Selective Deletion of the Brain-Specific Isoform of Renin Causes Neurogenic Hypertension

Keisuke Shinohara, Xuebo Liu, Donald A. Morgan, Deborah R. Davis, Maria Luisa S. Sequeira-Lopez, Martin D. Cassell, Justin L. Grobe, Kamal Rahmouni, Curt D. Sigmund

Abstract—The renin–angiotensin system (RAS) in the brain is a critical determinant of blood pressure, but the mechanisms regulating RAS activity in the brain remain unclear. Expression of brain renin (renin-b) occurs from an alternative promoter-first exon. The predicted translation product is a nonsecreted enzymatically active renin whose function is unknown. We generated a unique mouse model by selectively ablating the brain-specific isof orm of renin (renin-b) while preserving the expression and function of the classical isof orm expressed in the kidney (renin-a). Preservation of renal renin was confirmed by measurements of renin gene expression and immunohistochemistry. Surprisingly, renin-b–deficient mice exhibited hypertension, increased sympathetic nerve activity to the kidney and heart, and impaired baroreflex sensitivity. Whereas these mice displayed decreased circulating RAS activity, there was a paradoxical increase in brain RAS activity. Physiologically, renin-b–deficient mice exhibited an exaggerated depressor response to intracerebroventricular administration of losartan, captopril, or aliskiren. At the molecular level, renin-b–deficient mice exhibited increased expression of angiotensin-II type 1 receptor in the paraventricular nucleus, which correlated with an increased renal sympathetic nerve response to leptin, which was dependent on angiotensin-II type 1 receptor activity. Interestingly, despite an ablation of renin-b expression, expression of renin-a was significantly increased in rostral ventrolateral medulla. These data support a new paradigm for the genetic control of RAS activity in the brain by a coordinated regulation of the renin isoforms, with expression of renin-b tonically inhibiting expression of renin-a under baseline conditions. Impairment of this control mechanism causes neurogenic hypertension. (Hypertension. 2016;68:1385-1392. DOI: 10.1161/HYPERTENSIONAHA.116.08242.) • Online Data Supplement

Key Words: angiotensin II • brain • hypertension • renin • sympathetic nervous system

It is well known that the renin–angiotensin system (RAS) plays a crucial role in regulating blood pressure (BP) and fluid homeostasis. Many tissues express all components of the RAS and have the capacity for the synthesis and action of angiotensin-II (ANG). The importance of the tissue RAS as an autocrine and paracrine pathway and should remain intracellular. Renin-b has a cysteine-rich prosegment. Consequently, renin-b cannot enter the secretory pathway and should remain intracellular. Renin-b has been reported to be enzymatically active. Although renin-b is the brain-specific isof orm of renin in the brain under normal conditions, the function of renin-b in the brain and whether it encodes a functional intracellular renin are unknown.5,7

There is a long-held hypothesis that ANG acts as a neurotransmitter. Although this is supported by functional evidence, the criteria for de novo intracellular synthesis of ANG have yet to be satisfied.1,11 Renin is expressed in neurons along with its substrate angiotensinogen in regions of the brain controlling cardiovascular function.12,13 Therefore, the hypothesis for an intracellular renin in neurons is particularly compelling because it may offer the missing mechanistic link defining predicted that translation initiates at the next ATG in exon 2, a codon that is both in frame and evolutionarily conserved.6 This translation product (termed renin-b) is virtually brain-specific and lacks both the signal peptide and the first third of the prosegment. Consequently, renin-b cannot enter the secretory pathway and should remain intracellular. Renin-b has been reported to be enzymatically active. Although renin-b is the dominant isof orm of renin in the brain under normal conditions, the function of renin-b in the brain and whether it encodes a functional intracellular renin are unknown.5,7

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ANG as a neurotransmitter. There is a second hypothesis that expression of renin-a and renin-b is differentially but coordinately regulated in the brain.14 At baseline, renin-a expression in brain is undetectable, and the predominant isoform is renin-b (although expressed at a level orders of magnitude lower than in the kidney). However, in response to deoxycorticosterone acetate (DOCA)-salt, there is an induction of renin-a mRNA expression concomitant with a suppression of renin-b mRNA expression. This induction of renin-a occurs concurrently with a state of brain RAS activation. Thus, the balance between these 2 isoforms may dictate brain RAS activity, which could have profound effects on BP. This is further complicated by the fact that the protein products of renin-a and renin-b mRNAs likely differ. In brain, expression of renin-a should produce and release prorenin, whereas expression of renin-b should support production of a nonsecreted intracellularly retained form of active renin. Herein, we used an unconventional genetic approach to generate mice that selectively lack renin-b in the brain to investigate the importance of renin-b and differentiate between these 2 hypotheses. A notable feature of the model is the preservation of renin-a expression by the kidney and secretion of renin. This is critical because renin-a-deficient mice exhibit postnatal lethality.15

Methods

Generation of Renin-b\textsuperscript{null} Mice

A targeting vector was designed to delete 500 bp upstream and downstream of exon 1b using a bacterial artificial chromosome clone carrying the mouse Ren\textsuperscript{b} gene as a template. Gene targeting was performed in C57BL/6 inbred embryonic stem cells (IC1) by the inGenious Targeting Laboratory (Ronkonkoma, NY). Neomycin resistance and Herpes Simplex Virus thymidine kinase gene were used for positive and negative selection, respectively. Recombinant clone ITL4D5 successfully passed all quality control tests for the presence of loxP and FRT sites and was used for blastocyst injection. Chimeras were bred to C57BL/6J mice. Positive offspring were bred to C57BL/6J congenic B6.129S4-Gt(Rosa)26Sortm1(FLP1)Dym/RainJ (a FLP deleter strain, Jax 009086) to eliminate the neomycin gene and then to C57BL/6J to ensure removal of FLP. Offspring were bred to C57BL/6J congenic B6.FVB-Tg(EIIa-cre)C5779Lmgd/J (Jax 003724) to generate the Ren-b\textsuperscript{null} allele. Mice carrying the Ren-b\textsuperscript{null} allele were maintained by backcross breeding to C57BL/6J, and heterozygotes were intercrossed to generate the renin-b\textsuperscript{null} mice.

In this first study, aged-matched male Ren-b\textsuperscript{null} mice were examined. Control littermates carrying both wild-type alleles were used as controls in all experiments. Studies are currently in progress to assess sex differences in both cardiovascular and metabolic parameters. All mice were fed standard laboratory chow (NIH-31 modified 6% mouse diet; Harlan Teklad) and tap water ad libitum. All studies were approved by the University of Iowa Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Design

Measures of cardiovascular parameters, spectral analysis of heart rate, sympathetic nerve activity (SNA; performed in chloralose-anesthetized mice), plasma renin and ANG levels, isolation of specific brain regions and gene expression, and renin immunohistochemistry are detailed in the online-only Data Supplement. The doses of drugs used in this study were propranolol (5 mg/kg, IP), methylatropine (2 mg/kg, IP), hexamethonium bromide (1 mg/kg, IP), losartan (5 µg/h, ICV), and captopril (5 µg/h, ICV).

Statistics

Data were analyzed using t tests or ANOVA with repeated measures as appropriate, followed by Tukey multiple comparison procedures. Differences were considered significant if P<0.05. All data are presented as mean±SEM.

Results

We used a gene-targeting strategy to selectively delete renin-b while preserving renin-a, by ablating exon 1b, its promoter,
and surrounding sequences in the mouse renin gene, whereas exon 1a and the common portions (exon 2–9) of the renin gene were retained (Figure 1A). The selective deletion of exon 1b in renin-bNull mice was confirmed by Southern blot (Figure S1 in the online-only Data Supplement). Importantly, there was no decrease in survival of renin-bNull mice to adulthood as has been reported for renin-aNull mice.15

Renin-b mRNA has a limited tissue distribution and is the predominant isoform of renin mRNA in the brain.5,15 Thus, renin-bNull mice are essentially brain-specific knockouts of renin-b. Real-time quantitative reverse transcription-polymerase chain reaction established a loss of renin-b mRNA in the brain (Figure 1B), but a preservation of renin-a mRNA in the kidney (Figure 1C). Immunostaining studies revealed normal expression and distribution of renin protein in juxtaglomerular areas in the kidneys of both control and renin-bNull mice (Figure 1D). For reasons explained below, plasma levels of renin and ANG were modestly but significantly decreased in renin-bNull mice (Figure 1E). We conclude that renin-b was selectively eliminated from the brain without altering expression of renin-a in the kidney.

We predicted loss of renin-b to decrease BP. In contrast to our expectation, renin-bNull mice exhibited a significant increase in systolic BP compared with controls (Figure 2). There was no difference in diastolic BP during the day or night between groups. The differential effect on systolic and diastolic BP was reflected in a large increase in pulse pressure in renin-bNull mice (Figure S2). The increased BP likely caused feedback inhibition, which decreased plasma renin and angiotensin peptides as noted above.

There was no difference in baseline heart rate between renin-bNull and control mice (Figure 3A). Power spectral analysis of heart rate variability showed increased indices of sympathetic outflow and decreased indices of parasympathetic tone (Figure 3B). To test this directly, we treated a separate cohort of mice with the β-adrenergic antagonist propranolol and the parasympathetic agonist atropine. We observed a larger bradycardic response after propranolol (Figure 3C) and a blunted tachycardic response induced by atropine in renin-bNull mice (Figure 3D). Baroreflex gain was decreased in renin-bNull mice, indicating impaired baroreflex sensitivity and autonomic dysfunction (Figure 3E).

Direct measurement of SNA subserving the kidney, a key cardiovascular organ, revealed higher baseline renal sympathetic tone in renin-bNull mice (Figure 4A and 4B). A higher resolution recording is shown in Figure S3. The sensitivity of ganglionic blockade to the elevated BP in renin-bNull mice was investigated using a dose of hexamethonium that did not change BP in control mice. Renin-bNull mice exhibited increased sensitivity to hexamethonium as illustrated by a 30-mm Hg decrease in BP (Figure 4C) and a similar decrease in heart rate (data not shown). A comparison of the effect of hexamethonium on systolic, diastolic, and mean BP is shown in Figure S4. Collectively, these data suggest that mechanistically, hypertension in renin-bNull mice is sympathetically mediated.

The increased BP and SNA are phenotypes consistent with increased brain RAS activity. Although antithetical to our initial hypothesis, we next considered the possibility that deletion of renin-bNull in the brain was causing a paradoxical increase in brain RAS activity. To test this, we measured BP in mice chronically treated with the angiotensin-II type 1 (AT1) receptor antagonist losartan or the angiotensin-converting enzyme inhibitor captopril. Chronic intracerebroventricular (ICV) losartan not only abolished the elevated BP in renin-bNull mice but also reduced it below the baseline in untreated or losartan-treated control mice (Figure 5A). Similarly, chronic ICV infusion of captopril caused an exaggerated BP reduction in renin-bNull mice (Figure 5B). Summary data for both treatments are shown in Figure 5C, and a comparison of systolic, diastolic, and mean BP is shown in Figure S5. Interestingly, inhibition of the brain RAS normalized the impairment of baroreflex sensitivity (Figure 5D) and
autonomic nervous function (Figure 5E) in renin-b\textsuperscript{null} mice. We conclude that the hypertension is because of increased activity of the brain RAS in renin-b\textsuperscript{null} mice, likely reflecting increased local synthesis of ANG and action at the AT\textsubscript{1} receptor within the brain.

To identify the potential nuclei underlying the effects evoked by brain RAS activation in renin-b\textsuperscript{null} mice, we measured expression of RAS genes in several cardiovascular control regions of the brain. There was no significant difference in expression of angiotensinogen, prorenin receptor, or Mas receptor mRNA (Table S1 in the online-only Data Supplement). Expression of angiotensin-converting enzyme was increased in the subfornical organ but not in other nuclei. Expression of AT\textsubscript{1a} receptor mRNA was significantly increased in the paraventricular nucleus, and there was a trend for an increase in the rostral ventrolateral medulla (Figure 6A). There was no change in expression of AT\textsubscript{1a} receptor mRNA in the subfornical organ, arcuate nucleus, and nucleus tractus solitarius.

To address the functional importance of increased AT\textsubscript{1a} receptor, we took advantage of our previous observation that increased renal SNA in response to leptin is dependent on AT\textsubscript{1a} receptors in the brain.\textsuperscript{16} Consistent with this, the renal SNA response to ICV leptin was markedly augmented in renin-b\textsuperscript{null} mice compared with controls (Figure 6B and 6C). Importantly, this augmented response was ablated by pretreatment with losartan (Figure 6D). These data suggest that increased sympathoexcitation in renin-b\textsuperscript{null} mice is because of increased activity of AT\textsubscript{1a} receptors.

Finally, given the coordinated regulation of renin-a and renin-b mRNA in the brain of DOCA-salt mice, we considered the possibility that the loss of renin-b mRNA might trigger an increase in renin-a mRNA.\textsuperscript{14} We used a sensitive TaqMan assay designed to detect total renin mRNA instead of an isof orm-specific assay because (1) the level of renin-a mRNA within individual regions of the brain is extremely low and below the level of detection, (2) the assays measuring each renin isof orm selectively are inefficient because the respective exons are small and primers cannot be optimized, and (3) renin-b mRNA is the dominant form under baseline conditions. Thus, any renin mRNA detected in renin-b\textsuperscript{null} mice must be, by definition, renin-a. Like AT\textsubscript{1a} receptor, expression of renin-a mRNA was significantly increased in the rostral ventrolateral medulla, and there was a trend for an increase in the paraventricular nucleus in renin-b\textsuperscript{null} mice (Figure 7A). There was no increase in renin mRNA in the subfornical organ, arcuate nucleus, or nucleus tractus solitarius. Given the increase in renin mRNA, which in renin-b\textsuperscript{null} mice must be renin-a mRNA, we tested whether the hypertensive response is renin-dependent. Acute ICV injection of aliskiren, a renin inhibitor, significantly reduced BP in renin-b\textsuperscript{null} but not control mice (Figure 7B).

**Discussion**

Physiological phenotypes in renin-b\textsuperscript{null} mice, such as hypertension, elevated renal SNA, and the enhanced renal SNA response to leptin, are consistent with increased brain RAS activity.\textsuperscript{2} Deletion of renin-b paradoxically results in increased brain RAS activity strongly suggesting that expression of renin-b acts as an endogenous inhibitor of the brain RAS. Mechanistically, our data suggest that in the brain, expression of renin-b tonically inhibits expression of renin-a, which encodes secreted renin. The processes controlling renin-a in the kidney have been extensively studied, but there are no data
on the regulation or mechanisms of renin-b expression.\textsuperscript{17} That this mechanism occurs naturally is supported by our previous result showing the concomitant increase in renin-a and decrease in renin-b in the brain in DOCA-salt hypertension.\textsuperscript{14} A mechanism controlling the regulation of secreted renin in the brain is conceptually interesting because the ANG precursor and renin substrate, angiotensinogen, is widely expressed in and constitutively released from astrocytes and glia.\textsuperscript{18,19} Thus, the extracellular space is essentially bathed with the precursor to ANG. Renin and angiotensinogen are synthesized in neighboring cells in the rostral ventrolateral medulla.\textsuperscript{13} Deletion of angiotensinogen specifically in the brain of mice and rats decreases BP and SNA.\textsuperscript{20–23} Thus, without a control mechanism, the generation of extracellular ANG may directly
activate AT1 receptors in this region of the brain, leading to increased sympathetic outflow. Consequently, expression of renin-b might provide a counter regulatory control mechanism, preventing widespread conversion of extracellular angiotensinogen to ANG peptides.

Our original provocative hypothesis was that renin-b encodes an intracellular isoform of renin, which functions as part of the cellular machinery synthesizing ANG from angiotensinogen within neurons. Evidence supporting intracellular ANG synthesis could support the hypothesis that ANG functions as a neurotransmitter. The main weakness of the intracellular ANG hypothesis is in modeling how intracellular renin could interact with angiotensinogen within the cell. Angiotensinogen is a secretory protein, whereas renin-b lacks the signal peptide needed for incorporation of the protein into the secretory apparatus. Although we predicted that deletion of an intracellular mediator for ANG synthesis would be associated with decreased BP, it was in fact associated with increased BP. However, an argument can be made that intracellular renin might control an inhibitory neural circuit, and indeed, ANG-dependent inhibitory circuits have been reported. Nevertheless, the strongest data implicating ANG as a neurotransmitter are in the subfornical organ-paraventricular nucleus axis where ANG is strongly stimulatory. Moreover, disinhibition of an inhibitory neural circuit would not be expected to be RAS-dependent. Consequently, our data does not provide supporting evidence for a role for intracellular renin. Rather, our data support a model in which the synthesis of ANG in the brain is controlled at the level of renin transcription (Figure 7C). We hypothesize that under normal conditions, expression of renin-b inhibits expression of renin-a, thus limiting the synthesis and action of ANG (left). In the lifelong absence of renin-b (as in the renin-bNull mice), or in normal mice under conditions where renin-b is suppressed (eg, DOCA-salt hypertension), the renin-a transcript is induced and renin (most likely prorenin) is synthesized and released, which converts angiotensinogen (in the presence of angiotensin-converting enzyme) to ANG with consequent activation of neuronal AT1 receptors. Activation of AT1 receptors in the paraventricular nucleus and rostral ventrolateral medulla increases activity of the sympathetic nervous system. The mechanism converting prorenin to renin in the brain and the requirement, if any, of (pro)renin receptor remains undefined. This model is supported by data showing that AT1 receptor blockade in the brain does not affect BP in normotensive models or under baseline conditions, wherein renin-b inhibits brain RAS activity. Similarly, knockout of renin-a specifically in the brain does not affect BP in the absence of a hypertension-inducing stress because renin-a is already silent. Both data are consistent with a state of renin-b synthesis and tonic inhibition of brain RAS activity under normal conditions. Hypertension-inducing signals, such as DOCA-salt, cause a reprogramming of renin synthesis from renin-b to renin-a leading to local secretion of renin and local ANG synthesis and action.

**Perspectives**

Perhaps the most important question to ask is whether our results have implications for BP control in humans and whether this pathway is involved in human hypertension. Two experimental findings suggest that our results may have applicability to humans. First, the initial identification of renin-b expression in the brain was in a transgenic animal model in which expression of the human renin gene was exquisitely regulated and responsive to physiological cues. This suggests that encoded in the human renin gene is the capacity...
for expression of both renin-a and renin-b. Our second experimental finding is that renin-b (not renin-a) is the primary isoform of renin expressed in human brain. Thus, humans, like mice, express renin-b as the predominant isoform in the brain. Therefore, the main question that remains unresolved is whether the molecular switch we propose herein is active in humans. Future mechanistic studies in cultured neurons or neuronal cell lines may help to address this issue.

Are our findings applicable to human hypertension? It is interesting to note that increased sympathetic outflow has been attributed to be the basis of the failure of antihypertensive therapy in refractory hypertension. Refractory hypertension differs from resistant hypertension. Resistant hypertension is defined as uncontrolled high BP, despite the use of effective doses of 3 or more different classes of antihypertensive agents, including a diuretic. Refractory hypertension defines an extreme phenotype of antihypertensive treatment failure most recently classified by uncontrolled BP with optimal dose of 5 or more different classes of antihypertensive agents, including chlorthalidone and a mineralocorticoid receptor antagonist. Whereas persistent intravascular fluid retention is a characteristic commonly associated with resistant hypertension, this is not the case in refractory hypertension. Instead, increased heart rate has been observed in refractory hypertension suggesting a neurogenic cause. Although RAS blockers effectively lowered BP in renin-b–/– mice, it is important to recall that the drugs were administered intracerebroventricularly and therefore had direct access to the compartment where ANG II was being generated and where it acted. We did not determine whether systemic administration of RAS blockers lowered BP. In retrospect, it would have been interesting to make this assessment. Antihypertensive agents differ in their ability to access the central nervous system through the blood-brain barrier, which may differ if the blood-brain barrier becomes compromised in hypertension. Future studies will clearly be needed to determine whether the renin-b/renin-a regulatory pathway is active in the central nervous system in humans and to determine whether it plays a role in some patients such as those with refractory hypertension. Unfortunately, we expect that assessing the importance of this pathway in humans may be a challenging undertaking.

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Disclosures
None.

References


**Novelty and Significance**

**What Is New?**

- There are 2 forms of renin, renin-a, which is expressed in the kidney and encodes preprorenin, and renin-b, which is expressed in the brain and has been proposed to encode a nonsecreted form of active prorenin.
- We generated a unique mouse model by selectively ablating the brain-specific isoform of renin-b while preserving expression and function of renin-a in kidney.
- Selective deletion of renin-b results in hypertension and increased sympathetic nerve activity because of activation of the brain RAS in response to an isoform switch, which induces renin-a in the absence of renin-b in the brain.

**What Is Relevant?**

- Expression of renin-b provides a sensitive control mechanism, which limits activity of the brain renin-angiotensin system under normal conditions.

**Summary**

These data support a new paradigm for the genetic control of RAS activity in the brain by coordinated regulation of the renin isoforms, with expression of renin-b tonically inhibiting expression of renin-a under baseline conditions. This control mechanism becomes impaired under hypertension-inducing conditions. Impairment of this control mechanism causes neurogenic hypertension.
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Supplemental Materials
1. Detailed Methods and References
2. Supplemental Table S1
3. Supplemental Figures S1-S5
Supplemental Methods

Cardiovascular evaluation. BP and heart rate was recorded by radiotelemetry. Mice were anesthetized with ketamine/xylazine, and a radiotelemetric BP probe (TA11PA-C10, DSI) was implanted into the common carotid artery. The mice were allowed to recover for 7 days. BP and heart rate were recorded for 10 seconds every 5 minutes for 5 consecutive days. Data from each animal were averaged hourly, and corresponding times across the 5 days were averaged for each animal to create a single composite 24-hour tracing for each animal.

Separately, BP and heart rate were recorded continuously for 2 hours (from 9-11 AM) in a higher frequency (2000 Hz) to evaluate the autonomic nervous system activity and the baroreflex sensitivity, as previously described.

Sympathetic and parasympathetic control of heart rate were assessed by autonomic blockade with propranolol (5 mg/kg, i.p.) and methyl-atropine (2 mg/kg, i.p.), respectively. On the following day, the protocol was repeated inverting the sequence of the autonomic blockers. Heart rate was measured for 1 hour before and after each drug was administered starting at 9 AM and end at 11 AM. The values shown are the maximum change in each animal. The effect of ganglionic blockade on BP was tested using hexamethonium bromide (1 mg/kg, i.p.) during the daytime period.

Mice were instrumented with an i.c.v. cannula (Alzet Brain Infusion Kit III) connected to an osmotic minipump for 28 days-treatment (Alzet Model 1004) for chronic i.c.v. administration of losartan (5 µg/h, Sigma-Aldrich, #61188) or captopril (5 µg/h, Sigma-Aldrich, #C4042), 7 days after the radiotelemetry surgery. Under anesthesia with intraperitoneal ketamine/xylazine, the mice were placed in a stereotaxic frame and the cannula was inserted using the following coordinates: 1.0 mm lateral, 0.3 mm caudal to Bregma and 2.5 mm ventral from the dorsal surface of the skull. The cannula was secured to the skull with Vetbond (3M) and dental cement and the minipump was implanted under the dorsal skin. For acute i.c.v. administration of aliskiren (5 µg/2 µl, Tocris Bioscience, #5491), we used a different type of i.c.v. cannula (PlasticsOne Inc.) using the same coordinates as described above. The cannula was anchored in place with a stainless steel machine screw and dental cement. A stylet was inserted to seal the cannula until use.

Spectral Analysis of Heart Rate. Beat-by-beat heart rate time series were derived from the blood pressure waveforms and converted to an equidistant sampling rate using cubic spline interpolation. Those equidistant heart rate time series were subjected to a fast Fourier transform to calculate spectral power in the very low frequency (VLF, 0.02-0.20 Hz), low frequency (LF, 0.2-0.6 Hz, reflecting mainly sympathetic cardiac modulation) and high frequency (HF, 1.0-5.0 Hz, reflecting para-sympathetic cardiac modulation) bands. Relative LF and HF were calculated as the relative value of each power component in proportion to the total power minus the VLF component. Baroreflex sensitivity was determined from the blood pressure basal recording using the sequence technique. Spectral analysis and baroreflex testing were performed using HemoLab software, freely available from the following Website:
Sympathetic nerve responses to intracerebroventricular (i.c.v.) leptin. For the placement of an i.c.v. cannula, mice were anesthetized with intraperitoneal ketamine/xylazine. A stainless steel 25-gauge, 9-mm long cannula was implanted into the left lateral cerebral ventricle under the following coordinates: 1.0 mm lateral, 0.3 mm caudal, and 3.0 mm ventral to Bregma. The cannula was anchored in place with a stainless steel machine screw and dental cement. A stylet was inserted to seal the cannula until use. After 7 days of recovery, sympathetic nerve recording were performed. Mice were anesthetized with ketamine/xylazine and instrumented for measurement of BP and heart rate (left carotid artery catheter) and for maintenance of anesthesia (right jugular vein catheter) with intravenous α-chloralose. Body temperature was maintained at 37.5°C with a temperature controlled surgical table and lamp. The left kidney was exposed and a renal nerve was carefully dissected free and placed on a bipolar 36-gauge platinum-iridium electrode. When an optimal recording of renal SNA was obtained, the electrode was covered with silicone gel (World Precision Instruments). Electrodes were attached to a high-impedance probe (HIP-511, Grass Instruments), and the nerve signal was amplified to $10^5$ times with a Grass P5 AC preamplifier, filtered at a 100 and 1000 Hz cutoff, and routed to a speaker system and to an oscilloscope (model 54501A; Hewlett-Packard) for both auditory and visual confirmation of the nerve signal. The amplified, filtered nerve signal was also directed to a MacLab analog-digital converter (model 8S ADInstruments) to a cursor modular that would count the action potentials (spikes/sec) that exceeded the background noise threshold and to a resetting voltage integrator (RVI, model B600C, University of Iowa Bioengineering) that sums the total voltage output (background noise plus nerve activity) to a unit of 1 V*sec/min before resetting to zero. All surgical preparations were performed by the same individual (DAM). Basal recordings of SNA between the control and the REN-bNull mice were kept consistent using the same recording conditions throughout the study. At the end of the study, the background noise was determined after death and were subtracted from the total SNA. Data were recorded and analyzed using a PowerLab unit and associated Chart software (ADInstruments). Renal SNA, arterial pressure, and heart rate were recorded at baseline and for 4 hours after i.c.v. injection of leptin (5 µg).

Plasma renin and angiotensin II levels. Blood sample was collected into a microcentrifuge tube containing EDTA, gently mixed, and immediately centrifuged for 5 min at 5000 g in 4 °C to isolate plasma from whole blood. Plasma renin and angiotensin II levels were measured by ELISA (ELM-Renin1, RayBiotech for renin; EIAM-ANGII, RayBiotech for angiotensin II).

ELISA protocol for plasma renin: The Mouse Renin ELISA kit is an in vitro enzyme-linked immunosorbent assay. According to the manufacturer, the assay uses an antibody specific for mouse renin coated on a 96-well plate. Standards and samples are pipetted into the plate, the wells are washed and biotinylated anti-mouse Renin antibody is added. After washing, HRP-conjugated streptavidin is added and the wells are washed. TMB substrate solution is added, followed by Stop Solution, and the intensity is measured at 450 nm.
ELISA protocol for plasma angiotensin II: According to the manufacturer, a biotinylated angiotensin II peptide is spiked into samples and standards which are added to the plate, where the biotinylated angiotensin II peptide competes with endogenous (unlabeled) angiotensin II for binding to the anti-angiotensin II antibody. The wells are washed, incubated with horseradish peroxidase (HRP)-streptavidin.

Isolation of specific brain regions and gene expression. Brains were removed and immediately frozen in liquid nitrogen, and each brain was later frozen in Tissue-Tek O.C.T. Compound (Sakura Fintek). In a cryostat, specific brain regions (subfornical organ, paraventricular nucleus, arcuate nucleus, nucleus tractus solitarius, and rostral ventrolateral medulla) were isolated using a brain punch kit (Stoelting) following the coordinates and landmarks described previously. The punch size for SFO, PVN, and NTS are 0.50 mm, and that for ARC and RVLM are 0.25 mm.

Total RNA was extracted using TRizol (Invitrogen). RNA was then treated with DNase I (Invitrogen), and cDNA generated by reverse transcriptase using SuperScript III using oligo-dT as primer (Invitrogen). β-actin was used as internal control. The forward primer for sREN and iCREN mRNA anchors to exon 1a and exon 1b, respectively. In the quantitative RT-PCR, gene expression was determined by the Livak method.

Primers and probes used in this study were as follows.

**RT-PCR primers:**
renin-a
Forward: 5’-ACCTTCAGTCTCCCAACACGCACC-3’
Reverse: 5’-GGGAGGTAAGATTGGTCAAGGAAGG-3’
renin-b
Forward: 5’-TTTGATGAGAGGATACGCATAGCACTTC-3’
Reverse: 5’-GGGAGGTAAGATTGGTCAAGGAAGG-3’

**Quantitative real-time RT-PCR primers and probes:**
Renin-a
Forward: 5’-GCACCTTCAGTCTCCCAACAC-3’
Reverse: 5’-TCCCCGACAGGAAGCATTTC-3’;
renin-b
Forward: 5’-CCGGCTGCTTTGAAGATTTGAT-3’
Reverse: 5’-ATGCCAATCTCGCCGTAGTA-3’
PRR
Forward: 5’-TCTCTCCGAACTGCAAGTGCAACA-3’
Reverse: 5’-CCAAACCTGCCAGCTCCAATGAAT-3’;
β-actin
Forward: 5’-CATCCTCTTCCTCCCTGGAGAAGA-3’
Reverse: 5’-ACAGGATTCCATACCCAAGAAGGAAGG-3’;

Taqman Probe Systems
Total REN probe, Applied Biosystems, Ren1, Mm02342888_gH;
AT1aR probe, Applied Biosystems, Agtr1a, Mm01166161_m1;
β-actin probe, Applied Biosystems, actb, Part# 4532341E.

Southern blotting probes:
5’ probe
Forward 5’-TGCCAGCCAGGGTCAGGT-3’
Reverse 5’-GGCAGGCATGGGGGTGTG-3’

3’ probe
Forward 5’-GTTATGACGAGGGCATCCTCA-3’
Reverse 5’-AAACAAAGGCATGAAGCTGGC-3’.

Renin immunohistochemistry in kidney. Mice were perfused transcardially with Bouin’s solution under deep anesthesia (ketamine/xylazine). The kidneys were removed and post-fixed overnight in the same fixative and embedded in paraffin. 5 µm paraffin sections were cut using a Leica RM 2155 microtome and dried overnight on a hot plate at 42 ºC. Immuno-staining for renin was performed using a 1:500 dilution of rabbit anti-mouse renin polyclonal antibody as previously described.7,8 The antibody is an affinity purified custom anti-renin peptide antibody made in rabbit by Alpha Diagnostic International. This antibody was fully characterized by the Gomez laboratory over the last 13 years. It shows the same pattern as Dr. Inagami’s anti-renin antibodies made in goat or rat. It follows the same developmental pattern as the one observed by in situ hybridization or using fluorescent reporters driving renin expression.9,10 The immunocytochemical signal completely disappears in the total renin KO mouse.11 A rabbit Vectastain ABC kit (Vector Laboratories) was used as previously described.7,8 Normal rabbit IgG (Millipore Calbiochem, cat. # NI01 and Cell Signaling Technology, cat. #2729) was used as a negative control. Images were captured using a Leica CTR HS microscope and LAS 3.8 software.
Supplemental References


Table S1: Expression of RAS Genes in Brain Regions

<table>
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<tr>
<th>Gene</th>
<th>PVN</th>
<th>RVLM</th>
<th>SFO</th>
<th>ARC</th>
<th>NTS</th>
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<td>CON</td>
<td>Ren-b*</td>
<td>CON</td>
<td>Ren-b*</td>
<td>CON</td>
</tr>
<tr>
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<tr>
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<td>1.0±0.2</td>
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<tr>
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<tr>
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<td>1.0±0.4</td>
<td>0.9±0.5</td>
<td>1.0±0.2</td>
</tr>
</tbody>
</table>

*, P<0.05 vs Control

Table S1: Expression of RAS genes in the brain. There was no significant difference in expression of angiotensinogen, prorenin receptor (PRR), or Mas (Ang-(1-7) receptor) mRNA, and ACE2 mRNA was undetectable in all regions. Expression of ACE was only increased in the subfornical organ (SFO). Unpaired t test was used. Control n = 8-10, icREN-KO n = 9-10. All data represent mean ± SEM. PVN, paraventricular nucleus of hypothalamus; RVLM, rostral ventrolateral medulla; SFO, subfornical organ; ARC, arcuate nucleus; NTS, nucleus tractus solitarius.
Figure S1. *Generation of Renin-b<sup>Null</sup> Mice.*
Southern blotting of null and littermate control mice. 5’ and 3’ Southern probes were used to confirm the deletion of exon 1b. EcoR1 digestion produces an 8762 bp band with both the 5’ and 3’ probe in the wildtype renin allele. In the renin-b<sup>Null</sup> allele, EcoR1 digestion generates a 2547 bp band with the 5’ probe and 5232 bp band with the 3’ probe.
Figure S2. Arterial Pressure Phenotyping.
Pulse pressure is plotted hourly and averaged across the light, dark, and 24-hour phases. Shaded areas indicate the dark phase (control n=7, renin-\(b^{\text{Null}}\) mice n=8). *, P<0.05 vs control.
Figure S3. *Basal Renal Nerve Activity in Control and Renin-b<sup>Null</sup> Mice*. Representative raw tracing of renal sympathetic nerve activity in chloralose-anesthetized control and renin-b<sup>Null</sup> mice.
Figure S4. Effect of Ganglionic Blockade
Systolic, mean and diastolic BP responses to ganglionic blockade (control n=5, renin-bNull mice n=8). Base, baseline; HEX, hexamethonium. *P<0.05 renin-bNull vs Control, **P<0.05 vs renin-bNull HEX vs Base. Gray bars are control mice, black bars are renin-bNull mice.
Figure S5. Activation of the Brain RAS.
Effect of chronic RAS inhibition in response to i.c.v. losartan (LOS) and captopril (CAP) on systolic, diastolic and mean BP. Sample size was 5 control vehicle, 6 control LOS, 5 control CAP, 7 renin-bNull vehicle, 6 renin-bNull LOS, 6 renin-bNull CAP. *P < 0.05 vs. control. **P < 0.05 vs. vehicle. Gray bars are control mice, black bars are renin-bNull mice.