT_{1A} transgenic (7 males and 8 females) and control littermates (6 males and 10 females) were fed with low-salt diet for 10 days with ad libitum access to water and NaCl (0.3 M). On *day 10*, mice were injected with furosemide (1 mg sc), and salt solution was removed (shaded area) until *day 12*. *A* and *C*: salt (squares) and water (circles) intakes were measured daily in control (open symbols) and NSE-AT_{1A} transgenic (solid symbols) mice before and after salt deprivation. *B* and *D*: following NaCl reintroduction (*day 12*), water and NaCl intakes were measured for 4 h in nontransgenic (open bars) and NSE-AT_{1A} transgenic (solid bars) mice. Values are means ± SE. Statistical significances vs. nontransgenic mice: *$P < 0.05$.

Table 3. *Fluid intake in NSE-AT$_{1A}$ mice*

		Normal Diet		Low-Salt Diet	
	n	Water	Salt	Water	Salt
Nontransgenic					
Males	6	2.39±0.07	N/A	2.46±0.16	0.45±0.16
Females	10	2.87±0.1†	N/A	2.55±0.16	1.19±0.24†
NSE-AT$_{1A}$					
Males	6	3.78±0.3*	N/A	2.78±0.25	1.06±0.28*
Females	10	3.56±0.2*	N/A	3.52±0.33†	1.66±0.47

Values are means ± SE; *n*, no. of mice. Water and NaCl (0.3 M) intakes (ml) were measured under both normal and low-salt (after 10 days) diets. *$P < 0.05$ vs. nontransgenic mice, †$P < 0.05$ vs. males.

the different mice. Figure 3 gives an example of the AT$_{1A}$-receptor staining observed in the SFO, showing increased immunolabeling in NSE-AT$_{1A}$ mice (Fig. 3*A*) compared with nontransgenic controls (Fig. 3*B*). In additional experiments, performed in transgenic mice, double labeling using the AT$_{1A}$-receptor antibody (Fig. 3*C*) and the neuronal marker, MAP-2 (Fig. 3*D*) confirmed the overexpression of AT$_{1A}$ receptors on neurons (Fig. 3*E*). Similar observations were previously made in the organum vasculosum laminae terminalis (OVLT) and the area postrema (AP) (23). In addition, the median preoptic area (MnPO), a brain region with a blood-brain barrier, and one that is interposed between the SFO and the OVLT also overexpressed AT$_{1A}$ receptors (23).

c-Fos expression, a marker of neuronal activation, was examined in the CVOs after sodium depletion. Figure 4 shows representative photomicrographs of c-Fos staining in the CVOs of male mice. The major areas exhibiting enhancement of c-Fos immunostaining are the OVLT (Fig. 4, *A* and *D*), the MnPO (Fig. 4, *B* and *E*), and, to a lower extent, the SFO (Fig. 4, *C* and *F*), with increased staining in NSE-AT$_{1A}$ mice compared with controls ($P < 0.05$). Other areas like the PVN and the lateral hypothalamus also exhibited increased c-Fos staining in transgenic animals, and similar results were observed in female NSE-AT$_{1A}$ (data not shown). In addition, the central nucleus of the amygdala and the bed nucleus of stria terminalis have been shown to be involved in mineralocorticoid- and steroid-induced salt appetite (20); however, under our experimental conditions, c-Fos immunostaining was not increased in these areas. In nontransgenic and NSE-AT$_{1A}$ mice, none of these regions displayed c-Fos immunoreactivity before salt depletion. Similarly, mice submitted to the same protocol but without sodium depletion exhibited none to very low neuronal activation (data not shown).

DISCUSSION

To better understand the role of the brain RAS in the regulation of cardiovascular function and body fluid homeostasis, we previously generated a new transgenic mouse model harboring widespread expression of the rat AT$_{1A}$ receptor throughout the central nervous system (23). The results of the present study demonstrate that brain-selective overexpression of AT$_{1A}$ receptors in NSE-AT$_{1A}$ mice lead to enhanced water and sodium intake, mediated by increased neuronal activation in brain regions involved in the neural control of thirst and salt appetite.

NSE-AT$_{1A}$ mice exhibit a high level of rat AT$_{1A}$ receptor, in addition to endogenous receptors, in areas controlling the regulation of thirst and salt appetite, like the SFO, the OVLT, and the MnPO, but also in other areas implicated in body fluid and cardiovascular regulation, like the PVN, the supraoptic nucleus (SON), and the AP (23). As in other models with upregulation of central AT$_1$ receptors, like the spontaneously hypertensive rats (17, 37, 51) and the obese Zucker rat (4, 8), spontaneous water intake is increased (up to 50%) in NSE-AT$_{1A}$. Water deprivation in rats has been shown to induce AT$_{1A}$ mRNA and AT$_{1A}$ receptor overexpression in the anterior pituitary and the SFO (2, 34), suggesting the importance of these receptors in thirst regulation. Furthermore, in transgenic models with neuron- and glia-targeted expression of RAS

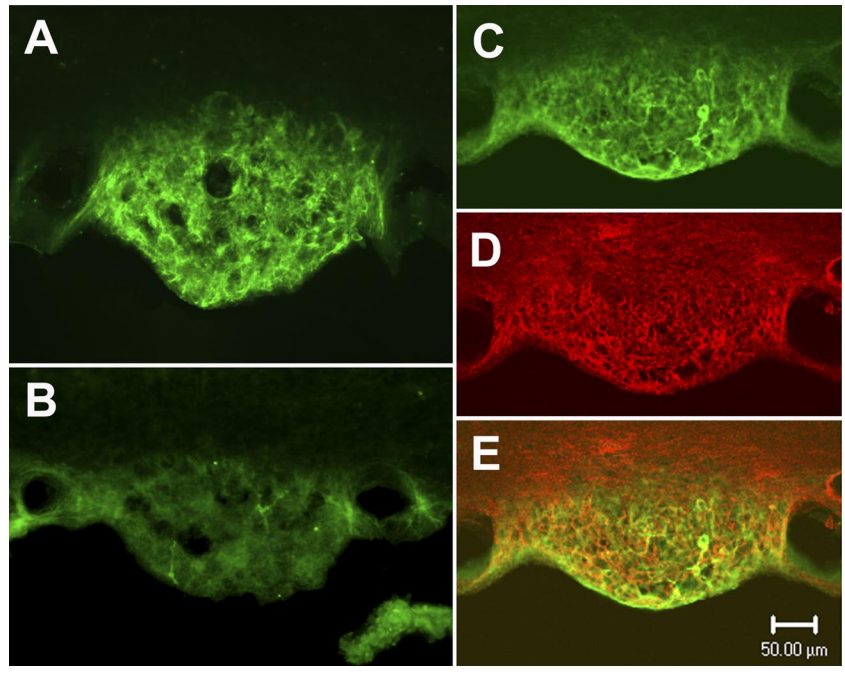

Fig. 3. Enhanced AT$_{1A}$ immunostaining in the subfornical organ (SFO) of NSE-AT$_{1A}$ transgenic mice. Confocal images of the SFO in NSE-AT$_{1A}$ transgenic mice (*A*) and nontransgenic controls (*B*) shows immunostaining for AT$_{1A}$ receptors. In additional experiments, sections from transgenic mice were double-stained for AT$_{1A}$ receptors (*C*) and MAP-2 antibody (*D*). *E*: the merged picture confirms the overexpression of AT$_{1A}$ receptors on neurons.

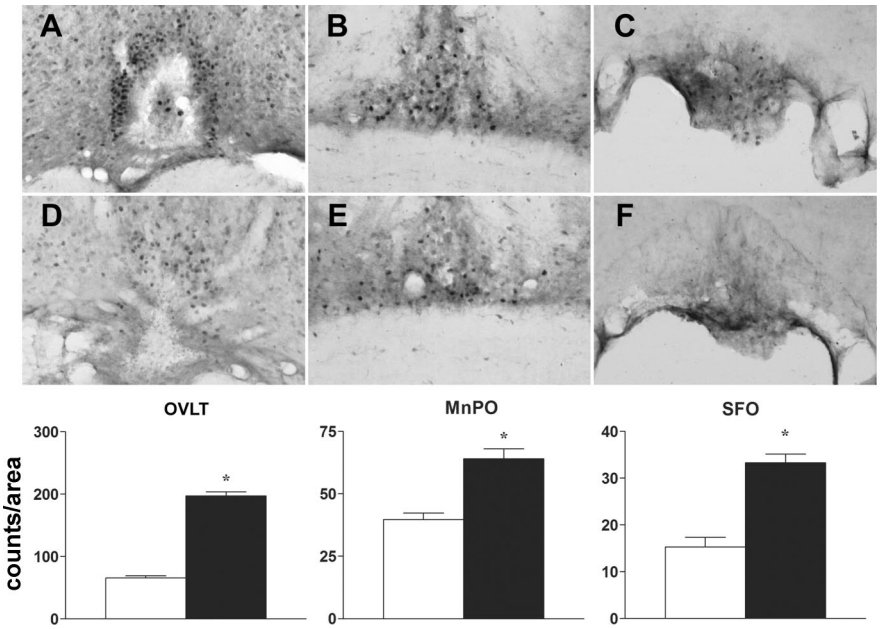

Fig. 4. Salt depletion induces enhanced c-Fos immunostaining of the circumventricular organs in NSE-AT$_{1A}$ transgenic mice. After being maintained on low-salt diet for 10 days, male NSE-AT$_{1A}$ transgenic mice (*A–C*) and nontransgenic littermates (*D–F*) were injected with furosemide (1 mg sc) and deprived of NaCl solution. Following 24 h of salt depletion, animals were killed, and their brain processed for c-Fos immunohistochemistry, as described in MATERIAL AND METHODS. NSE-AT$_{1A}$ mice exhibit enhanced c-Fos staining in the organum vasculosum laminae terminalis (OVLT; *A*), median preoptic area (MnPO; *B*), and SFO (*C*), compared with controls (*D–F*, respectively). Values are means ± SE. Statistical significances: $*P < 0.05$ vs. nontransgenics (open bars).

genes, overactivation of the brain RAS resulted in increased drinking and salt appetite (31), suggesting an involvement of increased ANG II production in areas such as the SFO, OVLT, and MnPO. Although the AT$_{1A}$ receptor appears to mediate thirst and salt appetite, our group also reported the participation of the AT$_{1B}$ subtype (13).

In mice, but also in other species, females drink more than males due to the influence of sexual hormones and eventually to meet the needs relative to gestation (15). In our study, nontransgenic females exhibit an increased spontaneous water intake vs. males, but this sex difference disappeared in NSE-AT$_{1A}$ mice; the fold increase in water intake in transgenic females is actually lower than that in transgenic males. Jonklaas and Buggy (22) reported similar sex-related alterations of the drinking response following ICV estrogen administration before ANG II and were able to localize the site of action of these sexual hormones in the medial preoptic area, which is also rich in AT$_{1A}$ receptors (21, 26). This observation suggests that female NSE-AT$_{1A}$ could be more sensitive to hormones or neuromediators inhibiting water intake, like estrogen (40) and serotonin (29). Another possibility would be that the systems are saturated, having reached a maximal stimulation of all AT$_{1A}$ receptors and preventing further water intake. However, this hypothesis is ruled out by the ANG II-induced water intake experiments, showing that baseline drinking in NSE-AT$_{1A}$ mice can still be increased in both males and females.

The antidiuretic hormone, vasopressin, is synthesized in the PVN and SON and stored in vesicles in the posterior pituitary from where it can be released in the circulation in response to hemorrhage, water deprivation, and osmotic stimuli. Both PVN and SON exhibit a high concentration of AT$_1$ receptors (1, 42), and utilization of AT$_1$ receptor antagonists (11) and AT$_1$ antisense oligonucleotides (30) suggests that vasopressin release is under control of these receptors. In addition to endogenous receptors, NSE-AT$_{1A}$ mice exhibit a high level of AT$_1$ receptors in the PVN and SON, which led us to hypothesize that our transgenic mice may exhibit increased levels of vasopressin. Hormone levels, assayed in urine and plasma, were not different between transgenic mice and control littermates, suggesting that vasopressin secretion may be normal in NSE-AT$_{1A}$ mice. Moreover, in situ hybridization shows that PVN and SON arginine vasopressin mRNA levels are not different between nontransgenic and NSE-AT$_{1A}$ mice (unpublished observations). Furthermore, despite an increase in water intake in NSE-AT$_{1A}$ mice, diuresis was not different between strains. Taken together, these observations suggest that, in NSE-AT$_{1A}$ mice, in basal conditions, vasopressin levels are not altered by increased AT$_{1A}$-receptor level and that additional mechanisms may participate in the elimination of water. Among them, sweating, salivation, and lacrimation have been reported in mice and are believed to be modulated by the RAS (18, 46, 47).

A large range of experiments involving special diets (high and low salt), pharmacological manipulations of receptors (peripheral and central AT$_1$ receptors), electrolytic lesions (anteroventral third ventricle), and immunohistochemistry markers (c-Fos) demonstrated that central areas involved in maintenance of sodium homeostasis are, like for water intake, associated with the lamina terminalis and are under control of ANG II receptors located in the CVOs (15, 20). In support of these observations, NSE-AT$_{1A}$ mice displayed a two- to three-fold increase in NaCl intake on the first day of low-sodium diet, suggesting that these mice may behave like sodium-depleted animals. Following furosemide treatment, leading to a massive loss of salt and water, c-Fos immunohistochemistry revealed an increase in neuronal activation in the core of the SFO, the periphery of the OVLT, and the MnPO of the transgenic mice. The areas involved and the distribution of staining are in accordance with previous reports (48, 50). In NSE-AT$_{1A}$ mice, c-Fos immunostaining was particularly dense in the periphery of the OVLT and to a lower level in the SFO. In response to sodium depletion, the juxtaglomerular cells in the kidney secrete renin, which, in turn, will lead to the formation of ANG II. The peptide then enters the brain via the CVOs and stimulates AT$_1$ receptors in the SFO to activate forebrain nuclei in the lamina terminalis. In parallel, stimula-

tion of osmoreceptors located in the CVOs (SFO and AP) can activate the brain RAS, leading to local ANG II production, which, in turn, by acting locally, will promote sodium and water intake.

Although sodium-depletion-induced increases in circulating ANG II levels have been reported to trigger neuron activation in the OVLT and MnPO (15, 20), a controversy still exists concerning the role of the SFO. Our study shows that there is some activation, although small, in the SFO, which could relay signaling to downstream nuclei. Indeed, other areas, such as the PVN (9), SON (38), as well as brain stem structures like the AP (35) and the NTS (44), have been reported to play a role in salt appetite.

NSE-AT_{1A} mice provide an interesting tool to address several hypotheses regarding the dissociation between water intake, sodium appetite, and increased BP. Recent studies suggested that different intracellular pathways, downstream of the AT_1 receptor, are mediating water and salt intake, with thirst resulting from the activation of protein kinase C, while salt appetite would involve a MAP kinase pathway (12). It is interesting to note that, in NSE-AT_{1A} mice, while basal water and salt intakes are increased, BP remains unaffected. Possible explanations for this observation could be that the neuroanatomical processes involving water and salt intake are taking place mostly in the lamina terminalis, which has little influence over BP control. This hypothesis is supported by our observation of a low neuronal activation in the SFO of NSE-AT_{1A} mice, a pivotal region for BP regulation (45). Other hypotheses suggest that BP is regulated by a larger number of neuroendocrine systems and/or that the activation threshold is higher in neurons involved in BP control. Clearly, additional experiments are needed to address these hypotheses.

One concern about NSE-AT_{1A} mice is that they potentially express AT_{1A} receptors on neurons usually devoid of these receptors. A study investigating the expression of AT_1 receptors during furosemide-induced sodium deficit (10) reported that two mechanisms compensate for sodium depletion: *1*) an increase in AT_1-receptor turnover on neurons, and *2*) activation of AT_1 genes in neurons that usually do not express those receptors. This observation suggests that, under certain conditions, neurons normally devoid of AT_1 receptors express those receptors in response to a physiological need. In addition, neural plasticity triggered by salt craving was recently observed in brain regions not involved in body fluid homeostasis (33). The physiological significance of our model is apparent from these studies, demonstrating that changes in dietary NaCl can alter AT_1 expression in multiple brain regions. Using autoradiography, we estimated that, in the PVN, the level of AT_1 receptors in NSE-AT_{1A} mice is ~40-fold higher than in control littermates (unpublished data). Similarly, chronic heart failure has been shown to increase AT_1 receptor expression by 85% in rabbits (16), and similar increases have been observed in rats following 5 days of water deprivation (43). However, in hypertensive rats, the level of AT_1 receptor upregulation was reported to be fourfold compared with that of normotensive Wistar-Kyoto rats (39). Although the AT_1-receptor level of expression in our model appears to be significantly higher than in these models, comparison is difficult due to the lack of data in mice and to the different species sensitivity to ANG II. Consequently, NSE-AT_{1A} mice provide an interesting new model to study the activation of brain AT_1 receptors under conditions of disrupted body fluid and cardiovascular homeostasis.

In conclusion, our results suggest that activation of central AT_{1A} receptors by endogenous ANG II in NSE-AT_{1A} mice lead to chronic water intake and salt appetite similar to those observed after water deprivation. In addition, overexpression of AT_{1A} receptors in this model provides a new tool for studying the mechanisms of brain AT_1-dependent water and salt intake behaviors.

Perspectives and Significance

The involvement of AT_{1A} receptors in mediating the water intake and salt appetite behaviors has been well documented in various species. Similarly, the signaling mechanisms downstream of this ANG II receptor have been fairly well characterized in vitro. However, although recent studies started to investigate the participation of specific pathways (activated protein kinase C, MAP kinases, etc.) in ANG II-mediated behavioral responses, our understanding of these mechanisms is almost inexistent. NSE-AT_{1A} mice in which baseline water intake and salt appetite are reinforced provide an ideal tool to study these different intracellular pathways in vivo.

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REFERENCES

1. **Allen AM, Zhuo J, Mendelsohn FAO.** Localization and function of angiotensin AT1 receptors. *Am J Hypertens* 13: 31S–38S, 2000.
2. **Barth SW, Gerstberger R.** Differential regulation of angiotensinogen and AT1A receptor mRNA within the rat subfornical organ during dehydration. *Brain Res Mol Brain Res* 64: 151–164, 1999.
3. **Blair-West JR, Burns P, Denton DA, Ferraro T, McBurnie MI, Tarjan E, Weisinger RS.** Thirst induced by increasing brain sodium concentration is mediated by brain angiotensin. *Brain Res* 637: 335–338, 1994.
4. **Bokil HS, Porter JP.** Brain angiotensin type 1 receptor expression and function in the Zucker obese rat. *Neurosci Lett* 281: 139–142, 2000.
5. **Bourque CW, Oliet SH, Richard D.** Osmoreceptors, osmoreception, osmoregulation. *Front Neuroendocrinol* 15: 231–274, 1994.
6. **Bourque CW, Voisin DL, Chakfe Y.** Stretch-inactivated cation channels: cellular targets for modulation of osmosensitivity in supraoptic neurons. *Prog Brain Res* 139: 85–94, 2002.
7. **Brefel C, Lazartigues E, Tran MA, Gauquelin G, Geelen G, Gharib C, Montastruc JL, Montastruc P, Rascol O.** Central cardiovascular effects of acetylcholine in the conscious dog. *Br J Pharmacol* 116: 2175–2182, 1995.
8. **Brock JW, Truett GE, Ross KD, Kloster CA.** Quantitative analysis of abnormal spontaneous behavior and clinical assessment of the stargazer rat. *Lab Anim Sci* 45: 276–280, 1995.

9. **Camargo LA, Saad WA.** Role of the alpha(1)- and alpha(2)-adrenoceptors of the paraventricular nucleus on the water and salt intake, renal excretion, and arterial pressure induced by angiotensin II injection into the medial septal area. *Brain Res Bull* 54: 595–602, 2001.
10. **Charron G, Laforest S, Gagnon C, Drolet G, Mouginot D.** Acute sodium deficit triggers plasticity of the brain angiotensin type 1 receptors. *FASEB J* 16: 610–612, 2002.
11. **Culman J, von Heyer C, Piepenburg B, Rascher W, Unger T.** Effects of systemic treatment with irbesartan and losartan on central responses to angiotensin II in conscious, normotensive rats. *Eur J Pharmacol* 367: 255–265, 1999.
12. **Daniels D, Yee DK, Fluharty SJ.** Hydromineral neuroendocrinology: angiotensin II receptor signalling. *Exp Physiol* 92: 523–527, 2007.
13. **Davisson RL, Oliverio MI, Coffman TM, Sigmund CD.** Divergent functions of angiotensin II receptor isoforms in the brain. *J Clin Invest* 106: 103–106, 2000.
14. **DiNicolantonio R, Hutchinson R, Mendelsohn FA.** Exaggerated salt appetite of spontaneously hypertensive rats is decreased by central angiotensin-converting enzyme blockade. *Nature* 298: 846–848, 1982.
15. **Fitzsimons JT.** Angiotensin, thirst, and sodium appetite. *Physiol Rev* 78: 583–686, 1998.
16. **Gao L, Wang W, Li YL, Schultz HD, Liu D, Cornish KG, Zucker IH.** Sympathoexcitation by central ANG II: roles for AT$_1$ receptor upregulation and NAD(P)H oxidase in the RVLM. *Am J Physiol Heart Circ Physiol* 288: H2271–H2279, 2005.
17. **Gutkind JS, Kurihara M, Castren E, Saavedra JM.** Increased concentration of angiotensin II binding sites in selected brain areas of spontaneously hypertensive rats. *J Hypertens* 6: 79–84, 1988.
18. **Imai K, Hayashi Y, Hashimoto K.** Acute toxicological studies of captopril in rats and mice. *J Toxicol Sci* 6: 179–188, 1981.
19. **Imanishi K, Nonoguchi H, Nakayama Y, Machida K, Ikebe M, Tomita K.** Type 1A angiotensin II receptor is regulated differently in proximal and distal nephron segments. *Hypertens Res* 26: 405–411, 2003.
20. **Johnson AK, Thunhorst RL.** The neuroendocrinology of thirst and salt appetite: visceral sensory signals and mechanisms of central integration. *Front Neuroendocrinol* 18: 292–353, 1997.
21. **Johren O, Inagami T, Saavedra JM.** AT1A, AT1B, and AT2 angiotensin II receptor subtype gene expression in rat brain. *Neuroreport* 6: 2549–2552, 1995.
22. **Jonklaas J, Buggy J.** Angiotensin-estrogen central interaction: localization and mechanism. *Brain Res* 326: 239–249, 1985.
23. **Lazartigues E, Dunlay SM, Loihl AK, Sinnayah P, Lang JA, Espelund JJ, Sigmund CD, Davisson RL.** Brain-selective overexpression of angiotensin (AT1) receptors causes enhanced cardiovascular sensitivity in transgenic mice. *Circ Res* 90: 617–624, 2002.
24. **Lazartigues E, Lawrence AJ, Lamb FS, Davisson RL.** Renovascular hypertension in mice with brain-selective overexpression of AT1a receptors is buffered by increased nitric oxide production in the periphery. *Circ Res* 95: 523–531, 2004.
25. **Lazartigues E, Zimmerman MC, Sinnayah P, Dunlay RP, Davisson RL.** Importance of circumventricular organs in enhanced cardiovascular sensitivity observed in a transgenic model with brain-selective expression of exogenous angiotensin (AT1) receptors (Abstract). *FASEB J* 15: A786, 2001.
26. **Lenkei Z, Corvol P, Llorens-Cortes C.** The angiotensin receptor subtype AT1A predominates in rat forebrain areas involved in blood pressure, body fluid homeostasis and neuroendocrine control. *Brain Res* 30: 53–60, 1995.
27. **Liedtke W, Friedman JM.** Abnormal osmotic regulation in trpv4-/- mice. *Proc Natl Acad Sci USA* 100: 13698–13703, 2003.
28. **Liu L, Leonard AS, Motto DG, Feller MA, Price MP, Johnson WA, Welsh MJ.** Contribution of Drosophila DEG/ENaC genes to salt taste. *Neuron* 39: 133–146, 2003.
29. **Menani JV, Colombari DS, Beltz TG, Thunhorst RL, Johnson AK.** Salt appetite: interaction of forebrain angiotensinergic and hindbrain serotonergic mechanisms. *Brain Res* 801: 29–35, 1998.
30. **Meng H, Wielbo D, Gyurko R, Phillips MI.** Antisense oligonucleotide to AT1 receptor mRNA inhibits central angiotensin induced thirst and vasopressin. *Regul Pept* 54: 543–551, 1994.
31. **Morimoto S, Cassell MD, Sigmund CD.** Glial- and neuronal-specific expression of the renin-angiotensin system in brain alters blood pressure, water intake, and salt preference. *J Biol Chem* 277: 33235–33241, 2002.
32. **Morris M, Li P, Callahan MF, Oliverio MI, Coffman TM, Bosch SM, Diz DI.** Neuroendocrine effects of dehydration in mice lacking the angiotensin AT1a receptor. *Hypertension* 33: 482–486, 1999.
33. **Morris MJ, Na ES, Johnson AK.** Salt craving: the psychobiology of pathogenic sodium intake. *Physiol Behav* 94: 709–721, 2008.
34. **Nazarali AJ, Gutkind JS, Saavedra JM.** Regulation of angiotensin II binding sites in the subfornical organ and other rat brain nuclei after water deprivation. *Cell Mol Neurobiol* 7: 447–455, 1987.
35. **Osborn JW, Collister JP, Carlson SH.** Angiotensin and osmoreceptor inputs to the area postrema: role in long-term control of fluid homeostasis and arterial pressure. *Clin Exp Pharmacol Physiol* 27: 443–449, 2000.
36. **Osborn JW, Fink GD, Sved AF, Toney GM, Raizada MK.** Circulating angiotensin II and dietary salt: converging signals for neurogenic hypertension. *Curr Hypertens Rep* 9: 228–235, 2007.
37. **Perez-Delgado MM, Carmona-Calero E, Marrero-Gordillo N, Perez-Gonzalez H, Castaneyra-Perdomo A.** Effect of hypertension on the angiotensin II fibres arriving at the posterior lobe of the hypophysis of the rat. An immunohistochemical study. *Histol Histopathol* 15: 73–77, 2000.
38. **Pietranera L, Saravia FE, McEwen BS, Lucas LL, Johnson AK, De Nicola AF.** Changes in Fos expression in various brain regions during deoxycorticosterone acetate treatment: relation to salt appetite, vasopressin mRNA and the mineralocorticoid receptor. *Neuroendocrinology* 74: 396–406, 2001.
39. **Reja V, Goodchild AK, Phillips JK, Pilowsky PM.** Upregulation of angiotensin AT1 receptor and intracellular kinase gene expression in hypertensive rats. *Clin Exp Pharmacol Physiol* 33: 690–695, 2006.
40. **Rosas-Arellano MP, Solano-Flores LP, Ciriello J.** Co-localization of estrogen and angiotensin receptors within subfornical organ neurons. *Brain Res* 837: 254–262, 1999.
41. **Sakai RR, Chow SY, Epstein AN.** Peripheral angiotensin II is not the cause of sodium appetite in the rat. *Appetite* 15: 161–170, 1990.
42. **Sakai RR, Ma LY, He PF, Fluharty SJ.** Intracerebroventricular administration of angiotensin type 1 (AT1) receptor antisense oligonucleotides attenuate thirst in the rat. *Regul Pept* 59: 183–192, 1995.
43. **Sanvitto GL, Johren O, Hauser W, Saavedra JM.** Water deprivation upregulates ANG II AT1 binding and mRNA in rat subfornical organ and anterior pituitary. *Am J Physiol Endocrinol Metab* 273: E156–E163, 1997.
44. **Schreihofer AM, Anderson BK, Schiltz JC, Xu L, Sved AF, Stricker EM.** Thirst and salt appetite elicited by hypovolemia in rats with chronic lesions of the nucleus of the solitary tract. *Am J Physiol Regul Integr Comp Physiol* 276: R251–R258, 1999.
45. **Sinnayah P, Lazartigues E, Sakai K, Sharma RV, Sigmund CD, Davisson RL.** Genetic ablation of angiotensinogen in the subfornical organ of the brain prevents the central angiotensinergic pressor response. *Circ Res* 99: 1125–1131, 2006.
46. **Takeda H, Kondo S.** Immunohistochemical study of angiotensin receptors in normal human sweat glands and eccrine poroma. *Br J Dermatol* 144: 1189–1192, 2001.
47. **Vilches JJ, Ceballos D, Verdu E, Navarro X.** Changes in mouse sudomotor function and sweat gland innervation with ageing. *Auton Neurosci* 95: 80–87, 2002.
48. **Vivas L, Pastuskovas CV, Tonelli L.** Sodium depletion induces Fos immunoreactivity in circumventricular organs of the lamina terminalis. *Brain Res* 679: 34–41, 1995.
49. **Watanabe E, Fujikawa A, Matsunaga H, Yasoshima Y, Sako N, Yamamoto T, Saegusa C, Noda M.** Nav2/NaG channel is involved in control of salt-intake behavior in the CNS. *J Neurosci* 20: 7743–7751, 2000.
50. **Weisinger RS, Burns P, Colvill LM, Davern P, Giles ME, Oldfield BJ, McKinley MJ.** Fos immunoreactivity in the lamina terminalis of adrenalectomized rats and effects of angiotensin II type 1 receptor blockade or deoxycorticosterone. *Neuroscience* 98: 167–180, 2000.
51. **Wright JW, Roberts KA, Harding JW.** Drinking to intracerebroventricularly infused angiotensin II, III and IV in the SHR. *Peptides* 9: 979–984, 1988.