Supplemental Material

Microglia participate in neurogenic regulation of hypertension

Xiao Z. Shen[1,3], You Li[2], Liang Li[2], Kandarp H. Shah[1], Kenneth E. Bernstein[1,3], Patrick Lyden[2] and Peng Shi[2]

[1]Department of Biomedical Sciences; [2]Department of Neurology, [3]Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, CA, USA

Short title: Microglia and neurogenic hypertension

Corresponding author:

Peng Shi, Ph.D.

Department of Neurology

Cedars-Sinai Medical Center

127 S San Vicente Blvd, AHSP 8306

Los Angeles, CA 90048

Email: Peng.Shi@cshs.org

Phone: 310-423-7373

Fax: 310-967-0601

Methods

Mice and hypertension models. C57BL/6 and CD11b-DTR mice were purchased from Jackson Laboratories. All mice were maintained in micro isolator cages, and all experimental protocols were approved by the Institutional Animal Care and Use Committee at Cedars Sinai Medical Center. We used 8-10 weeks old male C57BL/6 mice for studying microglial activation (Figures 1, 2, S1), microglial adoptive transfer (Figure 5) and DT effect (Figure S2B). For microglial depletion studies, both male and female CD11b-DTR mice (8-10 weeks old) were identically mixed in each group. Hypertension was induced by *s.c.* infusion of Ang II (1000 ng/kg/min) (Pheonix Pharmaceuticals) via an osmotic minipump (Alzet) or by L-NAME treatment (1.5 mg/ml in the drinking water) (Bachem). Blood pressure (BP) was monitored in conscious mice using a computerized non-invasive tailcuff system (Visitech Systems, BP-2000 series II, Apex), and invasively using radiotelemetry as described[1, 2]. For the former, mice were trained for 5 continuous days before data acquisition. BP was determined by averaging 20 measurements, with tracings manually reviewed to verify proper BP determination. For the latter, mice were anesthetized with isoflurane, and a catheter connected to a radiotelemetry device (HD-X11; Data Sciences International) was inserted in the left carotid artery. After a 10-14-day recovery phase, baseline BP and HR were recorded before osmotic pump implantation, followed by sampling every 2-3 days to the end of the protocol. Data were collected, stored, and analyzed using Dataquest A.R.T. 4.0 software (Data Sciences International). For acute BP recording, mice were anesthetized (2% isoflurane mixed with oxygen, 1 l/min). Blood pressure was recorded through a cannulated PE-50 catheter into the right common carotid artery. The head was positioned onto the stereotaxic frame. After baseline blood pressure and heart rate recording, a single dose of Ang II (50 ng in 1 µl) was injected through a 5 µl Hamilton syringe driven by a digital stereotaxic injector (Steolting). BP and heart rates were recorded via a Statham Transducer (P23XL), continuously monitored and digitally recorded at 100 Hz using PowerLab software (ML870, AD Instrument). Body temperature was maintained at 37^0C with a water-circulating pad throughout the procedure.

Microglial depletion. CD11b-DTR Mice were anesthetized and positioned in the stereotaxic frame. A small piece of skull was removed according to the following coordinates (0.3-0.5 mm post Bregma, 1.5-2 mm lateral to the midline). A 2-wk osmotic mini-pump infusing DT (Bio Academia) was connected to a brain infusion kit (Alzet), and the tip of the cannula was positioned in the left cerebroventricle with a depth of 3 mm, and then stabilized on the skull surface using bio-adhesive glue. The osmotic mini-pump was placed along the neck pocket.

Microglial dissociation. Following transcardiac perfusion with sterile heparinized PBS (100 ml, 4°C), mice were decapitated and the brains were removed. The procedures have been documented previously with slight modification[3]. In brief, whole brains were chopped to small pieces followed by enzymatic digestion with 1.6 mg/ml collagenase IV (Worthington) and 15 units of DNase I (Sigma) at 37°C for 1 h. Digested tissues were passed through 70 µm strainer (BD) and centrifuged in 37% and 70% Percoll (GE Healthcare) gradients. Microglia were enriched from the interface between 37% and 70% Percoll.

Antibodies and Flow Cytometry. The following antibody clones were used: M1/70 (anti-CD11b), 30-F11 (anti-CD45), AF6-120.1 (anti-I-A^b), mIL4R-M1 (anti-IL-4Rα), 4B12 (anti-CCR7), 2E2 (anti-IFNγR), MR5D3 (anti-mannose receptor), TEK4 (anti-Tie2), 72-1 (anti-CD36), MP6-XT22 (anti-TNFα), NJTEN3 (anti-IL-1β) and MP5-20F3 (anti-IL-6). All the antibodies above were purchased from either eBioscience, BioLegend or Pharmingen. For staining intracellular proteins, fixation and permeabilization buffers were purchased from eBioscience. PE-labeled anti-iNOS was from Santa Cruz Biotechnology. Microglia was defined as $CD11b^+CD45^{low}$. To detect microglia cytokine expression, cells were cultured for 6 h in dish in the presence of brefeldin A (eBioscience). Some cells were stimulated with LPS (Sigma) in the 6-h culture. The stained samples were analyzed on a Beckman Coulter CyAn ADP and data were analyzed by FlowJo software.

Immunohistochemistry. After being deeply anesthetized with 5% isoflurane mixed with oxygen, mice were perfused transcardially with heparinized saline followed by 4% paraformaldehyde. Brains were cut into 30 μm coronal sections. Brain sections were incubated with monoclonal mouse anti-NeuN (Millipore, MAB377) antibody followed by Alexa 488-labeled goat anti-mouse IgG for detecting neurons; sections were incubated with polyclonal rabbit anti-Iba1 antibody (Wako; 019-19741) followed by Alexa 594-labeled goat anti-rabbit IgG for detecting microglia; sections were incubated with chicken anti-GFAP (Millipore, AB5541) antibody followed by Alexa 594-tagged goat anti-chicken IgG for detecting astrocyte; and sections were incubated with cocktail of goat anti-CD31 (R&D Systems) and rat anti-F4/80 (AbD Serotec) antibodies followed by cocktail of donkey anti-goat Alexa 594 and donkey anti-rat Alexa 488 IgG for detecting endothelial cells and brain macrophages, respectively. Sections were imaged using a laser scanning confocal microscope (Olympus, FV10i).

Fractional area analysis. The morphological changes of microglia, identified by Iba1 immunoreactivity, were analyzed by measuring the fractional area as described previously[2, 3]). The fractional areas were defined as the fraction of area immunoreactive for Iba1 antigen. In brief, the sections that covered the entire PVN (Bregma -0.82 to -1.06 mm) were analyzed based on the ratio of the calculated area of Iba1-positive staining to the entire images (0.2x0.2 mm^2 area), and 10 fields from 3 different sections of each animals were analyzed. Motor cortex was selected on the dorsal part of the same brain section.

Western blot. PVN tissues were homogenized in RIPA buffer (Thermo) mixed with protease inhibitor cocktail (Thermo), loaded on 4-12% gradient Bis-tris gels (Invitrogen) followed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes in a conventional method as described previously. The membrane was blocked (Odyssey) and incubated with rabbit anti-GluN2A (Cell signaling) and mouse anti-α-tubulin (Developmental Studies Hybridoma Bank) 4°C overnight, and then reacted with the fluorescence-conjugated goat anti-rabbit or goat anti-mouse antibodies (Li-Cor). The fluorescent intensities were visualized by Odyssey Clx imaging system (Li-Cor). Fluorescent signals were captured and stored digitally; and quantified using Odyssey software (Li-Cor, v3.0).

ELISA. The ELISA kits used in this research are listed below: vasopressin (Enzo; ADI-900-017), norepinephrine (Abnova; KA1891), IL-1β (eBioscience; 88-7013-88) and

TNFα (eBioscience; BMS607/2INST). ELISA was performed according to the manufacturers' instructions.

Primary astrocyte culture. Astrocytes were dissociated from one-day-old C57BL/6J mouse pups as previous stated[4]. In brief, brains were removed, mechanically and enzymatically dissociated, and plated in 75-cm^2 flask. When the cells reached 100% confluency, cells were suspended and re-seeded to a new flask for culture for another 7 days. The attached cells, of which 99% are astrocytes, were suspended, washed and re-seeded to 35-mm dishes one day before Ang II treatment (100 nM, 12 hr).

Adoptive transferring of primed microglia or primary astrocytes. After being stimulated with Ang II (100 nM) or LPS (10 ng/ml, Sigma) with or without minocycline (100 μM, Sigma; M9511), N9 cells[3, 5] or primary astrocytes were harvested, washed once with DMEM medium and re-suspended in PBS. Each recipient mouse received $5x10^5$ cells via a pump-driven Hamilton microsyringe based on the following coordinates: 0.3-0.35 mm posterior to Bregma; 1.5-2 mm lateral to midline; 2 mm below the dura). In this process, cells were injected in a volume of 1 μl at 0.1 μl/min.

Statistics. Data are summarized as mean ± S.E.M.. Statistical analysis was performed with GraphPad Prism version 6. Differences between groups were compared by unpaired t-test, one- or two-way ANOVA analysis by Newman-Keuls Multiple comparison test, accordingly.

References

1. Ong FS, Lin CX, Campbell DJ, Okwan-Duodu D, Chen X, Blackwell WL, Shah KH, Gonzalez-Villalobos RA, Shen XZ, Fuchs S, Bernstein KE. Increased angiotensin II-induced hypertension and inflammatory cytokines in mice lacking angiotensin-converting enzyme N domain activity. *Hypertension*. 2012;59:283-290.

2. Shi P, Diez-Freire C, Jun JY, Qi Y, Katovich MJ, Li Q, Sriramula S, Francis J, Sumners C, Raizada MK. Brain microglial cytokines in neurogenic hypertension. *Hypertension*. 2010;56:297-303.

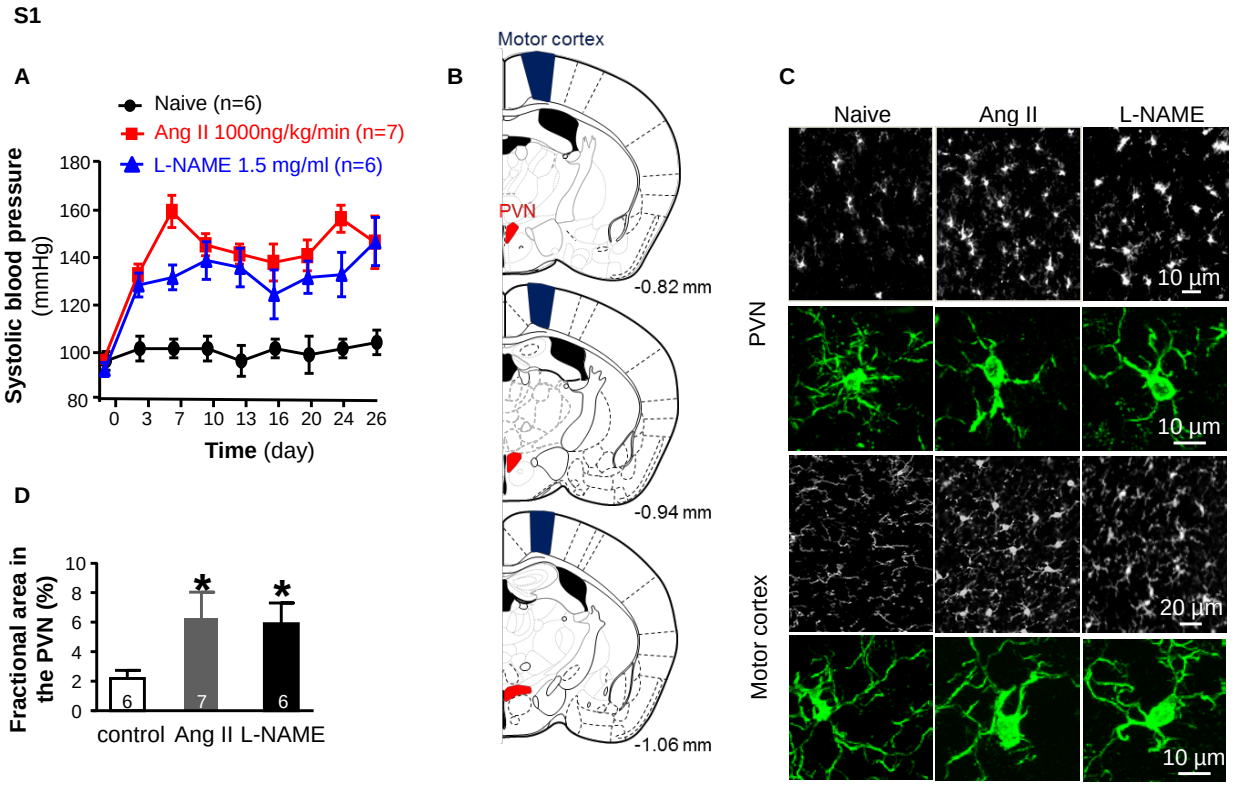
3. Shi P, Grobe JL, Desland FA, Zhou G, Shen XZ, Shan Z, Liu M, Raizada MK, Sumners C. Direct pro-inflammatory effects of prorenin on microglia. *PLoS One*. 2014;9:e92937.

4. Shan Z, Cuadra AE, Sumners C, Raizada MK. Characterization of a functional (pro)renin receptor in rat brain neurons. *Exp Physiol*. 2008;93:701-708.

5. Sedgwick JD, Schwender S, Imrich H, Dorries R, Butcher GW, ter Meulen V. Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. *Proc Natl Acad Sci U S A*. 1991;88:7438-7442.

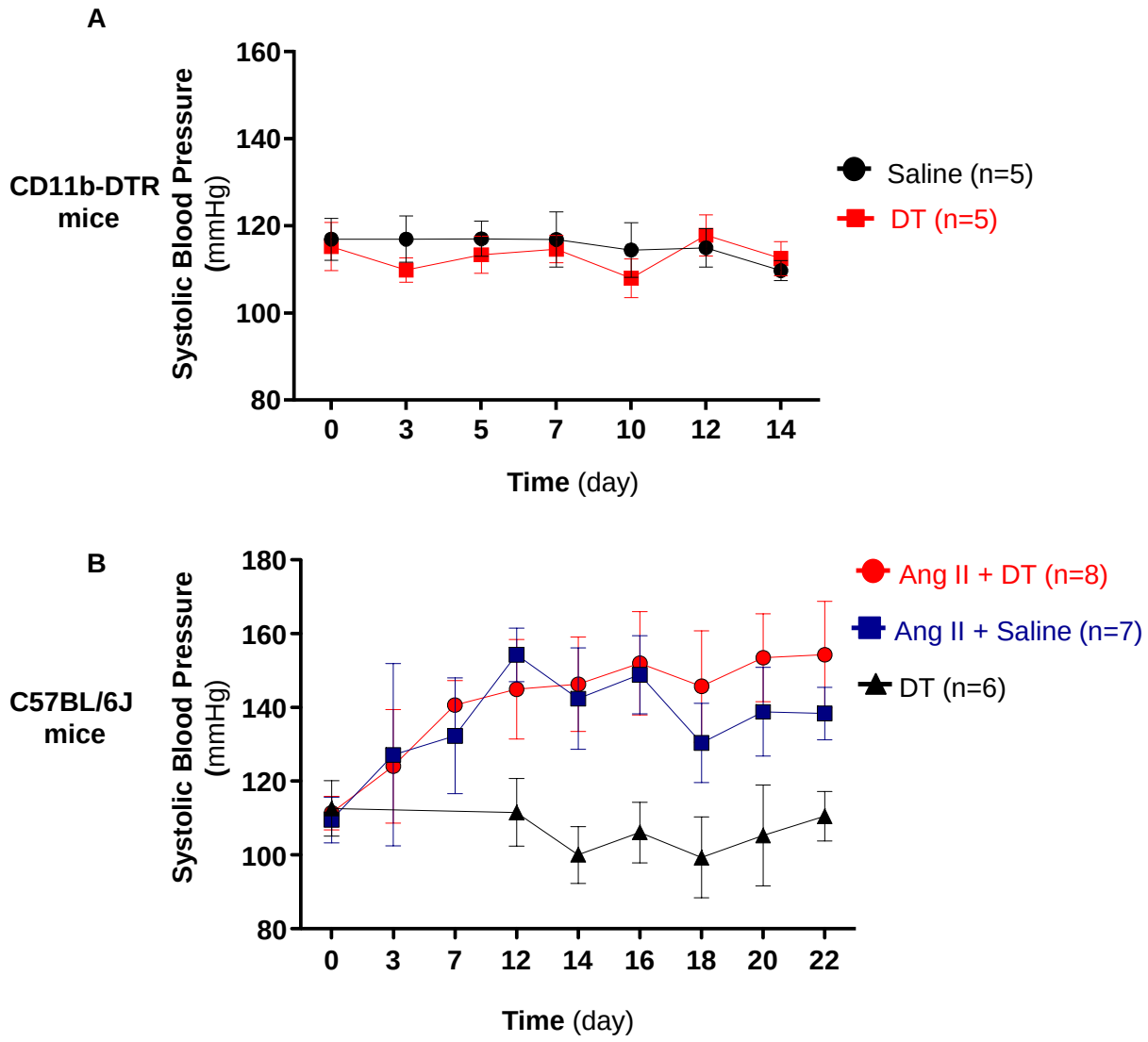
Table S1. Baseline mean arterial pressure (MAP) and heart rate (HR) in the mice receiving *i.c.v.* transfer of saline (Sham) or pre-treated microglia or astrocytes.

	No cells	**Microglia**					**Astrocyte**
Treatment	sham	Naive	Ang II	Ang II + Minocycline	LPS	LPS + Minocycline	Ang II
n	8	7	10	9	7	7	8
MAP (mmHg)	81±1	85±1	85±2	81±1	82±1	82±1	85±1
HR (beat/min)	327±10	327±20	303±10	327±8	311±9	340±12	332±20



S1. Characterization of microglia associated with hypertension. (A) Systolic blood pressure of naïve mice or mice treated with Ang II (1000 ng/kg/min) or L-NAME (1.5 mg/ml in drinking water). (B) Schematic outline of motor cortex and PVN in mouse brain. Coordinates are in reference to the Bregma based on coronal sections adapted from the atlas of Paxinos and Watson. (C) Iba1 staining of microglia in the PVN and motor cortex of normotensive mice or the mice treated with Ang II or L-NAME for 4 weeks. (D) Fractional area analysis of microglia in the PVN of above three groups. * P<0.05 vs. control by One-way ANOVA.

S2



S2. Blood pressure responses to *i.c.v.* DT. (A) Systolic blood pressure was measured after *i.c.v.* saline or DT infusion into CD11b-DTR mice. The two groups have no difference observed. (B) Systolic blood pressure of C57BL/6J mice treated with or without *s.c.* Ang II infusion (1000 ng/kg/min). The group without Ang II infusion and one group with Ang II infusion received *i.c.v.* DT (1000 pg/g body weight) treatment. DT treatment did not alter the blood pressure patterns of C57BL/6J mice.