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

Zeng-You Ye, De-Pei Li, Hui-Lin Pan

Abstract—Increased glutamatergic input in the hypothalamic paraventricular nucleus (PVN) plays an important role in the development of hypertension. Group II metabotropic glutamate receptors are expressed in the PVN, but their involvement in regulating synaptic transmission and sympathetic outflow in hypertension is unclear. Here, we show that the group II metabotropic glutamate receptors agonist 2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV) produced a significantly greater reduction in the frequency of spontaneous and miniature excitatory postsynaptic currents and in the amplitude of electrically evoked excitatory postsynaptic currents in retrogradely labeled spinally projecting PVN neurons in spontaneously hypertensive rats (SHRs) than in normotensive control rats. DCG-IV similarly decreased the frequency of GABAergic inhibitory postsynaptic currents of labeled PVN neurons in the 2 groups of rats. Strikingly, DCG-IV suppressed the firing of labeled PVN neurons only in SHRs. DCG-IV failed to inhibit the firing of PVN neurons of SHRs in the presence of ionotropic glutamate receptor antagonists. Lowering blood pressure with celiac ganglionectomy in SHRs normalized the DCG-IV effect on excitatory postsynaptic currents to the same level seen in control rats. Furthermore, microinjection of DCG-IV into the PVN significantly reduced blood pressure and sympathetic nerve activity in SHRs. Our findings provide new information that presynaptic group II metabotropic glutamate receptor activity at the glutamatergic terminals increases in the PVN in SHRs. Activation of group II metabotropic glutamate receptors in the PVN inhibits sympathetic vasomotor tone through attenuation of increased glutamatergic input and neuronal hyperactivity in SHRs. (Hypertension. 2013;62:00-00.) ● Online Data Supplement

Key Words: hypertension ▪ hypothalamus ▪ receptors, glutamate ▪ sympathetic nervous system ▪ synaptic transmission

The paraventricular nucleus (PVN) of the hypothalamus contributes to the development of hypertension.1–3 A population of PVN neurons projects to preganglionic sympathetic neurons in the spinal cord and rostral ventrolateral medulla and is likely involved in regulating sympathetic outflow and blood pressure.4–7 Elevated sympathetic nerve activity has been shown in patients with essential hypertension,8–10 and the hyperactivity of PVN presympathetic neurons has been demonstrated in animal models of essential hypertension, such as spontaneously hypertensive rats (SHRs).11–13 However, the precise mechanisms underlying the hyperactivity of PVN presympathetic neurons in hypertension are not fully known.

Glutamate is the major excitatory neurotransmitter in the PVN, and increased glutamatergic input into PVN presympathetic neurons leads to elevated sympathetic vasomotor tone in SHRs.3,12 In addition to fast-acting ionotropic glutamate receptors, G protein–coupled metabotropic glutamate receptors (mGluRs) are also activated by glutamate. Eight different mGluRs have been cloned and classified into 3 groups.16 Group I mGluRs (mGluR1 and mGluR5) are coupled to Gq/11 proteins to activate phospholipase C and protein kinase C, which increase neuronal firing activity and synaptic transmission. In contrast, group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, and mGluR8) mGluRs are coupled to Gi/o proteins, which reduce neuronal excitability and synaptic transmission.17 Although mGluR2/3 are widely distributed in the brain, including the PVN18,19 and median preoptic nucleus,20 the functional significance of group II mGluRs in the PVN in the control of glutamatergic input and sympathetic vasomotor tone in hypertension has not been determined.

In the present study, we tested the hypothesis that group II mGluRs control the excitability of PVN presympathetic neurons in hypertension by regulating glutamatergic input. In addition, we determined the role of group II mGluRs in the PVN in the regulation of sympathetic outflow in hypertension. Our findings provide new information that the activity of group II mGluRs at the glutamatergic terminals in the PVN

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increases in hypertension, which may provide a mechanism to dampen excessive glutamate release. Activation of group II mGluRs restrains the excitability of PVN presympathetic neurons and sympathetic vasomotor tone in hypertension.

Methods
Male Wistar-Kyoto (WKY) rats and SHRs (13 weeks old; Harlan, Indianapolis, IN) were used in this study. We used 89 SHRs and 79 Wistar-Kyoto rats for the entire study. The surgical procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee 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 noninvasive tail-cuff system. The systolic ABP was significantly higher in SHRs (208±3.01 mm Hg; n=25) than in age-matched WKY rats (122.18±2.56 mm Hg; n=25).

The detailed methods for retrograde labeling of spinally projecting PVN neurons, electrophysiological recordings of glutamatergic excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) in brain slices, PVN microinjections and lumbar sympathetic nerve recording in vivo, and data analysis are described in the online-only Data Supplement.

Results
Group II mGluR Activity Is Increased to Regulate Glutamate Release to PVN Presympathetic Neurons in Hypertension
To determine the role of group II mGluRs in controlling glutamatergic input to PVN presympathetic neurons in hypertension, we examined the effect of (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV), a highly specific group II mGluR agonist, on spontaneous EPSCs (sEPSCs) of spinally projecting PVN neurons in SHRs and WKY rats. The mean basal frequency of sEPSCs in labeled PVN neurons was significantly higher in SHRs than in WKY rats (Figure 1A and 1B). Bath application of DCG-IV (0.1, 0.3, 1.0, and 3.0 μmol/L; each applied for about 4 minutes) significantly decreased the frequency, but not the amplitude, of sEPSCs in a concentration-dependent manner in both WKY rats and SHRs (Figure 1A–1C). Notably, DCG-IV caused a significantly greater reduction in the frequency of sEPSCs in SHRs than in WKY rats (Figure 1C). Bath application of 100 nmol/L LY341495, a highly selective group II mGluR antagonist, had no significant effect on the basal frequency of sEPSCs. LY341495 abolished the inhibitory effect of DCG-IV (1 μmol/L) on the frequency of sEPSCs in labeled PVN neurons in both WKY rats and SHRs (Figure 1D).

To further determine the activity of presynaptic group II mGluRs at glutamatergic terminals in hypertension, we examined the effect of DCG-IV on miniature EPSCs (mEPSCs) of spinally projecting PVN neurons in SHRs and WKY rats. Bath application of 1 μmol/L DCG-IV significantly inhibited the frequency, but not the amplitude, of mEPSCs in labeled PVN neurons in both WKY rats and SHRs (Figure 2A–2D). The cumulative probability analysis of mEPSCs revealed that the distribution pattern of the interevent interval of mEPSCs was shifted toward the right in response to DCG-IV (Figure 2B and 2C). The inhibition of the frequency of mEPSCs by

Figure 1. DCG-IV inhibition of spontaneous excitatory postsynaptic currents (sEPSCs) of spinal projecting paraventricular nucleus (PVN) neurons was greater in spontaneously hypertensive rats (SHRs) than in Wistar-Kyoto (WKY) rats. A, Representative traces show sEPSCs recorded in labeled PVN neurons during control and application of DCG-IV (0.1 and 1 μmol/L) in 1 SHR and 1 WKY rat. B, Summary data show the concentration-dependent inhibitory effect of DCG-IV on the frequency and amplitude of sEPSCs in WKY rats (n=8) and SHRs (n=7). C, Comparison of the inhibitory effect of DCG-IV on the frequency of sEPSCs of PVN neurons (shown in B) in WKY rats and SHRs. D, Group data show that LY341495 (100 nmol/L) blocked the effect of DCG-IV (1 μmol/L) on the frequency of sEPSCs of labeled PVN neurons in SHRs and WKY rats (n=7 neurons in each group). *P<0.05, compared with the respective baseline control. #P<0.05, compared with the corresponding value in WKY rats.
DCG-IV in labeled PVN neurons was significantly greater in SHRs than in WKY rats (Figure 2C).

We next confirmed the presynaptic effects of DCG-IV on evoked EPSCs by including a general G protein inhibitor, guanosine 5′-[β-thio]diphosphate (GDP-β-s) (1 mmol/L), in the pipette solution to block the postsynaptic effect of DCG-IV.22 In WKY rats, bath application of 1 μmol/L DCG-IV significantly decreased the amplitude of evoked EPSCs in the labeled PVN neurons (44.09±4.23% reduction; n=8). In SHRs, DCG-IV caused a much larger reduction in the amplitude of evoked EPSCs (60.74±5.68% reduction; n=8; P<0.05; Figure 3A). Bath application of 100 nmol/L LY341495 abolished the inhibitory effects of DCG-IV on evoked EPSCs of PVN neurons in both WKY rats and SHRs (Figure 3B). In addition, bath application of 1 μmol/L DCG-IV had no significant effect on the amplitude of currents elicited by puff α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, 100 μmol/L) application onto labeled PVN neurons in WKY rats and SHRs (Figure 3C).

It has been shown that group II mGluRs are present on glia in some brain regions.24 To determine the possible contribution of these group II mGluRs located on glia to the inhibitory effect of DCG-IV on evoked EPSCs of PVN neurons in both WKY rats and SHRs (Figure 3B). In addition, bath application of 1 μmol/L DCG-IV had no significant effect on the amplitude of currents elicited by puff α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, 100 μmol/L) application onto labeled PVN neurons in WKY rats and SHRs (Figure 3C).

Increased Presynaptic Group II mGluR Activity in the PVN in Hypertension Is an Adaptive Response to Increased ABP

To determine whether the increased group II mGluR activity is the cause of hypertension or a secondary adaptive response to increased ABP, we examined the effects of DCG-IV on evoked EPSCs (recorded with GDP-β-s included in the pipette solution) of spinally projecting PVN neurons in SHRs 2 weeks after lowering the ABP with celiac ganglionectomy (CGx).14 Compared with the sham surgery, CGx significantly lowered the mean ABP, measured with radiotelemetry, for ≥2 weeks in SHRs (Figure 3E). CGx had no effects on the food or water intake and body weight in all rats studied. DCG-IV (1 μmol/L) produced a significantly smaller inhibitory effect on evoked EPSCs in labeled PVN neurons in SHRs undergoing CGx than in the sham group (Figure 3F). Notably, the effect of DCG-IV on evoked EPSCs of labeled PVN neurons in SHRs subjected to CGx was not significantly different from that in WKY rats.

Group II mGluR Activity Controlling GABAergic Input to PVN Presympathetic Neurons Does Not Change in Hypertension

The activity of PVN presympathetic neurons is finely tuned by both excitatory glutamatergic and inhibitory GABAergic inputs.12,26,27 To determine whether the control of GABAergic input by the activity of group II mGluRs in the PVN is altered in hypertension, we compared the effect of DCG-IV on GABAergic IPSCs of spinally projecting PVN neurons in WKY rats and SHRs. The basal frequency of spontaneous IPSCs (sIPSCs) was significantly lower in the PVN in SHRs than in WKY rats (Figure 4A and 4B), and the amplitude of sIPSCs was significantly smaller in SHRs than in WKY rats (Figure 4A and 4B). Bath application of DCG-IV (1 μmol/L) significantly decreased the frequency, but not the amplitude, of sIPSCs in both WKY rats and SHRs (Figure 4A and 4B). The inhibitory effect of DCG-IV on the frequency of sIPSCs did not differ significantly between WKY rats and SHRs (Figure 4B).
The basal frequency and amplitude of miniature IPSCs (mIPSCs) in labeled PVN neurons were also significantly reduced in SHRs compared with WKY rats (Figure 4C). Bath application of 1 μmol/L DCG-IV significantly decreased the frequency, but not the amplitude, of mIPSCs in both WKY rats and SHRs. The inhibitory effect of DCG-IV on the frequency of mIPSCs in labeled PVN neurons did not differ significantly between WKY rats and SHRs (Figure 4C).

We also examined the effect of DCG-IV on evoked IPSCs (recorded with GDP-β-s included in the pipette solution) of spinally projecting PVN neurons in WKY rats and SHRs. The basal amplitude of evoked IPSCs was significantly smaller in SHRs than in WKY rats (Figure 4D and 4E). DCG-IV (1 μmol/L) produced a similar magnitude of reduction in the amplitude of evoked IPSCs in WKY rats and SHRs (Figure 4E). These data suggest that the activity of presynaptic group II mGluRs at GABAergic terminals in the PVN is not altered in hypertension.

Group II mGluRs Regulate the Firing Activity of PVN Presympathetic Neurons in Hypertension

To determine the role of group II mGluRs in the control of the firing of PVN presympathetic neurons in hypertension, we tested the effect of DCG-IV on the firing activity of labeled PVN neurons with cell-attached recordings in SHRs and WKY rats. The basal firing rate of PVN neurons in SHRs was significantly higher than that in WKY rats. Bath application of DCG-IV (1 μmol/L) had no significant effect on the firing activity of PVN neurons in WKY rats. In contrast, DCG-IV significantly decreased the firing rate of PVN neurons in SHRs (Figure 5A and 5B). The group II mGluR antagonist LY341495 abolished the inhibitory effect of DCG-IV on the firing activity in SHRs (Figure 5C).

In additional labeled PVN neurons in SHRs, blocking N-methyl-D-aspartate (NMDA) and AMPA receptors with their respective antagonists, d-2-amino-5-phosphonovalerate (AP-5, 50 μmol/L) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μmol/L), significantly reduced their basal firing activity (Figure 5D). In the presence of AP-5 and CNQX, DCG-IV failed to further inhibit the firing activity of these PVN neurons in SHRs (Figure 5D). These results suggest that activation of group II mGluRs inhibits the firing of PVN presympathetic neurons through attenuation of glutamatergic input in hypertension.

Group II mGluRs in the PVN Control Sympathetic Vasomotor Tone in Hypertension

To determine the role of group II mGluRs in the PVN in controlling sympathetic outflow in hypertension, we microinjected DCG-IV directly into the PVN. Bilateral microinjection of DCG-IV (10–20 pmol/50 nL) slightly increased lumbar sympathetic nerve activity (LSNA) and mean ABP in WKY rats (n=7 rats; Figure 6A and 6B). In contrast, in SHRs, microinjection of DCG-IV (5–20 pmol/50 nL) into the PVN significantly decreased LSNA, mean ABP, and heart rate (HR) in a dose-dependent manner (n=7 rats). Bilateral microinjection of LY341495 (6 pmol/50 nL) had no significant effect on LSNA, mean ABP, and HR in WKY rats and sham controls.
rats or SHRs (Figure 6C). LY341495 abolished the effect of DCG-IV on LSNA, mean ABP, and HR in SHRs and WKY rats (Figure 6C). These data suggest that activation of group II mGluRs in the PVN inhibits sympathetic outflow in hypertension.

During the course of this study, 2 WKY rats and 3 SHRs were excluded from the data analysis because of the misplacement of microinjections (Figure 6D). Microinjection of DCG-IV (20 pmol/50 nL) had minimal effect on ABP (WKY: 85.8±4.6 versus 84.3±3.7 mm Hg; SHR: 138.5±8.5 versus 140.1±6.2 mm Hg), LSNA (WKY: 100% versus 101.5±0.5%; SHR: 100% versus 101.4±1.7%), and HR (WKY: 278.5±10.5 versus 282.0±9.0 bpm; SHR: 340.8±12.1 versus 344.8±12.3 bpm) in these 2 WKY rats and 3 SHRs.

**Discussion**

The major objective of our study was to determine the role of group II mGluRs in the control of synaptic input to PVN presympathetic neurons and sympathetic outflow in a commonly used animal model of essential hypertension. At the present time, there are no available methods to unequivocally identify PVN neurons involved only in ABP control in brain slices. We acknowledge that not all spinally projecting PVN neurons are exclusively involved in ABP regulation. Group II mGluRs are located at presynaptic terminals, postsynaptic neurons, and glia in several brain regions.28 In this study, we found that activation of group II mGluRs with DCG-IV significantly inhibited the frequency, but not the amplitude, of sEPSCs and mEPSCs in spinally projecting PVN neurons in both WKY rats and SHRs. DCG-IV also significantly reduced the amplitude of electrically evoked EPSCs when GDP-β-s was intracellularly dialyzed to inhibit the postsynaptic G proteins. Furthermore, DCG-IV had no significant effect on postsynaptic AMPA receptor currents elicited by puff AMPA application. In addition, we found that the inhibitory effect of DCG-IV on evoked EPSCs of PVN neurons was not altered when brain slices were treated with fluorocacetate to inhibit glial function, which suggests that glial cells are not involved in the control of glutamatergic input by group II mGluRs in the PVN. Our findings indicate that activation of group II mGluRs inhibits glutamatergic input to PVN presympathetic neurons through a presynaptic mechanism.

An important finding of our study is that the activity of group II mGluRs on glutamatergic terminals is increased in the PVN in hypertension. This conclusion is supported by our data showing that the effects of DCG-IV on the frequency of sEPSCs and mEPSCs and the amplitude of evoked EPSCs in the PVN were significantly greater in SHRs than in WKY rats. Interestingly, lowering ABP in SHRs with CGx normalized the inhibitory effect of DCG-IV on evoked EPSCs of PVN neurons to the same level observed in WKY rats. This finding suggests that the increased activity of group II mGluRs at the presynaptic glutamatergic terminals in the PVN in SHRs is a secondary adaptive response to increased ABP. Although it is not clear whether this increased activity of presynaptic group II
The increase in excitatory glutamatergic input to PVN presympathetic neurons in SHRs is caused by augmentation of pre- and postsynaptic NMDA receptor activity by the protein kinase CK2.12,31 Interestingly, we found that DCG-IV inhibited the firing activity of spinally projecting PVN neurons only in SHRs, although DCG-IV inhibited synaptic glutamate release in both SHRs and WKY rats. The lack of an inhibitory effect of DCG-IV on the firing of PVN neurons in WKY rats is probably because of the fact that group II mGluR activation also removed inhibitory GABAergic input to PVN neurons in WKY rats. Because of augmented excitatory glutamatergic input and diminished inhibitory GABAergic input in the PVN in SHRs,3,11,12,30 activation of group II mGluRs could reduce the excitability of PVN presympathetic neurons by attenuation of increased glutamatergic input in hypertension. This hypothesis is supported by our finding that the inhibitory effect of DCG-IV on the firing activity of PVN neurons in SHRs was abolished when NMDA and AMPA receptors were blocked. Although the presynaptic glutamatergic neuronal soma may not be present within the PVN, group II mGluRs present on the presynaptic terminals are directly involved in controlling the firing activity of postsynaptic (spinally projecting) neurons in the PVN in SHRs.

Another significant finding of our study is that activation of group II mGluRs in the PVN inhibited sympathetic vasomotor tone only in SHRs. Microinjection of DCG-IV into the PVN induced a significant and dose-dependent reduction in the ABP and LSNA in SHRs. These results are consistent with our brain slice recordings showing an inhibitory effect of DCG-IV on the firing activity of PVN neurons only in SHRs. However, we unexpectedly found that microinjection of DCG-IV into the PVN slightly increased the ABP and LSNA in WKY rats at high doses. It is not clear how DCG-IV in the PVN increases ABP and sympathetic nerve activity in WKY rats. It is unlikely that DCG-IV produces a nonspecific effect, because blocking group II mGluRs with LY341495 abolished the DCG-IV effect in both SHRs and WKY rats. Because GABAergic inhibition of PVN neuronal excitability is more pronounced in WKY rats than in SHRs,11,15,30 removal of GABAergic input by the group II mGluR agonist may disinhibit PVN presympathetic neurons in vivo, leading to a small increase in sympathetic outflow in WKY rats. Although we did not observe a stimulatory effect of DCG-IV on neuronal firing in brain slices of WKY rats, it is possible that certain inhibitory interneurons might have been removed in our thin brain slice preparation.

**Perspectives**

We found that activation of group II mGluRs inhibits both glutamatergic and GABAergic transmission at a presynaptic site in the PVN. The activity of group II mGluRs at glutamatergic terminals is increased in hypertension, which may limit excessive glutamate release through a negative feedback mechanism. This finding provides further evidence of synaptic and neuronal plasticity in the PVN associated with hypertension. We also show that activation of group II mGluRs inhibits the hyperactivity of PVN presympathetic neurons and sympathetic vasomotor tone only in SHRs and not in normotensive rats. On the basis of these findings, group II mGluRs may represent a
potential therapeutic target for the treatment of neurogenic hypertension. It should be noted that because the group II mGluR agonist was microinjected into the PVN, it can inhibit sympathetic outflow by affecting neurons other than just spinally projecting neurons. Further studies are also needed to determine changes in presynaptic group II mGluRs in the PVN in other models of hypertension and to examine the long-term effect of the group II mGluR agonist in conscious animal models of hypertension.

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**Novelty and Significance**

- Presynaptic group II metabotropic glutamate receptors (mGluRs) regulate excitatory and inhibitory synaptic input to presympathetic neurons in the hypothalamus.
- Group II mGluR stimulation inhibits hyperactivity of hypothalamic presympathetic neurons in hypertension by reducing glutamatergic input.
- Group II mGluR activation in the hypothalamus reduces sympathetic nerve discharges and arterial blood pressure in hypertension.

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

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**What Is New?**

- Presynaptic group II metabotropic glutamate receptors (mGluRs) regulate excitatory and inhibitory synaptic input to presympathetic neurons in the hypothalamus.
- Group II mGluR stimulation inhibits hyperactivity of hypothalamic presympathetic neurons in hypertension by reducing glutamatergic input.
- Group II mGluR activation in the hypothalamus reduces sympathetic nerve discharges and arterial blood pressure in hypertension.

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

**What Is Relevant?**

- Presynaptic group II metabotropic glutamate receptors (mGluRs) regulate excitatory and inhibitory synaptic input to presympathetic neurons in the hypothalamus.
- Group II mGluRs may be targeted for treatment of hypertension by reducing the sympathetic vasomotor tone.

**Summary**

Activation of group II mGluRs in the hypothalamus inhibits sympathetic drive and hyperactivity of presympathetic neurons by reducing synaptic glutamate release.
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Short title: Hypothalamic mGluR2/3 in SHRs

Methods

Animal models

Male Wistar-Kyoto (WKY) rats and SHRs (13 weeks old; Harlan, Indianapolis, IN) were used in this study. We used 89 SHRs 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 SHRs (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 1,2. 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 MgCl2, 2.4 CaCl2, 1.2 NaH2PO4, 10 glucose, and 26 NaHCO3 saturated with 95% O2 and 5% CO2. 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 1,2. 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
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-glucuronate, 5.0 KCl, 2.0 MgCl₂, 0.5 CaCl₂, 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. 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. 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. 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. (2S,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 SHRs anesthetized with 2-3% isoflurane, as we have described previously. 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 SHRs, 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. 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.7,8

For microinjection of DCG-IV, the brain was exposed at the level of the hypothalamus. A glass microinjection pipette (tip diameter 20–30 μm) 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.7,8 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 μm 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 ± 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