Brain-Targeted (Pro)renin Receptor Knockdown Attenuates Angiotensin II–Dependent Hypertension

Wencheng Li, Hua Peng, Theresa Cao, Ryosuke Sato, Sarah J. McDaniels, Hiroyuki Kobori, L. Gabriel Navar, Yumei Feng

Abstract—The (pro)renin receptor is a newly discovered member of the brain renin-angiotensin system. To investigate the role of brain (pro)renin receptor in hypertension, adeno-associated virus-mediated (pro)renin receptor short hairpin RNA was used to knockdown (pro)renin receptor expression in the brain of nontransgenic normotensive and human renin-angiotensinogen double-transgenic hypertensive mice. Blood pressure was monitored using implanted telemetric probes in conscious animals. Real-time PCR and immunostaining were performed to determine (pro)renin receptor, angiotensin II type 1 receptor, and vasopressin mRNA levels. Plasma vasopressin levels were determined by ELISA. Double-transgenic mice exhibited higher blood pressure, elevated cardiac and vascular sympathetic tone, and impaired spontaneous baroreflex sensitivity. Intracerebroventricular delivery of (pro)renin receptor short-hairpin RNA significantly reduced blood pressure, cardiac and vasomotor sympathetic tone, and improved baroreflex sensitivity compared with the control virus treatment in double-transgenic mice. (Pro)renin receptor knockdown significantly reduced angiotensin II type 1 receptor and vasopressin levels in double-transgenic mice. These data indicate that (pro)renin receptor knockdown in the brain attenuates angiotensin II–dependent hypertension and is associated with a decrease in sympathetic tone and an improvement of the baroreflex sensitivity. In addition, brain-targeted (pro)renin receptor knockdown is associated with downregulation of angiotensin II type 1 receptor and vasopressin levels. We conclude that central (pro)renin receptor contributes to the pathogenesis of hypertension in human renin-angiotensinogen transgenic mice. (Hypertension. 2012;59:1188-1194.) ● Online Data Supplement

Key Words: hypertension ■ (pro)renin receptor ■ adeno-associated virus ■ renin-angiotensin system ■ central nervous system

Many studies demonstrate the importance of the brain renin-angiotensin (Ang) system (RAS) in the development of hypertension. However, uncertainty regarding the functionality of the intrinsic brain RAS persists because the brain renin activity may not be sufficient to effectively catalyze formation of Ang II. Recent studies show that renin and its precursor prorenin can bind the (pro)renin receptor (PRR), suggesting a more complex contribution of renin and prorenin via PRR. The PRR gene is widely expressed in the brain, heart, kidney, liver, and pancreas. Binding of renin and prorenin to the PRR increases the catalytic efficiency for Ang II formation and initiates Ang II–independent intracellular signaling pathways. Transgenic rats with human PRR expression targeted to vascular smooth muscle cells exhibit hypertension and increased plasma aldosterone, suggesting a pathological role of the PRR in raising blood pressure (BP).

Ubiquitous expression of the human PRR in rats results in proteinuria, glomerulosclerosis, and cyclooxygenase 2 up-regulation. Inhibition of the PRR by a “handle” region peptide prevents the development of nephropathy in diabetic rats and attenuates the development of cardiac fibrosis in spontaneously hypertensive rats.

Despite evidence of PRR involvement in cardiac, renal, and vascular pathophysiology, the role of the neural PRR in the central regulation of BP and cardiovascular function remains largely unknown. In the present study, we found that PRR expression is upregulated in the subfornical organ (SFO) and the paraventricular nucleus (PVN) of human renin-angiotensinogen transgenic (RA) hypertensive mice. Brain-targeted PRR knockdown attenuates hypertension and is associated with downregulation of Ang II type 1 (AT1) receptor and arginine vasopressin (AVP) levels.

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

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Animal
Human renin and human angiotensinogen transgenic mice were generated at the University of Iowa Transgenic Animal Facilities (a generous gift from Dr Curt D. Sigmund). Double-transgenic mice (RA) expressing both human renin and AGT transgenes were generated by breeding human renin mice with human angiotensinogen mice. RA mice and nontransgenic (NT) litters were used in this study. All of the procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees at Tulane University School of Medicine.

Construction and Production of Adeno-Associated Virus-PRR-Short-Hairpin RNA
Short-hairpin RNA (shRNA) was designed to target mRNA encoding for mouse PRR. Targets were ligated and cloned into an adeno-associated virus 2 vector tagged with green fluorescent protein (GFP). All of the vectors were packaged in the University of Iowa Gene Transfer Vector Core using the baculovirus system.

Physiological Recordings
RA mice and NT mice were anesthetized and instrumented with radiotelemetry transmitters. After a 14-day recovery, mice underwent ICV injection with 100 nL of adeno-associated virus (AAV)-PRR-shRNA or AAV-enhanced GFP (eGFP). BP was continuously recorded in conscious mice for 2 weeks. Autonomic function was assessed by determining BP or heart rate response to β-receptor blockade, muscarinic receptor blockade, and ganglionic blockade. Spontaneous baroreflex sensitivity (SBRS) was calculated using the “sequence method.”

RNA Isolation and Real-Time RT-PCR
Brain nuclei, including the subfornical organ (SFO), paraventricular nucleus (PVN), nucleus of the tractus solitarius (NTS), and rostral ventrolateral medulla (RVLM), were micropunched from NT and RA mice to isolate total RNA. Specific primers for mouse PRR, mouse AT1 receptor, mouse AVP, and, as an internal control, mouse GAPDH, were designed using PrimerQuest Software (Integrated DNA Technologies, Coralville, IA). The expression levels of targeted mRNAs were normalized based on the expression levels of GAPDH mRNA.

Immunofluorescence
Brain tissues were harvested and processed for PRR, AT1 receptor, neuron-specific nuclear protein,12 and glial fibrillary acidic protein13 staining. Quantification was performed using National Institutes of health Image J software in a blinded manner.

Western Blot Analysis
Brain tissues were harvested and lysed. Protein lysates (25 μg) were used for SDS-PAGE and blotting. Quantification was performed using National Institutes of Health Image J software in a blinded manner. The expression levels of targeted proteins were normalized based on the expression levels of β-actin protein.

ELISA
Peptide was extracted and concentrated from plasma using a Sep-Pak extraction kit (Waters Corporation). Plasma vasopressin concentrations were measured using AVP Fluorescent Immunoassay kit (Phoenix Pharmaceuticals).

Statistical Analysis
Data are expressed as mean±SEM and analyzed by Student t test (2 groups) or 2-way ANOVA (multiple groups) followed by a Bonferroni post hoc test to compare replicate means when appropriate. Statistical comparisons were performed using Prism5 (GraphPad Software). Differences were considered statistically significant at P<0.05.

Results

PRR Is Expressed in Neurons and Upregulated in Ang II–Dependent Hypertensive Mice
Double immunostaining for PRR and neuron-specific nuclear protein or glial fibrillary acidic protein revealed that the PRR was mainly located in neurons (Figure 1A through 1F). The PRR was detected throughout the brain in cardiovascular regions including the SFO, PVN, nucleus of raphe pallidus, NTS, and RVLM, as well as in noncardiovascular regions, such as brain cortex (Figure 1G through 1L). Sections incubated with preabsorbed peptide showed a complete lack of immunostaining (Figure S2, available in the online-only Data Supplement), confirming the specificity of PRR staining.

To examine PRR expression in normotensive and hypertensive conditions, coronal brain sections were immunostained with PRR antibody. Quantification of immunostaining showed that PRR expression was upregulated in the SFO and PVN of RA mice compared with NT mice (Figure 2A through 2C and 2E through 2G; P<0.01). PRR mRNA levels in the SFO and PVN were also significantly increased in the RA mice compared with NT mice (Figure 2D and 2H; P<0.01). Other areas of the brain, including the NTS, RVLM, and cortex, did not exhibit significant differences in PRR expression between RA and NT mice (Figure S3). PRR mRNA expression was not uniform throughout the brain. PRR levels were significantly higher in the SFO and PVN and lower in the NTS compared with those in the cortex (Figure S7).

AAV-PRR-shRNA Decreases PRR Expression Both in Neuronal Cells and SFO of the Brain
AAV-PRR-shRNA decreased PRR mRNA and protein expression by 60% to 70% compared with control virus in Neuro-2A cells (P<0.01; Figure 3A and 3B). We observed high levels of GFP fluorescence 3 days after virus infection in Neuro-2A cells (Figure 3C) and in the SFO (Figure 3D) after ICV administration of virus. A less intense GFP staining was observed in the ependymal cells of the lateral and third ventricular walls but not in other regions, including cortex, PVN, NTS, RVLM, median preoptic nucleus, vascular organ of the lamina terminalis, and area postrema (Figure S4).

PRR levels were measured 2 weeks after AAV-PRR-shRNA or AAV-eGFP administration to further confirm PRR knockdown efficiency in vivo. ICV injection of AAV-PRR-shRNA significantly reduced PRR mRNA levels in the SFO of both NT and RA mice compared with AAV-eGFP treatment (P<0.05; Figure 4A). Although the PVN also exhibited upregulated PRR expression in RA mice, PRR mRNA levels were not significantly decreased (P=0.341) by AAV-PRR-shRNA (Figure 4B).

Brain-Targeted PRR Knockdown Attenuates Hypertension in RA Mice
RA mice with AAV-eGFP exhibited higher mean arterial BP compared with NT mice (P<0.05). AAV-PRR-shRNA significantly reduced mean arterial BP (P<0.05) in RA mice compared with control virus. PRR knockdown did not alter BP in NT mice (Figure 5A). RA mice exhibited a
lower SBRS (1.5±0.1 ms/mmHg) than NT mice (3.2±0.3 ms/mmHg; P<0.05) after AAV-eGFP injection. PRR knockdown markedly improved SBRS in RA mice (2.2±0.2 ms/mmHg; P<0.05); however, it did not affect SBRS in NT mice (Figure 5B). RA mice exhibited significantly higher cardiac (Figure 5C) and vasomotor (Figure 5D) sympathetic tone (heart rate to propranolol: −146±27 versus −42±6 bpm and mean arterial BP to chlorisondamine: −75.9±1.2 versus −38.2±1.4 mmHg; P<0.05) than NT mice with AAV-eGFP injection. AAV-PRR-shRNA significantly decreased cardiac and vasomotor sympathetic tone (heart rate: −91±17 bpm and mean arterial BP: −58.6±6.6 mmHg; P<0.05) in RA mice; however, AAV-PRR-shRNA did not alter sympathetic tone in NT mice. Parasympathetic tone (response to methylatropine) and intrinsic heart rate (response to methylatropine and propranolol) were not altered in any of the groups (Figure 5E and 5F).

**PRR Knockdown Decreases AT<sub>1</sub> Receptor and AVP Level in the RA Mice**

To elucidate the possible mechanisms involved in the attenuation of hypertension after brain PRR knockdown, we investigated AT<sub>1</sub> receptor, plasma AVP, and AVP mRNA levels in the PVN. AT<sub>1</sub> receptor mRNA levels were significantly higher in RA compared with those of NT mice (Figure 6A; P<0.01). AAV-PRR-shRNA significantly reduced AT<sub>1</sub> receptor mRNA levels in RA mice compared with control virus but not in NT mice. Western blot data also revealed upregulated AT<sub>1</sub> receptor expression in RA mice (Figure 6B; P<0.01). PRR knockdown significantly decreased AT<sub>1</sub> receptor expression in RA mice but had no effect on NT mice. Plasma AVP and AVP mRNA levels in the PVN were increased in the RA mice (Figure 6C and 6D). AAV-PRR-shRNA significantly reduced AVP mRNA levels in the PVN and plasma levels in RA mice (P<0.05) but not in NT mice.

**Discussion**

Although previous studies have shown the importance of the PRR in diabetic nephropathy, cardiac fibrosis, and heart failure, the physiological and pathophysiological roles of the PRR in the central nervous system remain largely unknown. The present study reveals that PRR protein is highly expressed in the brain and primarily located in neurons. Green staining represents the neuron marker neuron-specific nuclear protein (NeuN; A) or astroglial cell marker glial fibrillary acidic protein (GFAP; D); red staining represents the PRR (B and E), and merged pictures (C and F) show that the PRR is mainly expressed in neurons. Double-staining pictures show PRR expression in the subfornical organ (SFO; G), paraventricular nucleus (PVN; H), nucleus of raphe pallidus (NPR; I), nucleus of the tractus solitarius (NTS; J), rostral ventrolateral medulla (RVLM; K), and brain cortex (L).

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Figure 1. The (pro)renin receptor (PRR) is highly expressed in the brain and primarily located in neurons. Green staining represents the neuron marker neuron-specific nuclear protein (NeuN; A) or astroglial cell marker glial fibrillary acidic protein (GFAP; D); red staining represents the PRR (B and E), and merged pictures (C and F) show that the PRR is mainly expressed in neurons. Double-staining pictures show PRR expression in the subfornical organ (SFO; G), paraventricular nucleus (PVN; H), nucleus of raphe pallidus (NPR; I), nucleus of the tractus solitarius (NTS; J), rostral ventrolateral medulla (RVLM; K), and brain cortex (L).
and noncardiovascular regions. Although brain renin activity is below the detection threshold of most standard assays, renin and prorenin mRNA are expressed in the neurons of regions controlling cardiovascular function. In addition, circulating renin can reach the brain via the circumventricular organs that lack a blood-brain barrier, interacting with PRR located in these areas. Because the PRR binds both renin and prorenin leading to Ang II production, the finding of PRR in organs that lack a blood-brain barrier, interacting with PRR in non-(pro)renin synthesizing organs, this interaction might occur in prorenin-synthesizing tissues like brain, as showed in Figure S8. Our data indicate that PRR is upregulated in the SFO and PVN of RA mice. The upregulated PRR may be attributed to higher Ang II levels in the transgenic mice. In fact, Siragy and Huang demonstrated that AT$_1$ receptor blockade decreases PRR expression in diabetic rats, supporting a role for Ang II in regulating PRR expression in vivo. In addition, we found that Ang II stimulated PRR mRNA expression in neuronal cells, which could be completely blocked by the AT$_1$ receptor blocker losartan (Figure S6).

Previous studies have shown that ICV administration of the viral vectors successfully infects the SFO. Thus, in this study, we used ICV injection to deliver the AAV. By tracing GFP expression and measuring PRR levels, we found that the virus profoundly infected the SFO 2 weeks after injection. The dominant expression of GFP is probably attributed to the proximity of the ICV injection site to the SFO. In addition, because of the tight junction between the ependymal cells covering the SFO, losartan (Figure S6).

To clarify the role of the PRR in BP regulation, we delivered AAV-PRR-shRNA into the mouse brain. Our data provide novel evidence that PRR knockdown in the brain attenuated hypertension, which was associated with improvement of SBRS and decrease in sympathetic tone. In contrast, PRR knockdown did not affect BP in normotensive mice. These findings are consistent with a previous study showing that PRR knockdown in supraoptic nuclei (SON) of the spontaneously hypertensive rats is associated with attenuation of hypertension development without affecting BP of normotensive control rats. The nonalteration of BP in normotensive mice after PRR knockdown could be attributed to normal PRR activity and redundant mechanisms in maintaining BP in physiological conditions. In this study, we found that PRR
expression was also upregulated in the PVN of RA mice. The role of the PVN in Ang II–dependent hypertension has been well documented.23 We also noticed that ICV injection of AAV-PRR-shRNA did not completely normalize BP in the RA mice, indicating a possible role for PRR in the PVN in this model.

Brain AT1 receptor is well known for stimulating sympathetic tone leading to the development of hypertension.24–26 We observed that brain-targeted PRR knockdown reduced AT1 receptor expression in the SFO. Although the exact mechanism by which the PRR regulates the AT1 receptor remains unknown, the interaction between the 2 receptors tends to appear in pathological conditions, because the AT1 receptor does not decrease in NT mice after PRR knockdown. A previous study showed that brain RAS activation enhances AVP release, which suggests chronically increased AVP as a possible mechanism of the elevated BP in RA mice.24 Recently, it was reported that PRR knockdown in the SON decreases plasma AVP level associated with attenuation of hypertension in the spontaneously hypertensive rat.22 The reduced synthesis and subsequent release of AVP after PRR knockdown observed in our study indicate that this might be another physiological mechanism for the contribution of PRR to BP regulation.

**Perspectives**

The identification of neural PRR as a potential activator of AT1 receptor and AVP release suggests a novel mechanism contributing to the development of hypertension. Knockdown of the PRR in the central nervous system reduces BP and

**Figure 4.** ICV administration of adeno-associated virus (AAV)-(pro)renin receptor (PRR)-short-hairpin RNA (shRNA) mediates PRR knockdown in subfornical organ (SFO). AAV-PRR-shRNA significantly decreases PRR mRNA levels in the SFO of both nontransgenic (NT) and renin-angiotensinogen transgenic (RA) mice (A; n=4 per group) but not in the paraventricular nucleus (PVN; B; n=8 per group). *P<0.01 vs NT mice with AAV- enhanced green fluorescent protein (eGFP); #P<0.01 vs RA mice with AAV-eGFP (2-way ANOVA).

**Figure 5.** Brain-targeted (pro)renin receptor (PRR) knockdown attenuates hypertension in renin-angiotensinogen transgenic (RA) mice. Continuously telemetric blood pressure (BP) was recorded in conscious nontransgenic (NT) and RA mice for baseline and after ICV injection (A). Spontaneous baroreflex sensitivity (SBSR) was calculated using the sequence method (B). Cardiac sympathetic tone (C), vasomotor sympathetic tone (D), parasympathetic tone (E), and intrinsic heart rate (F) were determined after administration of propranolol, chlorisondamine, and methylatropine; n=4 per group. *P<0.05 vs NT mice with adeno-associated virus (AAV)-enhanced green fluorescent protein (eGFP). #P<0.05 vs RA mice with AAV-eGFP (2-way ANOVA).
findings is of importance because blockade of the AT1 receptor has been proven an effective way to treat hypertension. PRR blockade in the central nervous system may represent a novel approach for the treatment of neurogenic hypertension.

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Disclosures

None.

References


Figure 6. Angiotensin II type 1 (AT1) receptor and arginine vasopressin (AVP) levels are decreased after (pro)renin receptor (PRR) knockdown in the renin-angiotensinogen transgenic (RA) mice. AT1 receptor mRNA levels (A; n=4 per group) were detected by real-time PCR and protein levels (B; n=10 per group) were detected by Western blotting in nontransgenic (NT) and RA mice with ICV delivery of adeno-associated virus (AAV)-PRR-short-hairpin RNA (shRNA) or control virus. C and D (n=5 per group) represent AVP mRNA level in the paraventricular nucleus (PVN) and plasma AVP level of NT and RA mice after ICV delivery of AAV-PRR-shRNA or control virus. *P<0.01 in A and B, P<0.05 in C and D vs NT mice with AAV-eGFP (2-way ANOVA).


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BRAIN-TARGETED (PRO)RENNIN RECEPTOR KNOCKDOWN ATTENUATES ANGIOTENSIN II-DEPENDENT HYPERTENSION.

Short Title: (Pro)renin receptor knockdown in hypertension.

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Methods

Animal preparation: Human renin transgenic mice (R) and human angiotensinogen transgenic mice (A) were generated at the University of Iowa Transgenic Animal Facilities by microinjection into fertilized C57BL/6JxSJL/J (B6SJLF2) mouse embryos. These transgenic mice express human renin and angiotensinogen (AGT) gene respectively everywhere in the body.\(^1\)\(^2\) Double transgenic mice (RA) expressing both human renin and human AGT transgenes were generated by breeding heterozygous R transgenic mice with heterozygous A transgenic mice as previously described.\(^3\) In the double transgenic mice, the major phenotype is a chronic hypertension, concomitant with high plasma and tissue Ang II levels. RA mice and non-transgenic littermates (NT) were used our in experiments. All mice were fed standard mouse chow and water ad libitum. All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Tulane University School of Medicine.

Development of PRR antibody: For this study, a new mouse PRR antibody was raised using a customized service (Open Biosystems). Mouse-specific PRR antibodies were raised in rabbits against a synthetic peptide GLDELGKRYGEDSEQFRD which was chosen based on the extracellular domain of PRR. Affinity purification was also done by Open Biosystems. PRR antibody specificity was verified by immunostaining and western blotting with or without preabsorbed peptide (10µM) and the absence of signal with PRR knockout tissues.

Construction and production of AAV-PRR-shRNA: The short hairpin RNA was designed to target the mRNA encoding mice PRR at sequence GGGCTTCTCTGTAAAGGAAGA (GenBank Accession No. NM-027439). Targets were ligated and cloned into an adeno-associated virus (AAV2) vector, under the control of human U6 promoter. A green fluorescent reporter gene under the control of the cytomegalovirus (CMV) promoter was ligated downstream of the PRR-shRNA to directly trace the virus expression. Before virus preparation, plasmids were sequenced and in vitro transgene expression was confirmed. All vectors were packaged at the University of Iowa Gene Transfer Vector Core using a baculovirus system.\(^4\)\(^5\)

Physiological recordings: RA mice and littermate NT mice (n=4), 8-10 weeks old, were anesthetized using continuous isoflurane inhalation and instrumented with a radiotelemetry transmitter into the carotid artery as described before.\(^6\)\(^7\) After 14 days recovery, mice were intracerebroventricularly (ICV) injected with 100nl AAV-PRR-shRNA (3.5x10\(^{11}\) Vg/100nl) or control virus (AAV-eGFP). Mouse was placed in a stereotaxic apparatus (Stoelting). The coordinates for the ICV injection was as following: 3.0 mm below the skull surface, 0.3 mm posterior to bregma and 1.0 mm lateral to the midline. After the injection, BP was continuously recorded in conscious freely moving mice for 2 weeks. The ICV injection was verified pharmacologically based on the pressor and bradycardic response to carbachol (50ng), and histologically after completion of the protocol. Autonomic function was assessed by using a standard pharmacological method involving random intraperitoneal injection of a β-blocker (propranolol, 6mg/kg), a muscarinic receptor blocker (methylatropine, 1mg/kg) and a ganglionic blocker (chlorisondamine, 5mg/kg).\(^8\) Changes in HR to propranolol and methylatropine and in
BP to chlorisorbamine were calculated following administration of the antagonists. Spontaneous baroreflex sensitivity (SBRS) was calculated using the sequence methods.9, 10

**RNA isolation and real-time reverse transcription PCR:** Brain nuclei including SFO, PVN, nucleus of the tractus solitarius (NTS) and rostral ventrolateral medulla (RVLM) from NT and RA mice 14 days after AAV-PRR-shRNA or AAV-eGFP injection, were micro-punched using cryostat and total RNA was isolated using a standard RNA extraction procedure with RNeasy mini kit (Qiagen Technologies, Hilden, Germany). Total RNA was quantified using a Spectrophotometer (Thermo Scientific Nanodrop 2000). Total RNA (200 ng) was used for cDNA synthesis using cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Specific primers for mouse PRR (Fwd: 5'-TCT CTC CTA ACT GCA AGT GCA ACA-3'; Rev: 5'-CCA AAC CTG CCA GCT CCA ATG AAT-3'), for mouse AT1 receptor (Fwd: 5'-TCA CCA GAT CAA GTG CAT TTT GA-3'; Rev: 5'-AGA GTT AAG GGC CAT TTT GCT TT-3') and for an internal control, mouse GAPDH (Fwd: 5'-AAT ATG TCC TGC GTG CAT 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 reverse transcription PCR was performed with the Mx3000P System (Stratagene, La Jolla, CA) using the SYBR green qPCR master mix (USB Corporation, Cleveland, OH) following the manufacturer’s instruction. Cycling conditions were 95°C for 10 min and then 40 cycles consisting of 15 sec at 95°C, 60 sec at 60°C and 30 sec at 72°C. Relative expression was calculated using MxPro software according to the $2^{-\Delta\Delta CT}$ method.11 Values were expressed as the ratio relative to NT mice with control virus administration. The expression levels of targeted mRNAs were normalized based on the expression levels of GAPDH mRNA.

**Immunofluorescence** The brain tissues were harvested after transcardial perfusion with 4% paraformaldehyde and then placed in 20% sucrose solution overnight. Brains were sectioned at 30 µm thickness on a cryostat, and sections were collected in PBS. After non-specific binding was blocked with 5% bovine serum albumin, free-floating coronal sections were incubated with self-designed rabbit anti-mouse PRR antibody (1:200) and fluorescein-conjugated anti-mouse neuron-specific nuclear protein antibody (Alexa 488, 1:100, Millipore, Billerica, MA) for 48 hours at 4 °C or rabbit anti-mouse AT1 receptor antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. This was followed by fluorescence-conjugated goat anti-rabbit antibody (Alexa 594, 1:1000, Invitrogen, Carlsbad, CA) incubation at room temperature for 1 hour. Images were captured by using a fluorescent microscope. Additionally, to assess specificity of the AT1 receptor or PRR staining, control sections were incubated without primary or secondary antibodies, or with the addition of preabsorbed peptide to assure the specificity of the antibody. To quantify the immunostaining, 5-10 pictures were taken from each interested site of one mouse. Quantification was performed by another investigator in a blinded fashion using NIH Image J software.

**Western blot analysis** Brain tissues were harvested and homogenized with a glass pestle in a lysis buffer (Thermo Scientific, Waltham, MA) containing a protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO). The lysate was centrifuged at 13,000 rpm for 10 min and the supernatant was transferred to a clean tube. Protein concentration was measured using a BCA assay kit (Thermo Scientific, Waltham, MA). Protein lysates (25 µg) were mixed with SDS-PAGE
sample buffer (Invitrogen, Carlsbad, CA), heated at 95 °C for 5 min and loaded onto a 4-20% Tris-Glycine gel (Invitrogen, Carlsbad, CA) for electrophoresis. Proteins were transferred to nitrocellulose membrane for 7 min by iBlot Gel Transfer Device (Invitrogen, Carlsbad, CA). Membranes were blocked with 5% non-fat milk in PBS-T for 2 hours at room temperature and incubated with a rabbit-anti-mouse AT1 receptor antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at room temperature. Membranes were then incubated with IRDye 800 CW goat anti-rabbit IgG antibody (1:10000, Li-Cor Bioscience, Lincoln, NE) for 1 hour at room temperature. Specific bands were detected by chemiluminescence with a Li-Cor Odyssey system. Membranes were then stripped with Western Blot Stripping Buffer (Thermo Scientific, Waltham, MA) for 30 minutes at room temperature, and incubated with rabbit anti-mouse β-actin antibody (Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature which followed by incubation with IRDye 800 CW goat anti-rabbit IgG antibody (1:10000, Li-Cor Bioscience, Lincoln, NE) for 1 hour at room temperature. Membranes were visualized under a Li-Cor Odyssey system for a second time. Quantification was performed by another investigator in a blinded fashion using NIH Image J software. The expression levels of targeted proteins were normalized based on the expression levels of β-actin protein.

**Plasma vasopressin** Enzyme-Linked Immuno Sorbent Assay was performed to determine the plasma vasopressin concentrations in NT and RA mice with AAV-PRR-shRNA or AAV-eGFP injection. Mice (n=5/group) were decapitated, and trunk blood was collected in chilled tubes containing EDTA. Peptide was then extracted and concentrated from plasma using a Sep-pak extraction kit (Waters). Plasma vasopressin concentration was then measured using an AVP Fluorescent Immunoassay kit (Phoenix Pharmaceuticals) following the company’s instruction. Briefly, samples were incubated with primary antibody in the secondary coated immunoplate at 4 °C overnight. This was followed by incubation with biotinylated peptide at room temperature for 1.5 hours. After sufficient washing for 3 times, streptavidin-horseradish peroxidase was added into the plate and incubated for at room temperature. At the end of the protocol, substrate solution was added for the generation of fluorescence. The relative fluorescence unit was measured by a fluorescence reader with wavelength 340nm for excitation and 405nm for emission.
References


Figure S1. PRR expression in Neuro-2A cells. The specificity of PRR antibody was verified by western blots. Protein samples were extracted from Neuro-2A cells infected with AAV-eGFP or AAV-PRR-shRNA. Samples incubated with PRR antibody showed strong bands at 37kD. PRR-shRNA significantly decreased the signal at 37kD suggesting the specificity of the PRR antibody.
**Figure S2.** PRR immunostaining was performed with or without pre-absorbed peptide (10µmol/l) to verify the specificity of PRR staining. Sections from SFO (A), PVN (C), NTS (E) and RVLM (G) totally are lack of immunofluorescence incubated with pre-absorbed peptide. However, high levels of fluorescence were observed in these nuclei without pre-absorption (B, D, F and H).
Figure S3. PRR expression in NT and RA mice. PRR immunostaining was performed in both NT and RA mice to investigate PRR expression in different brain areas including NTS, RVLM and cortex. Quantification of immunostainings shows that PRR expression is not significantly different in NTS, RVLM and cortex between NT and RA mice (A, C and E). Real-time PCR does not reveal different PRR mRNA levels between these two groups (B, D and F).
Figure S4. Three days after ICV administration of AAV-PRR-shRNA, mice brains were harvested and sectioned to verify viral infection. Some GFP expression is found in ependymal cells around the third ventricle of the brain. However, there is no GFP expression in the cortex (A), PVN (B), NTS (C), RVLM (D), MnPO, OVLT (E) and AP (F).
Figure S5. PRR mRNA levels in the SON. There is no significant difference of PRR mRNA levels between NT and RA mice (n=4), and ICV injection of AAV-PRR-shRNA does not affect PRR levels in the SON of either NT mice or RA mice.
Figure S6. Ang II increases PRR expression via AT1 receptor in neurons. Neuro-2A cells were stimulated with Ang II (10nmol/l). Cells were then harvested at different time points for PRR mRNA measurement. PRR mRNA increased as soon as 10 minutes after Ang II stimulation, and remained significantly higher than non-stimulated cells for 2 hours (A). Cells were pretreated with Losartan (10µmol/l) for 30 minutes, then stimulated with Ang II for 1 hour (B). Ang II mediated PRR up-regulation is completely blocked by Losartan. (n=6/group)* P<0.05 vs. non-treated, #<0.05 vs. Ang II-treated.
Figure S7. PRR mRNA expression in different brain regions. Different brain regions from NT mice were micro-punched for PRR mRNA measurement. PRR mRNA levels are significantly higher in the SFO and PVN and lower in the NTS compared to brain cortex. * P<0.05 vs. brain cortex.
Figure S8. (Pro)renin protein expression in C57Bl/6J mouse brain. The existence of renin and prorenin protein in the brain was detected by western blots. Protein samples were extracted from whole brain lysate. Samples incubated with (pro)renin antibody (Santa-Cruz: H-105) showed strong bands at 46 kD for prorenin and thin bands at 38kD for matured renin.