Supplementary Materials

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

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Methods

Animals

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

Generation of neuron-specific PRR conditional knockout mice

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

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

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

Immunofluorescence staining

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

**ICV cannulation**

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

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

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

**Telemetry recordings**

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

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

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

**Statistical analysis**

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

**Reference**


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