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

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## 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 (RVLM) 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 ganglionectomy in SHRs. Inhibition of Src kinase activity with 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2) completely normalized the higher amplitudes of evoked NMDAR-mediated excitatory postsynaptic currents (EPSCs) and puff NMDA-elicited currents of RVLM-projecting PVN neurons in SHRs. PP2 treatment also attenuated the higher frequency of NMDAR-mediated miniature EPSCs of these neurons in SHRs. However, PP2 had no effect on NMDAR-EPSCs or miniature EPSCs of RVLM-projecting PVN neurons in WKY rats. NMDAR activity increased by a 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.

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**Competing Interest/Disclosures**

None.

**Keywords**

Autonomic nervous system; hypertension; hypothalamus; NMDA receptor; sympathetic nerve discharges; synaptic plasticity

## Introduction

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[1–3] and in hypertensive patients[4–7]. The paraventricular nucleus (PVN) of the hypothalamus is an important brain region controlling sympathetic outflow[8–10]. Presympathetic neurons in the PVN project to vasomotor neurons in the rostral ventrolateral medulla (RVLM) in the brainstem and sympathetic preganglionic neurons in the intermediolateral cell column in the spinal cord, which in turn regulate sympathetic nerve discharges[11, 12]. Electrolytic lesion or pharmacological inhibition of the PVN decreases arterial blood pressure (ABP) and sympathetic nerve activity in spontaneously hypertensive rats (SHRs)[1, 2, 13, 14]. Also, transplantation of embryonic hypothalamic tissue containing the PVN from SHRs to normotensive rats leads to hypertension in the normal rats[15, 16]. Although hyperactivity of PVN presympathetic neurons is a major source of elevated sympathetic outflow in SHRs[1, 2], 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[2, 17]. The synaptic NMDAR activity of PVN presympathetic neurons is tightly regulated by phosphorylation via serine/threonine kinases and phosphatases, including casein kinase II (CK2) and calcineurin[18–20]. In addition, increasing the phosphorylation level of tyrosine residues in NMDARs can increase NMDAR activity[21–24]. The Src family of non-receptor protein tyrosine kinases has at least nine 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 PVN 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[27, 28]. 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 Online Supplements.

### Brain slice preparation and recordings

Coronal hypothalamic slices (300 μm thick) containing the PVN were obtained from FluoSphere-injected rats using a vibrating microtome. Whole-cell patch-clamp recordings were performed in labeled neurons in the PVN of the slices (Supplemental Fig. S1). Excitatory postsynaptic currents (EPSCs) were elicited by electrical 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 (CNQX). 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. 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.

### Western immunoblotting

Hypothalamic slices were sectioned 1.08–2.12 mm caudal to the bregma, and PVN tissues were micro-punched bilaterally with a slice punch. 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. 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 (LSNA) 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 LSNA and ABP were recorded using a 1401-PLUS analog-to-digital converter and Spike2 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 ± S.E.M. 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.

## 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, Fig. 1A,B). 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, Fig. 1A,B). 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. Fig. 1C,D).

We then determined whether the higher Src protein level in the PVN of SHRs was a secondary change due to 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 (Fig. 2A,B; Supplemental Fig. S2). Immunoblotting showed that the Src protein level in the PVN of SHRs did not differ significantly between rats receiving CGx and sham surgery (Fig. 2C,D). In addition, CGx induced a small decrease in ABP in WKY rats (Fig. 2A,B). 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 (Fig. 2C,D). 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 PP2, a highly selective Src kinase inhibitor[30–32], on evoked AMPAR- 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, DMSO) or PP2 (10 μM) for 1–2 hours before electrophysiological recording. PP2 at 10 μM 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, Fig. 3A,B). 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, Fig. 3A,B). Also, in vehicle-treated slices, the ratio of NMDAR-EPSCs to AMPAR-EPSCs was significantly higher 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 (Fig. 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 μM) induced significantly greater NMDAR currents in SHRs (n = 7 neurons) than in WKY rats (n = 9 neurons, Fig. 3D,E). PP2 treatment profoundly reduced NMDAR currents of labeled PVN neurons in SHRs but had no effect on puff NMDA-elicited currents in WKY rats (Fig. 3D,E). 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[17, 20]. 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[17, 34]. In these experiments, MK-801 (1 mM), an NMDAR channel blocker, was added to the pipette solution to block postsynaptic NMDAR activity[35]. In vehicle-treated slices, the baseline frequency of mEPSCs was significantly higher in SHRs than in WKY rats (n = 8 neurons in each group, Fig. 4A–E), but the baseline amplitude of mEPSCs did not differ significantly between the two groups. To confirm that the increase in the frequency of mEPSCs in SHRs was due to increased NMDAR activity, we bath applied the NMDAR antagonist AP5 (50 μM). In vehicle-treated slices, AP5 application significantly reduced the frequency of mEPSCs of labeled PVN neurons of SHRs, but not WKY rats (Fig. 4A–E).

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 (Fig. 4A–F). Furthermore, bath application of 50 μM AP5 had no effect on the frequency of mEPSCs in SHR brain slices that had been pretreated with PP2 (Fig. 4C–E). These data suggest that increased Src kinase activity plays a critical role in the enhanced presynaptic NMDAR activity of PVN presympathetic neurons in SHRs.

## 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[20]. 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[36, 37], through the recording pipette. Intracellular dialysis of (pY)EEI (100 μM) for 15 min caused a large increase in NMDA puff-elicited currents in labeled PVN neurons of WKY rats (n = 9 neurons, Fig. 5A,B). In brain slices pretreated with PP2 (10 μM), 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 (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, Fig. 5A,B).

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, Fig. 5C,D). 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/or 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[2]. The baseline integrated LSNA (averaged over 30 s) was 0.12 ± 0.05 μVs in SHRs, which was significantly higher in SHRs than in WKY rats (0.06 ± 0.06 μVs, $P < 0.05$). We microinjected PP2 (40 pmol, 50 nL)[38] bilaterally into the PVN, which significantly decreased the LSNA and mean ABP of SHRs (n = 7 rats, Fig. 6A–E). The LSNA and ABP started to decrease at a mean time of 3.1 ± 0.5 min after PP2 injection, and this effect lasted for 29.7 ± 1.8 min. In SHRs injected with PP2, subsequent microinjection of AP5 (1.0 nmol, 50 nL)[2, 20] into the PVN failed to decrease LSNA, ABP, and HR. In contrast, microinjection of vehicle (0.5% DMSO, 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 (Fig. 6A–E). 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[39, 40] and can interact with postsynaptic density proteins such as PSD-95[41–43]. 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 in SHRs but not in WKY rats. Although AMPARs can also be phosphorylated by the Src kinases[44], 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[45, 46]. Presynaptic NMDARs in the hypothalamus and spinal cord are latent and not functionally active under physiological conditions[18, 20]. However, these receptors become tonically activated and promote synaptic glutamate release to PVN presympathetic neurons in hypertension[17, 20]. 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 due to 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 depression[48] 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 LSNA and ABP in SHRs. Importantly, subsequent microinjection of AP5 reduced

LSNA 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.

Increased Src kinase activity in SHRs may directly increase the phosphorylation level of certain tyrosine residues of NMDARs. For example, various tyrosine residues in GluN2A (Tyr-1292, Tyr-1325, and Tyr-1387) and GluN2B (Tyr-1252, Tyr-1336, and Tyr-1472) can be phosphorylated by Src kinases[43, 50–53]. However, we cannot exclude the possibility that Src kinases increase NMDAR activity by phosphorylating tyrosine residues in NMDAR-interacting proteins, such as NADH dehydrogenase subunit 2[54]. CK2 is also involved in increased NMDAR activity of PVN presympathetic neurons in SHRs[20]. In this study, we found that intracellular application of a Src-activating peptide readily increased the NMDAR activity of RVLM-projecting PVN neurons. Although the potentiating effect of the Src-activating peptide on NMDAR activity was blocked by PP2, it was not affected by the CK2 inhibitor DRB. Furthermore, DRB had no further effect on NMDAR activity of PVN neurons that had already been reduced by PP2 in SHRs. Our data suggest that although both CK2 and Src kinases may contribute to increased NMDAR activity of PVN presympathetic neurons in SHRs through different phosphorylation sites, their roles are not mutually exclusive. Interestingly, PP2 has no effect on spinal NMDAR activity increased by CK2[55]. It has been shown that CK2 phosphorylates Ser-1480 residue in GluN2B[56], whereas Src kinases phosphorylate Tyr-1325 in GluN2A[48]. 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[57]. 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 presympathetic 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 presympathetic neurons in SHRs. Our study provides new insight into the molecular mechanism underlying elevated sympathetic vasomotor tone in an animal model of essential hypertension. The prevalence of resistant hypertension has been estimated to be 20% to 30%[58], making it a common and challenging clinical problem. On the basis of our findings, we suggest that Src kinases are potential targets for treating patients with neurogenic hypertension. Further studies are needed to determine the dynamic changes in the Src kinases and their various isoforms in the PVN in different animal models of hypertension and to examine the long-term effect of the Src kinase inhibition in conscious hypertensive animals.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Novelty and Significance

**1) What Is New**

- Src tyrosine kinase is upregulated in the hypothalamus in a rat model of hypertension
- Src inhibition normalizes the increased NMDAR activity of hypothalamic presympathetic neurons in hypertensive animals
- Inhibition of Src-mediated NMDAR activity reduces sympathetic vasomotor tone in hypertensive animals

**2) What Is Relevant**

- Increased glutamatergic input and excitability of presympathetic neurons in the hypothalamus contribute elevated sympathetic output in hypertension
- Src kinases may be targeted for treatment of hypertension by reducing the sympathetic vasomotor tone

**3) Summary**

- Increased Src kinase activity augments sympathetic outflow in hypertension by potentiating glutamatergic input to hypothalamic presympathetic neurons

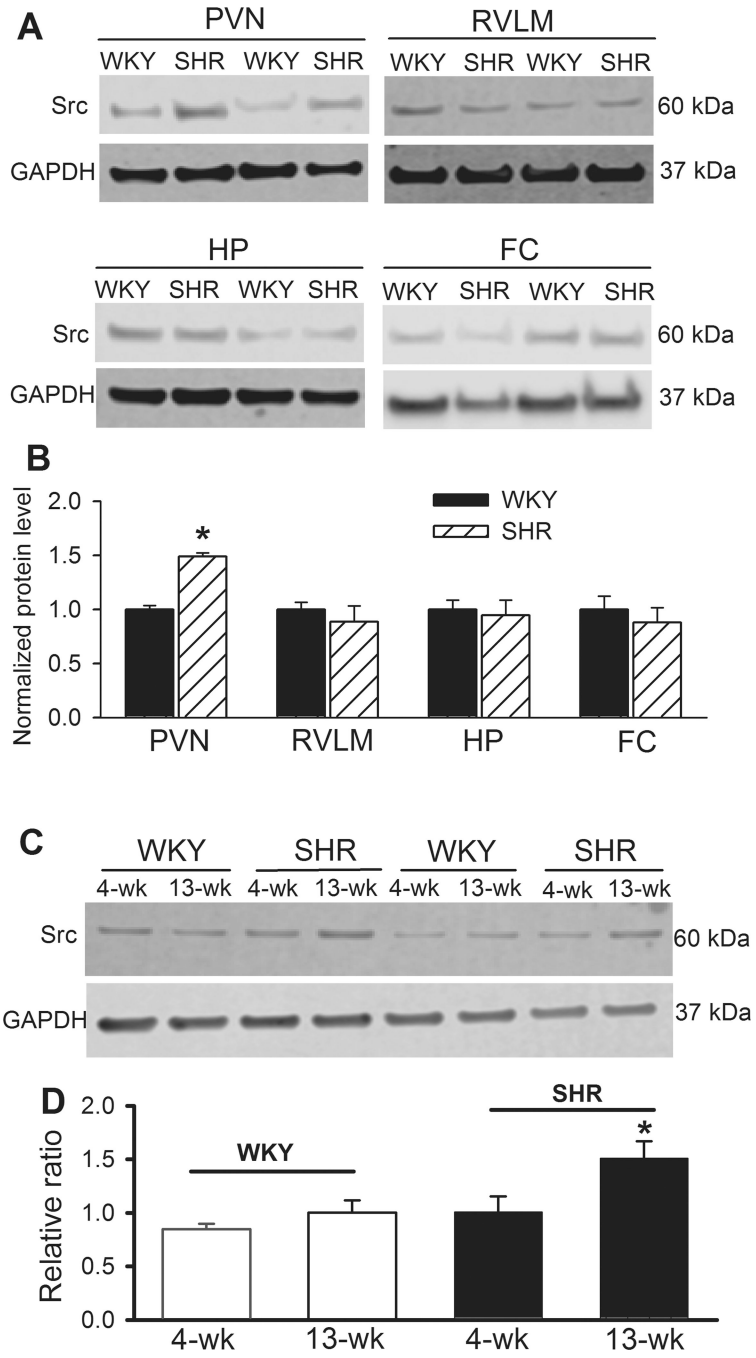


**Figure 1. Changes in Src protein levels in the PVN, rostral ventrolateral medulla, frontal cortex, and hippocampus in WKY rats and SHRs**

A and B: Representative gel images (**A**) and summary data (**B**) show the Src protein level (normalized to GAPDH) in the PVN, rostral ventrolateral medulla (RVLM), frontal cortex (FC), and hippocampus (HP) in WKY rats and SHRs (n = 6 rats in each group). C and D: Representative gel images (C) and group data (D) show the Src protein level in the PVN in 4-week-old and 13-week-old WKY and SHRs (n = 6 rats in each group). *P < 0.05, compared with WKY rats.

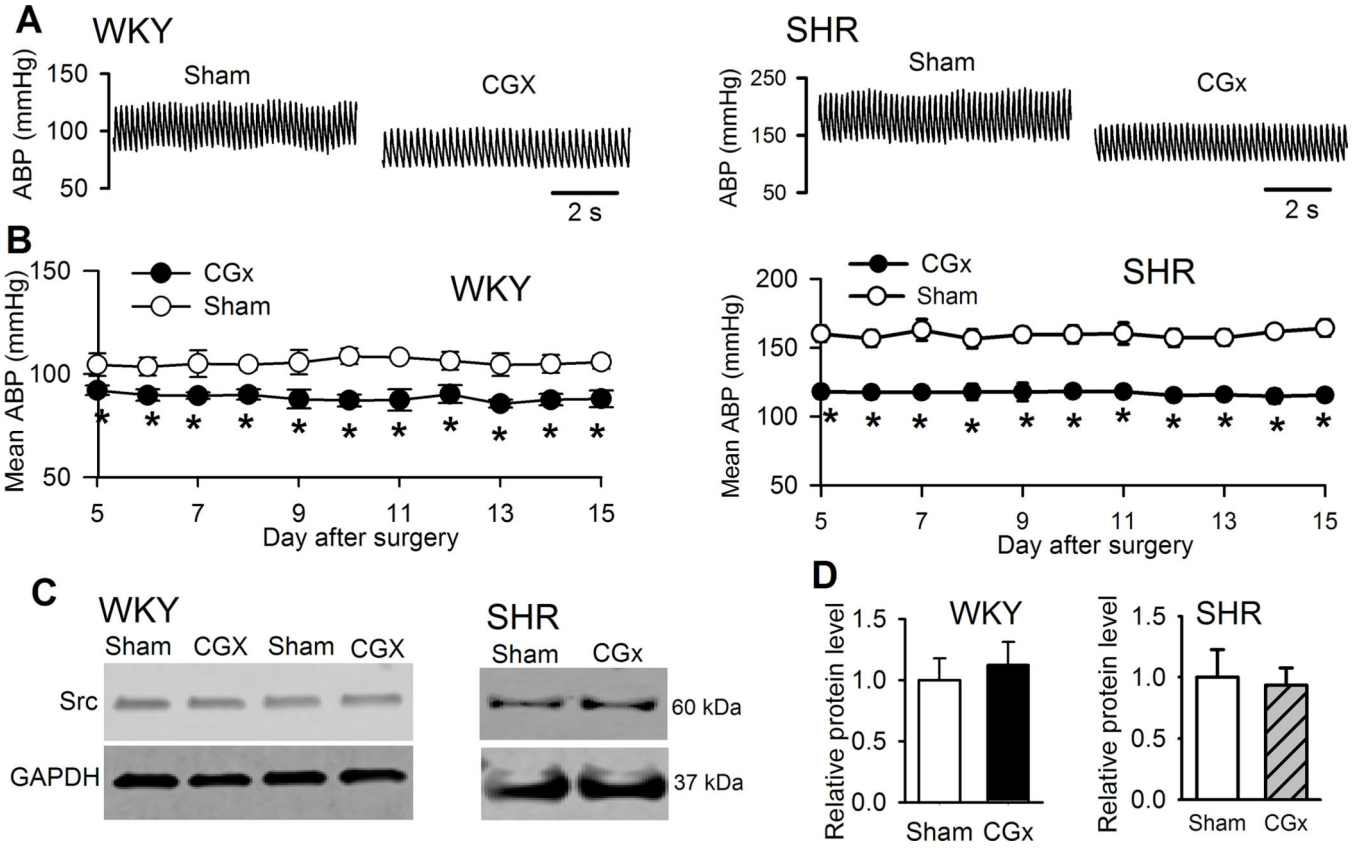


**Figure 2. Effect of lowering ABP on the Src protein level in the PVN in WKY rats and SHRs**

A and B, Original ABP traces (**A**) and group data (**B**) show the effect of CGx and sham surgery on the mean ABP in WKY rats and SHRs (n = 6 rats in each group). C and D, Representative gel images (**C**) and summary data (**D**) show the Src protein levels (normalized to GAPDH) in the PVN in WKY rats and SHRs subjected to CGx or sham surgery (n = 6 rats in each group). *P < 0.05 compared with the sham group.

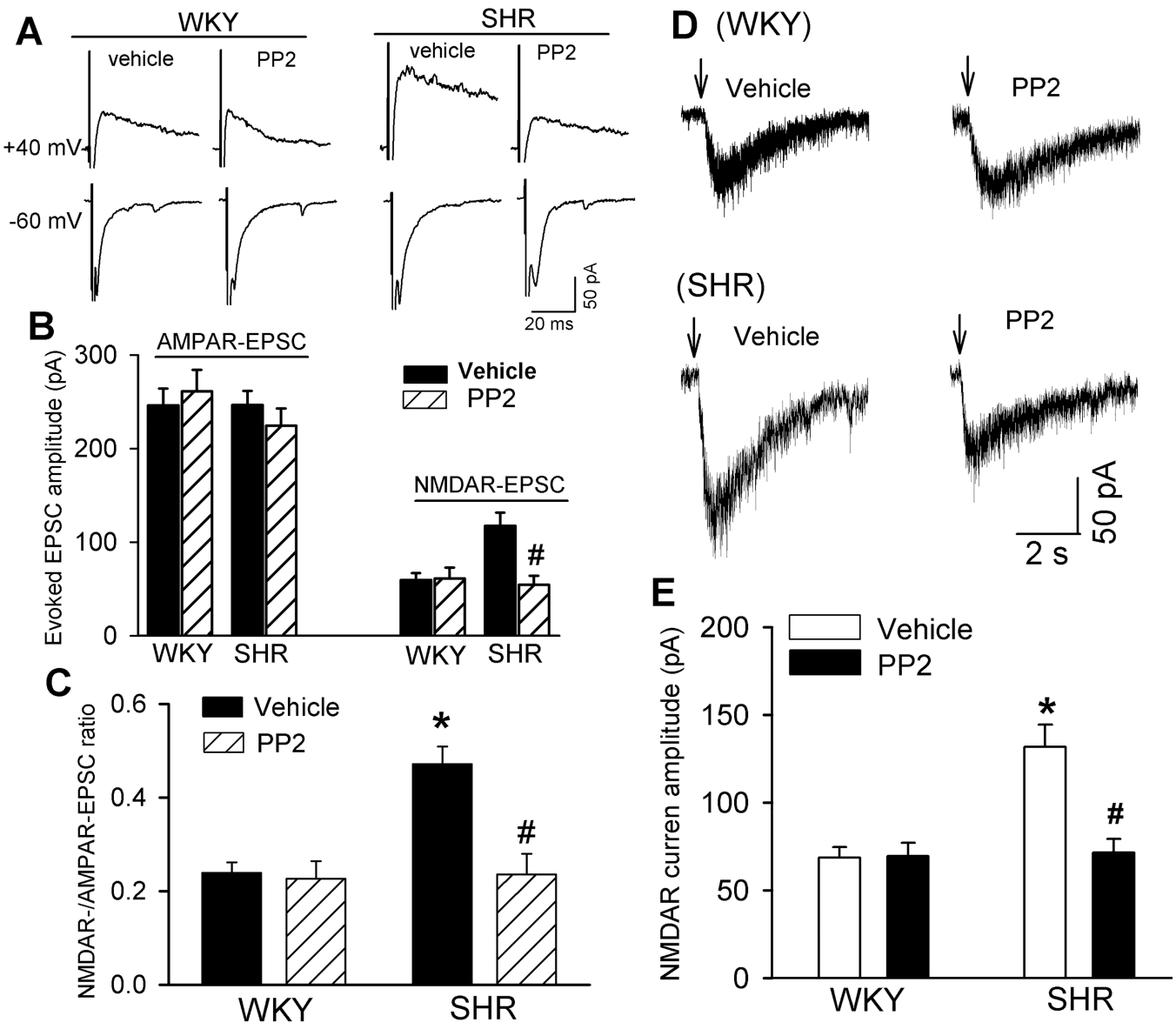


**Figure 3. Src is involved in increased synaptic NMDAR activity of RVLM-projecting PVN neurons in SHRs**

A, Representative current traces of evoked AMPAR-EPSCs (at −60 mV) and NMDAR-EPSCs (at +40 mV) recorded in labeled neurons from WKY rats and SHRs treated with vehicle or 10 μM PP2. B, Summary data of evoked AMPAR-EPSCs and NMDAR-EPSCs in neurons recorded from WKY rats or SHRs pretreated with vehicle or PP2 (n = 7 neurons in each group). *P < 0.05 compared with WKY rats. #P < 0.05 compared with the SHR vehicle group. C, Group data show the ratio of NMDAR-EPSCs to AMPAR-EPSCs in neurons recorded from WKY rats or SHRs treated with vehicle or PP2 (n = 7 neurons in each group). D and E, Original current traces (D) and summary data (E) show the effect of 10 μM PP2 on puff NMDA (100 μM)-elicited currents of labeled PVN neurons recorded from 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.

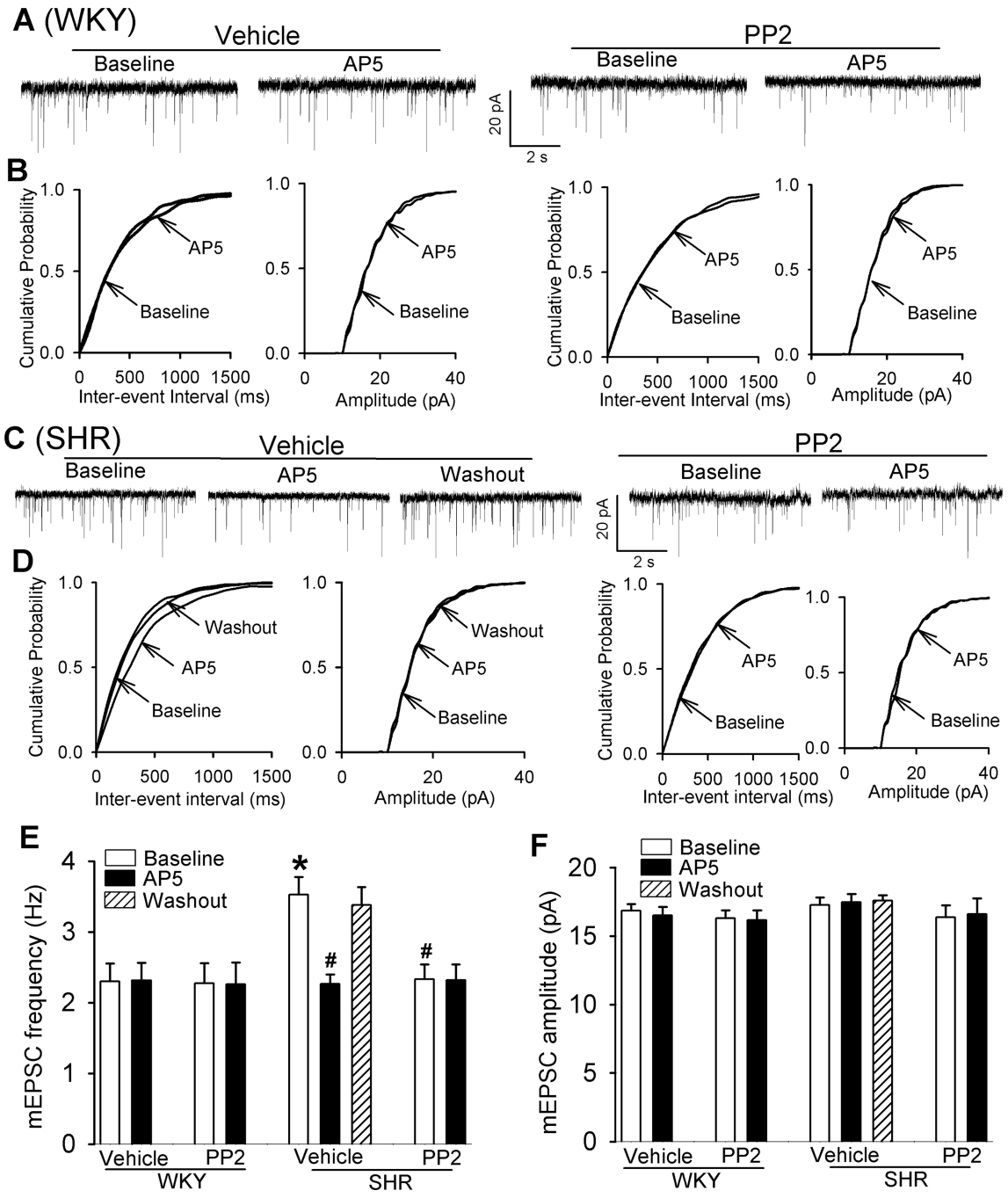


**Figure 4. Src contributes to increased presynaptic NMDAR activity of RVLM-projecting PVN neurons in SHRs**

A–D: Original traces and cumulative probability plots show the effect of bath application of 50 μM AP5 on mEPSCs of labeled PVN neurons recorded from WKY rats and SHRs pretreated with vehicle or 10 μM 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.

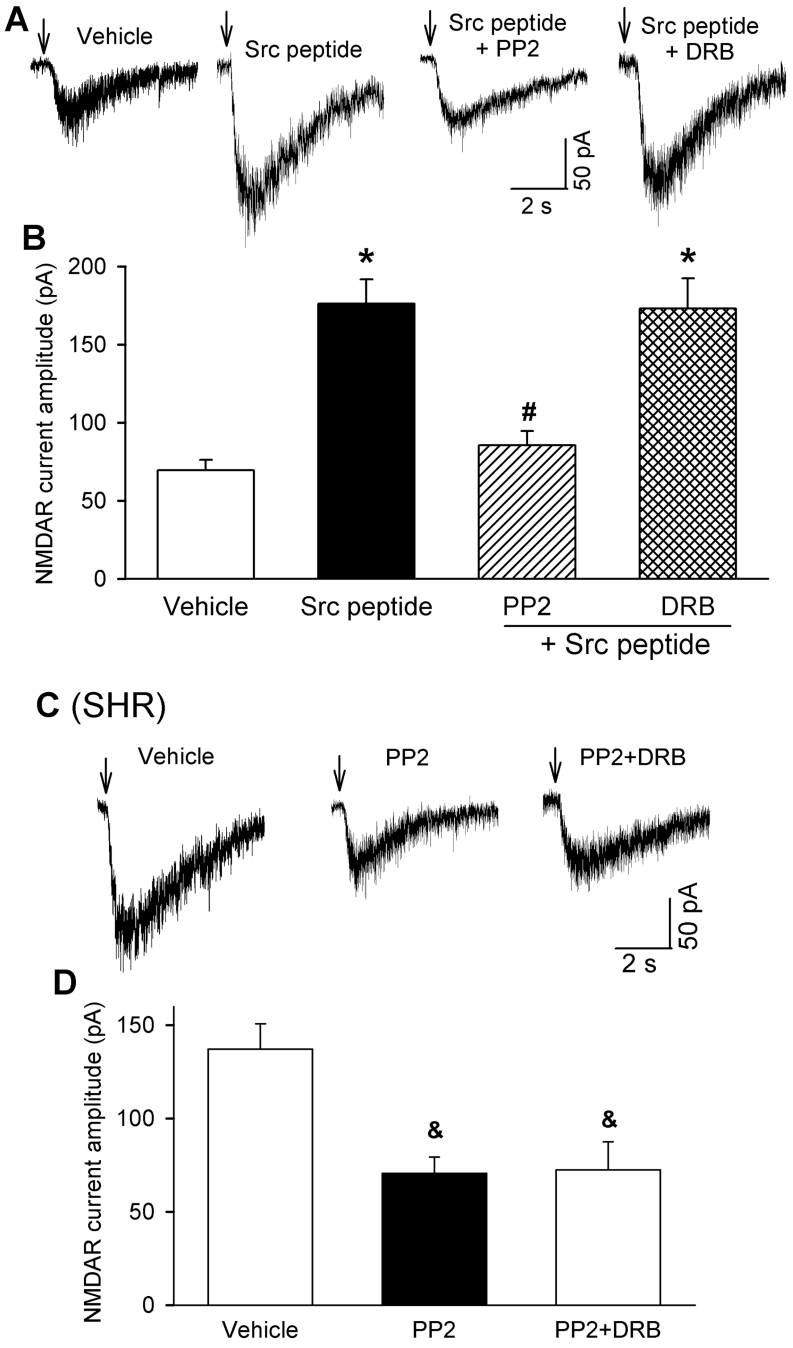


**Figure 5. CK2 and Src kinases differentially potentiate NMDAR activity of RVLM-projecting PVN neurons**

A and B, Original recording traces (A) and group data (B) show the effect of intracellular dialysis of the Src-activating peptide (pY)EEI (100 μM) on puff NMDA-elicited currents of labeled PVN neurons of WKY rats pretreated with vehicle (n = 9 neurons), 10 μM PP2 (n = 9 neurons) or 100 μM DRB (n = 11 neurons). C and D, Representative traces (C) and summary data (D) show the effect of vehicle, PP2 (10 μM) alone, and PP2 plus DRB (100 μM) on puff NMDA-elicited currents of labeled PVN neurons of SHRs (n = 7 neurons in each group). *P < 0.05 compared with neurons recorded without Src peptide (vehicle). #P <

0.05 compared with neurons recorded with Src peptide alone. $^{\&}P < 0.05$ compared with neurons pretreated with vehicle.

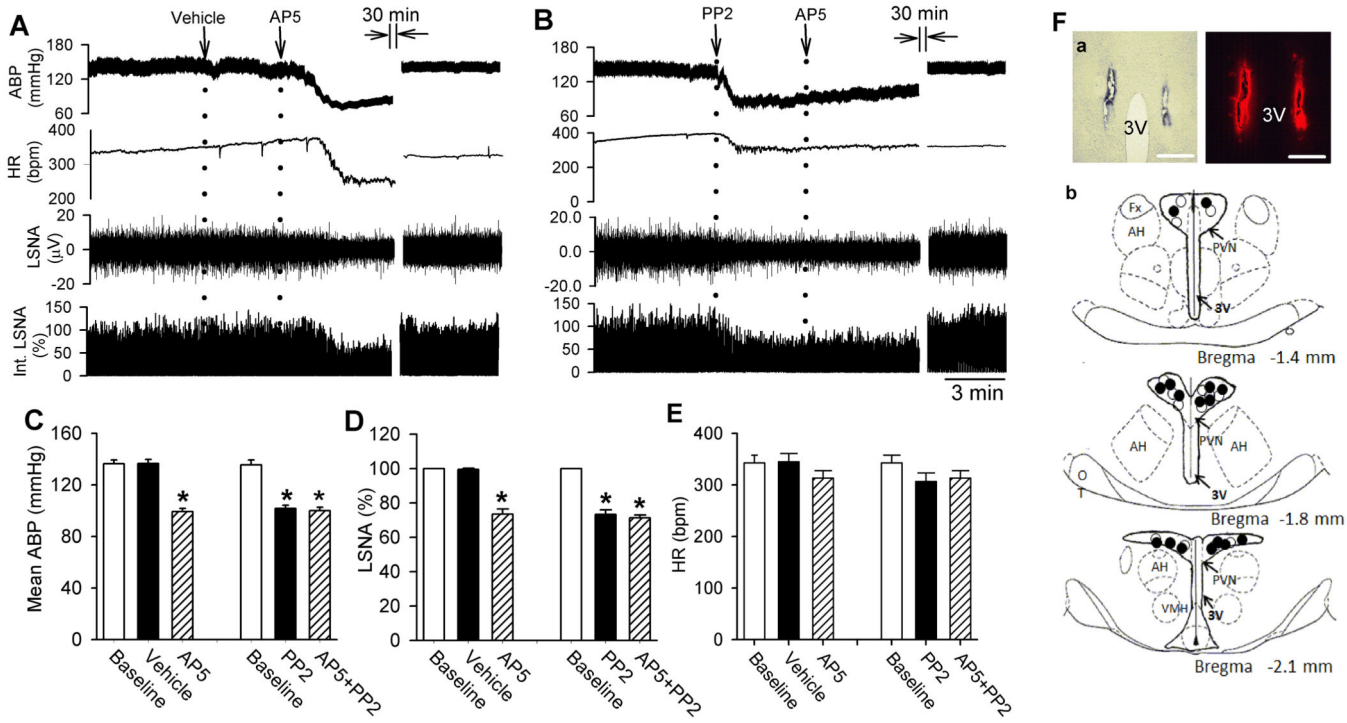


**Figure 6. Src-mediated NMDAR activity in the PVN maintains elevated sympathetic outflow in SHRs**

A and B, Representative recording traces show the effect of bilateral microinjection of vehicle (A) or PP2 (B) and AP5 into the PVN on ABP, LSNA, and HR in SHRs. C–E, summary data show changes in mean ABP, LSNA, 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. AH, anterior hypothalamus; 3V, third ventricle; Fx, Fornix; VMH, ventromedial hypothalamus.