Enhanced water and salt intake in transgenic mice with brain-restricted overexpression of angiotensin (AT₁) receptors

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Lazariguës E, Sinnayah P, Augoyard G, Gharib C, Johnson AK, Davison RL. Enhanced water and salt intake in transgenic mice with brain-restricted overexpression of angiotensin (AT₁) 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 (AT₁A) to blood pressure and volume homeostasis, we generated a transgenic mouse model [neuron-specific enolase (NSE)-AT₁A] with brain-restricted overexpression of AT₁A 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-AT₁A compared with nontransgenic littermates, and bolus intracerebroventricular injections of ANG II (200 ng in 200 nl) caused further enhanced water intake in NSE-AT₁A. 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-AT₁A mice compared with wild types. For immunohistochemistry, used to assess neuronal activation, demonstrated sodium depletion-enhanced activity in the anteroventral third ventricle region of the brain in NSE-AT₁A mice compared with control animals. The results show that brain-selective overexpression of AT₁A receptors results in enhanced salt appetite and altered water intake. This model provides a new tool for studying the mechanisms of brain AT₁A-dependent water and salt consumption.

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 (AT₁) 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 AT₁ 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 AT₁ receptor in various areas of the brain, including the subfornical organ (SFO) and the anterior pituitary (19). In addition, water and sodium intake can be abolished by pretreatment with selective antagonists (3, 11) and antisense oligonucleotides (42) for the AT₁ receptor. A significant role for brain AT₁ receptors in volume regulatory mechanisms has been suggested in both AT₁A- and AT₁B-receptor knockout mice (13, 32). Interestingly, increased neuronal activation and vasopressin expression were observed in the paraventricular nucleus (PVN) of AT₁A⁻/⁻ mice exposed to dehydration (32), supporting a role for the AT₁B-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 AT₁ receptors [neuron-specific enolase (NSE)-AT₁A] (23). These transgenic mice exhibit widespread overexpression of the AT₁ 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-AT₁A 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 AT₁ receptors in the brain of these mice would lead to increased water intake and salt appetite. We investigated whether NSE-AT₁A 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-AT₁A 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 AT₁ 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-AT1A 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-AT1A transgenic (n = 12) and control littersmates (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 AT1-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-AT1A transgenic (n = 30) and control littersmates (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 as described (23). Following 2–3 days recovery from surgery, mice 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 AT1-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).

**Vasopressin and electrolytes.** Urine from NSE-AT1A transgenic (n = 30) and control littersmates (n = 36) was analyzed for vasopres- sin 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-AT1A transgenic (n = 15) and control littersmates (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 furo-semide (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 (ENN Life Science Products) for 30 min at room temperature. Sections were then incubated at 4°C with the primary antibody; rat AT1A-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 AT1A-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 avii-din-biotinylated-horseradish peroxidase complex was performed with a diaminobenzidine/H2O2 reaction, as described previously (48).

Sections were mounted with buffered glycerol (Molecular Probes, Eugene, OR). AT1A 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 immuno- staining 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-AT1A mice from both sexes compared with nontransgenic littersmates (Fig. 1A, P < 0.05). While a typical enhanced baseline water intake was observed in control females vs. males, no sex differences were noticed in NSE-AT1A mice.
mice (Fig. 1A). 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. 1B) and female (1.5 ± 0.3 ml/day) NSE-AT~1~A mice to a similar level seen in nontransgenic littermates (Fig. 1B, P > 0.05).

Following ICV saline, a small drinking response was observed in all groups (Fig. 1, C and D), and this dipogenic response was significantly increased in transgenic mice vs. controls (Fig. 1, C and D, P < 0.05). In nontransgens, ANG II induced a typical dipogenic effect, but the magnitude of this response was dramatically increased in NSE-AT~1~A 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 osmolality 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 transgens 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~1~A 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~1~A mice compared with 15% in nontransgenic mice (Table 3). Water and salt intake under both diets indicates that male NSE-AT~1~A mice exhibit an enhanced sodium intake compared with control littermates, representing, after 10 days, ~30% of the total daily fluid intake in male NSE-AT~1~A mice compared with 15% in nontransgenic mice (Table 3). Water and salt intake under both diets indicates that male NSE-AT~1~A mice have changed the source of their fluid intake by reducing the amount of water ingested, preferring the NaCl solution. Female NSE-AT~1~A 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~1~A 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~1~A (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~1~A-receptor expression was investigated in the sensory circumventricular organs (CVO) of

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<th>Table 2. Food and water intakes during low-salt diet</th>
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<td><strong>Food intake, g/24 h</strong></td>
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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.

Values are expressed as mean ± SE; n = 36 nontransgenic and n = 30 neuron-specific enolase (NSE)-angiotensin II receptor type 1A (AT~1~A) mice. Nos. in parentheses are n values. *P < 0.05 vs. nontransgenic mice.
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Table 3. Fluid intake in NSE-AT1A mice

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<td>Water</td>
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<td>Nontransgenic</td>
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<td>Males</td>
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<td>2.39±0.07</td>
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<td>Females</td>
<td>10</td>
<td>2.87±0.1†</td>
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<tr>
<td>NSE-AT1A</td>
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<tr>
<td>Males</td>
<td>6</td>
<td>3.78±0.3*</td>
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<tr>
<td>Females</td>
<td>10</td>
<td>3.56±0.2*</td>
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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.

Fig. 3. Enhanced AT1A immunostaining in the subfornical organ (SFO) of NSE-AT1A transgenic mice. Confocal images of the SFO in NSE-AT1A transgenic mice (A) and nontransgenic controls (B) shows immunostaining for AT1A receptors. In additional experiments, sections from transgenic mice were double-stained for AT1A receptors (C) and MAP-2 antibody (D). E: the merged picture confirms the overexpression of AT1A receptors on neurons.

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 AT1A receptor throughout the central nervous system (23). The results of the present study demonstrate that brain-selective overexpression of AT1A receptors in NSE-AT1A 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-AT1A mice exhibit a high level of rat AT1A 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 AT1 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-AT1A. Water deprivation in rats has been shown to induce AT1A mRNA and AT1A 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

The different mice. Figure 3 gives an example of the AT1A-receptor staining observed in the SFO, showing increased immunolabeling in NSE-AT1A mice (Fig. 3A) compared with nontransgenic controls (Fig. 3B). In additional experiments, performed in transgenic mice, double labeling using the AT1A-receptor antibody (Fig. 3C) and the neuronal marker, MAP-2 (Fig. 3D) confirmed the overexpression of AT1A receptors on neurons (Fig. 3E). 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 AT1A 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-AT1A 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-AT1A (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-AT1A 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).
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 AT1A receptor appears to mediate thirst and salt appetite, our group also reported the participation of the AT1B 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-AT1A 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 AT1A receptors (21, 26). This observation suggests that female NSE-AT1A 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 AT1A 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-AT1A 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 mass. 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 AT1 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-AT1A 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 AT1 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-AT1A 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-AT1A 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-AT1A mice is that they potentially express AT1A receptors on neurons usually devoid of these receptors. A study investigating the expression of AT1 receptors during furosemide-induced sodium deficit (10) reported that two mechanisms compensate for sodium depletion: 1) an increase in AT1-receptor turnover on neurons, and 2) activation of AT1 genes in neurons that usually do not express those receptors. This observation suggests that, under certain conditions, neurons normally devoid of AT1 receptors express these 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 AT1 expression in multiple brain regions. Using autoradiography, we estimated that, in the PVN, the level of AT1 receptors in NSE-AT1A mice is ~40-fold higher than in control littermates (unpublished data). Similarly, chronic heart failure has been shown to increase AT1 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 AT1 receptor upregulation was reported to be fourfold compared with that of normotensive Wistar-Kyoto rats (39). Although the AT1-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 different species sensitivity to ANG II. Consequently, NSE-AT1A mice provide an interesting new model to study the activation of brain AT1 receptors under conditions of disrupted body fluid and cardiovascular homeostasis.

In our results, our results suggest that activation of central AT1A receptors by endogenous ANG II in NSE-AT1A mice lead to chronic water intake and salt appetite similar to those observed after water deprivation. In addition, overexpression of AT1A receptors in this model provides a new tool for studying the mechanisms of brain AT1-dependent water and salt intake behaviors.

**Perspectives and Significance**

The involvement of AT1A 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 nonexistent. NSE-AT1A 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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