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

Xin Qiao,* Jing-Jing Zhou,* De-Pei Li, Hui-Lin Pan

Abstract—The elevated sympathetic outflow associated with hypertension is maintained by increased N-methyl-d-aspartate receptor (NMDAR) activity in the paraventricular nucleus (PVN) of the hypothalamus. Synaptic NMDAR activity is tightly regulated by protein kinases, including the Src family of tyrosine kinases. We determined whether Src kinases play a role in increased NMDAR activity of PVN neurons projecting to the rostral ventrolateral medulla and in elevated sympathetic vasomotor tone in spontaneously hypertensive rats (SHRs). The Src protein level in the PVN was significantly greater in SHRs than in normotensive Wistar–Kyoto (WKY) rats and was not significantly altered by lowering blood pressure with celiac ganglionection in SHRs. Inhibition of Src kinase activity with 4-amino-5-(4-chlorophenyl)-7-(dimethylthyl)pyrazolo[3,4-d]pyrimidine (PP2) completely normalized the higher amplitudes of evoked NMDAR-mediated excitatory postsynaptic currents and puff NMDA–elicited currents of rostral ventrolateral medulla–projecting PVN neurons in SHRs. PP2 treatment also attenuated the higher frequency of NMDAR-mediated miniature excitatory postsynaptic currents of these neurons in SHRs. However, PP2 had no effect on NMDAR-excitatory postsynaptic currents or miniature excitatory postsynaptic currents of rostral ventrolateral medulla–projecting PVN neurons in WKY rats. NMDAR activity increased by an Src-activating peptide was blocked by PP2 but not by inhibition of casein kinase 2. In addition, microinjection of PP2 into the PVN not only decreased lumbar sympathetic nerve discharges and blood pressure but also eliminated the inhibitory effect of the NMDAR antagonist on sympathetic nerve activity and blood pressure in SHRs. Collectively, our findings suggest that increased Src kinase activity potentiates presynaptic and postsynaptic NMDAR activity in the PVN and sympathetic vasomotor tone in hypertension. (Hypertension. 2017;69:154-162. DOI: 10.1161/HYPERTENSIONAHA.116.07947.) ● Online Data Supplement

Key Words: autonomic nervous system ● hypertension ● hypothalamus ● NMDA receptor ● sympathetic nerve discharges ● synaptic plasticity

Hypertension affects approximately one third of adults in the United States and is a well-recognized risk factor for stroke, coronary artery disease, and renal failure. Although essential (primary) hypertension is the most prevalent form of hypertension, its root cause and underlying mechanisms remain poorly understood. Elevated sympathetic outflow is clearly associated with the development of essential hypertension in animal models and in hypertensive patients. The paraventricular nucleus (PVN) of the hypothalamus is an important brain region controlling sympathetic outflow. Presympathetic neurons in the PVN project to vasomotor neurons in the rostral ventrolateral medulla (RVLM) in the brain stem and sympathetic preganglionic neurons in the intermediolateral cell column in the spinal cord, which in turn regulate sympathetic nerve discharges. Electrolytic lesion or pharmacological inhibition of the PVN decreases arterial blood pressure (ABP) and sympathetic nerve activity in spontaneously hypertensive rats (SHRs). Also, transplantation of embryonic hypothalamic tissue containing the PVN from SHRs to normotensive rats leads to hypertension in the normal rats. Although hyperactivity of PVN presynaptic neurons is a major source of elevated sympathetic outflow in SHRs, the molecular mechanisms responsible for the increased excitability of these neurons are not fully known.

Glutamate is the major excitatory neurotransmitter in the PVN, and increased N-methyl-d-aspartate receptor (NMDAR) activity is critically involved in the augmented sympathetic vasomotor tone in SHRs. The synaptic NMDAR activity of PVN presynaptic neurons is tightly regulated by phosphorylation via serine/threonine kinases and phosphatases, including CK2 (casein kinase II) and calcineurin. In addition, increasing the phosphorylation level of tyrosine residues...
in NMDARs can increase NMDAR activity.21–24 The Src family of nonreceptor protein tyrosine kinases has at least 9 members, and Src is actively expressed in the adult hypothalamus.25,26 However, it remains unclear whether the Src kinases contribute to the increased NMDAR activity of PVN presympathetic neurons observed in SHRs.

In the present study, we determined the role of Src kinases in the increased synaptic glutamatergic input to RVLM-projecting neurons in SHRs using in vivo retrograde tracing and in vitro brain slice recordings. We also studied whether Src-mediated NMDAR activity in the PVN is involved in maintaining elevated sympathetic output in SHRs. Our findings suggest that increased Src kinase activity in the PVN plays a pivotal role in the potentiation of pre- and postsynaptic NMDAR activity of PVN presympathetic neurons and in augmented sympathetic outflow in SHRs. Our study provides new insight into the molecular mechanism of the synaptic plasticity associated with sustained sympathetic outflow in hypertension.

**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.2,23 For the entire study, data were collected from 59 WKY rats and 77 SHRs. The experimental procedures 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.

The detailed methods for retrograde labeling of PVN neurons, electrophysiological recordings in brain slices, celiac ganglionectomy (CGx), Western blotting, PVN microinjections and lumbar sympathetic nerve recording in vivo, and data analysis are described in the online-only Data Supplement.

**Brain Slice Preparation and Recordings**

Coronal hypothalamic slices (300-μm thick) containing the PVN were obtained from Fluorosphere-injected rats using a vibrating microtome. Whole-cell patch-clamp recordings were performed in labeled neurons in the PVN of the slices (Figure S1 in the online-only Data Supplement). Excitatory postsynaptic currents (EPSCs) were elicited by electric stimulation through a bipolar tungsten electrode connected to a stimulator. 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. Miniature EPSCs (mEPSCs) were recorded at a holding potential of −60 mV in the presence of 1 μM/μL tetrodotoxin and 10 μM/μL bicuculline. To record postsynaptic NMDAR currents, we puffed NMDA (10 μM) directly onto the recorded neuron at a holding potential of −60 mV. Miniature inhibitory postsynaptic currents were recorded using a 1401-PLUS analog-to-digital converter and Spiker2 system (Cambridge Electronic Design, Cambridge, UK). A glass microinjection pipette (tip diameter 20–30 μm) was advanced into the PVN. The location of the pipette tip and diffusion of the

**Western Immunoblotting**

Hypothalamic slices were sectioned 1.08 to 2.12 mm caudal to the bregma, and PVN tissues were micropunched bilaterally with a slice punch. The samples were subjected to 4% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and incubated with a mouse anti-Src antibody (1:1000, catalog no 2578064; Millipore, Bedford, MA) for 24 hours. 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.

**PVN Microinjection and Recording of Lumbar Sympathetic Nerve Activity and ABP**

Rats were anesthetized with a mixture of α-chloralose (60–75 mg/kg IP) and urethane (800 mg/kg IP). 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 lumbar sympathetic nerve activity (LSNA) and ABP were recorded using a 1401-PLUS analog-to-digital converter and Spiker2 system (Cambridge Electronic Design, Cambridge, UK). A glass microinjection pipette (tip diameter 20–30 μm) was advanced into the PVN. 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.2,29

**Data Analysis**

Data are presented as mean±SEM. We used the Student’s *t* test or Mann–Whitney *U* test to determine the significant differences between the 2 groups. One-way analysis of variance with Dunnett’s or Tukey’s post hoc test was used to determine the significant differences involving more than 2 groups. *P*<0.05 was considered statistically significant.

**Results**

**SHRs Show Higher Src Protein Level in the PVN**

We determined the Src protein levels in the PVN, RVLM, hippocampus, and frontal cortex in WKY rats and SHRs. Western immunoblotting showed a single band of Src proteins in all of the brain tissues, and the total Src protein level in the PVN was significantly greater in SHRs than in WKY rats (n=6 rats in each group; Figure 1A and 1B). In contrast, Src protein levels in the RVLM, hippocampus, and frontal cortex did not differ significantly between WKY rats and SHRs (n=6 rats in each group; Figure 1A and 1B). Furthermore, the Src protein level did not significantly differ between 4-week-old and 13-week-old WKY rats. However, the Src protein level in 13-week-old SHRs was significantly higher than that in 4-week-old SHRs and age-matched WKY rats (n=6 rats in each group; Figure 1C and 1D).

We then determined whether the higher Src protein level in the PVN of SHRs was a secondary change because of high ABP in SHRs. We performed CGx to lower ABP and then measured Src protein levels in the PVN of SHRs. CGx caused a large reduction in ABP, monitored by telemetry system, in SHRs compared with those subjected to sham surgery. CGx-induced decreases in ABP in SHRs occurred within 5 days after surgery and lasted for at least 2 weeks (Figure 2A and 2B; Figure S2). Immunoblotting showed that the Src protein level in the PVN of SHRs did not differ significantly between rats receiving CGx and sham surgery (Figure 2C and 2D). In addition, CGx induced a small decrease in ABP in WKY rats (Figure 2A and 2B). The Src protein level in the PVN was not significantly altered in WKY rats subjected to CGx compared with that in sham-operated WKY rats (Figure 2C and 2D). These data suggest that Src upregulation in the PVN is independent of ABP changes in SHRs.

**Src Contributes to Increased Postsynaptic NMDAR Currents of PVN Presympathetic Neurons in SHRs**

To determine the functional significance of increased Src kinase activity in regulating NMDARs of PVN presympathetic neurons in SHRs, we examined the effects of 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2), a highly selective Src kinase inhibitor,30–32 on evoked AMPAR-EPSCs and NMDAR-EPSCs in retrograde labeled RVLM-projecting PVN neurons. The hypothalamic slices from WKY rats and SHRs were incubated with either vehicle control (0.05% dimethyl sulfoxide) or PP2 (10 μmol/L) for 1 to 2 hours before electrophysiological recording. PP2 at 10 μmol/L can potently inhibit the Src kinase activity in the hippocampus.33 The amplitude of evoked NMDAR-EPSCs of labeled PVN neurons was significantly higher in neurons from SHRs than in those from WKY rats in vehicle-treated slices (n=7 neurons in each group; Figure 3A and 3B). PP2 treatment significantly reduced the amplitude of evoked NMDAR-EPSCs of labeled PVN neurons in SHRs but not in WKY rats (n=7 neurons). However, PP2 treatment did not significantly alter the amplitude of evoked AMPAR-EPSCs of labeled PVN neurons in WKY rats or SHRs (n=7 neurons in each group; Figure 3A and 3B). Also, in vehicle-treated slices, the ratio of NMDAR-EPSCs to AMPAR-EPSCs was significantly higher in neurons from SHRs than in those from WKY rats in vehicle-treated slices (n=7 neurons in each group; Figure 3A and 3B).
in SHRs than in WKY rats. PP2 treatment normalized the ratio of NMDAR-EPSCs to AMPAR-EPSCs in SHRs but had no effect on this ratio in WKY rats (Figure 3C).

To directly determine the role of Src kinases in the regulation of postsynaptic NMDAR activity, we examined the effect of PP2 on puff NMDA–induced currents in labeled PVN neurons. In vehicle-treated slices, puff application of NMDA (100 μmol/L) induced significantly greater NMDAR currents in SHRs (n=7 neurons) than in WKY rats (n=9 neurons; Figure 3D and 3E). PP2 treatment profoundly reduced NMDAR currents of labeled PVN neurons in SHRs but had no effect on puff NMDA–elicited currents in WKY rats (vehicle, n=9 neurons; PP2, n=7 neurons) and SHRs (vehicle, n=7 neurons; PP2, n=10 neurons) pretreated with vehicle or PP2. *P<0.05 compared with WKY rats. #P<0.05 compared with the SHR vehicle group.

These results suggest that increased Src kinase activity critically contributes to the increased postsynaptic NMDAR activity of PVN presympathetic neurons in SHRs.

**Src Is Involved in Tonic Activation of Presynaptic NMDARs of PVN Presympathetic Neurons in SHRs**

Presynaptic NMDARs in the PVN are latent and not functional in physiological conditions, but they become tonically active to increase synaptic glutamate release in SHRs. To determine whether Src kinases contribute to increased presynaptic NMDAR activity in the PVN in SHRs, we measured the frequency of mEPSCs, which reflects spontaneous quantal release of glutamate from presynaptic terminals. In these experiments, MK-801 (1 mmol/L), an NMDAR channel blocker, was added to the pipette solution to block postsynaptic NMDAR activity. In vehicle-treated slices, AP5 application significantly reduced the frequency of mEPSCs of labeled PVN neurons of SHRs, but not WKY rats (Figure 4A–4E). PP2 treatment did not significantly change the baseline frequency or amplitude of mEPSCs of labeled PVN neurons in WKY rats. In contrast, treatment with PP2 significantly
reduced the frequency of mEPSCs without changing their amplitude in SHRs (Figure 4A–4F). Furthermore, bath application of 50 μmol/L AP5 had no effect on the frequency of mEPSCs in SHR brain slices that had been pretreated with PP2 (Figure 4C–4E). These data suggest that increased Src kinase activity plays a critical role in the enhanced presynaptic NMDAR activity of PVN presympathetic neurons in SHRs.

**Figure 4.** Src contributes to increased presynaptic N-methyl-d-aspartate receptor (NMDAR) activity of rostral ventrolateral medulla (RVLM)-projecting paraventricular nucleus (PVN) neurons in spontaneously hypertensive rats (SHRs). A–D. Original traces and cumulative probability plots show the effect of bath application of 50 μmol/L AP5 on miniature excitatory postsynaptic currents (mEPSCs) of labeled PVN neurons recorded from Wistar-Kyoto (WKY) rats and SHRs pretreated with vehicle or 10 μmol/L 4-amino-5-(4-chlorophenyl)-7-(dimethylamino)pyrazolo[3,4-d]pyrimidine (PP2). E and F, Summary data show the effects of PP2 and AP5 on the frequency and amplitude of mEPSCs in labeled PVN neurons of WKY rats (vehicle, n=8 neurons; PP2, n=8 neurons) and SHRs (vehicle, n=8 neurons; PP2, n=9 neurons). *P<0.05 compared with WKY rats. #P<0.05 compared with the baseline in SHR vehicle group.

**CK2 and Src Kinases Are Differentially Involved in the Increased NMDAR Activity of PVN Presympathetic Neurons in SHRs**

We have shown previously that CK2, a constitutively active protein serine/threonine kinase, plays a role in increased NMDAR activity of PVN presympathetic neurons in SHRs. To determine whether CK2 and Src kinases play overlapping roles in the control of NMDAR activity in the PVN, we applied (pY)EEI, a synthetic Src-activating peptide, through the recording pipette. Intracellular dialysis of (pY)EEI (100 μmol/L) for 15 minutes caused a large increase in NMDA puff–elicited currents in labeled PVN neurons of WKY rats (n=9 neurons; Figure 5A and 5B). In brain slices pretreated with PP2 (10 μmol/L), dialysis of the (pY)EEI peptide failed to significantly alter the NMDA puff–elicited currents in labeled PVN neurons (n=9 neurons). In contrast, in brain slices pretreated with the CK2 inhibitor DRB (5,6-dichloro-1-β-d-ribofuranosylbenzimidazole; 100 μM), intracellular application...
of the Src-activating peptide still caused a large increase in NMDA puff–elicited currents in labeled PVN neurons of WKY rats (n=11 neurons; Figure 5A and 5B).

Furthermore, we determined the possible interaction between CK2 and Src kinases in SHRs. Treatment with PP2 alone and PP2 plus DRB similarly reduced the amplitude of NMDA puff–elicited currents of labeled PVN neurons in SHRs (n=7 neurons in each group; Figure 5C and 5D). The amplitude of NMDAR currents of labeled PVN neurons did not significantly differ between SHR brain slices treated with PP2 alone and PP2 plus DRB. These results suggest that although both CK2 and Src kinases regulate NMDAR activity of PVN presympathetic neurons, they probably affect different phosphorylation sites of NMDARs and their interacting proteins.

### Src-Mediated NMDAR Activation in the PVN Plays a Role in the Maintenance of Sympathetic Vasomotor Tone in SHRs

We determined the functional significance of Src-mediated NMDAR activity in the PVN in controlling sympathetic vasomotor tone in SHRs. We did not perform PP2 injection in WKY rats because blocking NMDARs in the PVN has no effect on ABP or sympathetic nerve activity in normotensive rats. The baseline integrated LSNA (averaged over 30 s) was 0.12±0.05 μV in SHRs, which was significantly higher in SHRs than in WKY rats (0.06±0.06 μV; P<0.05). We microinjected PP2 (40 pmol, 50 nL) bilaterally into the PVN, which significantly decreased the LSNA and mean ABP of SHRs (n=7 rats; Figure 6A–6E). The LSNA and ABP started to decrease at a mean time of 3.1±0.5 minutes after PP2 injection, and this effect lasted for 29.7±1.8 minutes. In SHRs injected with PP2, subsequent microinjection of AP5 (1.0 nmol, 50 nL) into the PVN failed to decrease LSNA, ABP, and HR. In contrast, microinjection of vehicle (0.5% dimethyl sulfoxide, 50 nL) into the PVN had no significant effect on ABP and LSNA in another set of SHRs (n=6 rats). Subsequent AP5 microinjection into the PVN significantly decreased LSNA and ABP in the SHRs receiving prior vehicle microinjection (Figure 6A–6E). These data suggest that the increased sympathetic vasomotor tone in SHRs is sustained by Src-mediated NMDAR activation in the PVN.

### Discussion

The most salient finding of our study is that Src kinases critically contribute to the increased NMDAR activity, at both pre- and postsynaptic sites, of RVLM-projecting PVN neurons in a rat model of essential hypertension. Src is highly expressed in various brain regions and can interact with postsynaptic density proteins, such as PSD-95. In the present study, we found that inhibition of Src kinase activity with PP2 significantly decreased the amplitude of evoked NMDAR-EPSCs and puff NMDA–induced currents in RVLM-projecting PVN neurons but not in WKY rats. Although AMPARs can also be phosphorylated by the Src kinases, we found that PP2 had no significant effect on the amplitude of mEPSCs or evoked AMPAR-EPSCs in PVN neurons in SHRs or WKY rats. Thus, Src regulation of AMPAR activity seems to be uninvolved in regulating glutamatergic input in the PVN in SHRs.

Src is also expressed at nerve terminals and is associated with synaptic vesicles. Presynaptic NMDARs in the hypothalamus and spinal cord are latent and not functionally active under physiological conditions. However, these receptors become tonically activated and promote synaptic glutamate release to PVN presympathetic neurons in hypertension. We found that PP2 significantly reduced the baseline frequency of mEPSCs of RVLM-projecting PVN neurons in...
SHRs. Furthermore, blocking NMDARs with AP5 decreased the frequency of mEPSCs in vehicle-treated, but not PP2-treated, brain slices of SHRs. Interestingly, we showed that PP2 produced no effect on puff NMDA-elicited currents or the mEPSC frequency in normotensive WKY rats. This differential effect of Src kinase inhibition on synaptic NMDAR activity is probably because of the low basal Src kinase activity in the PVN in WKY rats. Our findings suggest that increased Src kinase activity in the PVN may facilitate glutamate release by enhancing presynaptic NMDAR activity in SHRs. The sources of endogenous glutamate for tonic activation of presynaptic NMDARs in SHRs may come from the same terminals at which the NMDARs are expressed or from excitatory interneurons in the PVN that result from increased excitability and reduced synaptic inhibition.34,47

We showed that the Src protein level was significantly elevated in the PVN in adult SHRs compared with 4-week-old SHRs and adult WKY rats. However, the Src protein level in the RVLm, hippocampus, and frontal cortex did not significantly differ between WKY rats and SHRs, suggesting that Src upregulation is not a general phenomenon throughout all brain regions in SHRs. Because the Src protein level in the PVN was not altered by lowering ABP in SHRs with CGx, this suggests that Src upregulation in the PVN may not result from increased ABP but may instead contribute to hypertension development in SHRs. Altered tyrosine phosphorylation of NMDARs in the brain has been implicated in synaptic plasticity associated with depression48 and fear learning.49

Consistent with our in vitro slice recording data, we found that microinjection of the Src kinase inhibitor PP2 into the PVN significantly decreased LNSA and ABP in SHRs. Importantly, subsequent microinjection of AP5 reduced LNSA and ABP in vehicle-injected, but not in PP2-injected, SHRs. These in vivo data suggest that Src-mediated NMDAR activity in the PVN critically contributes to the elevated sympathetic vasomotor tone in SHRs.

Figure 6. Src-mediated N-methyl-D-aspartate receptor (NMDAR) activity in the paraventricular nucleus (PVN) maintains elevated sympathetic outflow in spontaneously hypertensive rats (SHRs). Representative recording traces show the effect of bilateral microinjection of vehicle (A) or 4-amino-5-(4-chlorophenyl)-7-(dimethylamino)pyrazolo[3,4-d]pyrimidine (PP2; B) and AP5 into the PVN on arterial blood pressure (ABP), lumbar sympathetic nerve activity (LNSA), and HR in SHRs. C–E, Summary data show changes in mean ABP, LNSA, and HR in response to injection of AP5 after microinjection of PP2 (n=7 rats) or vehicle (n=6 rats) into the PVN in SHRs. F, A representative image and schematic drawing show the microinjection sites for vehicle plus AP5 (●) and PP2 plus AP5 (○) in the PVN in SHRs. *P<0.05 compared with the baseline control. 3V indicates third ventricle; AH, anterior hypothalamus; Fx, Fornix; and VMH, ventromedial hypothalamus.
sites, their roles are not mutually exclusive. Interestingly, PP2 has no effect on spinal NMDAR activity increased by CK2. It has been shown that CK2 phosphorylates Ser-1480 residue in GluN2B, whereas Src kinases phosphorylate Tyr-1325 in GluN2A. Alternatively, NMDAR phosphorylation may involve a coordinated interaction between CK2 and Src kinases. In this regard, the catalytic subunits of CK2 can be directly tyrosine-phosphorylated by the Src kinases, leading to increased CK2 activity. The overall activity of NMDARs in the PVN likely depends on their phosphorylation levels controlled by both CK2 and Src kinases. Nevertheless, the precise interaction between CK2 and Src kinases in the regulation of NMDAR activity of PVN presynaptic neurons in hypertension remains to be delineated.

Perspectives

We present novel evidence that increased Src kinase activity in the PVN plays a pivotal role in increased pre- and postsynaptic NMDAR activity of PVN presynaptic neurons in SHRs. Our study provides new insight into the molecular mechanism of hypertension and to examine the long-term effect of the hypothalamic paraventricular and supraoptic nuclei.

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Disclosures

None.

References


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Novelty and Significance

**What Is New?**

- Src tyrosine kinase is upregulated in the hypothalamus in a rat model of hypertension.
- Src inhibition normalizes the increased N-methyl-D-aspartate receptor activity of hypothalamic presynaptic neurons in hypertensive animals.
- Inhibition of Src-mediated N-methyl-D-aspartate receptor activity reduces synaptic vasomotor tone in hypertensive animals.

**What Is Relevant?**

- Increased glutamatergic input and excitability of presynaptic neurons in the hypothalamus contribute to elevated sympathetic output in hypertension.

- Src kinases may be targeted for the treatment of hypertension by reducing the sympathetic vasomotor tone.

**Summary**

Increased Src kinase activity augments sympathetic outflow in hypertension by potentiating glutamatergic input to hypothalamic presynaptic neurons.
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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 MgCl₂, 2.4 CaCl₂, 1.2 NaH₂PO₄, 10 glucose, and 26 NaHCO₃ saturated with 95% O₂ and 5% CO₂. 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 Cs₂SO₄, 2.0 MgCl₂, 0.5 CaCl₂, 5.0 EGTA, 5.0 Mg-ATP, 0.5 Na₂-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. 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. 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. Because NMDARs are voltage-dependently blocked by Mg²⁺ at a negative holding potential and co-activated by glycine, puff NMDA-induced currents were recorded in Mg²⁺-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.

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.

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. 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. 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. 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. 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 O₂, 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% O₂. The expired CO₂ was continuously monitored by a CO₂ 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.

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. 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. 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.

**References**


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 (A).
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).