IFN-γ deficiency in the rostral ventrolateral medulla contributes to stress-induced hypertension by impairing microglial synaptic engulfment

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Supplemental Methods

Measurements of blood pressure (BP) and heart rate (HR)

Non-invasive BP and HR were measured on 0, 3, 6, 9, and 12 days using the tail-cuff system (ALC−NIBP, China). The rats were transferred to a standard setup with a heater for 30 min to ensure adaptation to the procedure. The BP and HR levels were always recorded by using a pressure transducer during inflation and deflation cycles. The signal from the pulse and pressure sensors were amplified and digitized. Measurements were repeated three times.

The BP and HR levels were also examined in anesthetized state through femoral artery annulation in each group. The rats were anesthetized with isoflurane (oxygen flowmeter, 0.8–1.5 L/min; isoflurane vaporizer, 1.5%–3%). Isoflurane level was adjusted as necessary to ensure appropriate depth of anesthesia. The right femoral vein and artery were exposed by blunt dissection and cannulated separately using two polyethylene catheters consisting of PE 50 tubing and filled with heparinized saline (50

U/mL). The distal end of the arterial cannula was attached to a pressure transducer for direct monitoring of BP. HR was calculated automatically by a computer in accordance with the BP phasic wave.

Renal sympathetic nerve activity (RSNA) recording

The rats were anesthetized as previously described. Following a left flank incision on rats, a bundle of left renal sympathetic nerves was identified and dissected free of surrounding tissue in the retroperitoneal space. Using an operating microscope, the nerve was placed onto platinum–iridium electrodes. The nerve–electrode complex was covered with silicone gel (Kwik-Sil, WPI, USA) for electrical insulation from surrounding tissues. The nerve activity was amplified (×100) and filtered (bandpass 100–3000 Hz) using a low-noise differential amplifier (AD Instruments, Australia). The signal was recorded using a Power Lab data acquisition system (AD Instruments, Australia). After the experiment, the signal recorded during euthanasia (pentobarbital: 200 mg/kg, i.p.) was regarded as the maximum signal, and after 30 min, the signal was regarded as background noise. The RSNA was calculated by subtracting the background noise from the recorded raw nerve activity, and then it was integrated (1 s time constant). RSNA was finally expressed as the percentage of the relative maximum signal.

Enzyme-linked immunosorbent assay (ELISA)

After sacrifice, plasma was collected in tubes using EDTA-Na_2 as an anticoagulant. Samples were centrifuged at 1000 g for 15 min at 4 °C. Changes in norepinephrine (NE) concentration were measured in supernatant using commercial ELISA kits (FineTest, China) according to the manufacturer's descriptions. Finally, absorbance was assessed at 450 nm using a SpectraMax iD5 microplate reader (Molecular Devices, USA).

Co-culture procedures

For co-culture experiments of microglia and neuron, rat microglial cells (GMI-R1) were added to rat neuroblastoma cells (B104) at a microglia-to-neuron ratio of 1.5:1. Then, cells were seeded on ploy-D-lysine-coated wells (Sigma, USA) in Dulbecco's Modified Eagle Medium containing 10% heat-inactivated fetal bovine serum (Gibco, USA), 0.6% glucose, 2 mM sodium pyruvate, and 1% penicillin–streptomycin (Lonza,

Switzerland). In a distinct set of experiments, B104 were plated in Transwell culture inserts (Biofil, China) at the same microglia-to-neuron ratio to prevent contact with GMI-R1. In investigating the mechanism of IFN-γ underlying microglial synaptic engulfment in vitro, the cells were cultured in the presence of 1000 U/mL of IFN-γ or 50 μM Bindarit (CCL2 inhibitor) for 48 h [1–3].

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA isolation and cDNA synthesis were performed using the TRIeasy Plus Total RNA Kit (Yeasen, China) and Hifair II cDNA Synthesis Kit (Yeasen, China), according to the manufacturer's instructions. qRT-PCR was performed using Hieff qPCR SYBR Green Master Mix (Yeasen, China) on CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The reaction mixture contained 0.4 μl of each primer (10 μM), 2 μl of cDNA, 7.2 μl of H_2O, and 10 μl of Hieff qPCR SYBR Green Master Mix. *Gapdh* served as an endogenous control. Sequences of custom-designed primers are shown in **Table S1**.

Western blotting

Total protein extracted from RVLM tissue homogenates and cells with RIPA Lysis Buffer (Beyotime, China) was used to analyze protein expression by Western blotting. Proteins were separated by 4%–12% SDS-PAGE and transferred onto PVDF membranes (0.45 μm; Millipore, USA). Membranes were placed in QuickBlock™ Blocking Buffer (Beyotime, China) for 30 min to block non-specific binding sites. Then, such membranes were incubated overnight at 4 °C with primary antibody solution and for 1 h at room temperature with secondary antibody solution. The primary antibody used for Western blot analysis included rabbit monoclonal antibody to IFN-γ (1:1000; R&D Systems, USA), rabbit polyclonal antibody to CCL2 (1:1000; Proteintech, China), rabbit polyclonal antibody to VGLUT1 (1:800; Abcam, UK), mouse monoclonal antibody to CD68 (1:600; Bio-Rad, USA), and mouse monoclonal antibody to GAPDH (1:2000; Abcam, UK). The secondary antibody included Goat Anti-Mouse IgG (HRP Conjugate; 1:5000; CST, USA) and Mouse Anti-Rabbit IgG (HRP Conjugate; 1:5000; CST, USA). Fluorescent signals were detected using ECL detection reagents (WBKLS0050; Millipore, USA) with a fully automatic chemiluminescence image

analysis system (Tanon-5200; Tanon Science & Technology, China). The amount of band protein was quantified as the ratio to GAPDH protein.

Immunofluorescence

For RVLM tissue staining, rats were anesthetized with pentobarbital sodium and perfused with 200 mL of heparinized saline through the ascending aorta followed by 200 mL of freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Brain tissues were removed and post-fixed in the same fixative for 12 h and then placed in 20% sucrose at 4 °C until they sunk to the bottom. Afterward, brain tissues were placed in 30% sucrose at 4 °C until they sunk to the bottom. Frozen 30 μm coronal sections were cut by using a cryostat (HM525; Microm, Germany). For cell staining, cell culture coverslips were fixed for 10 min at 4% paraformaldehyde in 0.1 M PBS. Sections or coverslips were incubated in 0.2 mg/mL of pepsin (Beyotime, China) at 37 °C for 10–15 min according to the procedure developed by Lorincz [4] and then in 0.3% Triton X-100 for 30 min. Next, they were incubated in QuickBlock™ Blocking Buffer (Beyotime, China) for 30 min for Immunol Staining to block non-specific binding sites and then in primary antibodies, including rabbit monoclonal antibody to IFN-γ (1:400; R&D Systems, USA), rabbit polyclonal antibody to CCL2 (1:600; Proteintech, China), mouse monoclonal antibody to NeuN (1:400; Abcam, UK), rabbit polyclonal antibody to VGLUT1 (1:100; Abcam, UK), goat monoclonal antibody to Iba1 (1:500; Abcam, UK), mouse monoclonal antibody to CD68 (1:400; Bio-Rad, USA), rabbit monoclonal antibody to PSD95 (1:300; Abcam, UK), mouse monoclonal antibody to SYN1 (1:500; Synaptic Systems, Germany), and rabbit monoclonal antibody to c-Fos (1:1000, Cell Signaling Technology, USA) overnight at 4 °C. In the following day, sections or coverslips were rinsed with 0.01 M PBS and incubated for 2 h with secondary antibody, including donkey anti-rabbit IgG H&L Alexa Fluor 555 (1:500; Abcam, UK), donkey anti-goat IgG H&L Alexa Fluor 647 (1:500; Abcam, UK), and donkey anti-mouse IgG H&L Alexa Fluor 594 (1:500; Abcam, UK) at room temperature. Fluorescence signals were monitored by using a confocal laser scanning microscope (LSM880; Zeiss, Germany).

Calcium imaging

Intracellular calcium concentration of B104 cells in co-cultures was measured by Fluo-4/AM (Beyotime, China). Cells were incubated in complete 5 μM Fluo-4/AM loading solution for 60 min at 37 °C. Then, cells were washed three times with PBS. Images were taken using a confocal laser scanning microscope at a 488 nm excitation ratio.

Image quantification procedures

Confocal Z-stack images were taken using a confocal laser scanning microscope (Zeiss, Germany) at ×40 or ×63 magnification consisting of at least 15 images. Images were transformed into 3D reconstruction files using IMARIS 9.7 (Oxford Instrument, UK). Images were subjected to maximum intensity projection, and only cells whose processes were entirely within the 3D Z-stack volume were quantified. We performed immunostaining for PSD95, VGLUT1, and SYN 1 to quantify the synaptic density. The number of PSD95 puncta, VGLUT1 puncta, and SYN1 puncta was determined using the spot module in IMARIS 9.7. We quantified the microglial engulfment of synapses by immunostaining PSD95, VGLUT1, and CD68 inside Iba1-positive cells. The ratio of PSD95-occupied, VGLUT1-occupied, and CD68-occupied areas in Iba1-positive cells was determined using the surface module in IMARIS 9.7.

Intra-RVLM microinjection

Each animal was placed in prone position, and the head was mounted in a Stereotaxic Instruments (RWD, China) under anesthesia with isoflurane. The skull was exposed with a midline scalp incision, and bregma and lambda were positioned on the same horizontal plane. IFN-γ (20 U/μl, Gibco, USA) [5,6] and Bindarit (CCL2 inhibitor, 200 μmol/L, MCE, USA) [7] were bilaterally microinjected into the RVLM (3.7–4.0 mm caudal to lambdoid suture, 2 mm lateral to the midline, and 8.0 mm ventral to the surface of the dura) [8] using a glass micropipette (the tip diameter was 60 μm). The microinjection volume was controlled at 0.5 μl/side. The micropipette was placed for approximately 3 min before suturing. Because the microinjection was implemented to test the effect of IFN-γ on BP, analgesics were not used after surgery to prevent the assessment from being skewed.

References

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Supplemental Figures

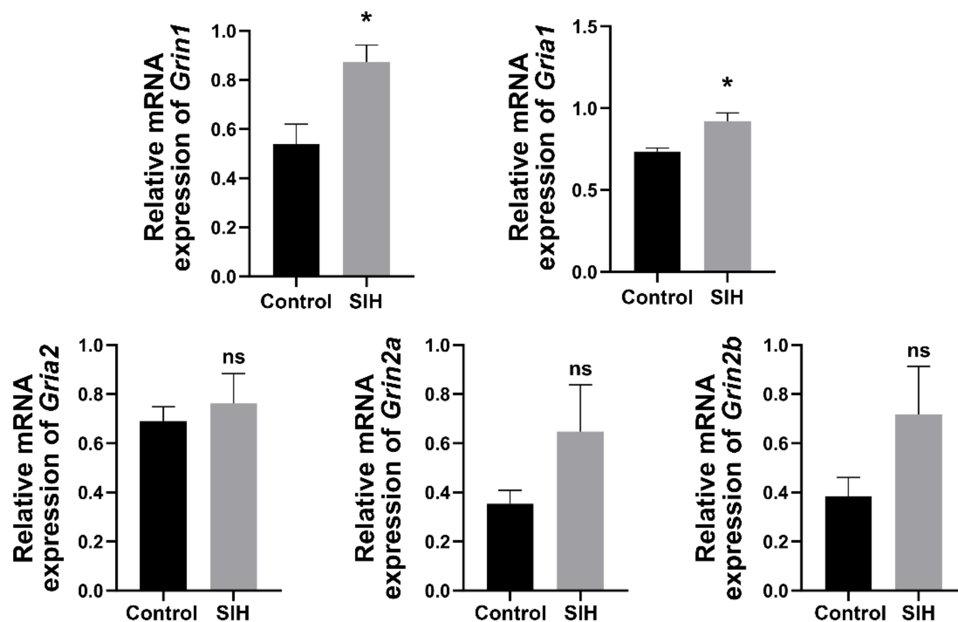

Fig. S1 qRT-PCR assays of *Grin1*, *Gria1*, *Gria2*, *Grin2a*, and *Grin2b* expression levels in the RVLM of the SIH and control rats. *Gapdh* was used as the internal

control. Data are expressed as mean ± SEM. Statistical significance was determined by unpaired two-tailed Student's *t*-test. n = 6 rats per group. $^*P < 0.05$ versus the control group. ns indicates nonsignificant versus the control group.

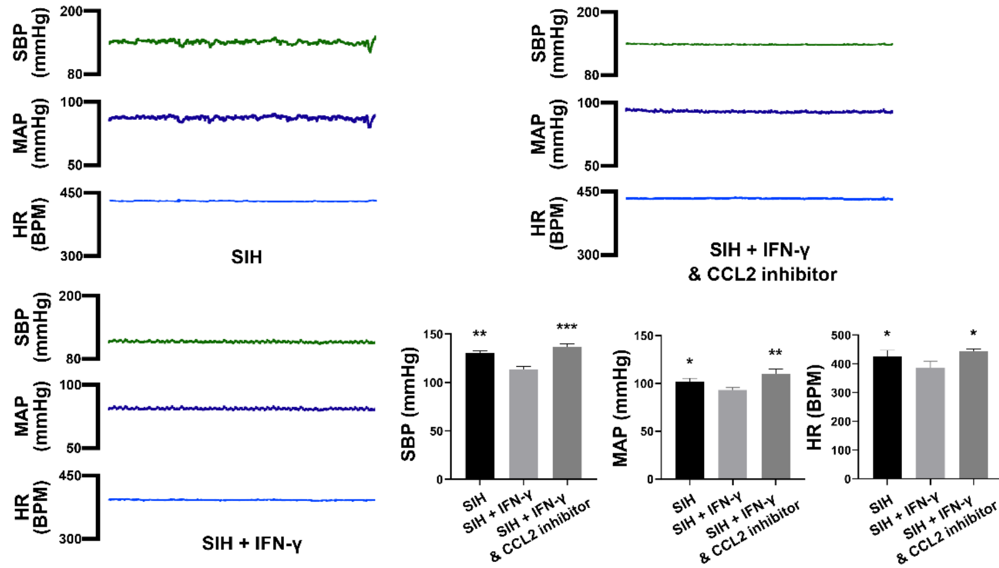

Fig. S2 The SBP, MAP, and HR levels of rats were detected by femoral artery cannulation methods. Data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by a post hoc Bonferroni test. n = 6 rats per group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the SIH + IFN-γ group.

Supplemental Table

Table S1 Primers used in qRT-PCR analysis.

Gene	GenBank accession No.	Primer sequence (5'-3')	Annealing temperature (℃)	The size of production (bp)
Grin1	NM_017010.2	F: CGTGAACGTGTGGAGGAA	60 ℃	145
		R: GGTGCTCGTGTCTTTGGA		
Gria1	NM_031608.2	F: CCAGACAACCAGTGACCA	60 ℃	145
		R: AGTGAAGAACCACCAGACG		
Grin2b	NM_012574.1	F: GAGAACTCGCCTCACTGG	60 ℃	152
		R: CCTACCACTCCGTGCTTC		
Grin2a	NM_012573.4	F: GGAGGGATGAAGGCTGTAA	60 ℃	133
		R: CCAACAAACTGGAGCAGAG		
Gria2	NM_001083811.1	F: TGAGGAAGGAAACAGAGGAG	60 ℃	120
		R: AAATGTCAAGCATCACCAAGT		
Ifn-γ	NM_138880.3	F: GGCAAAAGGACGGTAACA	60 ℃	188
		R: GAACTTGGCGATGCTCA		
Ccl2	NM_031530.1	F: CAGGTCTCTGTCACGCTTC	60 ℃	148
		R: AGTTCTCCAGCCGACTCA		
Gapdh	NM_017008.4	F: TCCCATTCTTCCACCTTTG	60 ℃	110
		R: TCCACCACCCTGTTGCT		