Title: Microglial activation induced by LPS mediates excitation of the hypothalamic paraventricular nucleus neurons projecting to the rostral ventrolateral medulla

Authors

Tae Hee Han, Heow Won Lee, Eun A Kang, Min Seok Song, So Yeong Lee, Pan Dong Ryu

Author affiliations

Laboratory of Veterinary Pharmacology, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul 151-742, Republic of Korea

Corresponding author

So Yeong Lee D.V.M. Ph.D
Laboratory of Veterinary Pharmacology
College of Veterinary Medicine
Seoul National University
1 Gwanak-Ro, Gwanak-Gu
Seoul 08826, Republic of Korea
Tel: +82-2-880-1283, Fax: +82-2-879-0378
E-mail: leeso@snu.ac.kr

Pan Dong Ryu D.V.M. Ph.D
Laboratory of Veterinary Pharmacology
College of Veterinary Medicine
Seoul National University
1 Gwanak-Ro, Gwanak-Gu
Seoul 08826, Republic of Korea
Tel: +82-2-880-1254, Fax: +82-2-879-0378
E-mail: pdryu@snu.ac.kr

EXPANDED MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 120-150g were purchased from Samtaco (Gyeonggi-do, Korea). The rats were maintained under a 12 h light/dark cycle (lights on at 9:00 A.M.) and were given free access to food and water until sacrifice. The rats were divided into two study groups: one used for the patch clamp recording and the other used for measure heart rate and plasma norepinephrine (NE) levels. Anesthesia was induced by intraperitoneal (ip) injecting an anesthetic cocktail (Zoletil 25 mg/kg and xylazine 10 mg/kg). All animal experiments were performed in accordance with the protocol (SNU-100617-2) for the care and use of animals approved by the Laboratory Animal Care Advisory Committee of Seoul National University.

Challenge

Minocycline (Sigma, St. Louis, MO) was dissolved in sterile saline and sonicated to ensure complete solubilization. Rats received an ip injection of a vehicle or minocycline (50 mg/kg) for three consecutive days. On the third day, the rats also received an ip injection of saline or Escherichia coli lipopolysaccharide (LPS; 055 B5, 5 mg/kg, Sigma, St, Louis, MO). One day after the last challenge, the rats were used in experimental studies (1).

Retrograde tracing

Retrograde dye (100 nl of FluoSphere-Red solution; Molecular Probe, Inc., OR, USA) was injected into the RVLM as reported previously (2). Under anesthesia, the rats' skulls were fixed into stereotaxic frames (SF-7, Narishige Inc., Setagaya-Ku, Tokyo, Japan). After exposing the surface of the skull by incising the skin of the head, a small hole was made with a dental drill. The injection point was 2.1 mm lateral to the midline, 11.3-12.0 mm from the bregma, and 8.0 mm below the dorsal surface. The dye solution was injected unilaterally with a pneumatic picopump (PV820-G, World Precision Instruments Inc., FL, USA). Rats were allowed to recover for 10 d before drug challenges. The location of the injections sites was confirmed histologically as shown in insets of Fig. 2C. The results from the rats showing a

misplaced injection site were excluded from the analysis.

Hypothalamic slice preparation

Hypothalamic brain slices were prepared according to methods previously described (3). The temperature of ACSF was kept at -2 ~ 2˚C throughout the sectioning period. The isolated brain was immersed in oxygenated (95% O_2; 5% CO_2), ice-cold ACSF. Two or three coronal hypothalamic slices (300 μm) were cut just caudally to the optic chiasm with a vibrating tissue slicer (Vibratome 1000 plus, Vibratome, St. Louise, MO, USA). These slices were incubated in oxygenated artificial cerebrospinal fluid (ACSF) for at least 1 hour at 32°C until recordings were made at 30-33 °C. The composition of the ACSF was (in mM) : 126 NaCl; 26 $NaHCO_3$; 5 KCl; 1.2 NaH_2PO_4; 2.4 $CaCl_2$; 1.2 $MgCl_2$; 10 glucose; and pH of 7.4.

Electrophysiological recording

A slice was transferred to a recording chamber (0.7 ml) and fixed with a grid of nylon stocking threads supported by an O-shaped silver wire weight while being perfused (4 ml/min) with oxygenated ACSF at 30-33°C. Labeled PVN neurons were selected for recording under an upright fluorescence microscope with a "green" filter cube (WG, Olympus) and visualized using differential interference contrast video microscopy. The patch electrode was located on a target neuron under bright light with the aid of a three-dimensional hydraulic micromanipulator (Narishige Co., Tokyo, Japan). Pipettes were pulled from borosilicate glass capillaries of 1.7 mm diameter and 0.5 mm wall thickness. The open resistance ranged from 2 to 5 MΩ, and the seal resistance from 1 to 5 GΩ. Patch pipettes were filled with K-gluconatate-rich solutions. The K-gluconate-rich solution contained (in mM) 135 K-gluconate, 5 KCl, 20 HEPES, 0.5 $CaCl_2$, 5 EGTA and 5 ATP-Mg, whereas KCl-rich solution contained (in mM) 140 KCl, 20 HEPES, 0.5 $CaCl_2$, 5 EGTA and 5 ATP-Mg. The pH was adjusted with KOH to 7.2. Electrical signals were recorded using an Axopatch 200B (Axon Instruments, Foster City, CA, USA). The signals were filtered at 1 kHz and digitized at 10 kHz by using an analog-digital converter (Digidata 1200B) and pClamp software (Version 8.0, Axon Instruments). Resting membrane potentials were corrected for the liquid junction potential (~ 14.4 mV). The membrane input resistance was calculated by dividing the potential changes (mV)

evoked by applied hyperpolarizing current pulses (−60 ~ −180 pA). Neurons showing a decrease in input resistance (〉 15%) during recordings were excluded from the analysis.

The frequency and coefficient of variation (CV) of spontaneous firing activity was measured from recording periods lasting 2 - 3 min. For simultaneous recording of spontaneous inhibitory postsynaptic currents and excitatory postsynaptic currents, synaptic currents were recorded in the whole cell mode at holding potentials near resting level with the use of pipettes filled with a K-gluconate-rich solution (4).

Analysis of firing activity and synaptic currents

The time course histograms and average of firing activity were analyzed using the Mini Analysis Program (Version 6.0, Synaptosoft Inc., Leonia, NJ, USA). Silent neurons, as well as those displaying a frequency of less than 0.1 Hz were not included in the analysis of firing frequency. The neurons with a firing frequency that had changed more than 20% after application of bicuculline were considered to be responsive to bicuculline (2). To compare the effect of bicuculline on the presympathetic PVN neurons in each group, all the neurons tested were used in analysis including un-responding neurons against bicuculline. CV of firing rate was calculated by dividing the standard deviation of the inter-spike intervals (ISIs) by the mean ISIs. The frequency, amplitude, and decay time constant of spontaneous synaptic currents were determined from a 3 - 5 min segments of current records according to the methods previously described using a Mini Analysis Program (2). The threshold for detection of synaptic current was normally set at ~10 pA. Decay time constants (10 - 90%) were obtained from the best-fit parameters with a double exponential equation.

Measurement of Heart rate and plasma norepinephrine

Under anesthesia, the heart rate and plasma norepinephrine (NE) levels of rats were measured the day after last drug injection. Bipolar electrocardiogram (ECG) was recorded continuously for 10 min with Power lab instruments (AD Instruments, Australia) and analysis was conducted using Chart 4 for Windows, v.4.2. (AD Instruments, Australia). The heart rate was evaluated as an ECG cycle length (RR

interval), which was determined using the peaks of QRS complex. Blood sample was collected from the caudal vena cava into EDTA-coated tubes. After centrifugation at 5000g for 40 min at 0 ˚C, plasma was collected and kept frozen (-80 ˚C) until assaying. Plasma NE was measured using a commercial ELISA kit (Labor Diagnostika Nord GmBH & Co. KG, Nordhorn, Germany). Assays were performed in duplicate and were sensitive to 2.6 pg/ml of plasma NE. The inter- and intra-assay coefficients of variation were less than 10%.

Immunohistochemistry and imaging acquisition

In brief, challenged rats were perfused with 0.01 M PBS and fixed with 4% paraformaldehyde. The brain was removed and placed in 30% sucrose solution until it sank. Using the cryostat, slices with a thickness of 30 μm containing the PVN were obtained and placed in a plate filled with PBS. Sections were rinsed and incubated in a blocking buffer containing 10% normal goat serum and 0.3% Triton X-100 for 30 min at room temperature. The sections were then incubated with diluted rabbit-Iba-1 (1:500, Wako, Japan) for 12 h at room temperature with mild shaking, followed by 48 h of incubation at 4 ˚C. Subsequently, the sections were rinsed with 0.01 M PBS and exposed for 10 min to anti-rabbit biotinylated secondary antibody from the Vectastain Elite ABC kit (Vector, Burlingame, CA, USA). Lastly, a Vector DAB substrate kit (Vector Labs, Burlingame, CA, USA) was used for color development. Slices were washed, mounted onto slides, and visualized using an Olympus IX70 microscope.

Image analysis and quantification

Images were captured with a CCD digital camera attached to the microscope using a 20× objective at a resolution of 2,600 × 2,000 pixels. The same anterior level (-2.0 to 2.1 mm from bregma) of the PVN from 3 consecutive slices were captured for all groups (N = 3, respectively). Three adjacent squares with a fixed area (0.2 × 0.2 mm2) covering a region of PVN were randomly positioned in the captured PVN image. The number of pixels and the ratio of area occupied by Iba-1 immunoreactive cells within a defined threshold were measured using Image J software [NIH Image, National Institutes of Health, Bethesda, MD] (5).

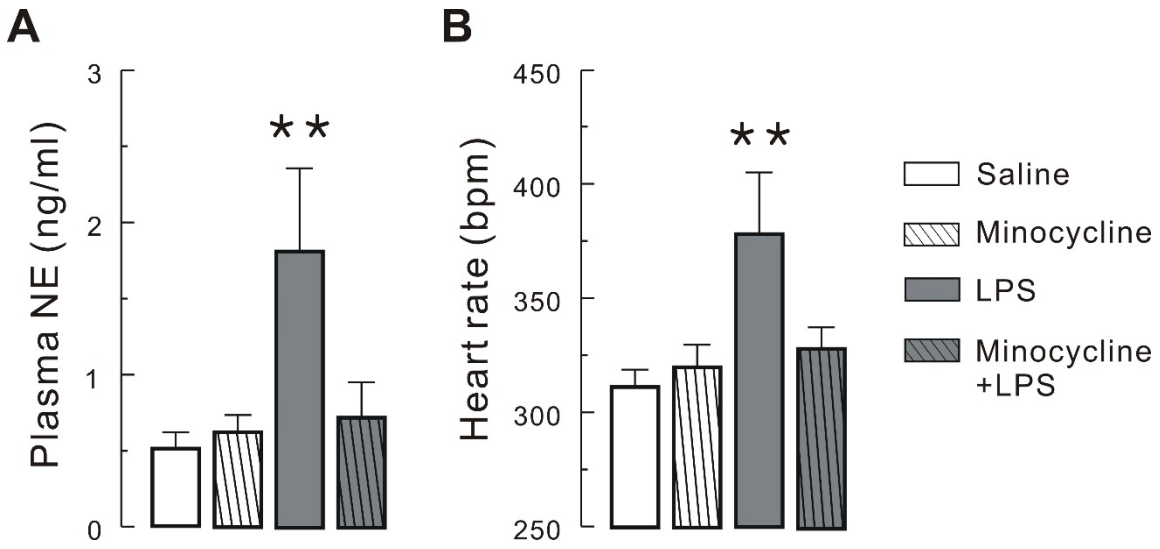
Statistics

Experimental data are expressed as means ± SEM and the number of neurons tested and analyzed is represented by 'n'. Statistical significance of the data was determined using independent or paired Student's t-tests and one-way ANOVAs followed by a Newman-Keuls multiple comparison test. A Fisher's exact test was used to detect a significant difference in the pattern of neuronal activity between each group. The level of significance was set at $P < 0.05$.

Table S1. Membrane properties of PVN–RVLM neurons in each group

	Saline (n = 22)	Minocycline (n = 18)	LPS (n = 15)	Minocycline + LPS (n = 17)
RMP (mV)	−62.6 ± 0.84	−62.2 ± 1.59	−63.5 ± 0.94	−63.3 ± 0.92
R_{in} (MΩ)	525 ± 48	480 ± 44	496 ± 45	475 ± 33
C_m (pF)	37.9 ± 3.51	36.8 ± 2.74	35.9 ± 2.84	36.7 ± 5.27

Data are presented as means ± SEM. Statistical analysis reveals that the three parameters
measured were not significantly different between other groups (P = 0.85, 0.82, and 0.98
by ANOVA for RMP, input resistance, and cell capacitance, respectively). RMP, resting
membrane potential; Rin, input resistance; Cm, membrane capacitance.



Supplementary Figure 1. Minocycline inhibits LPS-induced sympathetic activation. A, summary bar graphs showing the mean concentration of plasma NE from Saline (n = 7), Minocycline (n = 7), LPS (n = 5), and Minocycline + LPS group (n = 7), respectively. B, summary bar graphs showing the mean heart rate of Saline (n = 7), Minocycline (n = 5), LPS (n = 7), and Minocycline + LPS group (n = 7), respectively. Bars represent the mean ± SEM. **, $p < 0.01$ by one-way ANOVA followed by the Newman-Keuls multiple comparison test.

Table S2. Abbreviations

Abbreviation	Full Form of an Abbreviation
BP	blood pressure
GABA	gamma-aminobutyric acid
HF	heart failure
IL	interleukin
IML	intermediolateral cell column of the spinal cord
LPS	lipopolysaccharides
MI	myocardial infarction
NE	norepinephrine
PICs	pro-inflammatory cytokines
PVN	paraventricular nucleus
PVN-IML	presympathetic PVN neurons project to intermediolateral cell column of the spinal cord
PVN-RVLM	presympathetic PVN neurons project to the rostral ventrolateral medulla
RVLM	rostral ventrolateral medulla
sEPSC	spontaneous excitatory postsynaptic currents
sIPSC	spontaneous inhibitory postsynaptic currents
TNF	tumor necrosis factor

REFERENCES

1. Henry CJ, Huang Y, Wynne A et al (2008) Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. J Neuroinflammation 5, 15
2. Han TH, Lee K, Park JB et al (2010) Reduction in synaptic GABA release contributes to target-selective elevation of PVN neuronal activity in rats with myocardial infarction. Am J Physiol Regul Integr Comp Physiol 299, R129-139
3. Han TH, Jang SH, Lee SY and Ryu PD (2011) Adenosine reduces GABAergic IPSC frequency via presynaptic A(1) receptors in hypothalamic paraventricular neurons projecting to rostral ventrolateral medulla. Neurosci Lett 490, 63-67
4. Wuarin JP and Dudek FE (1993) Patch-clamp analysis of spontaneous synaptic currents in supraoptic neuroendocrine cells of the rat hypothalamus. J Neurosci 13, 2323-2331
5. Peters CM and Eisenach JC (2010) Contribution of the chemokine (C-C motif) ligand 2 (CCL2) to mechanical hypersensitivity after surgical incision in rats. Anesthesiology 112, 1250-1258