

## Online Supplements

### **Regulation of Hypothalamic Presympathetic Neurons and Sympathetic Outflow by Group II Metabotropic Glutamate Receptors in Spontaneously Hypertensive Rats**

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**Short title:** Hypothalamic mGluR2/3 in SHR

#### **Methods**

##### **Animal models**

Male Wistar-Kyoto (WKY) rats and SHR (13 weeks old; Harlan, Indianapolis, IN) were used in this study. We used 89 SHR and 79 WKY rats for the entire study. The surgical procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center and conformed to the National Institutes of Health guidelines for the ethical use of animals. The arterial blood pressure (ABP) of the rats was measured every day for 1 week before the final experiments, using a non-invasive tail-cuff system (IITC Life Science Inc., Woodland Hills, CA). The systolic ABP was significantly higher in SHR (208 ± 3.01 mmHg, n = 25) than in age-matched WKY rats (122.18 ± 2.56 mmHg, n = 25).

##### **Retrograde labeling of PVN presympathetic neurons**

Retrograde labeling of spinally projecting PVN neurons was performed as described previously<sup>1,2</sup>. Briefly, rats were anesthetized using intraperitoneal injection of ketamine (70 mg/kg) and xylazine (6 mg/kg), and their spinal cords between T2 and T4 were exposed. FluoSpheres (0.04 µm; Molecular Probes, Eugene, OR) were then pressure-ejected bilaterally into the vicinity of the intermediolateral cell column of the spinal cord at T2–T4 level (~500 µm from the midline and ~500 µm below the dorsolateral sulcus) using a Nanojector II (Drummond Scientific, Broomall, PA) via a glass pipette (3–4 separate 50-nl injections). The rats were returned to their cages for 3–5 days to allow the tracer to be transported to the PVN.

##### **Electrophysiological recordings in brain slices**

Rats were anesthetized with 2-3% isoflurane and decapitated, and the brain was quickly removed. Using a vibrating microtome, coronal hypothalamic slices were sectioned (300 µm thick) in ice-cold artificial cerebrospinal fluid (aCSF) solution containing (in mM) 126 NaCl, 3 KCl, 1.5 MgCl<sub>2</sub>, 2.4 CaCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose, and 26 NaHCO<sub>3</sub> saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The spinal cord was sectioned at the injection level and viewed under a microscope to verify the injection site and diffusion size of the FluoSpheres<sup>1,2</sup>. The brain slices were used for the following electrophysiological recordings only if the injection sites were located within the intermediolateral cell column of the spinal cord.

The brain slices were pre-incubated in the aCSF at 34°C for at least 1 h before being used for electrophysiological recordings. Tracer-labeled PVN neurons were identified under an upright

microscope (BX51WI, Olympus, Tokyo, Japan) with epifluorescence and infrared differential interference contrast optics. Whole-cell patch-clamp recording was then performed on the labeled neurons at 34°C using a borosilicate glass pipette (resistance, 4-6 MΩ). The pipette solution contained (in mM) 135 K-gluconate, 5.0 KCl, 2.0 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 5.0 EGTA, 5.0 Mg-ATP, 0.5 Na.GTP, and 10 HEPES adjusted to pH 7.3 with 1 M KOH (280-300 mOsm). For whole-cell recording, lidocaine *N*-ethyl bromide (10 mM), was included in the pipette solution to suppress the firing activity of the recorded neuron.

The glutamatergic excitatory postsynaptic currents (EPSCs) were recorded at a holding potential of -60 mV in the presence of 10 μM bicuculline<sup>1-3</sup>. The evoked EPSCs were elicited by electrical stimulation (0.1 ms, 0.8 mA, and 0.2 Hz) delivered through a bipolar electrode with the tip placed ~150 μm away from the recorded neuron. In some neurons, AMPA currents were elicited by puff application of 100 μM AMPA onto the recorded neuron in the presence of 1 μM tetrodotoxin<sup>4</sup>. Also, inhibitory postsynaptic currents (IPSCs) were recorded at a holding potential of 0 mV in the presence of 50 μM 2-amino-5-phosphonopentanoic acid (AP-5) and 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). The miniature EPSCs (mEPSCs) and miniature IPSCs (mIPSCs) were recorded in the presence of 1 μM tetrodotoxin. Neuronal firing activity was recorded using a cell-attached configuration<sup>5</sup>. Signals were processed using the Axopatch 700B amplifier (Molecular Devices, Foster City, CA), filtered at 1-2 kHz, and digitized at 20 kHz.

All drugs were freshly prepared in aCSF before the experiments and delivered via syringe pumps at their final concentrations. (2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV) and LY341495 were purchased from Tocris Bioscience (Ellisville, MO). AMPA was purchased from Sigma-Aldrich (St. Louis, MO). AP-5, CNQX, bicuculline, and tetrodotoxin were obtained from Ascent Scientific (Princeton, NJ).

### **Celiac ganglionectomy and ABP measurement with radiotelemetry**

Celiac ganglionectomy (CGx) surgery was performed aseptically in SHR<sub>s</sub> anesthetized with 2-3% isoflurane, as we have described previously<sup>4,6</sup>. Briefly, the celiac ganglion area was exposed and the celiac ganglion was identified near the superior mesenteric artery and celiac artery. The celiac plexus and all visible nerves were dissected and removed under a surgical microscope. In sham control rats, the celiac ganglion plexus was exposed but not disturbed.

To confirm the intended effect of CGx on ABP in SHR<sub>s</sub>, the Millar catheter of the telemetry system (Millar Instruments, Inc., Houston, TX) was inserted into the abdominal aorta below the bifurcation of the renal arteries<sup>4,6</sup>. The rats were injected with buprenorphine (0.3 mg/kg, i.m.) and penicillin (60,000 units, i.m.) for 3 days and housed singly after surgery. The ABP signal was recorded every 2 days in conscious rats through the receiver and analyzed using the LabChart data acquisition system (AD Instruments, Bella Vista, Australia). The rats were used for brain slice recordings about 2 weeks after surgery.

### **PVN microinjection and lumbar sympathetic nerve recording *in vivo***

Rats were anesthetized using a mixture of chloralose (60–75 mg/kg) and urethane (800 mg/kg) injected intraperitoneally. The right femoral vein was cannulated for intravenous administration of drugs. Supplemental doses of α-chloralose and urethane were administered as necessary to maintain an adequate depth of anesthesia. The trachea was cannulated for mechanical ventilation using a rodent ventilator. The ABP was recorded through a pressure transducer connected to a catheter in the left femoral artery. Heart rate (HR) signal was recorded by triggering from the pulsatile ABP. The left lumbar sympathetic chain was isolated under an operating microscope through a retroperitoneal incision. The lumbar sympathetic nerve activity (LSNA) and ABP were recorded using a 1401-PLUS and Spike2

system (Cambridge Electronic Design, Cambridge, UK), as we have described previously<sup>7,8</sup>.

For microinjection of DCG-IV, the brain was exposed at the level of the hypothalamus. A glass microinjection pipette (tip diameter 20–30  $\mu\text{m}$ ) was advanced into the PVN following the stereotactic coordinates: 1.6–2.0 mm caudal to the bregma, 0.5 mm lateral to the midline, and 7.0–7.5 mm ventral to the dura. The injection sites of the PVN were first verified by the depressor responses to microinjection of 5.0 nmol GABA (20 nl, 250 mM), as we have described previously<sup>7,8</sup>. A calibrated microinjection system (Nanoject II; Drumond Scientific, Broomall, PA) was used. The location of the pipette tip in the PVN was determined by including 5% rhodamine-labeled fluorescent microspheres in the injection solution. The rat brains were removed rapidly at the completion of the experiment and fixed in 10% buffered formalin solution overnight. Frozen coronal sections (40  $\mu\text{m}$  thick) were cut on a freezing microtome. Rhodamine-labeled fluorescent regions were identified using an epifluorescence microscope and plotted on standardized sections according to the Paxinos and Watson brain atlas. Rats were not included in the data analysis if the microinjection occurred outside the PVN.

### Data analysis

Data are presented as the mean  $\pm$  SEM. The action potential, mEPSCs, and mIPSCs were analyzed off-line using a peak detection program (MiniAnalysis, Synaptosoft, Leonia, NJ). The peak amplitude of evoked EPSCs was determined and analyzed using pClamp 10 (Molecular Devices). The LSNA, ABP, and HR were analyzed using the Spike2 software program. LSNA was rectified and integrated offline after subtracting the background noise. For comparisons of two data sets, Student's *t* test was used. For comparisons of more than two data sets, repeated-measures ANOVA with Dunnett's *post hoc* test or two-way ANOVA with Bonferroni's *post hoc* test was performed to compare differences within or between experimental groups. We used corresponding nonparametric analysis (i.e., Mann-Whitney or Kruskal-Wallis test) when data were not normally distributed.  $P < 0.05$  was considered to be statistically significant.

## References

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