Elevated Blood Pressure in Transgenic Mice With Brain-Specific Expression of Human Angiotensinogen Driven by the Glial Fibrillary Acidic Protein Promoter

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Abstract—In addition to the circulatory renin (REN)–angiotensin system (RAS), a tissue RAS having an important role in cardiovascular function also exists in the central nervous system. In the brain, angiotensinogen (AGT) is expressed in astrocytes and in some neurons important to cardiovascular control, but its functional role remains undefined. We generated a transgenic mouse encoding the human AGT (hAGT) gene under the control of the human glial fibrillary acidic protein (GFAP) promoter to experimentally dissect the role of brain versus systemically derived AGT. This promoter targets expression of transgene products to astrocytes, the most abundant cell type expressing AGT in brain. All transgenic lines exhibited hAGT mRNA expression in brain, with variable expression in other tissues. In one line examined in detail, transgene expression was high in brain and low in tissues outside the central nervous system, and the level of plasma hAGT was not elevated over baseline. In the brain, hAGT protein was mainly localized in astrocytes, but was present in neurons in the subfornical organ. Intracerebroventricular (ICV) injection of human REN (hREN) in conscious unrestrained mice elicited a pressor response, which was abolished by ICV preinjection of losartan. Double-transgenic mice expressing the hREN gene and the GFAP-hAGT transgene exhibited a 15–mm Hg increase in blood pressure and an increased preference for salt. Blood pressure in the hREN/GFAP-hAGT mice was lowered after ICV, but not intravenous losartan. These studies suggest that AGT synthesis in the brain has an important role in the regulation of blood pressure and electrolyte balance. (Circ Res. 2001;89:365-372.)

Key Words: blood pressure ■ renin-angiotensin system ■ brain ■ astrocyte ■ transgenic mouse

Angiotensin II (Ang II) has several actions on the central nervous system that may influence blood pressure (BP) regulation. These include increased sympathetic outflow and vasopressin release, as well as altered drinking and salt appetite.1 Ang II is formed by the enzymatic action of renin (REN) on angiotensinogen (AGT), followed by the action of angiotensin-converting enzyme (ACE) on angiotensin I (Ang I). Several lines of evidence point to a contribution of an overactive brain REN-angiotensin system (RAS) to the hypertensive state in experimental models such as spontaneously hypertensive rats (SHRs), DOCA-salt hypertensive rats, Dahl-salt sensitive rats, and renal hypertensive rats.2–8 For example, REN activity, Ang II, and Ang II binding are increased in the brains of SHRs.2,9–11 Acute and chronic intracerebroventricular (ICV) injection of ACE inhibitor, angiotensin antagonist, and antisense oligonucleotides to Ang II type 1 receptor (AT1) mRNA and AGT mRNA attenuates the development of hypertension in SHR.5,12,13 Overactivity of the brain RAS may play a role in the hypertension observed in a number of transgenic animal models.14,15 For example, transgenic mice overexpressing both the human REN (hREN) and human AGT (hAGT) genes are chronically hypertensive.15 ICV injection of the selective AT1 antagonist, losartan, in these mice causes a greater fall in BP than in control nontransgenic mice.16 It was hypothesized that overexpression of the brain RAS in this model caused systemic hypertension by a mechanism involving a central Ang II–dependent increase in circulating arginine vasopressin. Like the SHR model, however, this transgenic model exhibits both altered endocrine RAS (elevated circulating Ang II) and tissue RAS (elevated tissue Ang II) function, making it difficult to experimentally distinguish between them.

In the brain, AGT expression is localized mainly in astrocytes (glia).17 Glial AGT is detectable throughout the brain, with the highest expression in the hypothalamus and...
proeptic areas and moderate to high expression in the mesencephalon and myelencephalon. We have reported that transgenic mice expressing hAGT or a hAGT promoter–controlled reporter gene express the transgene in astrocytes in all regions of the brain and in selected neurons in important cardiovascular control centers.18 Neuronal expression of AGT has also been reported in the paraventricular nucleus and supraoptic nucleus in rats.19 Thus, although there is convincing evidence for primary expression of AGT mRNA and protein in the brain, its role in cardiovascular control remains unclear. Accordingly, because AGT synthesis is most abundant in astrocytes, we generated a transgenic mouse model expressing hAGT under the control of the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter to accomplish the following two specific goals: (1) to examine whether AGT synthesized in brain is able to raise BP and alter drinking and (2) to create a model of neurogenic hypertension resulting from brain-specific overexpression of the RAS.

Materials and Methods
Generation of Transgenic Mice
The transgene consists of a 2.2-kb fragment of human GFAP promoter fused to the hAGT gene. Human GFAP promoter was excised with EcoRI from the plasmid gfa2 and subcloned into pBluecript KS+ to form pGFAP-SK.20 The hAGT gene was cloned as a BglII-Nhel fragment into the BamHI-SpeI sites in pGFAP-SK to form pGFAP-hAGT.21 This fusion results in the utilization of DNA present in intron I as a 5′-untranslated region. Translation initiation starts in hAGT exon II. All cloning junctions were confirmed by sequencing. The transgene was excised by BssHI, purified by agarose gel electrophoresis, recovered using the Qia-Quick purification kit (Qiagen), and microinjected into 1-cell fertilized mouse (C57BL/6J × SJL/J) embryos.22 All transgenic mice were maintained by breeding with B6SJL F1 mice, and received standard mouse chow (LM-485; Teklad Premier Laboratory Diets) and water ad libitum. Care of the mice met the standards set forth by the NIH in their guidelines for the care and use of experimental animals. All procedures were approved by the University of Iowa Animal Care and Use Committee.

Analysis of Nucleic Acids
Genomic DNA was purified from tail biopsies and analyzed by Southern blot.21 Ten micrograms of genomic DNA was digested with EcoRI and probed with a segment of human AGT intron II. There was no cross-reactivity of this probe with mouse genomic DNA. A 9.5-kb band indicates the presence of the transgene. Polymerase chain reaction was performed using primers specific for hAGT, which amplified a 539-bp segment of exon II.23 Polymerase chain reaction was performed using primers specific for the AGT intron II.24 Southern blot.21 Ten micrograms of genomic DNA was digested with EcoRI and probed with a segment of human AGT intron II.

Plasma AGT Assay
Plasma mouse AGT (mAGT) and hAGT protein were determined as described previously.21,22 RIA was performed on plasma using the Ang I 125I-labeled RIA kit (NEN Life Science Products Inc). Samples were diluted with reagent blank to remain on the linear portion of the standard curve. Plasma AGT was extrapolated using the 1:1 molar relationship between Ang I and AGT.

Immunohistochemistry
Mice were killed and then perfused transcardially with 20 mL PBS followed by 50 mL 4% formaldehyde. The brain was removed, postfixed overnight (4°C) in the same fixative, and placed in 30% sucrose solution. The following day, the brain was frozen and cut coronally (30 μm, Reichert-Jung Cryostat). Sections were permeabilized with 0.1% Triton X-100 in PBS (25°C) and incubated 16 to 18 hours with a rabbit polyclonal antibody against hAGT (4°C, 1:100 dilution) provided by Dr Dwayne Tevksbury [Marshfield Medical Research Foundation, Marshfield, Wis]). Sections were also incubated with mouse monoclonal antiserum against GFAP (1:1000 dilution, Chemicon International Inc) or microtubule-associated protein-2 (MAP-2, 1:400 dilution, Chemicon International Inc).25,26 Samples were washed in 0.1% Triton X-100, incubated with fluorescein-conjugated anti-rabbit IgG (1:100 dilution, Chemicon International Inc) for 1 hour (25°C), rinsed, and then incubated with rhodamine-conjugated anti-mouse IgG (1:100 dilution, Chemicon International Inc).

Physiology
Mice were anesthetized with ketamine (90 mg/kg, IP) and acepromazine maleate (1.8 mg/kg, IP), shaved, and surgically instrumented with catheters in the carotid artery and jugular vein.16 Catheters were tunneled subcutaneously, exteriorized, and sutured in place between the scapulae. After catheterization, some mice were instrumented with IVC cannulae for microinjection into the lateral ventricle. Anesthetized mice were placed in a stereotoxic apparatus (David Kopf Instruments), and a 9-mm guide cannula (25 gauge, Small Parts Inc) was implanted.16 Injections were made using a 33-gauge stainless steel injector (Small Parts) connected to tubing (PE-10, drawn over heat) fitted with a 10-μL microsyringe (Hamilton Company). IVC and intravenous infusions were performed over 30 minutes. Compounds were dissolved in artificial cerebrospinal fluid (ACSF, in mmol/L, NaCl 136, KCl 5.6, NaHCO3 16.2, NaH2PO4 1.2, MgCl2 1.2, and CaCl2 2.2, pH 7.4) and saline, delivered in 0.5- and 10-μL volumes, respectively. Correct insertion of the IVC cannula was determined by the pressor and bradycardiac response to IVC Ang II (200 ng).10 Mice that did not respond (one GFAP-hAGT, three systemic hAGT, and one nontransgenic mouse) were excluded from further studies. Methylene blue dye (5 μL) was injected after finishing experiments, and the brains were fixed and sectioned. All mice that exhibited central Ang II–induced cardiovascular responses were confirmed to have correct cannula placement by histological examination.

After surgery, mice were allowed 48 hours to recover and were then tested in a conscious, unrestrained state in their home cages. Mice were allowed a 30-minute adaptation period after connection of the arterial line to a Transpac pressure transducer (Abbott Laboratory), venous line, or insertion of the microinjector into the guide cannula. Baseline BP and heart rate (HR) were measured continuously for 1 hour per day for 3 consecutive days. Each infusion was performed on different days after measuring basal BP and HR, and mice were monitored at least 1 hour after drug infusion. Mice were infused with IVC (10, 30, and 100 ng) or intravenous (500 ng) hREN (gift of Walter Fischli, Hoffman-LaRoche, Basel, Switzerland). Some mice were pretreated with intravenous or IVC losartan (10 μg, Merck Research Laboratories). Controls included IVC injection of ACSF and intravenous injection of saline. All hemodynamic data were collected and analyzed on a computer using Chart version 4.0.1 in Powerlab.

Measurement of Drinking Volume and Salt Preference
Mice were housed individually in metabolic cages and fed standard chow and tap water ad libitum. After a 3-day acclimatization period, water intake was measured daily for 3 days. To determine salt preference, mice were fed salt-deficient chow (Teklad Premier Laboratory Diets). Two burettes containing either 0.3 mol/L hyper-
tonic saline or tap water were provided to each mouse in a metabolic cage. Access to either burette was randomized and ad libitum. After a 3-day acclimatization period, intake of the hypertonic saline and tap water was measured daily for 3 days. The preference for hypertonic saline was calculated as a percentage determined by dividing the volume of saline consumed by the total volume of fluid consumed.

Statistical Analysis
Data are expressed as mean±SEM. Group comparisons were made with unpaired t tests and confirmed with repeated-measures ANOVA followed by the Student modified t test with Bonferroni correction. A value of P<0.05 was considered statistically significant.

Results
Transgenic mice were generated with a construct containing the human GFAP promoter fused to a genomic clone encompassing exons II, III, IV, and V; the intervening introns; and the native 3’-end of the hAGT gene containing the poly(A) site (Figure 1A). Of 53 live-born offspring, three transgenic founders were identified (Figures 1B and 1C). All founders were successfully bred to establish lines and each transmitted the transgene to ~50% of both males and females, indicating insertion in autosomes.

When controlled by its own endogenous promoter, hAGT expression was observed in the liver, kidney, and other tissues including the brain (Figure 2A). This transgene is termed the systemic hAGT transgene. However, expression of hAGT in GFAP-hAGT transgenic mice was more restricted, with the highest level of expression in brain, and lower-level expression in liver, heart, aorta, submandibular gland, diaphragm, and adipose tissue (Figure 2B). Note the clear shift in the ratio between liver and brain expression comparing systemic hAGT mice (Figure 2A) with GFAP-hAGT mice (Figure 2B). In all three lines, hAGT mRNA expression was consistently observed in the brain, but exhibited variable expression of hAGT mRNA in tissues outside the brain (Table 1).

The liver is the primary site of AGT release into the circulation. Therefore, given the presence of “ectopic” hAGT mRNA in liver of all three lines, we were concerned that hAGT protein may be released into the systemic circulation. Therefore, we measured circulating levels of mAGT and hAGT to determine whether transgene expression outside the brain resulted in a significant increase of AGT protein in the systemic circulation. The specificity of the assay was confirmed by the observation of baseline levels of mAGT, but high levels of hAGT protein in the plasma of systemic hAGT transgenic mice (Table 2). Plasma hAGT levels in GFAP-hAGT transgenic mice were extremely low compared with those of systemic hAGT mice. A statistically significant increase above baseline was only reached in the 10187/1 line, which exhibited the highest extrabrain transgene expression. Line 10176/3, which exhibited the most selective transgene expression in brain and had baseline plasma hAGT, was chosen for further analysis.
To examine the cell-specific expression of the transgene in the brain, double-labeling for hAGT and GFAP (a glial marker) or MAP-2 (a neuronal marker) was performed (Figure 3). hAGT staining was observed in the cell bodies and processes of astrocytes in all regions of the brain as confirmed by costaining with GFAP, but not with MAP-2 (Table 3). The only exception to this was the subfornical organ (SFO), where hAGT colocalized with both GFAP and MAP-2 (Figures 3F through 3I).

We next measured BP and HR to investigate whether functional hAGT protein was expressed in the brain in GFAP-hAGT mice. Consistent with the species specificity of the REN-AGT enzymatic reaction, there was no difference in baseline mean arterial pressure or HR among GFAP-hAGT transgenic mice (120±5 mm Hg, 598±16 bpm), systemic hAGT transgenic mice (118±5 mm Hg, 604±18 bpm), and nontransgenic mice (117±5 mm Hg, 617±21 bpm). In GFAP-hAGT mice, ICV infusion of purified hREN increased mean arterial pressure in a dose-dependent manner (Figure 4A). The pressor response to ICV hREN exhibited delayed onset and was of long duration, appearing in 5.7±1.0 minutes, reaching a peak in 24.3±4.5 minutes, and lasting 56.5±9.2 minutes. By comparison, the BP-elevating effect of ICV Ang II, initiated after 36±4 seconds, peaked quickly (4.1±0.5 minutes) and lasted only 15.4±1.0 minutes (P<0.0001 at all time points). The HR change by ICV hREN was variable and did not achieve statistical significance.

Neither ICV nor intravenous injection of losartan (10 mg) on its own caused a significant change in mean arterial pressure or HR, but the pressor response to ICV hREN (30 ng) was prevented by pretreatment with ICV losartan. Interestingly, administration of a large dose of hREN (500 ng) intravenously did not raise BP in GFAP-hAGT, whereas that same dose caused a 35-mm Hg rise in mean arterial pressure in systemic hAGT mice, further confirming the lack of hAGT protein in the systemic circulation of GFAP-hAGT mice. In nontransgenic mice, neither ICV hREN (30 ng) nor intravenous hREN (500 ng) altered mean arterial pressure or HR (data not shown).

### Table 1. Summary of hAGT Expression in GFAP-hAGT Transgenic Mice

<table>
<thead>
<tr>
<th>Line</th>
<th>Br</th>
<th>Lv</th>
<th>K</th>
<th>H</th>
<th>Lu</th>
<th>Ag</th>
<th>Ao</th>
<th>Sp</th>
<th>Sg</th>
<th>D</th>
<th>Wa</th>
<th>Ba</th>
<th>Sm</th>
<th>T</th>
<th>U</th>
<th>O</th>
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</thead>
<tbody>
<tr>
<td>10176/3</td>
<td>+++</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>10182/4</td>
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</tbody>
</table>

+++ indicates abundant expression; ++, moderate expression; +, easily detectable low-level expression; ±, expression at the limit of detection; −, no expression; Br, brain; Lv, liver; K, kidney; H, heart; Lu, lung; Ag, adrenal gland; Ao, aorta; Sp, spleen; Sg, submandibular gland; D, diaphragm; Wa, white adipose tissue; Ba, brown adipose tissue; Sm, skeletal muscle; T, testes; U, uterus; and O, ovary.

### Table 2. Plasma AGT Levels

<table>
<thead>
<tr>
<th></th>
<th>Nontransgenic</th>
<th>GFAP-hAGT Line 10176/3</th>
<th>GFAP-hAGT Line 10182/4</th>
<th>GFAP-hAGT Line 10187/1</th>
<th>Systemic hAGT</th>
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<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>mAGT</td>
<td>29.8±5.1</td>
<td>32.5±4.5</td>
<td>30.8±4.5</td>
<td>26.7±5.3</td>
<td>28.8±5.4</td>
</tr>
<tr>
<td>hAGT</td>
<td>19.1±4.6</td>
<td>33.3±4.9</td>
<td>33.1±5.8</td>
<td>49.5±5.5*</td>
<td>6070±1014†</td>
</tr>
</tbody>
</table>

Values are expressed in pmol/mL.

*P<0.05, †P<0.01 compared with nontransgenic mice.

### Table 3. Distribution of hAGT Staining in Brain of GFAP-hAGT Mice

<table>
<thead>
<tr>
<th>Glia</th>
<th>Neuron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain stem</td>
<td></td>
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<tr>
<td>Medulla</td>
<td>+</td>
</tr>
<tr>
<td>Nucleus of the solitary tract</td>
<td>+</td>
</tr>
<tr>
<td>Dorsal motor nucleus of the vagus</td>
<td>+</td>
</tr>
<tr>
<td>Area postrema</td>
<td>+</td>
</tr>
<tr>
<td>Ventrolateral medulla</td>
<td>+</td>
</tr>
<tr>
<td>Inferior olivary nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Pons</td>
<td></td>
</tr>
<tr>
<td>Locus coeruleus</td>
<td>+</td>
</tr>
<tr>
<td>Parabrachial nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Mesencephalic trigeminal nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Midbrain</td>
<td></td>
</tr>
<tr>
<td>Periaqueductal gray</td>
<td>+</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>+</td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Ventral posterior thalamus</td>
<td>+</td>
</tr>
<tr>
<td>Lateral septal nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Supraoptic nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Ventromedial nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Suprachiasmatic nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Median eminence</td>
<td>+</td>
</tr>
<tr>
<td>Organum vasculosum of the lamina terminalis</td>
<td>+</td>
</tr>
<tr>
<td>Periventricular anteroventral third ventricle</td>
<td>+</td>
</tr>
<tr>
<td>Median preoptic nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Cerebrum</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>+</td>
</tr>
<tr>
<td>SFO</td>
<td>+</td>
</tr>
<tr>
<td>Amygdala</td>
<td>+</td>
</tr>
<tr>
<td>Cortex</td>
<td>+</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>+</td>
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</tbody>
</table>
Finally, to examine the effect of chronic production of hAGT in astrocytes, we bred mice expressing hREN systemically with GFAP-hAGT mice. This hREN transgene is expressed in renal juxtaglomerular cells, in extrarenal tissues, and at a low level in the brain.16,23 Double-transgenic mice containing this hREN transgene and the systemically expressed hAGT exhibit hypertension that can be partially corrected by centrally administered losartan.16 The hREN/GFAP-hAGT transgenic mice exhibited moderately but significantly elevated mean arterial pressure on each of 3 days of measurement (mm Hg, 48 hours after surgery, 134.5 ± 2.6 versus 117.2 ± 0.6, P < 0.01 compared with saline injection; **P < 0.006; 72 hours after surgery, 134.8 ± 1.8 versus 117.2 ± 0.6, P < 0.010) (compiled in Figure 5A). HR was not significantly different between groups (hREN/GFAP-hAGT, 611 ± 17 bpm, n = 4; nontransgenic, 605 ± 17, n = 5). ICV injection of losartan caused a 15–mm Hg fall in mean arterial pressure in the double-transgenic mice (Figure 5B), reducing mean arterial pressure 15–mm Hg versus 15–mm Hg for losartan versus 15–mm Hg for saline) or HR in either group.

We also measured drinking volume and salt preference comparing hREN/GFAP-hAGT with nontransgenic littersmates. There was no significant difference in baseline water intake between groups (Figure 5C). In measuring salt preference, hREN/GFAP-hAGT mice drank (in mL/day per 10 g body weight) 1.3 ± 0.2 of water and 0.8 ± 0.1 of saline compared with 1.6 ± 0.2 water and 0.6 ± 0.1 saline in controls. When examined as a percentage of total volume intake, hREN/GFAP-hAGT mice exhibited a significantly greater preference for saline, drinking nearly 40% of their total volume as saline as compared with 26% in control mice (Figure 5D).

Discussion

The major site of AGT production in mammals is the liver, where it is released into the systemic circulation. AGT expression is also evident in several extrahepatic tissues including the heart, kidney, adipose tissue, and brain. There is a longstanding hypothesis that AGT synthesis in extrahepatic tissues may provide the precursor to locally produced Ang II, which may serve important functions at or near its site of synthesis. However, it remains unclear whether AGT synthesis in these tissues generates Ang II, which is important for cardiovascular function. We recently demonstrated that localized overproduction of AGT in the kidney causes systemic hypertension, providing strong evidence favoring the importance of extrahepatic AGT in cardiovascular function.27 In this report, we demonstrate that hREN/GFAP-hAGT double-transgenic mice are moderately hypertensive and have increased salt preference. The increased BP is due to stimulated production of central Ang II. These data clearly demonstrate that AGT protein synthesized in the brain is the substrate for local Ang II synthesis, which can then influence arterial pressure and electrolyte homeostasis.

In the brain, AGT mRNA and protein is most abundant in neuroglia (astrocytes) in the hypothalamus and preoptic region.
nuclei, and at lower levels in the mesencephalon, myelencephalon, and cerebellum.\textsuperscript{17,28} In addition, AGT can be detected in neurons in the cerebral cortex, midbrain, and medulla, including areas important for cardiovascular function.\textsuperscript{17} Imboden et al.\textsuperscript{19} reported that neurons containing both AGT and Ang II are present in hypothalamic paraventricular and supraoptic nuclei in rats. We reported that AGT-expressing neurons were identifiable in SFO, mesencephalic trigeminal nucleus, and parabrachial nucleus in hAGT transgenic mice.\textsuperscript{18} Despite strong evidence for the presence of AGT in the brain, its function remains poorly defined because of a lack of direct evidence demonstrating that Ang II is generated from AGT synthesized in the brain. We therefore generated a novel transgenic mouse model expressing hAGT under the control of the GFAP promoter to begin to unravel the functional significance of brain AGT. The GFAP promoter was chosen because it was shown to restrict expression to astrocytes throughout the brain.\textsuperscript{20} Use of the GFAP promoter eliminated neuronal hAGT expression in the parabrachial complex and mesencephalic nucleus, where hAGT is expressed when its endogenous promoter is used.\textsuperscript{18} Three individually derived transgenic lines exhibited hAGT mRNA expression in the brain, with variable expression in other tissues. In one line, transgene expression was observed primarily in the brain, and hAGT protein was expressed almost exclusively in astrocytes. Only in the SFO was AGT found to be colocalized with both GFAP- and MAP-2-containing cells. The colocalization of MAP-2 with hAGT is suggestive of neuronal localization of hAGT in the SFO. Neuronal expression of AGT has been reported previously in SFO, and both ACE and Ang II have been localized to neurons in this structure.\textsuperscript{18,29} However, MAP-2 is present in modified astrocytes in the neurohypophysis, and these cells are sensitive to water deprivation.\textsuperscript{30} It is possible that the cells containing both MAP-2 and hAGT in the SFO are not neurons but modified astrocytes.

IVC injection of hREN increased arterial pressure in GFAP-hAGT transgenic mice, and the pressor response to hREN was prevented by ICV pretreatment with the AT\textsubscript{1} antagonist losartan. The pressor response may be due to processing of brain or ventricular hAGT by hREN and the stimulation of AT\textsubscript{1}s. The absence of a pressor response after intravenous hREN infusion argues that AGT synthesized in the brain is not released into the circulation and supports our biochemical data showing that the level of plasma hAGT was not above baseline. This suggests that tissues outside the brain do not release significant amounts of AGT into the circulation, consistent with our previous examination of extrahepatic AGT in liver-specific hAGT knockout mice.\textsuperscript{31} Nevertheless, it is surprising that a large dose of hREN administered intravenously (15 times greater than administered ICV) did not exert any pressor effect, as we would have anticipated that plasma hREN would gain access to the circumventricular organs (CVOs). It remains unclear whether a sufficient dose of hREN (after intravenous administration) was present in the CVOs to mediate a pressor response.

It has been previously reported that in addition to processing by REN and ACE, AGT can be directly converted to Ang II by cathepsin G or tonin in the brain.\textsuperscript{32,31} At least in the context of this model, we feel this to be unlikely as single GFAP-hAGT and single systemic hAGT mice (which exhibit hAGT expression in astrocytes) exhibit normal resting arterial pressure.\textsuperscript{31} Thus, unless the species specificity of the AGT cleavage reaction extends to non-REN enzymes, such as cathepsin and tonin, then cleavage of AGT by endogenous non-REN enzymes in the brain may not occur in mice.

Double-transgenic mice containing both the hREN and GFAP-hAGT transgenes are modestly hypertensive. ICV losartan lowered arterial pressure in the double-transgenic mice, whereas the same dose administered intravenously did not affect BP. AT\textsubscript{1}s are widely distributed in the brain, including regions controlling cardiovascular function, hypothalamic nuclei such as the paraventricular, and brainstem nuclei such as the ventrolateral medulla and the nucleus of the solitary tract.\textsuperscript{34-37} In addition to these intrinsic brain sites, AT\textsubscript{1}s are densely localized at cardiovascular control centers located at the blood-brain interface surrounding the cerebroventricles, namely CVOs such as SFO, organum vasculosum of the lamina terminalis, and area postrema.\textsuperscript{34} ACE is observed throughout the brain, including all of those areas containing AT\textsubscript{1}s, and has been reported in cerebrospinal fluid.\textsuperscript{38,39} In GFAP-hAGT transgenic mice, hAGT-containing cells are widely distributed in areas containing AT\textsubscript{1}s. Therefore, our data are consistent with the notion that Ang II, produced via processing of hAGT by hREN and ACE, stimulates AT\textsubscript{1}s in the brain to increase arterial pressure and alter sodium appetite. Indeed, the inhibition of the pressor response to hREN, and the lowering of BP in the hREN/GFAP-hAGT mice after ICV losartan strongly suggests that the increase in BP is mediated by an AT\textsubscript{1}-dependent mechanism caused by chronic overproduction of Ang II in brain. Nevertheless, it is important to point out that the relative contribution of AT\textsubscript{1}s in the CVOs versus parenchyma of the brain remains undefined and will require additional experiments. Moreover, it is known that central Ang II increases arterial pressure by enhancing sympathetic nerve activity and vasopressin secretion, causing antidiuresis, and increases drinking and salt intake.\textsuperscript{1,40} Central infusion of Ang II and losartan also changes the sensitivity of the arterial baroreflex.\textsuperscript{41,42} Each of these mechanisms will have to be examined to determine which is responsible for the elevation in BP in the hREN/GFAP-hAGT model.

In the brain, it is unclear where the REN-AGT cleavage reaction occurs. Are both proteins synthesized in the same cells or are the proteins secreted into the extracellular space or cerebroventricles? It is interesting to note that we recently reported that an altered form of REN mRNA is expressed in the brain of transgenic mice carrying a large hREN transgene encoded on a P1 artificial chromosome.\textsuperscript{43} Importantly, the same form of REN mRNA was found in human fetal brain.\textsuperscript{43} If translated, this mRNA would encode an intracellular (nonsecreted) form of the protein, suggesting the intriguing possibility of an intracellular pathway of angiotensin production in brain. We are currently testing this hypothesis using transgenic mice designed to specifically express only the predicted nonsecreted form of REN in brain.

Our data are in general agreement with experiments performed in rats in which direct administration of antisen
oligonucleotides or RNAs directed against AGT into cerebroventricles lower BP. Similarly, lowered BP and diabetes insipidus were reported in transgenic rats containing an antisense RNA directed at AGT under the control of a glial-specific promoter. Although a 90% decrease in brain AGT was reported, it is unclear whether AGT in both glial cells and neurons was targeted. Transgenic rats containing the antisense are more sensitive to the dipsogenic actions of central Ang II, and when microinjected into the rostral ventrolateral medulla were more sensitive to the pressor actions of Ang II. AT density was increased in structures inside the blood–brain barrier, but decreased in the CVOs, suggesting a functional role for ATs within the blood–brain barrier in the control of water intake and BP.

In conclusion, the results of both overexpression and underexpression experiments suggest that AGT produced in the brain may play an important role in central regulation of BP and electrolyte balance.

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