Nervous System

Nucleus of the Solitary Tract (Pro)Renin Receptor-Mediated Antihypertensive Effect Involves Nuclear Factor-κB-Cytokine Signaling in the Spontaneously Hypertensive Rat

Jasenka Zubcevic,* Joo Y. Jun,* Gwyneth Lamont, Tatiane M. Murça, Peng Shi, Wei Yuan, Fan Lin, Jessica Marulanda Carvajal, Quihong Li, Colin Summers, Mohan K. Raizada, Zhiying Shan

Abstract—The importance of the (pro)renin receptor (PRR) in the function of the central nervous system is increasingly evident because PRR seems to play a role in neuronal control of cardiovascular function. PRR expression is elevated in the nucleus of the solitary tract (NTS) of spontaneously hypertensive rats (SHR). In this study, we tested the hypothesis that altered activity of PRR in the NTS is linked to hypertension. Eight weeks of chronic knockdown of the NTS PRR, using recombinant adeno-associated virus type 2 (AAV2)-PRR-small hairpin RNA (shRNA)–mediated gene transduction, caused a significant increase in mean arterial pressure (MAP) in the SHR (shRNA, 173±5; Control, 151±6 mm Hg) but not in Wistar Kyoto rats (shRNA, 108±7; Control, 106±6 mm Hg). The MAP elevation in the SHR was associated with decreased inflammatory markers tumor necrosis factor-α, interleukin-6, C-C motif ligand 5, and their transcription factor, nuclear factor-κB. Consistent with the pressor effects of the PRR knockdown, acute bilateral NTS injection of human renin (2 pmol/side) decreased MAP and heart rate (HR) in SHR (ΔMAP, −38±4 mm Hg; Δheart rate, −40±10 bpm), with negligible responses in Wistar Kyoto rats (ΔMAP, −4±3 mm Hg; Δheart rate, −12±7 bpm). These effects in SHR were attenuated (80%) by prorenin handle region peptide but were not affected by angiotensin II type 1 or angiotensin II type 2 receptor blockers. Finally, PRR activation in SHR neuronal cultures by prorenin activated nuclear factor-κB and increased mRNA levels of interleukin-1β (250-fold), tumor necrosis factor-α (32-fold), interleukin-6 (35-fold), C-C motif ligand 5 (12-fold), and interleukin-10 (7-fold) in a nuclear factor-κB–dependent but angiotensin II type 1 receptor–independent manner. Therefore, NTS PRR mediates antihypertensive effects via an angiotensin II–independent mechanism in SHR, which involves stimulation of the nuclear factor-κB–cytokine signaling pathway. (Hypertension. 2013;61:622–627.) ● Online Data Supplement

Key Words: antihypertensive • nuclear factor-κB signaling • nucleus of the solitary tract • prorenin receptor

Prorenin receptor (PRR) is highly expressed in many tissues, including those of the heart and brain,1,2 and plays an important role in the maintenance of cardiovascular homeostasis in the periphery.3,4 However, the role of PRR in the brain remains poorly understood. Despite intrinsically low renin concentrations in the brain, the ability of renin to signal in a similar manner to angiotensin (Ang)-II,5 coupled with the presence of PRR in the brain cardioregulatory areas,2,6 suggests a role for PRR in central cardiovascular control. Our previous study revealed that PRR expression is significantly increased in the nucleus of the solitary tract (NTS) and the supraoptic nucleus (SON) of spontaneously hypertensive rats (SHR) compared with normotensive Wistar Kyoto (WKY) controls.6 Genetic knockdown of PRR in the SHR SON6 and other cardioregulatory regions, such as the subfornical organ (SFO)7 in hypertensive rats, resulted in a reduction of blood pressure (BP). However, the role of NTS PRR in BP control and its precise mechanisms remain elusive.

Our interest in the role of NTS PRR derives from the following: (1) The NTS is the central termination site of baroreceptor input, regulating both the set-point of arterial pressure and the gain of the baroreflex, mechanisms essential for short- and long-term homeostatic control of arterial pressure6; (2) In the hypertensive model, the NTS exhibits an abnormal inflammatory state because the expression of many inflammatory mediators known to be involved in modulating synaptic transmission, including interleukin (IL)-6 and C-C motif ligand 5 (Ccl5), are downregulated in the SHR NTS compared with the WKY rats9,10,11; and (3) PRR mRNA in the SHR NTS is significantly increased compared with the WKY rats.6 We propose that altered activity of PRR in the NTS is linked to hypertension, and that the NTS PRR regulates BP...
via a cytokine-mediated mechanism. This study was designed to test this hypothesis.

Materials and Methods

The Methods section is available in the online-only Data Supplement. All animal experiments followed protocols approved by the University of Florida Institutional Animal Care and Use Committee.

Results

First, we investigated the effect of chronic PRR knockdown in the NTS on BP. Recombinant adeno-associated virus type 2 (AAV2)-PRR-small hairpin RNA (shRNA) gene transfer induced a significant increase in mean arterial pressure (MAP) in SHR (shRNA, 173±4; Control, 151±6 mm Hg; P<0.05; Figure 1A), but not in WKY rats (Figure S1 in the online-only Data Supplement). We observed a rightward shift in the SHR PRR-shRNA baroreflex curve, as the baroreflex gain reset to higher pressure, with no significant change in the maximum gain (PRR-shRNA, 3.05±0.42; vs Control, 2.33±0.26; Figure 1B; Table S1 in the online-only Data Supplement). Successful transduction of the NTS cells at the level of the calamus scriptorius with AAV viral vector was confirmed by the reporter gene green fluorescent protein (Figure S1B).

NTS significantly decreased mRNA levels of nuclear factor κB (nfb1, 43%), tumor necrosis factor-α (TNF-α, 32%), and interleukin-6 (IL-6, 35%) in the SHR compared with WKY rats, and PRR-shRNA–injected SHR. We observed significant changes only in the SHR restraining further decrease in NF-κB expression. However, PRR knockdown resulted in a 43% decrease in nfb1 mRNA expression compared with the control SHR (Figure S3).

Next, we studied the effect of acute NTS PRR activation by renin on MAP and heart rate (HR). Bilateral injections of human renin (0.02–2 pmol in 200 nL/side) in the SHR NTS resulted in an immediate dose-dependent decrease in MAP and HR: 0.02 pmol: ΔMAP, −16±4 mm Hg; ΔHR, −14±7 bpm; 0.2 pmol: ΔMAP, −33±8 mm Hg; ΔHR, −27±7 bpm; 2 pmol: ΔMAP, −38±4 mm Hg; ΔHR, −40±10 bpm (Figure 2A and 2B). In contrast, identical injections in the NTS of WKY rats produced negligible effects on MAP and HR: 0.02 pmol: ΔMAP, 7±1 mm Hg; ΔHR, 2±4 bpm; 0.2 pmol: ΔMAP, 1±4 mm Hg; ΔHR, −8±8 bpm; 2 pmol: ΔMAP, −4±3 mm Hg, ΔHR, −12±7 bpm; Figure 2A and 2B). Because we observed significant changes only in the SHR, further studies were carried out only in this rat strain. Preadministration of prorenin handle region peptide (200 pmol) into the SHR NTS attenuated the human renin-induced depressor and bradycardia effects by ≈80% (Figure 2C). However, preinjection of losartan (200 pmol), or Ang-II type 2 (AT2) receptor blocker PD123319 (2 nmol) did not influence the effects of human renin in the NTS of SHR (Figure 2C). Figure 2D shows the site of drug injections.

Figure 1. Effects of chronic knockdown of (pro)renin receptor (PRR) in the the nucleus of the solitary tract (NTS) of spontaneously hypertensive rat. PRR knockdown resulted in a significant increase in mean arterial pressure (MAP; A), and shifted the cardiac baroreflex curve to higher MAP with no change in the maximum gain (B). Recombinant adeno-associated virus type 2 (AAV2)-PRR-shRNA gene transfer in the NTS significantly decreased mRNA levels of nuclear factor κ B , interleukin (IL)-6, tumor necrosis factor (TNF)-α, C-C motif ligand 5 (C05L), and PRR (C). HR indicates heart rate.
We have previously demonstrated increased PRR expression in the NTS of SHR compared with WKY rats. Here, we report that chronic knockdown of PRR in the NTS exacerbated hypertension in SHR, despite having no effect in the WKY rats. Furthermore, acute PRR activation in the NTS produced negligible effects on MAP and HR in the WKY (250-fold), IL-6 (400-fold; Ccl5, 50-fold, and IL10, 20-fold; Figure S6). Finally, we selectively tested the effect of prorenin on IL-6 and TNF-α secretion into the cell culture medium. We observed that increases in the IL-6 and TNF-α mRNA after prorenin treatment were associated with NF-κB activation using primary neuronal cultures because PRR is Ang II–independent, and involve neuronal NF-κB–cytokine signaling.

The current study reports several novel findings: (1) The NTS PRR contributes to the maintenance of the set-point of arterial pressure; and (2) PRR cardiovascular effects in the NTS are Ang II–independent, and involve neuronal NF-κB–cytokine signaling.

We have previously demonstrated increased PRR expression in the NTS of SHR compared with WKY rats. Here, we report that chronic knockdown of PRR in the NTS exacerbated hypertension in SHR, despite having no effect in the WKY rats. Furthermore, acute PRR activation in the NTS produced negligible effects on MAP and HR in the WKY rats.
rats, different from previous observations. Additionally, we showed a profoundly augmented depressor effect of renin in the SHR, independent of a PRR-dependent Ang-II generation, as specific blockade of AT1 and AT2 receptors failed to abolish the effects of renin. Therefore, although the human renin, as used in this study, can bind to rat PRR with a comparable affinity, it is unable to act on the endogenous rat angiotensinogen to generate Ang-II. Furthermore, prorenin handle region peptide, a PRR inhibitor, attenuated renin actions by 80%. However, off-target effects of renin cannot be completely ruled out in view of the specificity of prorenin handle region peptide for the PRR. Development of a specific PRR antagonist must await this confirmation.

Because activation of PRR in the NTS resulted in a depressor effect, and silencing of this receptor in the NTS neurons further increased the SHR BP, the intrinsic baseline

![Figure 3](https://hyper.ahajournals.org/)

Figure 3. Effects of prorenin treatment on nuclear factor κB (NF-κB) activation in brain primary neuronal cultures. A, Left panel is a representative Western blot image of phosphorylated IκBα (top) and protein loading control GAPDH (bottom). Right panel shows ratio of phospho-IκBα to GAPDH (n=4; *P<0.05; B). Immunostaining of neuronal cultures with (pro)renin receptor (PRR; red) and NF-κB P65 (green) antibodies. Prorenin (100 nmol/L) treatment of neuronal cultures for 30 minutes induced NF-κB P65 translocation into the nucleus (blue). C, TransAM ELISA assay showing that prorenin treatment (30 minutes) increased nuclear content of NF-κB P65 (n=4; *P<0.05). D, Three hours of prorenin treatment resulted in an increase in mRNA levels of IκB kinase (IKK) subunit nfkbia and NF-κB subunit nfkb1 (n=6; *P<0.05).

![Figure 4](https://hyper.ahajournals.org/)

Figure 4. Prorenin treatment results in increased inflammatory markers. A, Neuronal cultures were coincubated with 100 nmol/L of prorenin, with or without different blockers for 3 hours. RNA, isolated from cells, was subjected to quantitative real-time polymerase chain reaction using interleukin (IL) 1β–, IL-6–, tumor necrosis factor (TNF)–α, C-C motif ligand 5 (Ccl5)–, and IL10-specific primers and probes. B, Released IL-6 and TNF–α protein levels were quantified by ELISA after treatment of prorenin (100 nmol/L) in the spontaneously hypertensive rat cultured neurons.
upregulation of endogenous PRR in the NTS of SHR suggests a compensatory mechanism of hypertension, acting to reduce further BP elevation. The restraining influence of certain neuronal phenotypes in the NTS on chronic BP has previously been reported. For example, the enzyme phosphoinositide 3-kinase (PI3K), thought to be involved in Ang-II–mediated neuronal signaling, is elevated in the NTS and paraventricular nucleus (PVN) of SHR. However, chronic neuronal blockade of PI3K in the NTS is prohypertensive, whereas it is antihypertensive in the PVN of SHR. Similarly, chronic silencing of catecholaminergic neurons in the NTS increases BP more profoundly in the SHR. The opposing effects of activation of the NTS versus the PVN/rostral ventrolateral medulla (RVLM)/SFO/SON are generally attributed to the notion that the NTS is considered an inhibitory cardioregulatory nucleus because its activation (by baro-input, for example) decreases the BP, whereas the activation of the PVN, RVLM, and SFO/SON (ie, the excitatory cardioregulatory nucleus) has an opposite cardiovascular effect. In line with this, PRR knockdown in the excitatory SFO and SON is sympathoinhibitory and alleviates hypertension. However, and similarly to the PI3K, upregulation of PRR in certain NTS neuronal subtypes, which seem to have a restraining influence on BP within the NTS, may promote compensatory activation of the NTS because the system attempts to restore the cardiovascular homeostasis and prevent even higher BP development. The question now, however, is why the SHR BP remains elevated, despite the intrinsic compensatory PRR upregulation in the NTS. From this, it follows that the NTS PRR upregulation, however beneficial, may be only 1 of the compensatory mechanisms attempting to re-establish the cardiovascular homeostasis in the SHR. Our current study also demonstrates the involvement of specific cytokines/chemokines in the NTS PRR signaling. Inflammatory status of the brain is now a well-established hallmark of essential hypertension. We observed a significant decrease in the inflammatory markers IL-6, TNF-α, and Ccl5, as well as their transcription regulator NF-κB, in the NTS of the SHR compared with the WKY rats. Consistent with this, IL-6 and Ccl5 exert cardiovascular effects via modulation of sympathetic neuronal outflow in the central nervous system. For example, acute microinjection of Ccl5 in the NTS induces a profound decrease in BP in the SHR but has only a minor effect on the WKY rats. Here, we showed that the chronic PRR knockdown in SHR resulted in further downregulation in the expression of Ccl5, while exacerbating hypertension in the SHR. Therefore, downregulation of Ccl5 in the NTS of SHR may be prohypertensive, as previously suggested.

Furthermore, activation of neuronal PRR in vitro resulted in increased NF-κB activation within 5 minutes of incubation, which is also the time frame of the acute depressor effects of a single renin injection in the NTS. Therefore, the acute effects of renin may also be mediated via the cytokine/chemokine signaling. However, we acknowledge the lack of evidence to support this hypothesis at present, and the NTS PRR activation may also have a cytokine-independent effect beyond the scope of the current study, warranting further investigation.

Collectively, these studies suggest that chronic PRR upregulation in the NTS of SHR acts as a compensatory mechanism attempting to normalize the levels of specific cytokines/chemokines. Considering the putative restraining role of the NTS neurons on chronic BP, without this mechanism of upregulation of the PRR in the NTS, the cytokine/chemokine levels may be even lower and the BP even higher in SHR. Therefore, the inherent overexpression of PRR in the NTS of SHR may exert a protective effect by preventing further increases in BP and HR.

**Perspectives**

PRR is the newest member of the renin-angiotensin system, the impact of which on pathophysiology of cardiovascular disease and diabetes mellitus is increasingly evident. However, the role of this receptor in neural control of cardiovascular function remains to be fully appreciated. Our study demonstrated that chronic knockdown of PRR in the SHR NTS exacerbates hypertension in an NF-κB–cytokine-mediated and AT1 receptor–independent manner. Thus, the current study provides support for a protective role of PRR in development of hypertension in SHR. Further studies should focus on the types of neurons involved in the NTS PRR signaling.

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**Disclosures**

None.

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What Is New?

- The nucleus of the solitary tract (NTS) (pro)renin receptor (PRR) regulates both short- and long-term cardiovascular homeostasis
- NTS PRRs present a protective effect against hypertension by an angiotensin II-independent pathway
- PRR signaling involves nuclear factor κB-cytokine pathway

What Is Relevant?

- Determination of the modulatory factors and investigation of the neurons involved in the NTS PRR activation may provide a pathway toward development of powerful intervention tools for lowering arterial pressure.

Summary

Chronic knockdown of NTS PRR in the spontaneously hypertensive rat elevates blood pressure and shifts the set-point of arterial pressure to a higher level; in line with this, acute prorenin injections in the NTS induce hypotensive and bradycardic effects. In addition, neuronal PRR signaling is nuclear factor κB-cytokine signaling-dependent. This study suggests that NTS PRR may be a compensatory mechanism of hypertension, acting to re-establish blood pressure homeostasis in a manner mediated by nuclear factor κB-cytokine signaling pathway.
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Supplement Materials

NTS (pro)renin receptor-mediated antihypertensive effect involves NF-KB-cytokine signaling in the spontaneously hypertensive rat (SHR)

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Short title: Brain prorenin receptor in hypertension

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Materials and Methods

Animals

All Wistar–Kyoto rats (WKY) and SHR 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.

Plasmid construction and production of AAV vectors containing PRR-shRNA

A synthetic complementary DNA encoding small hairpin RNA (shRNA) targeted rat PRR mRNA or scrambled (Sc) DNA, containing the same nucleotides as the PRR-shRNA DNA template but in a completely different arrangement and not targeted to any rat gene, was designed and cloned into an AAV vector, PTR-UF11, under the control of human U6 promoter. A GFP reporter gene under the control of chicken beta-actin (CBA) promoter was cloned upstream of the shRNA expression cassette to enable visualization of expression from the vector, confirming transgene delivery. Details of plasmid construction and generation of AAV2-PRR-shRNA are as described previously.

Chronic PRR knockdown using AAV2-PRR-shRNA in the Nucleus of the solitary tract (NTS)

PRR knockdown was performed using AAV2-PRR-shRNA mediated gene transfer. Eight-week-old male SHR were randomly divided into two groups. They were anesthetized and subjected to bilateral injection into the NTS with experimental virus, AAV2-PRR-shRNA, or control virus, AAV2-Sc-shRNA. AAV2 virus {2x10^8 genome containing virus particles (gc) in 200 nL} was injected into each injection site at the level of the calamus scriptorius ±400 µm from the midline, and 600 µm ventral to the dorsal surface. Eight weeks following microinjection, animals were subjected to BP and autonomic function measurement as described below.

Blood pressure (BP) and baroreflex measurement in PRR chronic knockdown SHR

All SHR were anaesthetized under urethane and implanted with catheters (PE-10 connected to a PE-50) into both the femoral artery, for BP measurement, and femoral vein, for drug delivery. The catheters were tunneled subcutaneously into the back of the neck to allow access when the animal was conscious. The arterial catheter was connected to a strain-gauge transducer coupled to a computer-based data acquisition system (PowerLab, ADInstruments) in order to record pulsatile arterial pressure (PAP). Mean arterial pressure (MAP) and heart rate (HR) were simultaneously calculated by the software LabChart (ADInstruments) and continuously displayed. After 24h the cardiovascular parameters were evaluated in unanesthetized animals.
The baroreflex control of HR was determined by recording reflex HR changes in response to transient increases or decreases in mean arterial pressure (MAP) produced by bolus injections of phenylephrine (0.5 and 1 µg/0.1 mL, iv; baroreflex bradycardia) or sodium nitroprusside (0.5 and 1 µg/0.1 mL, iv; baroreflex tachycardia) as previously described. In each animal, the ratio between changes in HR (as pulse interval, ΔPI, ms) and changes in MAP (ΔMAP, mmHg) was used as index of baroreceptor sensitivity (BSI). In addition, the baroreflex MAP-HR relationship was evaluated using the logistic sigmoid function curve for the best fit as described by the following equation,

\[ y = \frac{A_1}{1 + \exp\left[ A_2 \left( x - A_3 \right) \right]} + A_4, \]

where \( y \) is HR, \( x \) is MAP, \( A_1 \) is the \( y \) range, \( A_2 \) is the gain coefficient, \( A_3 \) is MAP at the midpoint (operating point), and \( A_4 \) is \( y \) at the bottom plateau. The function curves were constructed as described previously and the goodness of fit of each sigmoidal curve to the raw data points was measured by the correlation coefficient (\( R^2 \)). Only sigmoidal curves with \( R^2 > 0.91 \) were considered for further analysis. The results of the sigmoidal curves are represented in table S1.

**In vivo acute injection of recombinant human renin into the NTS**

Twelve-week-old male WKY rats and SHR were implanted with telemetry transmitters (DSI International) for BP measurement as described previously. Following one week of recovery, rats were anaesthetized with a urethane/a-chloralose mixture (800 mg/kg of urethane, 80 mg/kg of a-chloralose, i.p.; Sigma Aldrich), and the head was positioned in a stereotaxic frame. Bilateral microinjection (200 nL/side) of the vehicle (Tris-NaCl, pH 7.4), recombinant human renin (Innovative, Michigan, USA, 0.02-2 pmol in 200nL/side) and losartan (200 pmol in 200nL/side) were made at the level of the calamus scriptorius, ±400 µm from the midline, and 600 µm ventral to the dorsal surface, using a glass micropipette (Harvard Apparatus), with the head held firmly in the stereotaxic frame and the nose pointing downwards at 45º. Calamus scriptorius was used as a landmark to direct micropipettes into sites known to contain baroreceptive neurons in the rat. The losartan dose used here was previously shown to completely block Ang II effects in the NTS. The AT2R blocker (2 nmol in 200 nL/side), Mas receptor antagonist, A779 (200 pmol in 200 nL/side), or prorenin handle region peptide (HRP, 200 pmol in 200nL/side) were injected 5 minutes prior to injection of renin at the same site. The micropipette was placed into the NTS using a micromanipulator. The microinjection volume was determined by viewing the movement of the meniscus through a binocular microscope fitted with a calibrated eyepiece graticule. The microinjections were completed over a period of 1 minute. Immediate effects of the microinjections on the SBP and HR were monitored, and these returned to the baseline values within 5 minutes. Blue dye (60-80 nl) was microinjected unilaterally into the NTS at the end of each experiment to mark the injection site.

**PRR immunoactivity in the NTS**

Fresh brainstem c coronal sections containing the NTS was cut and PRR staining was performed as described previously.
RNA isolation from the NTS

Rats were euthanized, brains were dissected, and the NTS regions were punched out and subjected to RNA isolation, using RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions.

Preparation of neuronal cultures

Neuronal cultures were made from the hypothalamus and brainstem of one-day-old SHR as described previously\(^1\). Neuronal cultures contain more than 90% neurons (remaining cells are primarily astroglia). The cultures were maintained for 12-16 days prior to their use in experiments.

Measurement of phosphorylation of IKB\(\alpha\):

Western blot analysis was used to measure phosphorylated IKB\(\alpha\) and endogenous control GAPDH using the neuronal cultures from SHR as described previously\(^1\). Briefly, neuronal cultures were incubated with or without human prorenin (100 nmol/L) (Innovative Research, Novi, MI, USA) for differing time periods. Proteins were isolated, separated with 10% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with Phospho-IKB\(\alpha\) primary antibody (cell signaling, Boston, MA, USA). Hybrid protein bands were detected with ECL™ Western Blotting Detection Reagents. The membrane was stripped of phosphorylated ERK1/2 antibody and re-probed with GAPDH antibody to assay total protein levels. Protein bands representing Phospho-IKB\(\alpha\) were quantified using the NIH ImageJ program (Bethesda, Maryland, USA; http://rsb.info.nih.gov/ij) phosphorylated IKB\(\alpha\) were normalized to GAPDH.

Measurement of prorenin stimulation effect on cytokines and related genes

Neuronal cultures incubated with 100 nmol/L of prorenin or vehicle control for differing time periods (1h, 3h, 6h, 12h and 24 h). Cells were collected and quantitative real-time PCR was performed to measure mRNA levels of cytokines including: interleukin(IL)1\(\beta\), tumor necrosis factor-alpha (TNF\(\alpha\)), IL6, chemokine C-C ligand 5 (Ccl5), IL10, NF-KB subunit P50 (nfb1), I-kappa B kinase (IKK) subunit nfkbia using specific primers and probes (Applied Biosystems, Foster City, CA, USA) in the Step One Plus™ Real Time PCR System (Applied Biosystems). Based on the results from above time-course experiment, the maximum increase in cytokine mRNA levels occurred 3h following prorenin incubation. We then co-incubated neuronal cultures with 100 nmol/L of prorenin or 20 nmol/L of renin with or without AT1R blocker, losartan (2 \(\mu\)mol/L), NF-KB inhibitor quinazoline (15 \(\mu\)mol/L), or curcumin (50 \(\mu\)mol/L) for 3h, the mRNA levels of cytokines, NF-KB and IKK were determined by quantitative RT-PCR as described above.

Each set of experiments was performed using three separate culture dishes, and all cDNA samples were assayed in triplicate. The whole experiment was repeated at least 3 times. Data were normalized to GAPDH RNA.
NF-κB DNA binding ELISA

DNA binding activity of NF-κB p65 was measured using TransAM NF-κB p65 kits (Active Motif) following the manufacturer’s instructions. Briefly, 12-day-old primary neuronal cultures were incubated with either prorenin (100 nmol/L) or same volume of PBS for 30 min, the cells were collected and nuclear extract was isolated, and then 20 μg of nuclear protein samples was incubated for 1 h in a 96-well plate coated with an oligonucleotide containing a NF-κB consensus binding site (5’T–GGGACTTTCC−3’), to which NF-κB contained in nuclear extracts specifically binds. After washing, an antibody specific for p65 (1/1000 dilution) was added to these wells and incubated for 1 h. After incubation for 1 h with a secondary HRP-conjugated antibody (1/1000 dilution), specific binding was detected by colorimetric estimation at 450 nm with a reference wavelength of 655 nm.

Immunocytochemistry of PRR and NF-κB P65 in primary neuronal cultures

Immunocytochemistry of PRR and NF-κB P65 was performed as described previously. Briefly, neuronal cultures were fixed with 4% paraformaldehyde in PBS, incubated with 1% bovine serum albumin in PBS for 30 min to block non-specific binding, and then cultures were rinsed with PBS containing 0.3% Triton X-100 (PBST). They were then incubated with a 1:100 dilution of goat anti-ATP6AP2, a PRR specific antibody (NOVUS Biologicals, Littleton, CO, USA) and a 1:300 dilution of rabbit anti-NF-κB P65 antibody (cell signaling, Boston, MA, USA). This was followed by incubation with secondary antibodies. Cells were examined and photographed with the use of an Olympus BX41 microscope.

Assay of secreted cytokines

Neuronal cultures were pre-incubated with either AT1R blocker, losartan (1 μmol/L), or NF-κB activation inhibitor, quinazoline (15 μmol/L), or curcumin (50 μmol/L). Thirty minutes later, prorenin was added into the culture medium (100 nmol/L). Two further groups of neuronal cultures were treated with either identical concentrations of prorenin or vehicle control. Six hours following prorenin incubation, culture medium was collected, and processed to measure the production of TNFa and IL6 using ELISA kit (Invitrogen, Grand Island, NY, USA) exactly following the manufacturer's instructions. We selected incubation 6 h instead of 3h, which showed the maximum increase in the mRNA increase because the protein expression is slower than mRNA transcription.

Statistical analysis

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


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</table>

Values are represented as Mean±SEM. Maximum gain is represented by the gain of the sigmoidal curve at MAP corresponding to the operating point value. *P<0.05 vs. Sc-shRNA
Figure S1

A: Male WKY rats (8 weeks old) were randomly divided into two groups (n=5 each) following baseline blood pressure measurements using tail-cuff. The rats were bilaterally microinjected with either AAV2-PRR-shRNA, or control vector, AAV2-Scrambled (Sc)-shRNA into the NTS (2x10^8 gc in 200 nL/side). BP was monitored weekly by tail-cuff. No significant differences were observed in BP between the two groups. B: left panel is a diagrammatic sketch showing the NTS (red dotted line) and sites of injection (blue coloring) (at -13.68 mm from Bregma); right panel is a representative micrograph showing GFP reporter gene expression indicating successful transduction of AAV vector in the NTS. CC-central canal.
Figure S2: AAV2-PRR-shRNA decreased PRR expression in the NTS of SHR

A. representative immunofluorescence micrograph showing PRR (red) expression in the brain section containing the NTS in the control AAV2-Sc-shRNA-treated (A) and the AAV2-PRR-shRNA-injected SHR (B). The NTS is marked with white dotted line at approximately -13.40 mm from Bregma (A-B). Quantification of the PRR-positive cell numbers (C) and the mean fluorescence intensity (D), n=3 per group, *P<0.05.
Figure S3: comparison of inflammatory markers in the NTS of WKY rats, SHR and PRR-shRNA injected SHR

(A) IL1β, TNFα, IL6, Ccl5, Nfkb1 and nfkbia mRNA was quantified in the NTS of adult WKY rats, age-matched SHR, AAV2-PRR-shRNA -injected SHR, and control vector, AAV2-Sc-shRNA -injected SHR, by quantitative RT-PCR. *, †, # P<0.05
Figure S4: Prorenin treatment resulted in time-dependent increase in mRNA levels of IKK (nfkbia) and NF-KB (nfkb1) subunits.

Real-time PCR shows that prorenin treatment caused time-dependent increase in mRNA level of IKK subunit nfkbia (n=6, *P<0.05) (A) and NF-KB subunit nfkb1 (n=6 *P<0.05) (B).
Figure S5

Figure S5: Prorenin treatment resulted in time-dependent increase in mRNA levels of inflammatory markers

Co-incubation of neuronal cultures with human prorenin (100 nmol/L) or vehicle control for differing time periods (1h, 3h, 6h, 12h and 24h) increased mRNA expression of IL1β, IL6, TNFα, Ccl5 and IL10 in cultured neurons as quantified by quantitative RT-PCR. *P<0.05 compared to control
Figure S6: Renin treatment induced a robust increase in mRNA levels of cytokines

Neuronal cultures were co-incubated with renin (20 nmol/L), with or without losartan or quinazoline (3 h); mRNA levels of IL1β, IL6, TNFα, Ccl5 and IL10 were quantified by quantitative RT-PCR. * P<0.05 compared to control; † P<0.05 compared to renin group