Nervous System

Neuron-Specific (Pro)renin Receptor Knockout Prevents the Development of Salt-Sensitive Hypertension

Wencheng Li, Hua Peng, Eamonn P. Mehaffey, Christie D. Kimball, Justin L. Grobe, Jeanette M.G. van Gool, Michelle N. Sullivan, Scott Earley, A.H. Jan Danser, Atsuhiro Ichihara, Yumei Feng

Abstract—The (pro)renin receptor (PRR), which binds both renin and prorenin, is a newly discovered component of the renin–angiotensin system that is highly expressed in the central nervous system. The significance of brain PRRs in mediating local angiotensin II formation and regulating blood pressure remains unclear. The current study was performed to test the hypothesis that PRR-mediated, nonproteolytic activation of prorenin is the main source of angiotensin II in the brain. Thus, PRR knockout in the brain is expected to prevent angiotensin II formation and development of deoxycorticosterone acetate-salt–induced hypertension. A neuron-specific PRR (AT6AP2) knockout mouse model was generated using the Cre-LoxP system. Physiological parameters were recorded by telemetry. PRR expression, detected by immunostaining and reverse transcription–polymerase chain reaction, was significantly decreased in the brains of knockout mice compared with wild-type mice. Intracerebroventricular infusion of mouse prorenin increased blood pressure and angiotensin II formation in wild-type mice. This hypertensive response was abolished in PRR-knockout mice in association with a reduction in angiotensin II levels. Deoxycorticosterone acetate-salt increased PRR expression and angiotensin II formation in the brains of wild-type mice, an effect that was attenuated in PRR-knockout mice. PRR knockout in neurons prevented the development of deoxycorticosterone acetate-salt–induced hypertension as well as activation of cardiac and vasomotor sympathetic tone. In conclusion, nonproteolytic activation of prorenin through binding to the PRR mediates angiotensin II formation in the brain. Neuron-specific PRR knockout prevents the development of deoxycorticosterone acetate-salt–induced hypertension, possibly through diminished angiotensin II formation. (Hypertension. 2014;63:316-323.) ● Online Data Supplement

Key Words: angiotensin II ▪ central nervous system ▪ hypertension ▪ (pro)renin receptor

The brain renin–angiotensin system (RAS) plays an essential role in hypertension, mainly through the modulation of autonomic and neural endocrine activities. Angiotensin (Ang) II, the major bioactive peptide of the RAS, is unable to permeate the blood–brain barrier. Furthermore, brain Ang II content is significantly increased in bilaterally nephrectomized rats despite the fact that plasma Ang II is decreased to a low level. These observations suggest that brain Ang II is synthesized locally and can be regulated independently of peripheral Ang II. However, renin activity is extremely low in the central nervous system (CNS), although renin mRNA and its precursor prorenin are present. The (pro)renin receptor (PRR), which binds both renin and prorenin, is a newly discovered component of the RAS that is highly expressed in the brain. Binding of prorenin to the PRR leads to nonproteolytic activation of prorenin and mediates Ang II formation in vitro. These new findings suggest a possible alternative mechanism of Ang II formation in the brain without active renin.

Deoxycorticosterone acetate (DOCA)-salt–induced hypertension is a commonly used model to mimic low-renin hypertension, which accounts for 27% of essential hypertension in humans. The combination of DOCA and high salt markedly suppresses peripheral RAS in this model. In contrast, brain RAS signaling is elevated in this model, and several groups have demonstrated its importance in eliciting and maintaining DOCA-salt hypertension. We and others have reported that brain PRR knockout attenuates Ang II–dependent hypertension. However, the role of PRR in the DOCA-salt hypertension remains unknown.
In this study, we used a neuron-specific PRR (ATP6AP2)-knockout mouse model to investigate the function of brain PRRs in DOCA-salt hypertension. We demonstrated that intracerebroventricular infusion of prorenin induced robust pressor responses and promoted Ang II formation in wild-type (WT) mice. PRR deletion prevented the hypertensive response to prorenin and DOCA-salt–induced hypertension through inhibition of Ang II formation. These findings indicate that neuronal PRR-mediated, nonproteolytic activation of prorenin may be a major source of Ang II in the CNS.

Methods
An expanded Methods section is available in the online-only Data Supplement.

Animals
All animal procedures were approved by Institutional Animal Care and Use Committees at Tulane University School of Medicine and Colorado State University, and were in accordance with the National Institutes of Health Guidelines for the care and use of experimental animals.

Generation of Neuron-Specific PRR Conditional Knockout Mice
The PRR-floxed mouse was generated in Dr Atsuhiro Ichihara’s laboratory. Briefly, the exon 2 of PRR gene was flanked by loxP sites. Female PRR-floxed mice were bred with male Nefh-Cre+ mice (The Jackson Laboratory). The Nefh-Cre+ mice expressed the Cre-recombinase after embryonic day 18.5 under the control of the neuron filament promoter (Nefh). The resulting PRRloxp/y, Nefh-cre+ mice represent the neuron-specific PRR-knockout (Nefh-PRRKO) mice. The control mice (WT) were positive for Nefh-cre with WT PRR genes, thereby excluding Cre-mediated toxicity as the basis for phenotypic disparity.

Results
Characterization of Nefh-PRRKO Mice
Confocal imaging showed that the PRR was present on the neuronal plasma membrane and within the cytoplasm in brain tissue sections (Figure 1) with the relative distribution depending on preparation conditions. When Triton permeabilization of the plasma membrane was omitted, PRRs were primarily localized to the plasma membrane (Figure 1C, 1D, and 1G), with a membrane:cytoplasm intensity ratio of 5.2±0.4 (Figure 1H). In cells permeabilized with 0.2% Triton before immunostaining, the PRRs were detected in both cell membrane and cytoplasm (Figure 1E–1G), with a membrane:cytoplasm ratio of 2.6±0.2 (Figure 1H).

PRR deletion in neurons significantly reduced PRR mRNA levels in regions throughout the brain (Figure 2A). Double immunostaining of Nefh-PRRKO mice for PRR (red) and Cre-recombinase (green) revealed that the PRRs were absent in regions where Cre-recombinase was expressed (Figure 2D and 2E); this staining pattern was not observed in the WT (Nefh-Cre) mice, which showed overlapping expression of Cre-recombinase and PRRs (Figure 2B and 2C). PRR protein levels in Nefh-PRRKO mice were reduced in the subfornical organ, paraventricular nucleus, and rostral ventrolateral medulla (Figure 2I–2K) as well as other brain regions (Figure S1 in the online-only Data Supplement) compared with WT mice, which showed comparable expression of Cre-recombinase (Figure 2F–2H). Nefh-PRRKO mice exhibited normal baseline blood pressure (BP), heart rate (HR), locomotor activity, and body weight (Figure 3). Total neuron numbers, determined by counting cells positive for the neuron-specific nuclear protein, NeuN, were similar between Nefh-PRRKO and WT mice (Figure S2).

Neuron-Specific PRR Deletion Prevents the Prorenin-Induced Pressor Response and Ang II Formation
Intracerebroventricular infusion of artificial cerebrospinal fluid, used as a control, did not change BP or HR in either Nefh-PRRKO or WT mice (Figure 4A). Intracerebroventricular infusion of the acetylcholine receptor agonist carbachol increased BP and HR to a similar degree in both Nefh-PRRKO and WT mice (Figure 4A). Intracerebroventricular infusion of Ang II also similarly increased BP and HR in both groups, an effect that was blocked by the Ang II type 1 receptor antagonist losartan.
Intracerebroventricular infusion of mouse prorenin increased BP and HR in WT mice. This mouse prorenin-mediated pressor response was completely blocked by losartan or the angiotensin-converting enzyme inhibitor captopril. Interestingly, intracerebroventricular infusion of mouse prorenin did not increase BP or HR in Nefh-PRRKO mice (Figure 4C). Furthermore, intracerebroventricular infusion of mouse renin increased BP and HR similarly in both WT and Nefh-PRRKO mice (Figure 4D). These renin-mediated pressor effects were abolished by intracerebroventricular infusion of losartan or captopril.

Baseline Ang II levels were significantly decreased in the brain cortex, hypothalamus, and brain stem of Nefh-PRRKO mice compared with WT mice (Figure 4E). Intracerebroventricular infusion of mouse prorenin significantly increased Ang II levels in all 3 regions of WT mice, with the highest level observed in the hypothalamus. Intracerebroventricular infusion of prorenin also elevated Ang II levels in Nefh-PRRKO mice; however, Ang II levels remained significantly lower than those in WT mice. (Pro)renin (renin+prorenin) concentration and distribution in these regions after intracerebroventricular infusion of prorenin were measured by ELISA. Immunoreactive (pro)renin increased in all 3 regions after 10 minutes of intracerebroventricular infusion, with the highest level observed in the hypothalamus (Figure S3A).
Neuron-Specific PRR Deletion Prevents the Development of DOCA-Salt–Induced Hypertension

Three weeks of DOCA-salt treatment significantly upregulated PRR mRNA levels in various brain regions of WT mice (Figure 5A). Although DOCA-salt is known to markedly suppress plasma renin activity,14,21 we observed an increase in (pro)renin levels in the hypothalamus and brain stem after DOCA-salt treatment (Figure 5E) when measured by ELISA; no such
increase was seen using an enzyme kinetic assay for (pro)renin (Figure 5F). Brain Ang II levels were significantly increased in the cortex, hypothalamus, and brain stem after DOCA-salt treatment in WT mice (Figure 5B) despite a reduction of Ang II in kidney and plasma (Figure 5C and 5D). Ang II levels were significantly lower in the brains of Nefh-PRRKO mice compared with WT mice after 3 weeks of DOCA-salt treatment.

DOCA-salt treatment gradually increased BP in WT mice and maintained hypertensive levels until the end of the experiment (Figure 6A). Chronic intracerebroventricular infusion of losartan prevented the hypertension induced by DOCA-salt in WT mice. Interestingly, BP remained at normotensive levels throughout the protocol in Nefh-PRRKO mice. After DOCA-salt treatment, HR trended lower in Nefh-PRRKO and losartan-treated mice compared with WT mice, but these differences did not reach statistical significance (Figure 6B). Baseline cardiac (Figure 6C) and vasomotor (Figure 6D) sympathetic tone, cardiac parasympathetic tone (Figure 6E), and spontaneous baroreflex sensitivity (Figure 6F) were similar between WT and Nefh-PRRKO mice. DOCA-salt treatment significantly increased cardiac and vasomotor sympathetic tone, and decreased parasympathetic tone and spontaneous baroreflex sensitivity in WT mice. PRR knockout attenuated the change in autonomic function and spontaneous baroreflex sensitivity induced by DOCA-salt treatment, without altering the intrinsic HR (Figure S4).

Discussion

Although studies have shown the existence of Ang II in neuron bodies and fibers, its origin in the CNS remains a matter of controversy. The present study demonstrated that the PRR is a key factor in the formation of Ang II in the brain. The main findings of this study can be summarized as follows: (1) neuron-specific PRR knockout decreases baseline Ang II levels in the brain although cardiovascular parameters remain normal. (2) Intracerebroventricular infusion of prorenin elicits an Ang II–dependent pressor response via binding to the PRR. (3) DOCA-salt stimulation increases PRR expression and Ang II formation in the brain. (4) PRR knockout in neurons prevents the development of DOCA-salt hypertension by diminishing Ang II formation.

Global PRR knockout is lethal in mice, indicating an essential role of the PRR in embryonic development. Recently, several tissue-specific PRR-knockout mouse models have been generated; studies based on these mice indicate that the PRR is essential for embryonic development of the heart and kidney. In our animal model, Cre-recombinase was expressed after E18.5, circumventing the effects of PRR deletion during CNS development. These novel Nefh-PRRKO mice exhibited normal BP, HR, locomotor activity, autonomic function, and spontaneous baroreflex sensitivity. The difference in phenotypes between neuron-specific knockout and other cell-type knockouts probably reflects the early embryonic development of the CNS and the relatively late expression of Cre-recombinase in our model. Real-time reverse
transcription–polymerase chain reaction analyses of brain lysates showed a 40% to 50% reduction in PRR mRNA in Nefh-PRRKO mice. One possible explanation for this partial knockout is nonuniform Cre-recombinase expression; consistent with this, 76.3% to 87.3% of neurons in the brain stem, thalamus nucleus, and cerebral cortex of Nefh-Cre mice are reported to express Cre-recombinase. It is also possible that other cell types in the brain express PRR, and a truncated PRR mRNA still exists in the neurons.

Although the brain renin activity is extremely low, prorenin has been detected and successfully isolated from the brain. Methot et al reported that prorenin can be activated within pituitary glands nonproteolytically although the actual mechanism remains unknown. In the present study, we demonstrated that intracerebroventricular infusion of prorenin promotes Ang II formation and a pressor response through binding to the PRR. Prorenin was previously recognized as a precursor of renin, which can be converted to active renin only in the juxtaglomerular cells of the kidney. To confirm that the pressor response induced by intracerebroventricular infusion of mouse prorenin was not because of the unexpected conversion of prorenin to renin in the brain, we intracerebroventricularly infused mouse renin into both WT and Nefh-PRRKO mice. Renin infusion similarly increased BP in both WT and Nefh-PRRKO mice, whereas the prorenin increased BP only in WT mice, indicating that the pressor response to intracerebroventricular prorenin infusion was not because of a conversion of prorenin to renin but possibly reflects nonproteolytic activation via binding to the PRR. Evidence for nonproteolytic activation of PRR-bound prorenin has been previously reported for human proteins by Batenburg et al. Determining whether this is also true for mouse prorenin after binding to the mouse PRR requires assays that can distinguish open and closed prorenin. Unfortunately, such assays are currently not available for mouse prorenin. Evidence for the existence of open (ie, nonproteolytically activated) prorenin in rats from immunohistochemistry studies has been obtained by Ichihara et al. In the present study, we attempted to measure brain (pro)renin (ie, renin+prorenin) using both a commercial ELISA, which does not distinguish between renin and prorenin, and an in-house enzyme kinetic assay, which allows the measurement of renin activity before and after proteolytic prorenin activation, and thus provides information on the renin/prorenin ratio. Interestingly, although the enzyme kinetic assay confirmed that the majority of total brain renin was prorenin, it did not allow the detection of increased (pro)renin levels after DOCA-salt exposure, whereas the ELISA did. The reason for this difference is not clear. One possible explanation is that homogenizing the tissue to measure Ang I–generating activity destroys any link between prorenin and the PRR, preventing the detection of the increased prorenin activity by enzyme kinetic assay.

Although baseline brain Ang II levels were significantly decreased in the Nefh-PRRKO mice, BP, HR, and autonomic
function remained normal. This is not surprising given previous reports that intracerebroventricular infusion of Ang II type 1 receptor or an angiotensin-converting enzyme inhibitor does not alter BP or HR under physiological condition.34,35 Intracerebroventricular infusion of prorenin led to a slight increase in Ang II levels in Nefh-PRRKO mice compared with baseline, indicating that PRR expressed in other cell types, including the astroglia,29 the vascular endothelium36 or smooth muscle cells,37 may partially contribute to Ang II formation during prorenin infusion. In addition, the Nehf promoter is variably expressed in different brain regions including the cortex, hippocampus, hypothalamus, and brain stem,20 possible accounting for the increase in Ang II in Nehf-PRRKO mice. Nevertheless, our findings indicate that nonproteolytic activation of prorenin via neuronal PRRs may be a new mechanism for Ang II formation in the CNS. This conclusion is further supported by a previous report17 showing the localization of renin-expressing cells in the brain in regions controlling cardiovascular function, such as the subfornical organ, rostral ventrolateral medulla, and paraventricular nucleus, where the PRR is expressed.

We previously reported that PRR knockdown in the brain attenuates Ang II–induced hypertension in human renin–angiotensinogen double-transgenic mice,17 in association with a decrease in Ang II formation.29 In the present study, the DOCA-salt hypertensive model was used to determine whether brain Ang II formation in this pathophysiological condition is mediated by the PRR. It has previously been reported that activation of brain Ang II type 1 receptor is essential for the increased sympathetic activity and development of hypertension in the DOCA-salt model.15,16 A unique feature of the DOCA-salt model is that, despite overactivation of brain RAS, circulating RAS is suppressed,13,38 providing an ideal model for studying endogenous brain RAS activity without interference from circulating Ang II. Our data show that DOCA-salt treatment increased PRR expression and immunoreactive (pro)renin levels in the brain. This resulted in increased brain Ang II levels, despite the fact that we did not detect increased (pro)renin activity. As discussed above, these findings support the concept that the rise in brain immunoreactive (pro)renin predominantly concerns prorenin, the activity of which is unknown but might well depend on binding to the PRR. Indeed, the increase in Ang II was abolished in the Nehf-PRRKO mice during DOCA-salt hypertension because of the loss of PRR in the neurons. This observation supports our hypothesis that the neuronal PRR is a key factor for the Ang II formation in this pathophysiological condition. Although the mechanism by which PRR expression in the brain is upregulated during DOCA-salt hypertension needs further investigation, we and others previously reported that Ang II is a strong stimulator of PRR expression,21,39 suggesting positive feedback augmentation between Ang II formation and PRR expression.

In conclusion, prorenin increases BP through Ang II formation in the brain without being converted to renin, possibly through a nonproteolytic activation by binding to the PRR. Neuron-specific PRR knockout prevents the development of DOCA-salt hypertension by diminishing of Ang II formation in the CNS. PRR blockade in the CNS may represent a novel approach for treating hypertension and other cardiovascular diseases involving brain RAS activation.

**Perspectives**

Previous studies have shown that overactivation of brain RAS, especially activation of Ang II/Ang II type 1 receptor signaling, leads to autonomic dysfunction and the development of neurogenic hypertension. However, how brain angiotensin peptides formation is initiated has remained a missing piece of the puzzle. The present study provides a potential answer, by demonstrating that binding of prorenin to the PRR mediates Ang II formation and induces a pressor response in mice, suggesting a novel, and alternative pathway for Ang II formation in the CNS. Under pathophysiological conditions, knockout of the PRR in neurons prevents the development of hypertension, diminishes Ang II generation, and improves autonomic function. These findings support the pursuit of pharmacological PRR blockade in the CNS as a novel approach for the treatment of neurogenic hypertension.

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**Disclosures**

None.

**References**


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Neuron-specific (pro)renin receptor knockout prevents the development of salt-sensitive hypertension

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Methods

Animals

All animal procedures were approved by Institutional Animal Care and Use Committees at Tulane University School of Medicine and were performed in accordance with the National Institutes of Health Guidelines for the care and use of experimental animals.

Generation of neuron-specific PRR conditional knockout mice

The PRR-floxed mouse was generated in Dr. Atsuhiro Ichihara’s laboratory. Briefly, exon 2 of the PRR gene was flanked by loxP sites. Female PRR-floxed mice were bred with male Nefh-Cre mice (The Jackson Laboratory). Nefh-Cre mice expressed Cre-recombinase after embryonic day 18.5 under the control of the neuron filament promoter (Nefh). The resulting PRR<sup>loxp/loxp</sup>, Nefh-cre<sup>+</sup> mice correspond to neuron-specific PRR-knockout (Nefh-PRRKO) mice. Control wild-type (WT) mice were positive for Nefh-Cre and possessed wild-type PRR genes, thereby excluding Cre-mediated toxicity as the basis for phenotypic disparity.

RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

Brain nuclei, including the subfornical organ (SFO), paraventricular nucleus (PVN), nucleus tractus solitarius (NTS) and rostral ventrolateral medulla (RVLM), were micro-punched using a cryostat, and total RNA was isolated using an RNeasy mini kit (Qiagen Technologies, Hilden, Germany) as described by the manufacturer. cDNA was synthesized from 200 ng total RNA, quantified spectrophotometrically (Thermo Scientific Nanodrop 2000), using a cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Specific primers for mouse PRR (Fwd: 5'-TCT CTC CGA ACT GCA AGT GCA ACA-3'; Rev: 5'-CCA AAC CTG CCA GCT CCA ATG AAT-3') and internal control mouse GAPDH (Fwd: 5'-AAT GTG TCC GTC GTG GAT CTG A-3'; Rev: 5'-GAT GCC TGC TTC ACC ACC TTC T-3') were designed using PrimerQuest Software (Integrated DNA Technologies, Coralville, IA). Real-time RT-PCR was performed on an Mx3000P System (Stratagene, La Jolla, CA) using SYBR green qPCR master mix (USB Corporation, Cleveland, OH) following the manufacturer’s instructions. Cycling conditions were 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 60 s at 60°C, and 30 s at 72°C. The expression levels of target mRNAs were normalized to those of GAPDH mRNA in the same reaction and expressed relative to the average normalized values of PRR levels in WT mice.

Immunofluorescence staining

Brain tissues were harvested after transcardial perfusion with 4% paraformaldehyde and then fixed in 10% formalin overnight. After embedding in paraffin, brains were sectioned at 5-µm thickness and mounted onto slides. Sections were deparaffinized by heating the slides in an oven at 60°C for 1 hr. Slides were rehydrated using the following washes: two Xylene washed for 10 min each, 100% ethanol for 2 min, 95% ethanol for 2 min, 70% ethanol for 2 min, 50% ethanol for 2 min, 30% ethanol for 2 min and double-distilled water for 2 min. Antigen was recovered by boiling the slides in Na-
citrate buffer (10 mM, pH 6.5) for 45 min. After non-specific binding was blocked with 10% goat serum for 30 min, sections were incubated with a rabbit anti-mouse PRR antibody designed and made in-house\textsuperscript{3} (diluted 1:400 in phosphate-buffered saline containing 0.2% Triton) and anti-Cre mAb\textsuperscript{4} (diluted 1:100 in PBST) (COVANCE, Richmond, CA) for 48 hours at 4°C. This was followed by incubation with fluorescence-conjugated goat anti-rabbit antibody (Alexa 594 and Alexa 488, 1:1000; Invitrogen, Carlsbad, CA) at room temperature for 1 hr. Images were captured with a fluorescence microscope. The specificity of PRR and Cre staining was assessed by incubation of control sections without primary antibodies or by preabsorption with a blocking peptide.

**ICV cannulation**

Nefh-PRRKO and WT mice were anesthetized by isoflurane inhalation and placed in a stereotaxic apparatus. A 25-gauge stainless steel guide cannula was implanted in the lateral ventricle of the brain with the following coordinates: 0.3 mm posterior and 1 mm lateral relative to bregma and a depth of 3 mm. The cannula was fixed onto the skull using Loctite 454 (ALZET, Cupertino, CA). A 32-gauge inner needle was placed inside the cannula for ICV delivery. ICV infusion was verified pharmacologically based on the pressor response to carbachol (50 ng) and histologically after completion of the protocol.

**Deoxycorticosterone acetate (DOCA)-salt hypertension model and osmotic minipump implantation**

Mice were anesthetized by isoflurane inhalation and then subcutaneously implanted with a 50-mg pellet of DOCA (21-day release; Innovative Research of America) or sham implanted. After recovery from anesthesia, animals were housed singly in standard forced-air shoebox cages. Control animals were maintained on standard chow and provided ad libitum access to tap water. DOCA animals were maintained on standard chow and provided ad libitum access to a 0.15 mol/L (0.9%) NaCl solution. For the losartan-treated group, osmotic minipumps (Alzet) were subcutaneously implanted simultaneously with the DOCA pellet and connected to the ICV cannula to infuse the drug into the lateral ventricle.

**Telemetry recordings**

Nefh-PRRKO and WT mice (14-16 wk old) were anesthetized by isoflurane inhalation and instrumented with a radiotelemetry transmitter into the carotid artery as described previously\textsuperscript{5, 6}. After a 14-d recovery period, baseline blood pressure (BP), heart rate (HR), and locomotor activity were recorded. Autonomic function was assessed using a standard pharmacological method involving random intraperitoneal injection of a β-blocker (propranolol, 6 mg/kg), a muscarinic receptor blocker (methylatropine, 1 mg/kg) and a ganglionic blocker (chlorisondamine, 5 mg/kg)\textsuperscript{7}. Changes in HR to propranolol and methylatropine and changes in BP to chlorisondamine were calculated following administration of the antagonists. Spontaneous baroreflex sensitivity (SBRS) was calculated using sequence methods\textsuperscript{8, 9}. For acute studies, mice were ICV-infused with Ang II, prorenin, or renin (at a concentration of 100 ng/µl) through the cannula at a rate of 0.3 µl/min for 10 min. BP was continuously recorded in conscious, freely moving mice during the ICV infusion. For chronic studies, Nefh-PRRKO and WT mice were treated
with DOCA (50 mg) and 0.9% NaCl in the drinking water for 3 wk. A separate cohort of WT mice underwent ICV cannulation and connection to subcutaneous osmotic minipumps (Alzet) for chronic infusion of losartan (3 mg/kg/d; Sigma) into the lateral cerebral ventricle during DOCA-salt treatment.

**Ang II and (pro)renin measurements**

Ang II concentrations in the brain, kidney, and plasma samples were assayed with a commercially available enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals, Belmont, CA) as described by the manufacturer. Briefly, samples were immersed in cold methanol (100%) and homogenized immediately upon harvesting. Plasma and tissue homogenates were centrifuged, and the supernatants were dried overnight in a vacuum centrifuge. The dried fraction was re-suspended in EIA buffer, and Ang II levels were quantitated according to the manufacturer’s instructions. The concentrations of total renin in various brain areas following mouse prorenin infusion were quantified with a mouse total renin enzyme-linked immunosorbent assay (Molecular Innovations, Novi, MI). This assay does not distinguish between renin and prorenin; thus, total renin corresponds to renin + prorenin and is denoted as (pro)renin.

**Statistical analysis**

Data are expressed as means ±SEM. Data were analyzed by Student’s t-test or two-way analysis of variance (with Bonferroni post hoc tests to compare replicate means), as appropriate. Statistical comparisons were performed using Prism5 (GraphPad Software, CA). Differences were considered statistically significant at P<0.05.

**Reference**


Figure S1. PRR expression in neurons and was decreased in Nefh-PRRKO mice. Double immunostaining for the PRR (Red) and NeuN (Green, A-C) in the mouse brain sections. Double immunostaining for the PRR (Red) and Cre-recombinase (green, D-I) in the area postrema, brain cortex, and nucleus tractus solitaries of WT (D, E and F) and Nefh-PRRKO (G, H and I) mice.
Figure S2. Neuron numbers were the same in WT and Nefh-PRRKO mice. WT and Nefh-PRRKO mouse brains were stained with a neuron-specific nuclear protein antibody. Positive staining was quantified. N=5 mice/group.
Figure S3. ICV infusion of prorenin increased (pro)renin levels in the brain. (Pro)renin concentration was measured in the brain cortex, hypothalamus, and brainstem after ICV infusion of artificial CSF (aCSF) or mouse prorenin for 10 min. This assay does not distinguish between renin and prorenin, which are denoted collectively as (pro)renin). *P<0.05 vs. aCSF ICV; n=5/group.
Figure S4. Intrinsic HR did not change after DOCA-salt treatment. Intrinsic HR was measured after simultaneous injection of propranolol and methylatropine. No significant difference was observed among WT, Nefh-PRRKO, WT+DOCA-salt, and Nefh-PRRKO+DOCA-salt mice. WT mice, n=7; Nefh-PRRKO mice, n=9.