Involvement of the Brain (Pro)renin Receptor in Cardiovascular Homeostasis

Zhiying Shan,* Peng Shi,* Adolfo E. Cuadra, Ying Dong, Gwyneth J. Lamont, Qihong Li, Dale M. Seth, L. Gabriel Navar, Michael J. Katovich, Colin Sumners, Mohan K. Raizada

Rationale: Despite overwhelming evidence of the importance of brain renin–angiotensin system (RAS), the very existence of intrinsic brain RAS remains controversial.

Objective: To investigate the hypothesis that the brain (pro)renin receptor (PRR) is physiologically important in the brain RAS regulation and cardiovascular functions.

Methods and Results: PRR is broadly distributed within neurons of cardiovascular-relevant brain regions. The physiological functions of PRR were studied in the supraoptic nucleus (SON) because this brain region showed greater levels of PRR mRNA in the spontaneously hypertensive rats (SHR) compared with normotensive Wistar–Kyoto (WKY) rats. Adeno-associated virus (AAV)-mediated overexpression of human PRR in the SON of normal rats resulted in increases in plasma and urine vasopressin, and decreases in H₂O intake and urine output without any effects on mean arterial pressure and heart rate. Knockdown of endogenous PRR by AAV-shRNA in the SON of SHRs attenuated age-dependent increases in mean arterial pressure and caused a decrease in heart rate and plasma vasopressin. Incubation of neuronal cells in culture with human prorenin and angiotensinogen resulted in increased generation of angiotensin I and II. Furthermore, renin treatment increased phosphorylation of extracellular signal-regulated kinase 1/2 in neurons from both WKY rats and SHRs; however, the stimulation was 50% greater in the SHR.

Conclusions: The study demonstrates that brain PRR functions and plays a role in the neural control of cardiovascular functions. This may help resolve a long-held controversy concerning the existence of intrinsic and functional brain RAS. (Circ Res. 2010;107:934-938.)

Key Words: (pro)renin receptor ■ supraoptic nucleus ■ cardiovascular homeostasis ■ mean arterial pressure
Six-week-old male Sprague–Dawley (SD) rats or SHRs were implanted with telemetry transducers into the abdominal aorta. Following 10 days recovery, animals were randomly divided into 2 groups. SD rats were used for bilateral injection into the supraoptic nucleus (SON) (A/P: 1.4 mm, D/V: 9.2 mm, M/L: 1.8 mm) with either adeno-associated virus (AAV)–human (h)PRR or control virus, AAV–green fluorescent protein (GFP); similarly, SHRs were injected with either AAV–PRR–short hairpin (sh)RNA or control virus, AAV-Sc-shRNA. Mean arterial pressure (MAP) and heart rate (HR) were monitored; plasma and urinary vasopressin (AVP) and gene transduction were assayed as described in the Online Methods section.

**Results**

First, we compared PRR mRNA levels in the brains of WKY rats and SHR because its expression is shown to be abundant in cardiovascular-relevant brain regions. Compared with WKY rats, PRR mRNA levels were 70%, 45%, and 36% higher in the SON, the nucleus of the solitary tract, and the central amygdala, respectively (Figure 1a). Other brain regions tested did not show significant differences between these 2 strains of rats. PRR immunoreactivity was primarily localized in NeuN-positive cells (NeuN is a marker for neuronal nuclei); no significant staining of PRR was observed with glial fibrillary acidic protein (GFAP)-positive cells, an astroglial marker (Figure 1b and 1c). Predominant neuronal localization of PRR is supported by our in vitro data. Neuronal cultures from WKY hypothalamus/brain stem regions showed a 2.7-fold higher PRR mRNA than comparable astroglial cultures. In addition, neuronal cultures from the SHRs had ≈2-fold greater PRR mRNA compared with WKY neurons (Online Figure I).

Next, we studied the physiological role of PRR in the SON because this region exhibited the largest PRR expression difference between WKY rats and SHRs. We used AAV-hPRR to increase PRR in normotensive rats and AAV-PRR-shRNA to knockdown its expression in the SHRs. Bilateral microinjection of AAV-GFP showed a relatively restricted transduction of cells in the SON (Figure 2a). Transduction with AAV-hPRR demonstrated an increase in hPRR mRNA without influencing the levels of endogenous rat PRR (Figure 2b and 2c). Figure 3 shows that hPRR overexpression in the SON resulted in significant changes in the fluid homeostasis in SD rats. This increase was associated with a 21% decrease in daily water intake (PRR, 37.8 ± 2.4 mL; GFP: 48.0 ± 2.4 mL; P < 0.05), 51% decrease in daily urine excretion (PRR, 9.7 ± 1.8 mL; GFP, 19.7 ± 2.4 mL; P < 0.05), and 18% in-

### Non-standard Abbreviations and Acronyms

- **AAV**: adeno-associated virus
- **AT1R**: angiotensin II type 1 receptor
- **AVP**: vasopressin
- **GFAP**: glial fibrillary acidic protein
- **GFP**: green fluorescent protein
- **hPRR**: human (pro)renin receptor
- **HR**: heart rate
- **MAP**: mean arterial pressure
- **PRR**: (pro)renin receptor
- **RAS**: renin–angiotensin system
- **Sc**: scrambled
- **SD**: Sprague–Dawley
- **SHR**: spontaneously hypertensive rat
- **SON**: supraoptic nucleus
- **WKY**: Wistar–Kyoto

![Figure 1. PRR in the brain of WKY rats and SHRs.](http://circres.ahajournals.org/)

- **a**: PRR mRNA in WKY and SHR brains. MnPO indicates median preoptic nucleus; OVLT, organum vasculosum of lamina terminalis; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla. CA indicates central amygdala; NTS, nucleus of the solitary tract. *P < 0.05 vs WKY.**
- **b**: Representative immunofluorescence micrographs using anti-PRR and anti-NeuN antibodies reveal colocalization of PRR with neurons in the SON.**
- **c**: Representative immunofluorescence micrographs using anti-PRR and anti-GFAP antibodies reveal that PRR is little present on astroglia in the SON.
crease in urine osmolality (PRR, 2720±80 mOsm/kg; GFP, 2220±148 mOsm/kg; \(P<0.05\)). Furthermore, plasma AVP was increased by 2-fold (PRR, 2.6±0.58 pg/mL; GFP: 1.2±0.2 pg/mL; \(P<0.05\)), whereas urine AVP increased by 3-fold (PRR, 74.9±17 pg/mL; GFP, 25.5±7.9 pg/mL; \(P<0.05\)). Further support for the role of PRR in AVP secretion is provided from the data showing colocalization of PRR on AVP neurons in the SON (Online Figure II). However, PRR overexpression in the SON had no effect on MAP and HR (Online Figure III).

We constructed a shRNA-targeted PRR, which was cloned into the AAV vector. Infection of neuronal cultures with this PRR-shRNA caused \(\sim 60\%\) decreases in both PRR mRNA and renin stimulation of extracellular signal-regulated kinase (ERK)1/2 phosphorylation (Online Figure IV). Transduction of SHR SON with PRR-shRNA attenuated the age-dependent increases in MAP (\(\Delta\text{MAP}\)) and caused a decrease in HR (\(\Delta\text{HR}\)) compared with baseline MAP (PRR-shRNA, 124.6±7.6 mm Hg; Sc-shRNA: 121.2±3.0 mm Hg) and HR (PRR-shRNA, 400.5±5.1 bpm; Sc-shRNA, 388.6±5.9 bpm). Eight weeks
following microinjection, ΔMAP was 22.4±3.9 mm Hg in PRR-shRNA rats compared with 46.0±6.2 mm Hg in Sc-shRNA animals. Similarly, ΔHR was decreased in shRNA-treated SHRs (PRR-shRNA, −81.1±9.0 bpm; Sc-shRNA: −51.2±6.9 bpm; P<0.05) (Figure 4a). These changes were associated with a 38% decrease in PRR mRNA in the SON (Figure 4b). In addition, plasma AVP was decreased by 34% in PRR-shRNA animals (PRR-shRNA, 16.3±2.4 pg/mL; Sc-shRNA, 24.0±2.3 pg/mL; P<0.05) (Figure 4b). In contrast, transduction of WKY SON with PRR-shRNA failed to exert any effect on MAP (PRR-shRNA, 104.5±2.6 mm Hg; PRR-scRNA, 105.1±9.0 mm Hg; n=6 for each group).

Finally, we determined the cellular effects of prorenin and renin in neuronal cultures. A dose- and-time-dependent increase in angiotensin I and II formation was observed when the cultures were incubated with human prorenin and angiotensinogen (Online Figure V). Furthermore, incubation of neuronal cultures with renin in the presence of losartan, an angiotensin II antagonist, resulted in a rapid and transient increase in phosphorylated ERK1/2 in both strains of rats although this stimulation was 2-fold greater in the SHR neurons (Online Figure VI).

**Discussion**

Our study is significant in a number of ways. (1) It demonstrates that PRR in the brain is physiologically relevant because its overexpression in the SON stimulates AVP secretion and alters fluid balance in normal rats. In addition, chronic knockdown of PRR causes a significant attenuation of MAP and HR in SHRs. Thus, PRR may play a crucial role in the neuronal control of cardiovascular function by possibly regulating AVP secretion. (2) PRR mediates the generation of angiotensin II and stimulates intracellular signaling, similar to its action in the periphery. Therefore, its presence further strengthens the presence of a functional intrinsic brain RAS.

(3) Most previous studies implicate functions of PRR in pathophysiological states, such as cardiovascular disease and diabetes, or from in vitro situations. Our study provides data of functional PRR in a normotensive animal and demonstrates a beneficial outcome of PRR in hypertension by its chronic knockdown. Finally, our data demonstrate the role of SON in blood pressure control in the SHR model of hypertension.

PRR expression is widespread in brain cardioregulatory regions, consistent with the recent report of Contrepas et al. However, our study is novel in that it shows that expression levels of PRR are higher in the SON of SHR compared with WKY controls. This increase appears to be genetically linked because it is maintained in primary neuronal cultures of prehypertensive SHR. It is pertinent to point out that these cultures closely mimic the changes observed in the RAS activity of adult SHR. PRRs are primarily localized on neuronal cells because PRR-positive cells costain with NeuN-positive cells. Furthermore, GFAP-positive cells exhibit little PRR staining. This conclusion is further supported by our observation that PRR levels are several-fold lower in astroglial cultures than in neuronal cultures from the brains of both WKY rats and SHR.

Increased expression of PRR in the SHR SON appears to exert pathophysiological influences, a conclusion supported by our knockdown data. Long-term knockdown of PRR is associated with attenuation of hypertension development and a decrease in plasma AVP. Consistent with these data is our observation that overexpression of PRR in the SON of normotensive rat results in an increase in plasma and urinary AVP. However, increases in plasma AVP levels were not associated with an increase in MAP. There may be a number of explanations for this unexpected result. (1) Increases in plasma AVP observed in this study were insufficient to affect MAP. (2) Redundant physiological mechanisms in normal rats may be able to overcome increases in plasma AVP, a view consistent with the present dogma that AVP appears to exert little effect on MAP in normal individuals and animals.

(3) Circadian rhythmic release of AVP from the SON (rather than a continual increased secretion) is important in its cardiovascular effects. Transgenic overexpression of hPRR may not maintain this pattern of release, and (4) PRR may inhibit sympathetic nervous system activity in normal rats, thus counteracting potentially hypertensive effects of circulating AVP.

Finally, our data show that the neuronal PRR has dual functions. First, by binding to renin/prorenin, it increases the generation of angiotensin II. This solid-phase enzyme catalysis could provide thousands-fold higher rates of angiotensin formation than liquid phase occurring in the plasma. Thus, even a small amount of renin/prorenin would be capable of generating physiologically relevant concentrations of angiotensin II locally in the brain. We propose that this catalysis occurs on the surface of AT1R-expressing neurons. Evidence in support of this view are: (1) key components of angiotensin II actions, such as angiotensin-converting enzyme and AT1R, are present in the neurons; and (2) our preliminary data have shown that PRR and AT1R are colocized on the SON neurons (data not shown). Second, binding of renin/PRR
initiates signaling involving ERK1/2. These kinases have been shown to be involved in the regulation of neuronal activity. This signaling would complement AT1R mediated neuromodulation.

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**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is Known?**

- (Pro)renin receptor (PRR) is a new member of renin–angiotensin (RAS) system.
- PRR can bind either renin or prorenin, and this binding facilitates angiotensin II generation and stimulates signal transduction in the periphery.

**What New Information Does This Article Contribute?**

- PRR is more highly expressed in the supraoptic nucleus (SON) of spontaneously hypertensive rats (SHRs) than in normotensive Wistar–Kyoto (WKY) rats.
- Overexpression of PRR in the SON increases vasopressin (AVP) secretion and alters fluid balance in normal rats.
- Knockdown of this receptor significantly attenuates the age-dependent increases in blood pressure and causes a decrease in heart rate in the SHRs.

Our study shows that brain PRR is physiologically relevant because increase or decrease of this receptor in the brain affects physiological status. In addition, our data show that brain PRR mediates angiotensin II generation and stimulates signal transduction, which supports the presence of a functional intrinsic brain RAS. Most previous studies have implicated the functions of PRR in pathophysiological states or from in vitro situations. Our study supports the concept that even under physiological conditions, the PRR contributes to cardiovascular regulation in a normotensive animal. Moreover, our results also support the idea that increased activity of the PRR contributes to hypertension, because arterial pressure was reduced by its chronic knockdown. Finally, our data suggest, for the first time, that SON plays an important role in the control of blood pressure.
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SUPPLEMENT

Involvement of the brain (pro) renin receptor in cardiovascular homeostasis

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Short title: Increased PRR in the SHR brain

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Materials and Methods

Animals:
All Wistar–Kyoto rats (WKY), Spontaneously Hypertensive Rats (SHR) and Sprague-Dawley (SD) rats were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed individually and kept on a 12h:12h light–dark cycle in a climate-controlled room. Rat chow (Harlan Tekland) and water were provided ad libitum. All the animal experiments followed protocols approved by the University of Florida Institutional Animal Care and Use Committee.

Measurement of PRR mRNA levels:
Thirteen-week-old male SHR (MAP ~160mmHg) and WKY rats (MAP ~100mmHg) were euthanized, the brains dissected, and regions containing the paraventricular nucleus (PVN), supr-optic nucleus (SON), nucleus of the solitary tract (NTS), the rostral ventrolateral medulla (RVLM), organum vasculosum of lamina terminals (OVLT), median preoptic nucleus (MnPO), and central amygdala (CA) were punched out. These tissues were subjected to RNA isolation using RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions. RNA, ~500ng was reverse transcribed with iScript™ cDNA synthesis kit (BioRad, Hercules, CA, USA) in a 20 µL reaction system. The PRR mRNA levels were analyzed by quantitative real-time PCR using PRR specific primers and Taqman probe Rn01430718_m1 (Applied Biosystems, Foster City, CA, USA) in the PRISM 7000 sequence detection system (Applied
Biosystems). All cDNA samples were assayed in triplicate. Data were normalized to 18S RNA.

**PRR immunoreactivity in the brain:**

Immunofluorescence staining was performed using 10 µm fresh brain sections with the following protocols. Male WKY rats (~250g) were euthanized, brains removed, and frozen. Coronal sections were cut from the brainstem or hypothalamus areas, and were air dried overnight at room temperature. Prior to staining, slides were submerged in -20°C acetone for 5 minutes, permitted to air dry and then washed in TBS for 5 minutes. Sections were incubated with 3% horse serum in TBS for 20 minutes and then incubated overnight at 4°C in a cocktail consisting of 1:200 dilution of goat anti-ATP6AP2 (a PRR specific antibody (Abcam, Cambridge, MA, USA), and either 1:250 dilution of anti-neuronal nuclear antibody (NeuN, Chemicon, Temecula, CA, USA) or 1:500 dilution of rabbit anti-arginine vasopressin (Millipore, Billerica, MA, USA). Afterwards, sections were washed in TBS twice for 5 minutes and incubated for one hour at room temperature in a mixture of secondary antibodies (Alexa Fluor 594 donkey anti-goat IgG and Alexa Fluor 488 donkey anti-mouse IgG; or Alexa Fluor 488 goat anti- rabbit IgG; all diluted 500 fold). The sections were mounted in Vectashield (Vector Labs, Burlingame, CA) and images were taken with a Leica TCS SP2, laser-disk scanning, confocal microscope.
Plasmid construction and production of AAV vectors containing human PRR or rat PRR-shRNA

Human PRR cDNA or GFP cDNA was cloned into the AAV vector, PTR-UF22, under the control of acytomegalovirus enhancer and chicken β-actin (CBA) promoter, generating construct, AAV-hPRR or control vector, AAV-GFP. Our previous evidence has indicated that AAV-GFP is an appropriate control for AAV vector containing a transgene because GFP itself has no effect on blood pressure.

A synthetic complementary DNA encoding shRNA targeted rat PRR mRNA at 989-1007 region (CCTACAACCTTGCCTATAA, gene accession number: XM_217592) or scrambled DNA, containing the same nucleotides as the PRR-shRNA DNA template but in a completely different arrangement and not targeted any rat gene, was designed and cloned into a AAV vector, PTR-UF11, under the control of human U6 promoter (Online Figure IV). A GFP reporter gene under the control of CBA promoter was cloned upstream of the shRNA expression cassette in order to directly visualize expression from the vector after delivery, generating construct AAV-PRR-shRNA or control vector, AAV-scrambled shRNA (AAV-Sc-shRNA).

AAV-hPRR, AAV-PRR-shRNA and their control vectors, AAV-GFP, AAV-scRNA were packaged into AAV virus. Virus production and titer determination were performed as previously described. Briefly, HEK 293 cells were cotransfected
with the constructs and the helper plasmid pDG DNAs for 48-60 h. Cells were harvested, and the crude lysates purified through an iodixanol step gradient followed by Mono-Q FPLC chromatography. The vector genome (vg) titers of AAV particles were determined by real-time PCR.

**Implantation of telemetry transducers, *In Vivo PRR or PRR-shRNA delivery into the supraoptic nucleus (SON) and arginine vasopressin assay:***

Six-week-old male SD rats, WKY, or SHR were anesthetized and telemetry transducers consisting of a fluid-filled catheter attached to a PA-C40 transmitter (DSI, St. Paul, MN, USA) were implanted into the abdominal aorta as described previously. Following recovery for 10 days, animals were randomly divided into two groups. SD rats were used for bilateral injection into the SON with either experimental virus, AAV-hPRR or control virus, AAV-GFP (n=8 in each group). Similarly, SHR or WKY were injected with either AAV-PRR-shRNA or AAV-Sc-shRNA (n=6 in each group). 200 nl AAV viruses (2x10⁸ genome containing virus particles) were injected into each injection site (A/P: 1.4mm, D/V: 9.2mm, M/L: 1.8mm). Mean arterial pressure (MAP) and heart rate (HR) were monitored 8 hours during day time before microinjection as baseline, and were sampled once a week at the same time each day after the microinjection at indicated time periods.

At twelve weeks post microinjection, SD rats were transferred into metabolic cages and allowed 72 h to get used to the environment. Basal 24 h water intake, urine excretion and urine osmolality were then assayed for 3 consecutive days.
Urine was collected and kept at -80°C for AVP assay. All animals were euthanized one week after the metabolic experiment. Plasma was collected, and plasma AVP, plasma osmolality and urine AVP were then assayed.

AVP measurements from plasma and urine samples were performed using commercially available arg8-vasopressin EIA kits (Assay Designs, Michigan, USA) following the manufacturer’s instructions.

**Preparation of neuronal and astroglial cells in primary cultures from the WKY rat and SHR brains:**

Neuronal cells in primary culture from the brainstem and hypothalamus of one-day-old WKY and SHR were established as described previously ³, ⁴. Neuronal cultures contain more than 90% neurons (the remainder are primarily astroglia), while astroglial cultures contain>99% astroglial cells. The cultures were maintained for 12-14 days prior to their use in the experiments.

**Angiotensin I and II conversion in neuronal cultures:**

Fourteen day old primary neuronal cultures from the hypothalamus and brainstem of WKY rats were rinsed three times with 37°C PBS containing 2mM CaCl₂, 2mM MgCl₂ and 10mM glucose (PBSS). Cells were incubated in 0.5mL PBSS buffer with 2nM human prorenin and human angiotensinogen (AGT) for the indicated time periods and concentrations of AGT. Cultures incubated without AGT were used as control. Incubation media were collected to quantitate
Angiotensin I and Angiotensin II, which were determined by radio-immunoassay as described previously \(^5\).

**Measurement of phosphorylation of ERK1/2 MAP kinases:**

Western blot analysis was used to measure phosphorylated and total ERK1/2 MAP kinases using the neuronal cultures from WKY rats and SHR as described previously\(^4\). Briefly, neuronal cultures were incubated with or without recombinant human renin (Sigma-Aldrich, St Louis, MO, USA) in the presence of 2\(\mu\)M losartan, an AT1 receptor antagonist. Proteins were isolated, separated with 10\% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with mouse monoclonal antibody to phosphorylated ERK1/2. Hybrid protein bands were detected with ECL™ Western Blotting Detection Reagents. The membrane was stripped of phosphorylated ERK1/2 antibody and re-probed with ERK1 (C-16) and ERK2 (C-14) antibody to assay total ERK1/2 protein levels. Protein bands representing ERK1/2 MAP kinase were quantified using the NIH ImageJ program (Bethesda, Maryland, USA; [http://rsb.info.nih.gov/ij](http://rsb.info.nih.gov/ij)) phosphorylated ERK1/2 were normalized to total ERK1/2.

**Statistical analysis:**

All data are expressed as means ± S.E.M. Statistical significance was evaluated with the use of one-way ANOVA and unpaired Students t test. Differences were considered to be significant at \(p < 0.05\).
Figure Legends:

Online Figure I: Levels of PRR mRNA are increased in neuronal cultures from SHR brain

Neuronal and astroglial cells in primary cultures derived from the hypothalamus and brainstem regions of WKY and SHR were established as described previously. RNA was isolated from 12-14 day old cultures for quantitation of PRR mRNA. *p < 0.05 vs. corresponding astroglial sample. †p < 0.05 vs. WKY neurons.

Online Figure II: PRR immunoreactivity colocalizes with AVP in the SON of SD rats

Representative fluorescence images of PRR (left) and AVP (middle) immunostaining, and merged image (right), respectively.

Online Figure III: Increased PRR expression in the SON of SD rats does not influence mean arterial pressure or heart rate

MAP and HR were monitored for 8 hours via telemetry transducers, one day before microinjection of AAV-hPRR or AAV-GFP, in order to provide baseline values. After a one week period to recover from the procedure, MAP and HR were monitored twice a week at the same time, for up to twelve weeks, as described in the Methods. Data are means ± S.E.M from 8 rats in each group.

Online Figure IV: PRR-shRNA and its effect in WKY rat brain neurons

a. Map of AAV-PRR-shRNA (top) and control vector, AAV-Sc-shRNA (bottom).
Both PRR-shRNA and Sc-shRNA are under the control of human U6 promoter. A GFP reporter gene driven by CBA promoter was used to visualize expression from the vector after delivery.

b. PRR-shRNA decreases PRR mRNA level in neuronal cultures
Six-day-old neuronal cultures derived from hypothalamus and brainstem regions of WKY were treated with either AAV-PRR-shRNA (MOI=1:500) or AAV-Sc-shRNA (MOI=1:500). PRR mRNA level of neuronal cells were investigated at five days after virus infection. Data are means ± S.E.M (n=3 for each group). Experiments were repeated for twice. *P<0.05 compared to control and Sc-shRNA.

c. PRR-shRNA attenuates ERK1/2 phosphorylation in neuronal cultures.
Six-day-old neuronal cultures derived from hypothalamus and brainstem regions of WKY were infected with either AAV-PRR-shRNA or AAV-Sc-shRNA as described as Figure 4b. Cells were treated with 20nM of renin in the presence of 2 µM losartan for 2 minutes at seven days after infection. Western blotting was performed to evaluate p-ERK1/2 expression. Top is a representative image of Western blot. Bottom is ratio of phosphorylated ERK1/2 to total ERK1/2. ShRNA represent PRR-shRNA, ScRNA represent Sc-shRNA. *P<0.05 compared to control and Sc-shRNA.

**Online Figure V: Effect of PRR on angiotensin I and II formation in WKY brain neurons**
Fourteen-day old neuronal cultures were washed and incubated in PBS for 1 h. They were then treated with 2nM human prorenin in the presence or absence of increasing concentrations of human angiotensinogen substrate for 3 h (a) or for the indicated time period with 100µM angiotensinogen (b), media collected and used for measurement of angiotensin I and II by radioimmunoassay. Data are means ± S.E.M (n=3 for each group). This experiment was repeated for 3 times.

**Online Figure VI: Renin stimulates a time-dependent increase in ERK1/2 activation in WKY rat and SHR neurons**

WKY rat or SHR neuronal cultures were incubated with 20nM recombinant human renin in the presence of 2µM losartan for the indicated time periods. Proteins were separated and probed with total (T) ERK1/2 and phosphorylated (P) ERK1/2 antibodies. A. Top panel are representative Western blots showing P-ERK in neurons from WKY rats (left) and SHR (right). Bottom panel are representative Western blots showing T-ERK in the same neurons from WKY rats (left) and SHR (right). B. Quantification of P-ERK1/2 that has been normalized against T-ERK1/2 and compared with control, normalized to unity. Data are means ± S.E.M. *P< 0.05 vs. SHR control, # P<0.05 vs. WKY control; $ P< 0.05 vs. WKY in the same treatment.
References:


Online Figure I

![Bar chart showing PRR mRNA/18S expression levels in astroglia and neuron samples for WKY and SHR.](226977/R1)
Online Figure II
Online Figure III

![Graph showing blood pressure (MAP) and heart rate (HR) over time. The graph compares AAV-hPRR (n=8) and AAV-GFP (n=6). SON microinjection (Day 0) is marked on the x-axis. The y-axis for MAP ranges from 80 to 130 mmHg, and the y-axis for HR ranges from 250 to 450 beat/min. The data points are connected by lines, and error bars indicate variability.]
Online Figure IV

(a) Diagram showing the constructs with arrows indicating the direction of transcription. The constructs include ITR, PCBA, hGFP, SV40 pA, PU6, PRR-shRNA, bGH pA, and ITR.

(b) Bar graph showing the expression levels of PRR mRNA/18S in different conditions: Control, Sc-shRNA, and PRR-shRNA. The y-axis represents the expression levels in arbitrary units.

(c) Western blot images of P-ERK1/2 and T-ERK1/2 in different conditions: Control, Control + Renin, PRR-shRNA + Renin, Sc-shRNA + Renin. The images are accompanied by a graph showing the intensity of P-ERK/1/2 in arbitrary units for each condition.
Online Figure V

```
Time (Hr)

Ang (fmol/0.1 ml)
0 1 2 3 4 5 6
0 500 1000

AGT Ig(x)
-7 -6 -5 -4 -3

Ang I (n=6)

Ang II (n=6)

Ang (fmol/0.5 ml)
0 2000 4000

Ang (fmol/0.1 ml)
0 500 1000

0 1 2 3 4 5 6

226977/R1
```
Online Figure VI

(a) 

WKY

SHR

P-ERK1/2

T-ERK1/2

Time (Min) → C  0  2  5  10  30  60

(b) 

P-ERK/T-ERK1/2

WKY (n=6)

SHR (n=6)

Time (min)