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Running head: Brain-specific human renin expression

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Key Words: cardiovascular system, central nervous system, sympathetic nervous system, glia, astrocytes
Abstract

The purpose of this study is to examine the regulation of blood pressure and fluid and electrolyte homeostasis in mice over-expressing Angiotensin II (Ang-II) in the brain, and to determine if there are significant physiologic differences in Ang-II production in neurons or glia. Therefore, we generated and characterized transgenic mice over-expressing human renin (hREN) under the control of the GFAP promoter (GFAP-hREN) and Synapsin-I promoter (SYN-hREN) and bred them with mice expressing human angiotensinogen (hAGT) under the control of the same promoters (GFAP-hAGT and SYN-hAGT). Both GFAP-hREN and SYN-hREN mice exhibited highest hREN mRNA expression in the brain and had undetectable levels of hREN protein in the systemic circulation. In the brain of GFAP-hREN and SYN-hREN mice, hREN protein was observed almost exclusively in astrocytes and neurons, respectively. Transgenic mice over-expressing both hREN and hAGT transgenes in either glia or neurons were moderately hypertensive. In the glia targeted mice, blood pressure could be corrected by intracerebroventricular injection of the AT-1 receptor antagonist losartan; and intravenous injection of a ganglion blocking agent, but not an arginine vasopressin V1 receptor antagonist, lowered blood pressure. These data suggest that stimulation of AT-1 receptors in the brain by Ang-II derived from local synthesis of renin and angiotensinogen can cause an elevation in blood pressure via a mechanism involving enhanced sympathetic outflow. Glia and neuron targeted mice also exhibited an increase in drinking volume and salt preference, suggesting that chronic over-expression of renin and angiotensinogen locally in the brain can cause result in hypertension and alterations in fluid homeostasis.
Introduction

The peptide angiotensin II (Ang-II) is the main circulating effector hormone of the renin-angiotensin system (RAS), and is able to act not only on peripheral vascular structures, but also on the central nervous system (CNS) to increase blood pressure (BP) (1). Substantial evidence has accumulated that Ang-II has actions on the CNS including increasing sympathetic outflow, arginine vasopressin (AVP) release, water intake, and salt appetite (2). Existence of a local RAS in the CNS has been suggested since all components of this system have been reported in the brain. In addition, several lines of evidence point to a contribution of an overactive brain RAS to the hypertensive state in spontaneously hypertensive rats, DOCA-salt hypertensive rats, Dahl-salt sensitive rats, and renal hypertensive rats (3-6).

Despite numerous studies demonstrating the important cardiovascular effects of Ang-II or Ang-II receptor antagonists injected into the brain, it remains unclear whether RAS components synthesized in the brain, in particular renin and angiotensinogen (AGT), have an important role in the local synthesis of Ang-II. In the brain, AGT expression is localized mainly in astrocytes (glia) (7,8). To determine if locally synthesized AGT can be processed to Ang-II in the brain, we generated a transgenic mouse model expressing human AGT (hAGT) under the control of an astrocyte-specific (glial fibrillary acidic protein, GFAP) promoter (GFAP-hAGT) (9). These mice exhibit hAGT expression primarily in the brain, but have a normal BP because of the strict species-specificity of the enzymatic reaction between AGT and renin (10). Intracerebroventricular (ICV) injection of purified recombinant human renin (hREN) protein elicited a pressor response which was prevented by ICV pre-injection of an Ang-II type 1 (AT-1) receptor antagonist, losartan. Accordingly, we concluded that AGT synthesized in the brain can
be converted to Ang-II if renin is present. In addition to glia, AGT is expressed in select populations of neurons in regions of the brain, such as the subfornical organ (SFO) that can influence cardiovascular function (7,11). To determine the relevance of neuronal AGT, we also developed a transgene over-expressing hAGT from a neuronal promoter, synapsin-I (SYN)(12). As above, expression was high in the brain, and hAGT was exclusively localized in neurons co-expressing the neuronal marker NeuN. ICV infusion of purified hREN caused a transient pressor response suggesting that Ang-II release from either glial cells or neurons is functional in the brain.

ICV injection of homologous species renin increases BP in several animals (13,14), and early studies detected renin activity in the brain (15,16). Later, immunoreactive renin was found in the hypothalamus and cerebellar cortex in the mouse and rat brain, and in neurons in all areas of the human brain (17,18). Despite these observations and the clear documentation of the expression of the other RAS genes in the brain, the notion that renin is endogenously expressed in brain remains controversial. We recently reported hREN expression in the brain of a new transgenic model containing a tightly regulated hREN transgene (19). Renin containing cells were detected in neurons in the brain stem, and glial cells in the hypothalamus and cortex. We therefore hypothesize that local synthesis of renin may cleave locally synthesized AGT to produce brain Ang-II. To test this hypothesis and determine the functional role of glial and neuronal hREN expression, we generated two novel transgenic mouse model expressing hREN under the control of either the GFAP promoter (GFAP-hREN) or the SYN promoter (SYN-hREN).
Materials and Methods

Generation of GFAP-hREN and SYN-hREN Transgenic Mice: A cDNA encoding a modified hREN protein was removed from pRhR1100FM (generous gift from Dr. Timothy L. Reudelhuber, Clinical Research Institute of Montreal), modified by PCR to contain XhoI and MluI ends, and was cloned into the XhoI-MluI sites of pSTEC-1 (20,21). A 2.2 kb fragment of human GFAP promoter was excised with EcoRI from the plasmid gfa2 and was subcloned into pBluescript II SK+/- to form pGFAP-SK (22). The rat SYN promoter was amplified by PCR from pBL4.3 Syn-CAT to contain EcoRV and BamHI ends (23). The modified SYN promoter was cloned into the EcoRV-BamHI sites of pBluescript II SK+/- to create pSYN-SK. The intron, renin cDNA, and SV40 poly(A) site was removed from pSTEC-1 and ligated to pGFAP-SK to create GFAP-hREN, and to pSYN-SK to form SYN-hREN. All cloning junctions were confirmed by sequencing. The modifications consisted of replacing the normal prorenin converting enzyme cleavage site with the ubiquitous processing protease furin, and tagging the carboxy terminal of the protein with a myc-epitope (24). The myc epitope did not provide sufficient sensitivity to detect the protein using anti-myc antisera.

The transgenes were excised by digestion with BssHII and purified by agarose gel electrophoresis. Transgene DNA was microinjected into one-cell fertilized mouse embryos obtained from superovulated C57BL/6J X SJL/J (B6SJL F2) mice using standard procedures. All transgenic mice were heterozygous, and were maintained by breeding with B6SJL F1 mice. Transgenic mice carrying both GFAP-hREN and GFAP-hAGT transgenes were generated by breeding heterozygous GFAP-hREN with heterozygous GFAP-hAGT transgenic mice. The SYN-hREN/SYN-hAGT double transgenic mice were similarly bred.
Mice used for experiments were 15-20 weeks of age. Non-transgenic age-matched littermates from the same breeding were always used as controls in the studies described herein. All mice received standard mouse chow (LM-485; Teklad Premier Laboratory Diets, Madison, WI) and water *ad libitum* unless specified. Care of the mice used in the experiments met the standards set forth by the National Institutes of Health in their guidelines for the care and use of experimental animals, and all procedures were approved by the University Animal Care and Use Committee at the University of Iowa.

*Analysis of Nucleic Acids*: Existence of transgene(s) was identified by PCR of tail genomic DNA using hREN- and/or hAGT-specific primer sets as described previously (25). Results of PCR for GFAP-hREN and SYN-hREN founder mice were confirmed by Southern blot analysis. Tissues were harvested and snap-frozen in liquid nitrogen, and RNA was purified using TriReagent (Molecular Research Center Inc). RNase protection assays were performed using the RPA III kit (Ambion Inc., Austin, TX). Total RNA (50 µg) was hybridized to hREN and mouse β-actin probes labeled with [α-32P]UTP by in vitro transcription and purified through a Sephadex G-50 spin column (Boehringer Mannheim). Protected fragments for hREN and mouse β-actin were 300 and 245 bp, respectively.

*Plasma Renin Assay*: Plasma renin activity (PRA) and concentration (PRC) were determined as described previously (26). Radioimmunoassay was performed on plasma using the angiotensin-I (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.
Immunohistochemistry: Brain sections were permealized with 0.1% Triton X-100 in PBS at 25°C and incubated at 4°C 18 hours with a rabbit polyclonal antibody against hREN (1:2,000 dilution for GFAP-hREN, 1:100 for SYN-hREN, kindly provided by Professor Pierre Corvol, INSERM U36, College de France) which had been pre-adsorbed with non-transgenic brain sections at 4°C for 48 hours (19). Sections also incubated with mouse monoclonal antisera (Chemicon International Inc) against either GFAP (1:1,000 dilution), microtubule-associated protein-2 (MAP-2, 1:400 dilution), or neuronal nuclei (NeuN, 1:500)(Chemicon International Inc) as described (19). GFAP-hREN samples were incubated with biotinylated anti-rabbit IgG (1:250 dilution), avidin D (1:50), biotinylated anti-avidin (1:125), fluorescein avidin D (1:250, all from Vector Laboratories Inc), and rhodamine-conjugated anti-mouse IgG (1:100 dilution, Chemicon International Inc); each incubation was at 25°C for 1 hour followed by rinsing with 0.1% Triton X-100 in PBS except for the last rinse which used PBS.

Physiology: Measurement of arterial pressure (AP) and heart rate (HR) and, ICV and intravenous (IV) infusion studies were performed in conscious, unrestrained mice as described previously (9). Briefly, baseline AP and HR were measured continuously for 1 hour/day for 3 consecutive days starting 2 days after surgery. The effect of ICV losartan (10 µg, generous gift of Merck Research Laboratories, Rahway, NJ) and artificial cerebrospinal fluid (ACSF) on basal AP and HR was examined. IV injection of the same dose of losartan was also tested. The effects of IV injection of a ganglion blocker, hexamethonium (5 mg/kg IV), an antagonist of peripheral arginine vasopressin (AVP) V1 receptors (AVPX, Manning Compound [d(CH2)5Tyr2(ME)Arg8]-vasopressin, 10 µg/kg IV), and saline on baseline AP and HR were also determined. All hemodynamic data were collected and analyzed on a computer using Chart v 4.0.1 in Powerlab.
Drinking volume of water and salt preference were measured as described previously (9); water intake was measured individually in metabolic cages with standard chow and tap water ad libitum daily for 3 days. To determine salt preference, mice were fed salt-deficient chow and given 0.3 mol/L hypertonic saline and tap water in separate randomized burettes ad libitum for 3 days. Salt preference was calculated as a percentage by dividing the volume of saline consumed by the total volume of fluid consumed. Urine volume was measured by collecting 24 hour urine in metabolic cages with standard chow ground with tap water (100 g chow/150 mL water) and tap water ad libitum daily for 3 days. Urinary and plasma osmolality were measured by using a vapor pressure osmometer (Wescor Inc).

**Statistical Analysis:** Data were expressed as mean ± SEM. Group comparisons were made with unpaired t-tests and confirmed with repeated-measures ANOVA followed by the Student’s modified t-test with Bonferroni correction. A value of $P<0.05$ was considered statistically significant.
Results

GFAP-hREN and SYN-hREN transgenic mice were generated by fusing the GFAP and SYN promoters to a hREN cDNA. Since the mechanisms activating renin in the brain are unknown, we chose to use a hREN cDNA that was modified to contain a ubiquitous furin cleavage site in place of the prorenin converting enzyme site normally separating the prosegment from the active enzyme (Figure 1A). Three transgenic founders of each construct were identified; and two GFAP-hREN and three SYN-hREN founders were successfully bred to establish transgenic lines.

RNase protection assay was used to examine the tissue-specific expression of the transgenes. In GFAP-hREN line 10267/4, low but clearly detectable expression of hREN mRNA was observed in the brain with lower expression in other tissues such as heart, lung, spleen, submandibular gland, and white adipose tissue (Figure 1B). In SYN-hREN line 11110/2, robust expression of hREN mRNA was detect in the brain, with low level ectopic expression evident in brown adipose tissue (Figure 1C). Table 1 shows a quantitative summary of hREN mRNA expression in all lines of GFAP-hREN and SYN-hREN transgenic mice. GFAP-hREN line 10267/4, and Syn-hREN line 11110/2 was selected for further analysis as these exhibited the highest level of expression in the brain and retained the most brain-restricted pattern of hREN expression.

Given the presence of “ectopic” hREN mRNA expression in tissues outside the brain, we were concerned that hREN protein might be released into the systemic circulation. Therefore, we measured plasma hREN concentration to determine if transgene expression outside the brain results in a significant increase of hREN protein in the systemic circulation (Table 2). The
fidelity and specificity of the assay was confirmed by the observation of basal levels of mouse renin (24.5±3.7 vs. 30.3±3.4 ng Ang-I/mL/hr, transgenic vs control) and hREN protein (14.1±1.7 vs. 3.2±0.8 ng Ang-I/mL/hr, transgenic vs control) in the plasma of transgenic mice expressing hREN under the control of its own promoter (termed systemic-hREN transgenic mice)(27). Although all five lines of GFAP-hREN and SYN-hREN transgenic mice exhibited a slight increase in hREN concentration in the plasma, these values were not significantly different than in non-transgenic mice. Accordingly, it is unlikely that a significant amount of hREN protein is released into the systemic circulation in either the glial or neuronal transgenic models.

To examine the cell-specific expression of the transgene in the brain, double-labeling for hREN and GFAP (a glial marker) or MAP-2 or NeuN (neuronal markers) was performed. In GFAP-hREN transgenic mice, hREN staining was observed in the cell bodies and processes of astrocytes in almost all regions of the brain as confirmed by co-staining with GFAP, but not with MAP-2 (Figure 2A-D, Table 3). In non-transgenic mice, some very light hREN immunostaining of glial-like elements was observed in cortex (Figure 2E). This immunostaining was generally distributed throughout the brain, and showed no regional specificity. The only exception to this was the SFO (Figure 2F-I) and area postrema (data not shown) where hREN co-localized with both GFAP and MAP-2. We showed previously that the GFAP promoter targeted expression of hAGT to neurons in the SFO (9). In addition, hREN-positive radial astroglia were observed in cerebellum (Figure 2J). Strong non-specific staining was still detected in the Purkinje cell layer of the cerebellum in both controls and transgenic mice even though the antisera was preadsorbed with brain sections from control mice (Figure 2J-K). In SYN-hREN mice, hREN staining was observed in the cell bodies of neurons in all regions of the brain as confirmed by co-staining with
NeuN, but not with GFAP (Figure 3A-D, Table 3). Only low background staining was observed in sections from non-transgenic controls (Figure 3E).

To examine the physiological significance of the local production of renin and AGT in the brain on BP and electrolyte homeostasis, we generated GFAP-hREN/GFAP-hAGT and SYN-hREN/SYN-hAGT double transgenic mice. Because there is a strict species-specificity of the biochemical reaction between AGT and renin (10), there was no difference in baseline mean arterial pressure (MAP) or HR between single GFAP-hREN transgenic mice (lacking the human AGT gene) and their non-transgenic littermates (GFAP-hREN, 118±3 mmHg, 590±32 bpm, n=5; non-transgenic, 116±4 mmHg, 594±20 bpm, n=7) or between single SYN-hREN transgenic mice and their non-transgenic littermates (SYN-hREN, 114±3 mmHg, 628±34 bpm, n=5; non-transgenic, 119±5 mmHg, 609±28 bpm, n=5). In contrast, both the neuronal and glial double transgenic mice (containing both hREN and hAGT in brain) exhibited a moderate, but significant elevation in MAP on each of 3 days of measurement (compiled in Figure 4). Although statistically significant, the elevation in MAP is probably not enough to consider them overtly hypertensive as there are some strains of mice with higher baseline arterial pressure (28). Baseline HR was not significantly different between double transgenic and control groups.

ICV injection of losartan (10 µg) caused an approximate 11 mmHg fall in MAP in the GFAP-hREN/GFAP-hAGT mice, reducing MAP back to baseline (Figure 5A). Non-transgenic mice exhibited only a 5 mmHg decrease in MAP. The HR change induced by ICV losartan was variable and did not achieve statistical significance in either group. IV administration of the same amount of losartan injected ICV did not significantly affect MAP or HR in either group.
Because of the possible contribution of increased brain RAS to the elevated BP in these double transgenic mice, we wanted to investigate the mechanism of chronic BP elevation downstream of the CNS. We focused these studies in the GFAP-hREN/GFAP-hAGT mice because the blood pressure increase was larger than in SYN-hREN/SYN-hAGT. The major mechanisms of pressor response to ICV Ang-II are via activation of sympathetic nervous system and/or AVP secretion. Consequently, we examined effects of IV treatment with a ganglionic blocking agent, hexamethonium, and an AVP V1 receptor antagonist, AVPX, on BP. IV injection of hexamethonium caused a greater fall in MAP in glial-specific mice than non-transgenic mice (Figure 5B). Although the reduction in MAP by IV AVPX tended to be greater in the transgenic mice, the difference did not achieve statistical significance (Figure 5C).

Finally, we measured drinking volume, salt preference, and urinary volume in both the glial- and neuronal-specific double transgenic mice (Figure 6). Drinking volume and salt preference were significantly higher in both models compared with controls (Figure 6A and 6B). Concomitant with increased water intake, urinary volume was significantly higher in the double transgenics (Figure 6C). Urinary osmolality was lower in glial-specific mice (1,612±163 mosmol/KgH2O, n=11) than controls (2,240±156 mosmol/KgH2O, n=11, P<0.01). Urinary osmolality tended to be lower in neuron-specific mice (1,804±168 mosmol/KgH2O, n=11) but did not reach statistical significance. In this light, urine output was lower in the neuron-specific mice than in the glial-specific mice (Figure 6C). Plasma osmolality was not different between the groups (glial-specific 335±9, n=5; neuron-specific 325±6, n=9; controls 316±7, n=6, in mosmol/KgH2O).
**Discussion**

Renin is the rate-limiting regulator of the enzymatic cascade leading to the production of Ang-II in most mammals. The juxtaglomerular cells of the kidney are the primary site of synthesis, storage, processing, and release of renin. Classic dogma maintains that renin is released from the kidney into the circulation where it cleaves AGT derived from the liver to produce Ang-I, which is further converted into blood-borne Ang-II. In addition to this, renin is synthesized in extra-renal tissues such as brain and adrenal gland (29). Ganten *et al.* (15) and Fischer-Ferraro *et al.* (16) published the first reports demonstrating central renin activity. Renin activity is widely distributed in the brain, with the highest levels in the pineal gland, pituitary gland, and pons-medulla (30). Substantial controversy still remains as to the localization of renin containing cells in the brain. For example, immunoreactive REN was detected in most nerve cells throughout the human brain while it was detected only in hypothalamus and cerebellar cortex in the brain of rodents (17,18). We recently examined the cellular localization of renin in the brain of transgenic mice containing the hREN gene encoded on a large P1 artificial chromosome. This transgene, because it contains extensive amounts of 5’ and 3’ flanking DNA exhibits a tightly regulated and highly specific cell type-restricted expression of hREN (31). In these mice, hREN immunoreactivity was observed in glial cells and neurons in the brain stem, hypothalamus, and cerebrum (19).

Renin activity is increased in the brain of SHR, the most widely used animal model of human essential hypertension (32). Expression of hREN and hAGT is evident in the brain of hypertensive double transgenic mice over-expressing both hREN and hAGT transgenes (33). In SHR and hREN/hAGT double transgenic mice, ICV injection of Ang-II receptor antagonists is
effective in lowering BP (33,34). Therefore, it has long been suggested that renin synthesized in
the brain may also have an important role in the regulation of BP through the local generation of
Ang-II. However, since both SHR and hREN/hAGT double transgenic mice have systemic
factors affecting BP, it remains unclear whether brain-specific expression of renin \textit{per se} has an
important role in the regulation of cardiovascular function in these models.

To examine the physiological role of brain-derived Ang-II in the regulation of BP, and to begin
to distinguish between the effects of Ang-II derived from glial-cells and neurons we developed
transgenic models expressing hAGT from the astrocyte-specific GFAP promoter and the neuron-
specific SYN promoter (9,12). Double transgenic mice containing the GFAP-hAGT and an
hREN transgene expressed systemically (in plasma, kidney, brain and other tissues), but not
specifically in the brain resulted in hypertension (9). Interestingly, double transgenic mice
expressing SYN-hAGT and a systemically expressed hREN transgene were normotensive,
despite the pressor response caused by infusion of purified hREN in the brain (12). These
studies allowed us to conclude that AGT synthesized in brain is the substrate for local synthesis
of Ang-II. However, since hREN was present in the systemic circulation of that model and
could potentially gain access to regions of the brain outside the blood-brain interface, e.g. the
circumventricular organs (CVOs), we could not conclude that the Ang-II was derived from
cleavage of AGT from brain-derived renin.

In order to generate a model of exclusive synthesis of Ang-II in the brain, we generated two
additional transgenic models, expressing hREN under the control of the GFAP and SYN
promoters. As shown herein, hREN mRNA was expressed in the brain, and hREN protein was
localized mainly in astrocytes and neurons, respectively. Although some hREN expression was detected outside the CNS, such as in lung and adipose tissue, its expression was lower than in brain, and importantly no hREN protein was detected in the systemic circulation. Double transgenic mice containing both the GFAP-hREN and GFAP-hAGT and the SYN-hREN and SYN-hAGT transgenes exhibited a modest increase in arterial pressure. Whether this increase is clinically relevant will require additional studies to determine if these mice exhibit the typical sequelae associated with hypertension such as vascular and renal dysfunction and cardiac hypertrophy. It should be noted however, that a 10-15 mmHg rise in pressure is approximately what is anticipated of a single gene effect in polygenic hypertension.

In the glial model, the elevated blood pressure was corrected by ICV losartan, whereas the same dose of losartan administered intravenously did not affect BP. This suggests that the BP elevation was due to the local generation and action of Ang-II in the brain and not from any leakage into the systemic circulation. Angiotensin converting enzyme and AT1 receptors are present in the same region of the brain that express hREN and hAGT ensuring all components of the RAS are present for the generation and action of Ang-II in our model (35-37). Consequently, our data is consistent with the notion that Ang-II, chronically overproduced via processing of brain hAGT by brain hREN and ACE, stimulated local AT-1 receptors to raise BP.

In addition to the rise in BP, both double transgenic mice exhibited increased drinking volume and salt preference, suggesting that chronic overexpression of renin and AGT within the brain is capable of affecting water intake and salt appetite. Angiotensin-induced thirst is primarily mediated in the OVLT, SFO, and median preoptic nucleus and angiotensin-induced sodium
appetite originates in the OVLT and median preoptic nucleus where central osmoreceptors function to maintain constant extracellular osmotic pressure (2,38,39). It is tempting to speculate that increased concentration of Ang-II in these areas may be involved in the mechanism of altered behavior in these mice.

Mechanistically, the rise in BP in the glial-specific model may be due, at least in part, to an increase in sympathetic nerve activity (SNA). This is suggested by the greater fall in BP in double transgenic mice than control mice treated with hexamethonium. However, there was no significant difference in the reduction in BP in double transgenic and control mice treated with an AVP receptor antagonist. This differs from previous studies of double transgenic mice containing systemically expressed transgenes (R+/A+) where BP was significantly lowered by AVP receptor antagonist (33). These data suggest that the mechanisms maintaining elevated BP in the models may differ. It is possible that AVP release, and therefore the response to AVP receptor antagonists is blunted in the GR/GA model due to feedback inhibition caused by the elevated drinking. In the glial-specific model, two separate mechanisms may be operating to maintain an elevation in BP, increased sympathetic outflow and increased water intake, caused by the activation of AT-1 receptors from local over-production of Ang-II in distinct nuclei regulating each process.

This raises an important question regarding the physiological relevance of these transgenic models. In the GFAP-hAGT model, hAGT is widely expressed in glial cells throughout the brain, thus emulating the normal pattern of astrocytic expression of AGT in brain (8). With renin, however, we must recognize that hREN expression in the GFAP-hREN and SYN-hREN
model is very likely produced in both normal renin-expressing cells and by ectopic cells. It is important to remember that proven tools do not currently exist to specifically-target renin expression to highly restricted regions of the brain, thus leaving us with tools which may not totally allow us to completely recapitulate the normal pattern of gene expression. Moreover, it is equally important to point out that we are just beginning to understand some of the basic processes governing Ang-II production in the brain. For instance, we and others recently reported that an altered form of renin mRNA, deriving from the utilization of an alternative transcription site may be used in the brain (40,41). If translated, this mRNA would encode an intracellular (non-secreted) and constitutively active form of the protein suggesting the intriguing possibility of an intracellular pathway of angiotensin production in brain. Intracellular production of Ang-II has been proposed but remains controversial (42). The GFAP-hREN and SYN-hREN models described herein utilized a mutant renin that should essentially be constitutively active because of the cleavage of prorenin to renin by furin, a ubiquitous processing protease expressed in the brain (43). Studies are currently underway to examine the regulation of BP and electrolyte balance in a new model expressing only the intracellular (non-secreted) form of the protein in the brain.
Acknowledgment

The work described herein was funded by grants from the National Institutes of Health (HL58048, HL61446, HL55006). S.M. was funded by a Postdoctoral Fellowship from American Heart Association Heartland Affiliate. Transgenic mice were generated and maintained at the University of Iowa Transgenic Animal Facility, which is supported in part by the College of Medicine and the Diabetes and Endocrinology Research Center. DNA sequencing was performed at the University of Iowa DNA Core Facility. We would like to thank Norma Sinclair, Patricia Lovell, Brandon Campbell, Debbie Davis, and Xiaoji Zhang for their excellent technical assistance.
References


**Figure Legends**

*Figure 1: Schematic Map and Expression.*

A. A schematic map of the transgenes are shown. The start site of transcription is indicated as +1. The transgene was excised as a *Bss*HII (Bss) fragment for microinjection. The mutations made to the renin cDNA to replace the prorenin converting enzyme site (PCE) with furin are shown. B and C. RNase protection assay of RNA from a male GFAP-hREN (line 10267/4, panel B) and male SYN-hAGT (line 11110/2, panel C) transgenic mouse are shown. The hREN and β-actin transcripts are indicated. Key to abbreviations: +, kidney of a transgenic mouse expressing hREN under the control of its own promoter; -, liver of a known non-transgenic mouse; Br, brain; P, peripheral nerve; 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.

*Figure 2: Cell-Specific Expression of hREN Protein in the Brain of GFAP-hREN Mouse.*

Representative photomicrographs of double-labeling for hREN and GFAP or hREN and MAP-2 in brain. In cerebral cortex, GFAP-positive (B) but not MAP-2-positive cells (D) were co-stained with hREN (A and C) in GFAP-hREN mice. In subfornical organ, both GFAP-positive (G) and MAP-2-positive cells (I) were double-labeled with hREN (F and H, arrows). In cerebellum, hREN-positive radial astroglia were observed (J, white arrow). In non-transgenic mice, some very light immunostaining of glial-like elements was observed (E). However, this immunostaining was generally distributed throughout the brain, and showed no regional specificity, with the exception of Purkinje cell layer where strong immunostaining was observed.
in both GFAP-hREN and non-transgenic mice (J and K, respectively, yellow arrows). A and B, C and D, F and G, and H and I are the pairs of the same sections.

Figure 3: Cell-Specific Expression of hREN Protein in the Brain of SYN-hREN Mouse.

Representative photomicrographs of double-labeling for hREN and NeuN (neuronal marker) or hREN and GFAP (glial marker) in cerebral cortex. NeuN-positive (B) but not GFAP-positive cells (D) were co-stained with hREN (A and C) in SYN-hREN transgenic mice. No hREN staining was observed in non-transgenic mice (E). A and B, and C and D are pairs of the same sections.

Figure 4: Arterial Pressure in Double Transgenic Mice.

Resting MAP in GFAP-hREN/GFAP-hAGT (GR/GA, black bar, n=9), SYN-hREN/SYN-hAGT (SR/SA, hatched bar, n=8) and controls (open bar, n=12). *P<0.05, **P<0.01 compared with controls.

Figure 5: Mechanism of Hypertension in GFAP-hREN/GFAP-hAGT Double Transgenic Mice.

A. Blood pressure responses to ICV injection of losartan (10 µg) in GFAP-hREN/GFAP-hAGT transgenic mice (black bar, n=6) and controls (white bar, n=6). B. Blood pressure responses to IV infusion of hexamethonium in GFAP-hREN/GFAP-hAGT transgenic mice (black bar, n=5) and controls (white bar, n=5). C. Blood pressure responses to IV injection of AVPX in GFAP-hREN/GFAP-hAGT transgenic mice (black bar, n=5) and controls (white bar, n=5). *P<0.05, **P<0.01 compared with controls.
Figure 6. Volume Homeostasis in Double Transgenic Mice.

A. Drinking volume of water in GFAP-hREN/GFAP-hAGT (GR/GA, black bar, n=13), SYN-hREN/SYN-hAGT (SR/SA, crosshatched bar, n=7) and control mice (open bar, n=19). B. Salt preference in GFAP-hREN/GFAP-hAGT (GR/GA, black bar, n=12), SYN-hREN/SYN-hAGT (SR/SA, crosshatched bar, n=7) and control mice (white bar, n=12). C. Urinary volume in GFAP-hREN/GFAP-hAGT (GR/GA, black bar, n=11), SYN-hREN/SYN-hAGT (SR/SA, crosshatched bar, n=7) and control mice (white bar, n=12). *P<0.05, **P<0.01 compared with controls.
Table 1: Summary of hREN Expression in GFAP-hREN and SYN-hREN Transgenic Mice

<table>
<thead>
<tr>
<th>Line</th>
<th>Con N</th>
<th>Brain ±</th>
<th>Liver ±</th>
<th>Kidney ±</th>
<th>Heart ±</th>
<th>Lung ±</th>
<th>Adrenal ±</th>
<th>Aorta ±</th>
<th>Spleen ±</th>
<th>Salivary ±</th>
<th>Diaphragm ±</th>
<th>WAT ±</th>
<th>BAT ±</th>
<th>Skeletal ±</th>
<th>Testes ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>10267/4</td>
<td>G 3</td>
<td>130.4±48.3</td>
<td>4.7±3.5</td>
<td>4.3±3.1</td>
<td>19.1±12.8</td>
<td>0.8±0.5</td>
<td>0.8±0.5</td>
<td>3.3±2.7</td>
<td>11.3±2.4</td>
<td>1.1±0.7</td>
<td>53.2±22.7</td>
<td>0.9±0.5</td>
<td>13.2±11.4</td>
<td>4.0±4.0</td>
<td></td>
</tr>
<tr>
<td>10268/4</td>
<td>G 2</td>
<td>32.5</td>
<td>0.3</td>
<td>0.0</td>
<td>1.6</td>
<td>0.2</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
<td>17.9</td>
<td>5.7</td>
<td>14.7</td>
<td>32.5</td>
<td>17.9</td>
<td>1.1</td>
</tr>
<tr>
<td>11110/2</td>
<td>S 3</td>
<td>448.2±173.4</td>
<td>20.1±9.9</td>
<td>0.33±0.2</td>
<td>9.4±4.8</td>
<td>111.8±86.2</td>
<td>0.2±0.2</td>
<td>0.8±0.8</td>
<td>16.2±16.2</td>
<td>0±0</td>
<td>0±0</td>
<td>1.8±1.7</td>
<td>234.8±168.6</td>
<td>111.6±58.3</td>
<td>44.7±44.7</td>
</tr>
<tr>
<td>11114/3</td>
<td>S 1</td>
<td>78.0</td>
<td>11.1</td>
<td>30.5</td>
<td>7.3</td>
<td>3.2</td>
<td>1.6</td>
<td>1.8</td>
<td>6.2</td>
<td>9.0</td>
<td>2.4</td>
<td>65.1</td>
<td>21.6</td>
<td>54.6</td>
<td>6.1</td>
</tr>
<tr>
<td>11116/1</td>
<td>S 2</td>
<td>63.5</td>
<td>0.4</td>
<td>4.9</td>
<td>1.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>1.3</td>
<td>1.8</td>
<td>12.2</td>
<td>22.4</td>
<td>29.0</td>
<td>5.7</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Expression was quantified by scanning the RPA films and analyzed by NIH (SCION) IMAGE. Scans were first calibrated with an identical positive control sample run on each RPA gel and then were normalized for loading and sample loss using the actin band. Units are arbitrary. Samples with N=3 are mean ± SEM. Samples with N=2 are the average of both runs. Con, construct; G, GFAP-hREN; S, SYN-hREN; WAT, white adipose tissue; BAT, brown adipose tissue.
Table 2: Plasma hREN Concentration

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mouse PRA (ng Ang-I/mL/hr)</th>
<th>Human PRC (ng Ang-I/mL/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-transgenic</td>
<td>9</td>
<td>30.3 ± 3.4</td>
<td>3.2 ± 0.8*</td>
</tr>
<tr>
<td>GFAP-hREN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Line 10267/4</td>
<td>5</td>
<td>24.9 ± 4.8</td>
<td>4.1 ± 1.0</td>
</tr>
<tr>
<td>Line 10268/4</td>
<td>5</td>
<td>24.4 ± 3.8</td>
<td>4.2 ± 1.3</td>
</tr>
<tr>
<td>SYN-hREN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Line 11110/2</td>
<td>5</td>
<td>32.7 ± 5.9</td>
<td>5.3 ± 0.9</td>
</tr>
<tr>
<td>Line 11114/3</td>
<td>5</td>
<td>31.7 ± 3.3</td>
<td>6.6 ± 1.8</td>
</tr>
<tr>
<td>Line 11116/1</td>
<td>5</td>
<td>29.9 ± 1.6</td>
<td>3.8 ± 1.0</td>
</tr>
<tr>
<td>Systemic-hREN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Line 9</td>
<td>8</td>
<td>24.5 ± 3.7</td>
<td>14.1 ± 1.7*</td>
</tr>
</tbody>
</table>

*P<0.01 compared to non-transgenic mice. ¶, represents background of the assay.
Table 3: Distribution of hREN Staining in Brain of Transgenic Mice

<table>
<thead>
<tr>
<th></th>
<th>GFAP-hREN</th>
<th>SYN-hREN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glia</td>
<td>Neuron</td>
</tr>
<tr>
<td><strong>A. Brain Stem</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medulla</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nucleus of the solitary tract</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>dorsal motor nucleus of the vagus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>area postrema</td>
<td>+?</td>
<td>+?</td>
</tr>
<tr>
<td>ventrolateral medulla</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>inferior olivary nucleus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Pons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>locus coeruleus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>parabrachial nucleus</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>mesencephalic trigeminal nucleus</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td><strong>Midbrain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>periaqueductal gray</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>superior colliculus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Thalamus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>paraventricular nucleus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ventral posterior thalamus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>lateral septal nucleus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Hypothalamus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>paraventricular nucleus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>supraoptic nucleus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ventromedial nucleus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>suprachiasmatic nucleus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>median eminence</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>organum vasculosum of the lamina terminalis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>periventricular anteroventral third ventricle</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>median preoptic nucleus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>B. Cerebrum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hippocampus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>subfornical organ</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>amygdala</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>cortex</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>C. Cerebellum</strong></td>
<td>+</td>
<td>?</td>
</tr>
</tbody>
</table>
GFAP or SYN Promoter

INT

hREN

SV 40 Poly A

GFAP-hREN-

β-actin-

SYN-hREN-

β-actin-

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Glial- and neuronal-specific expression of the renin-angiotensin system in brain alters blood pressure, water intake, and salt preference
Satoshi Morimoto, Martin D. Cassell and Curt D. Sigmund

J. Biol. Chem. published online June 21, 2002

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