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Src Kinases Regulate Glutamatergic Input to Hypothalamic Presympathetic Neurons and Sympathetic Outflow in Hypertension

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Methods

Animal model

We used male normotensive Wistar-Kyoto (WKY) rats and SHRs (4- and 13-week-old, Harlan, Indianapolis, IN) in the present study. SHRs are the most commonly used and well-characterized animal model of essential hypertension [1, 2]. The experimental procedures and 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 on the ethical use of animals. Blood pressure was measured daily for at least 1 week before the electrophysiological experiments via a noninvasive tail-cuff system (IITC Life Science, Inc., Woodland Hills, CA) to confirm hypertension in a randomly selected group of SHRs and WKY rats. The systolic ABP in 13-week-old SHRs (205.16 ± 3.57 mmHg, n = 28 rats) was much higher than that in the age-matched WKY rats (129.43 ± 2.85 mmHg, n = 26 rats, $P < 0.05$).

Retrograde labeling of RVLM-projecting PVN presympathetic neurons

We identified RVLM-projecting PVN neurons using a retrograde labeling technique, as we described previously [3, 4]. The rats were anesthetized with intraperitoneal injection of a mixture of ketamine (70 mg/kg) and xylazine (6 mg/kg) and placed in a stereotactic frame. The skull was exposed, and two holes (1 mm diameter) were drilled bilaterally through the skull using a micromotor drill at 13 mm caudal to the bregma and 2 mm lateral to the midline. Then a glass micropipette filled with FluoSpheres (0.04 μm, Molecular Probes, Eugene, OR) was placed through each hole and positioned into the RVLM (7.5 mm ventral to the surface of the brain) through a micromanipulator. The FluoSpheres were pressure-injected (Nanojector II; Drummond Scientific, Broomall, PA) into the RVLM bilaterally in two separate 50-nL injections. The rats were returned to their home cages for 3–5 days to allow the FluoSpheres to be transported into the PVN.

Brain slice preparation

Coronal hypothalamic slices (300 μm thick) containing the PVN were obtained from FluoSphere-injected rats using a vibrating microtome. Each rat was rapidly decapitated while under anesthesia induced by 3% isoflurane. The brain was quickly removed and sliced in an ice-cold artificial cerebrospinal fluid (aCSF) solution containing (in mM) 126 NaCl, 3 KCl, 1.5 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 10 glucose, and 26 $NaHCO_3$ saturated with 95% O_2 and 5% CO_2. The slices were incubated in the aCSF at 34°C for at least 1 h before recording. To verify the injection sites of the FluoSpheres, the RVLM was sectioned at the injection level and viewed under a microscope immediately after the rat was killed. Data were excluded from the analysis if either of the injection sites were not located within the RVLM.

Electrophysiological recordings

Whole-cell patch-clamp recordings were performed in labeled neurons in the PVN of the slices. The recording chamber was continuously perfused with aCSF at 34°C. The labeled PVN neurons were identified under an upright microscope (BX51WI, Olympus, Tokyo, Japan) with epifluorescent and infrared differential interference contrast optics. Borosilicate glass microelectrodes (resistance, 3–7 MΩ) were pulled using a micropipette puller. The pipette solution contained (in mM) 110 Cs_2SO_4, 2.0 $MgCl_2$, 0.5 $CaCl_2$, 5.0 EGTA, 5.0 Mg-ATP, 0.5 Na_2-GTP, and 10 HEPES. The pH was adjusted to 7.2 with CsOH (280–300 mOsm). Signals were processed using an Axopatch 700B amplifier (Molecular Devices, Union City, CA), filtered at 1–2 kHz, and digitized at 20 kHz using a DigiData 1440 digitizer (Molecular Devices).

Excitatory postsynaptic currents (EPSCs) were initially elicited by electrical stimulation (0.2 ms, 0.8-1.0 mA at 0.2 Hz) through a bipolar tungsten electrode connected to a stimulator. The tip of the stimulation electrode was placed on the ventral side ~150 μm from the recorded neuron [5, 6]. Evoked α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-EPSCs were recorded at a holding potential of −60 mV in the presence of 10 μM bicuculline, and evoked NMDAR-EPSCs were recorded at a holding potential of +40 mV in the presence of 10 μM bicuculline and 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). We fixed the stimulation intensity (0.8 mA) for subsequent recording of AMPAR-EPSCs and NMDAR-EPSCs. We have shown that the evoked NMDAR-EPSCs at +40 mV were abolished by the NMDAR specific antagonist, confirming that these currents (regardless of permeable ions involved) are mediated by synaptic NMDARs [7, 8]. The EPSCs were not normalized by the capacitance, because the capacitance of labeled neurons was not significantly different between WKY rats and SHRs (42.5 ± 2.1 pF in WKY and 41.5 ± 1.9 pF in SHR). A sodium channel blocker, lidocaine N-ethyl bromide (10.0 mM), was included in the pipette solution to suppress the firing activity of the recorded neuron. Miniature EPSCs (mEPSCs) were recorded at a holding potential of –60 mV in the presence of 1 μM tetrodotoxin and 10 μM bicuculline.

To record postsynaptic NMDAR currents, we puffed NMDA (100 μM) directly onto the recorded neuron at a holding potential of –60 mV. The puff pipette (~10 μm tip diameter) was placed ~150 μm away from the recorded neuron. Positive pressure (4 psi) was applied to eject NMDA onto the recorded neuron for 150 ms [7, 8]. Because NMDARs are voltage-dependently blocked by Mg^{2+} at a negative holding potential and co-activated by glycine, puff NMDA-induced currents were recorded in Mg^{2+}-free aCSF in the presence of 10 μM glycine and 1 μM tetrodotoxin. Although puff application of NMDA can also result in presynaptic release of glutamate, the

currents elicited by presynaptic glutamate release are very small and negligible compared with currents produced by a large amount of puff glutamate agonists. For this reason, the current elicited by puff application of NMDA is generally considered a measure of postsynaptic NMDAR activity [7-9].

All drugs were freshly prepared in aCSF before the experiments and delivered via syringe pumps at their final concentrations. 4-Amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2) was purchased from Tocris Bioscience. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), glycine, and NMDA were purchased from Sigma-Aldrich. D-2-amino-5-phosphonopentanoate (AP5), CNQX, bicuculline, and tetrodotoxin were obtained from Ascent Scientific. Lidocaine N-ethyl bromide was purchased from Alomone Labs (Jerusalem, Israel). (pY)EEI peptide was purchased from Santa Cruz Biotechnology (Dallas, TX). Because the recording of synaptic NMDAR currents in tissue slices typically lasts only for 20-30 min (due to "run-down" associated with whole-cell recordings), we compared the effects of PP2 and vehicle in separate labeled PVN neurons, similar to previous studies [8-10].

Celiac ganglionectomy and measurement of ABP using telemetry

We performed celiac ganglionectomy (CGx) or sham surgery aseptically in WKY rats and SHRs that were under anesthesia with 2% isoflurane [8, 11]. After a midline laparotomy, the celiac ganglion plexus was identified near the superior mesenteric artery. Then the celiac plexus and all visible nerves connected to the celiac ganglion plexus were dissected and stripped completely. In the sham control rats, the celiac ganglion plexus was exposed but not disturbed. A transmitter-attached Millar catheter was inserted into the abdominal aorta, and the transmitter body was implanted in the abdominal cavity. The rats were housed individually, and the ABP was measured in freely moving rats by using the telemetry system (Telemetry Research Ltd.) that we described previously [8, 12]. The ABP data were recorded daily at three time points (8 am, 12 pm and 8 pm; each for 10 min) after surgery and analyzed with a data acquisition system (LabChart; AD Instruments). Two weeks after CGx or sham surgery, the rats were placed under anesthesia, and their brain tissues harvested below for Western blotting analysis.

Western immunoblotting

Rats were anesthetized with 3% isoflurane and then decapitated. Hypothalamic slices were sectioned 1.08–2.12 mm caudal to the bregma, and PVN tissues were micro-punched bilaterally with a slice punch (0.5 mm diameter) following stereotactic coordinates: 0.5 mm lateral to the midline and 1.7–2.5 mm ventral to the surface of the cortex [9, 13]. Inhibitors of proteases and phosphatase were added to the lysis buffer, and the total protein amount was quantified using a bicinchoninic acid assay. The samples were subjected to 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and incubated with a mouse anti-Src antibody (1:1,000, catalog #2578064, Millipore, Bedford, MA) for 24 h. Goat-anti-mouse HRP antibody (1:5,000, catalog #ab6789, Abcam, Cambridge, MA) was applied to the immunoblots for 2 h at 25°C. For protein loading control, we used a rabbit anti-GAPDH antibody (1:1,000, catalog #ab37168, Abcam). The monoclonal antibody against Src has been validated in previous studies [14, 15]. An ECL kit (ThermoFisher Scientific) was used to detect the Src protein band, which was visualized and quantified with the Odyssey Fc Imager (LI-COR Biosciences, Lincoln, NE) and normalized by the GAPDH protein band on the same blot.

Recording of lumbar sympathetic nerve activity and ABP

Rats were anesthetized using 2% isoflurane in O_2, and a mixture of α-chloralose (60–75 mg/kg) and urethane (800 mg/kg) was intraperitoneally injected. Before surgery, adequate depth of anesthesia was confirmed by the absence of both corneal reflexes and paw withdrawal responses to a noxious pinch. 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 (CWE, Ardmore, PA) using a rodent ventilator with 100% O_2. The expired CO_2 was continuously monitored by a CO_2 analyzer (Capstar 100; CWE) and maintained at 4–5% by adjusting the ventilation rate (50–70 breaths/min) or tidal volume (2–3 ml) throughout the experiment. ABP was monitored via a pressure transducer connected to a catheter placed into the left femoral artery. Heart rate (HR) was measured using the pulsatile ABP data. The right femoral vein was cannulated for intravenous administration of drugs. A small branch of the left lumbar postganglionic sympathetic nerve was isolated under an operating microscope through a retroperitoneal incision. The lumbar sympathetic nerve was cut distally to ensure that afferent activity was not recorded. The nerve was then immersed in mineral oil and placed on a stainless steel recording electrode. The nerve signal was amplified (20,000–30,000 ×) and band-pass filtered (100–3,000 Hz) by an alternating current amplifier (model P511; Grass Instruments), and the lumbar sympathetic nerve activity (LSNA) was monitored through an audio amplifier. The LSNA and ABP were recorded using a 1401-PLUS analog-to-digital converter and Spike2 system (Cambridge Electronic Design, Cambridge, UK). Background electrical noise was determined by a complete suppression of LSNA with administration of phenylephrine (20 μg/kg intravenously) both before and 5 min after the rats were euthanized by an overdose of sodium pentobarbital (200 mg/kg intravenously) at the end of each experiment. Respective electrical noise levels were subtracted from the integrated values of LSNA, and the percent change in LSNA from the baseline was calculated [4, 16].

PVN microinjection

For PVN microinjections, the rats were anesthetized as described above and placed in a stereotactic frame. Their brains were exposed at the level of the hypothalamus. A glass microinjection pipette (tip diameter 20–30 μm) was advanced into the PVN according to the following 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 verified by the depressor responses to microinjection of 5.0 nmol GABA (20 nL, 250 mM). The microinjection was done by using a calibrated microinjection system (Nanoject II; Drummond Scientific) and monitored using an operating microscope [4, 16]. GABA microinjections were separated by 10- to 15-min intervals to allow recovery of the depressor response. The PVN vasomotor site was considered to have been located when GABA injection decreased mean ABP by at least 10 mmHg. The stereotactic coordinates at which the prior GABA microinjection elicited the greatest depressor responses were used in the same rat for the subsequent microinjection of PP2 and AP5. In total, up to six microinjections of GABA in the PVN were performed in each rat. After microinjection of the drugs, the glass pipette was left in place for 1 to 2 min to ensure adequate delivery of the drug to the injection site.

The location of the pipette tip and diffusion of the drugs in the PVN were determined by including 5% rhodamine-labeled fluorescent microspheres (0.04 μm; Molecular Probes) in the injection solution [4, 16]. At the completion of the experiment, the rat brain was removed rapidly and

fixed in 10% buffered formalin solution overnight. Frozen coronal sections (40-μm thick) were cut on a freezing microtome and mounted on slides. Rhodamine-labeled fluorescent regions were identified using an epifluorescence microscope and plotted on standardized sections from the Paxinos and Watson rat brain atlas. Rats were excluded from the data analysis if they had one or more misplaced microinjections outside the PVN.

Data analysis

Data are presented as mean ± S.E.M. The peak amplitude of puff NMDA-induced currents and electrically-evoked EPSCs was determined and analyzed using pClamp 10 (Molecular Devices). The mEPSCs were analyzed off-line using a peak detection program (MiniAnalysis, Synaptosoft). Only one neuron was recorded in each brain slice, and at least four rats were used in each recording protocol. The mean ABP, LSNA, and HR were analyzed using Spike2 software. The mean ABP was derived from the pulsatile ABP and calculated as the diastolic pressure plus one-third of the pulse pressure. LSNA was rectified and integrated offline after subtracting the background noise, using the level obtained after the rats were euthanized with an overdose of sodium phenobarbital. Control values were obtained by averaging the signal over a 60-s period immediately before PVN microinjection. Response values after each intervention were averaged over 30 s when the maximal responses occurred [4, 16]. We used the Student *t* test or Mann-Whitney U test to determine the significant differences between the two groups. One-way ANOVA with Dunnett's or Tukey's *post hoc* test was used to determine the significant differences involving more than two groups. $P < 0.05$ was considered statistically significant.

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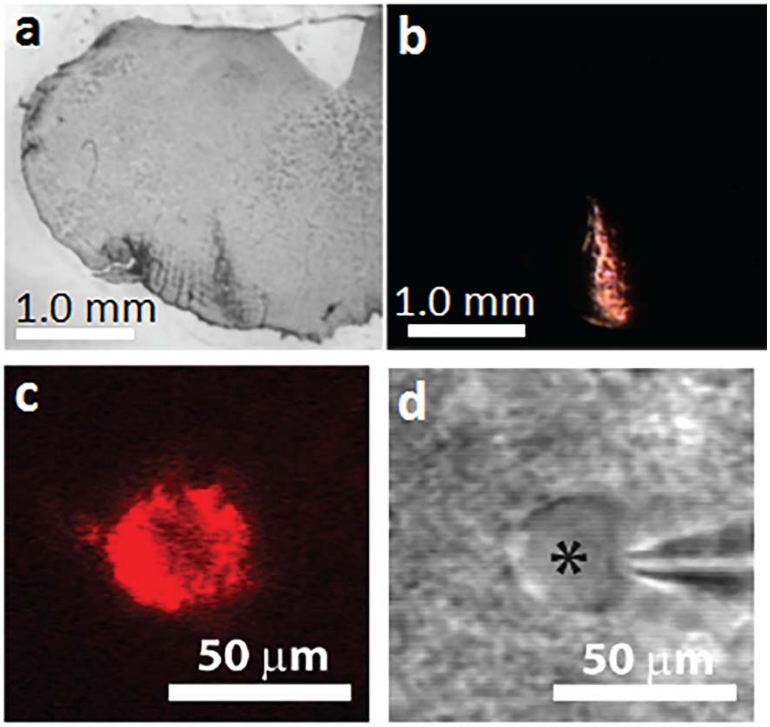
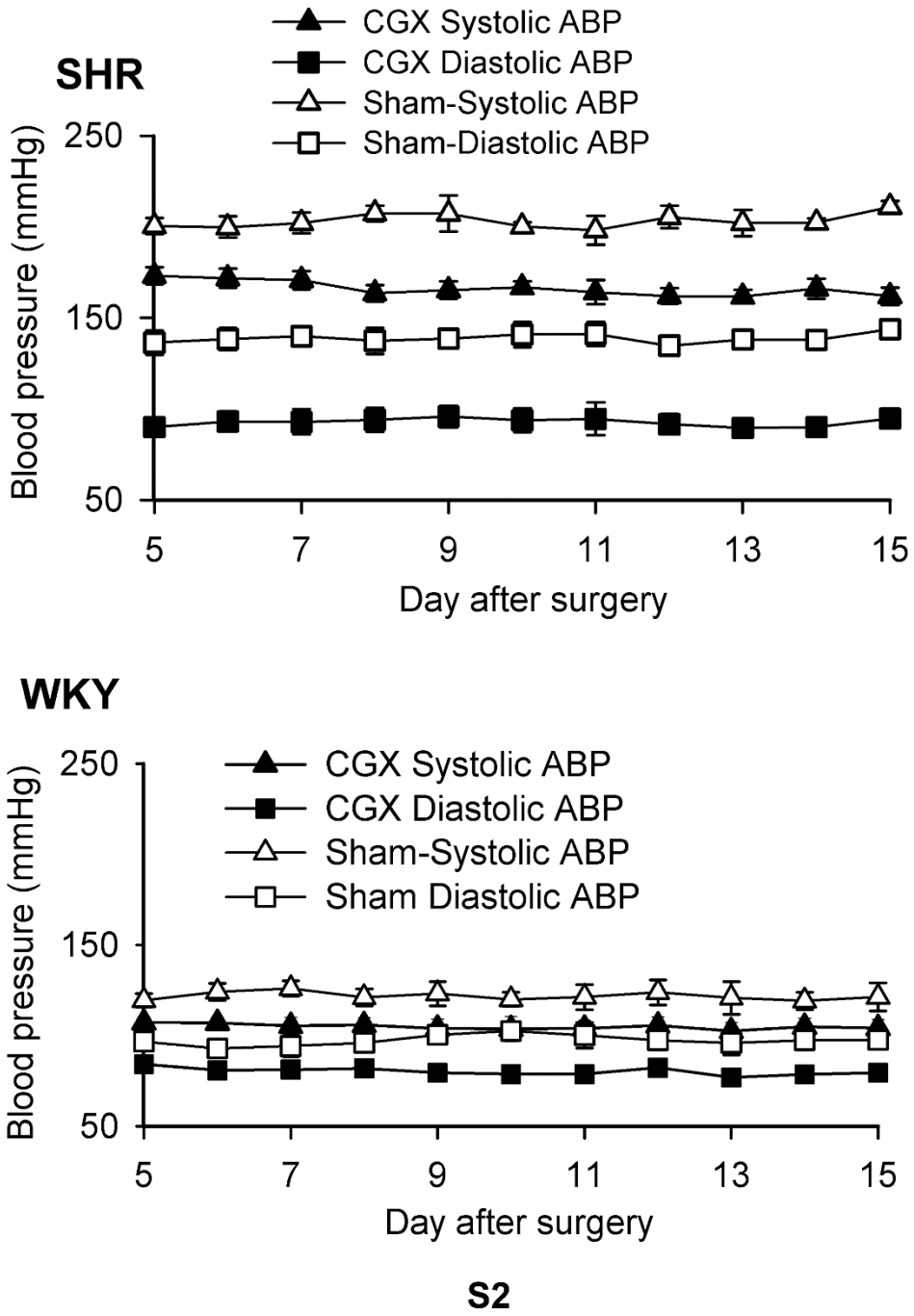


S1

Supplemental Figure S1. Identification of retrogradely labeled RVLM-projecting PVN neurons. A and B: photomicrographs show the FluoSphere injection site in the RVLM viewed under light (A) and fluorescence illumination (B). C and D: a FluoSphere-labeled PVN neuron in the brain slice viewed with fluorescence illumination (C) and the same neuron (*) shown in D with a recording electrode (∧).



S2

Supplemental Figure S2. Effect of CGx on the systolic and diastolic arterial blood pressure (ABP) in WKY rats and SHRs. Summary data show the time course of changes in the systolic and diastolic ABP after CGx and sham surgery in WKY rats and SHRs (n = 6 rats in each group).