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Interaction between glutamate and GABA systems in the integration of sympathetic outflow by the paraventricular nucleus of the hypothalamus

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Li, Yi-Fan, Keshia L. Jackson, Javier E. Stern, Brandon Rabeler, and Kaushik P. Patel. Interaction between glutamate and GABA systems in the integration of sympathetic outflow by the paraventricular nucleus of the hypothalamus. *Am J Physiol Heart Circ Physiol* 291: H2847–H2856, 2006. First published July 28, 2006; doi:10.1152/ajpheart.00625.2005.—The paraventricular nucleus (PVN) of the hypothalamus is a central site known to modulate sympathetic outflow. Excitatory and inhibitory neurotransmitters within the PVN dictate final outflow. The goal of the present study was to examine the role of the interaction between the excitatory neurotransmitter glutamate and the inhibitory neurotransmitter GABA in the regulation of sympathetic activity. In α-chloralose- and urethane-anesthetized rats, microinjection of glutamate and *N*-methyl-D-aspartate (NMDA; 50, 100, and 200 pmol) into the PVN produced dose-dependent increases in renal sympathetic nerve activity, blood pressure, and heart rate. These responses were blocked by the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (AP-5). Microinjection of bicuculline, a $GABA_A$ receptor antagonist, into the PVN (50, 100, and 200 pmol) also produced significant, dose-dependent increases in renal sympathetic nerve activity, blood pressure, and heart rate; AP-5 also blocked these responses. Using microdialysis and HPLC/electrochemical detection techniques, we observed that bicuculline infusion into the PVN increased glutamate release. Using an in vitro hypothalamic slice preparation, we found that bicuculline increased the frequency of glutamate-mediated excitatory postsynaptic currents in PVN-rostral ventrolateral medullary projecting neurons, supporting a $GABA_A$-mediated tonic inhibition of this excitatory input into these neurons. Together, these data indicate that *1*) glutamate, via NMDA receptors, excites the presympathetic neurons within the PVN and increases sympathetic outflow and *2*) this glutamate excitatory input is tonically inhibited by a $GABA_A$-mediated mechanism.

glutamate-GABA interaction; renal sympathetic nerve activity

THE PARAVENTRICULAR NUCLEUS (PVN) of the hypothalamus is well known as an important central site for integration of sympathetic nerve activity (32, 34). Morphological and functional studies have shown that the PVN is a major source of forebrain input to the sympathetic nervous system (25, 30). The PVN is reciprocally connected to other areas of the central nervous system that are involved in cardiovascular function (21, 32) and plays an important role in cardiovascular regulation (14, 33). However, the mechanisms under which different neurotransmitters within the PVN interact with each other to regulate sympathetic outflow have not been well established.

A number of excitatory and inhibitory neurotransmitters converge in the PVN to influence its neuronal activity (34). Among them are glutamate and GABA. Glutamate is a well-known excitatory neurotransmitter in the central nervous system. It has been reported that functional glutamate receptors are expressed in the PVN (3, 12, 13, 36). Functional studies have shown that glutamate receptors within the PVN are involved in cardiovascular reflexes (5, 23). Our recent study demonstrated that glutamate *N*-methyl-D-aspartate (NMDA) receptors within the PVN mediate an excitatory effect on sympathetic nerve activity (19), suggesting that a glutamatergic system within the PVN may play a role in regulating sympathetic nerve activity and, thus, cardiovascular function.

In contrast, GABA is a well-known inhibitory neurotransmitter in the central nervous system. A large body of evidence suggests that GABA plays an important role in central sympathetic and cardiovascular regulation. Intracerebroventricular injections of GABA agonists decrease arterial blood pressure (BP), heart rate (HR), and peripheral sympathetic nerve activity (1). Conversely, intracerebroventricular administration of GABA antagonists, such as bicuculline methiodide or picrotoxin, markedly increase BP and HR due to an increase in sympathetic nerve activity (4, 31). The cardiovascular effects of GABA on the central nervous system may occur at different levels in the brain, because GABA is widely localized within discrete autonomic centers of the brain. The PVN is one of the sites in which these effects of GABA are elicited. GABA has been reported to be a dominant inhibitory neurotransmitter within the PVN. GABA has been demonstrated to tonically restrain firing activity of PVN neurons, which innervate the rostral ventrolateral medulla (RVLM), an action driven in part by nitric oxide (16). Microinjection of bicuculline into the PVN significantly increases renal sympathetic nerve activity (RSNA), arterial BP, and HR (26). Taken together, these observations suggest that GABAergic mechanisms in the PVN may be tonically inhibitory and are important in control of sympathetic nervous system outflow.

It is conceivable that the interaction of these two opposing neurotransmitters within the PVN would significantly influence regulation of sympathetic nerve activity (6, 7) and neurosecretory function (6, 7). However, the details of the mechanism(s) of the interaction between GABA and glutamate within the PVN in the integration of sympathetic nerve activity remain to be elucidated. In the present study, we have investigated the interaction between these two neurotransmitters at

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the whole animal and the cellular level. We have tested the effect of blockade of glutamate NMDA receptors on GABA antagonist bicuculline-induced sympathetic excitation, as well as the effect of blockade of $GABA_A$ receptors on glutamate release and glutamate-mediated synaptic activity within the PVN.

METHODS

In Vivo Experiments

All rats were fed and housed according to institutional guidelines. This study was approved by the Institutional Animal Care and Use Committee and conformed to the guidelines for the care and use of laboratory animals of the National Institutes of Health and the American Physiological Society.

On the day of the experiment, rats were anesthetized with urethane (0.75 g/kg ip) and α-chloralose (70 mg/kg ip). The left femoral vein was cannulated and connected to a computer-driven data recording-and-analyzing system (PowerLab) via a pressure transducer (model P23 1D, Gould) for recording arterial BP and HR. The trachea was intubated to facilitate spontaneous ventilation.

Placement of microinjection and push-pull perfusion cannulas into the PVN. The anesthetized rat was placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). A longitudinal incision was made on the head, and the bregma was exposed. The coordinates for the PVN were determined from the atlas of Paxinos and Watson (24): 1.5 mm posterior to the bregma, 0.4 mm lateral to the midline, and 7.8 mm ventral to the dura. A small burr hole was made in the skull. For the microinjections, we lowered a thin (0.5 mm OD, 0.1 mm ID) needle connected to a 0.5-ml microsyringe (model 7000.5, Hamilton) into the PVN. For push-pull perfusion, we lowered a probe (0.7 mm OD, 0.2 mm ID) that led the inner (push) cannula into the PVN and collected the perfusate in the outer (pull) cannula. The push and pull cannulas were connected to a pump that infused and returned artificial cerebrospinal fluid (aCSF, in mM: 3.0 KCl, 0.65 $MgCl_2$, 1.5 $CaCl_2$, and 132 NaCl, with 24.6 mM $NaHCO_3$ and 3.3 mM glucose added immediately before use) at a constant rate of 3 μl/min. The returned perfusate was collected as a fraction every 20 min. The samples were rapidly frozen at −70°C for glutamate measurement.

Recording RSNA. The left kidney was exposed through a retroperitoneal flank incision. A branch of the renal nerve was isolated from the fat and connective tissue. The nerve was placed on a pair of thin bipolar platinum electrodes. The nerve-electrode junction was insulated electrically from the surrounding tissue with a silicone gel (Wacker Sil-Gel 604 A B). The electrical signal was amplified (10,000 times) with an amplifier (model P55, Grass) with high- and low-frequency cutoffs of 1,000 and 100 Hz, respectively. The output signal from the amplifier was directed to a computer-run data acquisition system (PowerLab) for recording and integration of the raw nerve discharge. The signal recorded at the end of the experiment (after the rat was dead) was deemed background noise. During the experiment, the value of the nerve activity was calculated by subtraction of background noise from the actual recorded value. The basal value of the nerve activity was defined by subtraction of background noise from the actual nerve activity before the administration of drugs into the PVN. The peak response of RSNA to the administration of drugs into the PVN during the experiment (averaged over 20–30 s) was subsequently expressed as percent change from baseline.

Measurement of glutamate and GABA release in perfusates from the PVN. Perfusate samples were subjected to measurement using an HPLC system consisting of a chromatographic pump (model PM-80, BAS, West Lafayette, IN), an electrochemical detector (model LC-44, BAS), and a column oven (model CTO-10Avp, Shimadzu, Kyoto, Japan). A 3-mM octadecylsilyl reverse-phase column (3.2 × 100 mm; Phase II, BAS) was used to separate glutamate and GABA from the perfusate. A guard column (model 1602, Upchurch Scientific, Oak Harbor, WA) was fitted upstream of the analytical column. The mobile phase [36% 0.1 M potassium phosphate (pH 7.0), 22% 0.1 M sodium acetate (pH 4.0), 42% acetonitrile, and 100 mg of sodium EDTA per liter of solution, with final pH 4.3] was filtered through a 0.22-mm filter (Millipore) and degassed before use. The flow rate was 0.5 ml/min. Twenty milliliters of perfusate were mixed with 5 ml of 75 mM aminovaleric acid as an internal standard, and 35 ml of derivatization reagent [16.75 mg of *o*-phthaldialdehyde, 12.5 ml of methanol, 14 ml of 2-methyl-2-propanethiol, 7.5 ml of double-distilled H_2O, and 3.75 ml of 1.0 M carbonated buffer, filtered through a 0.22-mm hydrophilic membrane filter (ALADN)]. A glassy carbon electrode set at a potential of 0.75 V was used for electrochemical detection of glutamate. Output data from the detector were integrated via a PowerChrom chromatograph data system (ADInstruments). The standard curve of glutamate was obtained from a series of freshly made L-glutamate solutions (0, 6.5, 13, 26, and 52 pmol) before measurement. The correlation between the above-mentioned concentrations and the integrated height of their peaks was linear, with correlation coefficients >0.99.

In Vitro Experiments

Male Wistar rats (250–350 g body wt; Harlan Laboratories, Indianapolis, IN) were housed in a 12:12-h light-dark cycle and given free access to food and water.

Retrograde labeling of PVN-RVLM projecting neurons. PVN-RVLM projecting neurons were retrogradely labeled as previously described (16). Briefly, rats were anesthetized by intraperitoneal injection of ketamine-xylazine (90 and 5 mg/kg, respectively), and the head of the rat was placed in a stereotaxic frame. A 4-mm burr hole was made in the skull, and rhodamine-labeled microspheres (Lumaflor, Naples, FL) were injected under pressure unilaterally (200 nl) into the RVLM as follows: bregma −11.96, lateral 2.0, and ventral to dura 8.0. The animals were allowed to recover, and 5–7 days after surgery they were killed and the brain was dissected for electrophysiological experiments. The location and extension of the injection sites were confirmed histologically, as previously described (16).

Slice preparation. Coronal hypothalamic slices (300 μm) containing the PVN were obtained using a Vibroslicer (DSK Microslicer, Ted Pella, Redding, CA), as previously described (29). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and perfused through the heart with an ice-cold standard aCSF solution (see below) in which NaCl was replaced by an equiosmolar amount of sucrose, a procedure known to improve the viability of neurons in adult brain slices. The standard aCSF solution contained (in mM) 120 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, 20 glucose, and 0.4 ascorbic acid (pH 7.4, 297–300 mosM). The hypothalamic slices were placed in a holding chamber containing standard aCSF solution and stored at room temperature (22–24°C). For recordings, a slice was transferred to a submersion-type recording chamber and continuously perfused (~2 ml/min) with a standard solution bubbled with 95% O_2-5% CO_2. All recordings were performed at 30–32°C.

Electrophysiology and data analysis. Patch pipettes (4–8 MΩ) were pulled from thin-walled (1.5 mm OD, 1.17 mm ID) borosilicate glass (model GC150T-7.5, Clark, Reading, UK) on a horizontal electrode puller (model P-97, Sutter Instruments, Novato, CA). The pipette internal solution contained (in mM) 140 cesium methanesulfonate, 10 HEPES, 5 EGTA, and 1 $CaCl_2$; pH was slowly titrated to 7.25–7.3. Patch recordings were obtained from retrogradely labeled PVN neurons under visual control with an upright microscope (Axioscop, Zeiss), as previously described (12). Electrical recordings were obtained using a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA). The voltage output was digitized at 16-bit resolution in conjunction with pClamp 8 software (Digidata 1320, Axon Instruments). Data were digitized at 10 kHz and transferred to a disk. The series resistance was monitored throughout the experiment, and data were discarded if series resistance during recordings doubled from that obtained at the beginning of the recording.

Spontaneous GABA inhibitory postsynaptic currents (sIPSCs) and glutamate excitatory postsynaptic currents (sEPSCs) were simultaneously recorded at a holding potential of −50 mV. At this membrane potential and on the basis of their different reversal potentials, IPSCs and EPSCs were distinguished by their outward and inward polarities, respectively (see Fig. 8). Synaptic events were detected using Mini Analysis (Synaptosoft, Leonia, NJ) or Axograph (Axon Instruments) software. The detection threshold was set to four times the baseline noise level (root mean square). Individual EPSCs were aligned at the 50% crossing of the rising phase before averaging. EPSC frequency and amplitude were analyzed using the same software. To study the effect of bicuculline on sEPSC frequency and properties, we analyzed events in 6-min periods before and after bath application of the drug. Results were analyzed and compared using a paired *t*-test.

Experimental Protocols

Experiment 1: excitatory effect of NMDA receptor stimulation in the PVN on sympathetic outflow. First, we determined the effect of NMDA receptor stimulation in the PVN on sympathetic outflow. In the first group of rats ($n = 6$), NMDA (50, 100, and 200 pmol in 50, 100, and 200 nl of 1 mM NMDA in aCSF, respectively) was injected into the PVN at 30-min intervals. In the second group ($n = 5$), glutamate (50, 100, and 200 pmol in 50, 100, and 200 nl of 1 mM glutamate in aCSF, respectively) was injected into the PVN at 30-min intervals. In the control group ($n = 6$), 50, 100, and 200 nl of aCSF were injected into the PVN at 30-min intervals.

Second, we determined the effect of blocking NMDA receptors in the PVN on the glutamate-induced excitatory effect on sympathetic outflow. In the first group ($n = 5$), glutamate (50, 100, and 200 pmol in 50, 100, and 200 nl of 1 mM glutamate in aCSF, respectively) was injected into the PVN at 30-min intervals. In the second group ($n = 4$), each dose of glutamate was administered 2 min after injection of 8 nmol of the glutamate antagonist DL-2-amino-5-phosphonovaleric acid (AP-5; in 50 nl of 0.16 M AP-5 in aCSF) into the PVN.

Third, we determined the effect of blocking NMDA receptors with AP-5 in the PVN on RSNA, BP, and HR. In the first group of rats ($n = 6$), the NMDA antagonist AP-5 (2, 4, and 8 nmol in 50, 100, and 200 nl of aCSF, respectively) was injected into the PVN at 30-min intervals. In the second group of rats ($n = 6$), 50, 100, and 200 nl of aCSF were injected into the PVN at 30-min intervals.

Experiment 2: effect of $GABA_A$ receptor blockade during NMDA receptor blockade in the PVN on RSNA, BP, and HR. In the first group of rats ($n = 6$), the NMDA antagonist AP-5 (2, 4, and 8 nmol of AP-5 in 50, 100, and 200 nl of aCSF, respectively) was injected into the PVN at 30- to 45-min intervals. In the second group ($n = 5$), the GABA antagonist bicuculline (100 pmol in 100 nl of 1 mM bicuculline in aCSF) was injected into the PVN. In the third group ($n = 6$), bicuculline was injected 2 min after injection of 8 nmol of AP-5 (50 nl of 0.16 M AP-5 in aCSF) into the PVN. In the fourth group ($n = 5$), another more specific $GABA_A$ antagonist, gabazine (50 pmol in 100 nl of 0.5 mM gabazine in aCSF), was injected into the PVN. At 1 h after the parameters had returned to normal values, 2 min after injection of 8 nmol of AP-5 (50 nl of 0.16 M AP-5 in aCSF) into the PVN, gabazine was injected (50 pmol). As shown above, these volumes of aCSF injections into the PVN did not significantly change RSNA, BP, or HR. The RSNA, BP, and HR responses were recorded after each application.

Second, we determined the effect of non-NMDA receptor blockade with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 2 nmol) in the PVN on RSNA, BP, and HR responses to bicuculline. In one group of rats ($n = 6$), bicuculline was injected 2 min after injection of 2 nmol (50 nl of 0.16 M CNQX in aCSF) of CNQX into the PVN. In a second group of rats ($n = 6$), glutamate (100 pmol)-induced changes in RSNA, BP, and HR were recorded before and after administration of the non-NMDA antagonist CNQX (2 nmol in 50 nl of aCSF).

Experiment 3: effect of microinjection of the $GABA_A$ receptor antagonist bicuculline into the PVN on glutamate release. After two 20-min baseline periods, bicuculline (50, 100, and 200 pmol in 50, 100, and 200 nl of 1 mM of bicuculline in aCSF, respectively) was injected into the PVN, and the rats ($n = 12$) were allowed three 20-min recovery periods. The push-pull technique was used to collect the perfusates from the PVN over each 20-min period. Glutamate concentration in the perfusates was measured as described above.

Experiment 4: effect of the $GABA_A$ receptor antagonist bicuculline on glutamatergic synaptic activity in PVN-RVLM projecting neurons. Spontaneous GABAergic and glutamatergic synaptic activities were recorded simultaneously from retrogradely labeled PVN-RVLM projecting neurons ($n = 10$ from 4 rats) in a hypothalamic slice preparation. The effects of bicuculline on the frequency, amplitude, and decay time constant of glutamate sEPSCs were assessed as described above.

Brain Histology

At the end of each experiment, 200 nl of pontamine blue dye solution (3% in 0.4 M sodium phosphate solution) were injected into the PVN to mark the microinjection/perfusion site. After the experiment, the rat was killed and the brain was removed and fixed in 10% formalin for ≥24 h. The brain was then frozen, and serial transverse sections (30 μm) were cut with a cryostat (−18°C). The sections were mounted on microscope slides and then stained using 1% neutral red. The location of the injection in the PVN was then verified under a microscope with ×40 magnification. Figure 1 illustrates the histological data at the termination of the microinjection and push-pull perfusion into the PVN. Those microinjections with termination in the boundaries of the PVN were considered to be effective injections. For purposes of analysis, only the animals in which dye deposits were located within, or <0.5 mm from, the boundaries of the PVN were considered to be histologically targeted. The 50- to 200-nl injection volumes targeting the PVN would be expected to distribute the drug in or within <0.5 mm from the rostrocaudal and mediolateral boundaries of the PVN (27).

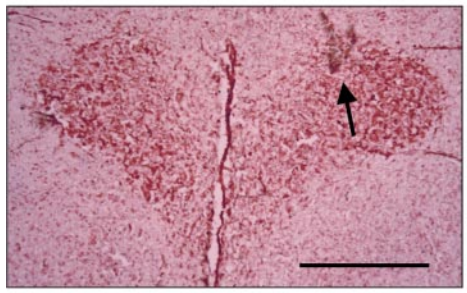
Data Analysis

Responses of RSNA to the various doses of drugs are expressed as percent change from baseline. Responses of arterial BP, HR, and glutamate level in the PVN are expressed as the difference between the basal value and the value after each dose of the drug. The data were statistically analyzed using two-way ANOVA followed by a Student-Newman-Keuls test. Changes in the frequency of EPSCs before and during application of bicuculline were analyzed using a paired *t*-test. $P < 0.05$ was considered to indicate statistical significance. Values are means ± SE.

RESULTS

Experiment 1: Effect of Stimulating NMDA Receptors in the PVN on RSNA, BP, and HR

Microinjection of glutamate or NMDA induced increases in RSNA, BP, and HR. An example of the responses to different doses of NMDA is illustrated in Fig. 2*A*. Figure 2*B* shows the group data of the responses to glutamate and NMDA. Microinjection of glutamate (50, 100, and 200 pmol) induced significant increases in RSNA, BP, and HR in a dose-dependent manner, reaching 47 ± 7%, 13 ± 3 mmHg, and 20 ± 4 beats/min, respectively, at the highest dose. Similarly, microinjection of NMDA (50, 100, and 200 pmol) induced significant increases in RSNA, BP, and HR in a dose-dependent manner, reaching 51 ± 6%, 17 ± 2 mmHg, and 22 ± 3 beats/min, respectively, at the highest dose (Fig. 2*B*). Previous work from our laboratory showed that RSNA, BP, and HR



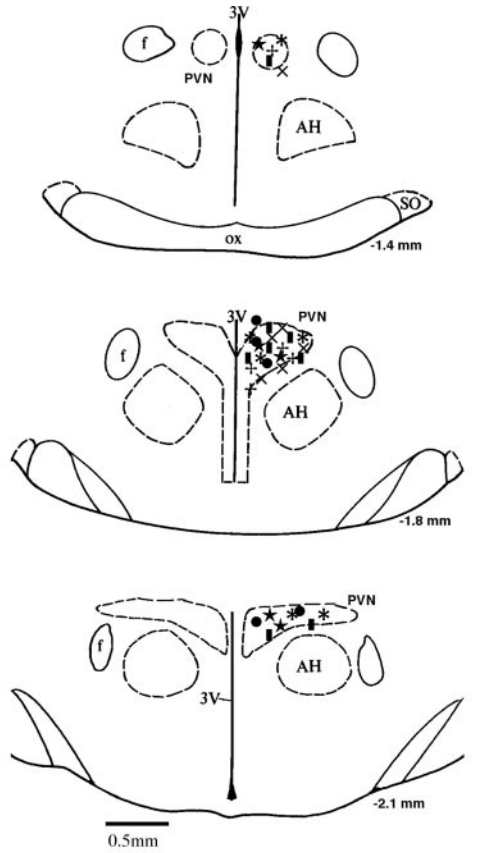


Fig. 1. Schematic representation of injection sites in serial sections from the rostral (−1.4) to the caudal (−2.12) extent of the region of the paraventricular nucleus (PVN). Distance posterior to bregma is shown for each section according to the atlas of Paxinos and Watson (24). Microinjections with *N*-methyl-D-aspartate (NMDA, $n = 6$), bicuculline (Bic) and bicuculline + DL-2-amino-5-phosphonovaleric acid (AP-5; $n = 6$), glutamate (Glu) and glutamate + AP-5 ($n = 5$), artificial cerebrospinal fluid (aCSF) push-pull ($n = 8$), bicuculline and bicuculline + 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; $n = 6$), and gabazine and gabazine + AP-5 ($n = 5$) within the PVN region were confirmed (total of 36 microinjections). AH, anterior hypothalamic nucleus; f, fornix; 3V, 3rd ventricle; SO, supraoptic nucleus. *Inset*: representative photomicrograph of injection into the PVN of a rat. Arrow, termination of microinjection within the PVN.

responses to NMDA are not due to peripheral action (19). Efficacy of NMDA receptor blockade was tested by recording RSNA, BP, and HR responses to microinjections of L-glutamate (100 pmol) before and after microinjection of AP-5 (8 nmol) into the PVN. A dose of 8 nmol of AP-5 significantly diminished the glutamate-induced increases in RSNA, BP, and HR (Fig. 3*A*).

However, as shown in Fig. 3*B*, administration of AP-5 alone induced minimal decreases in RSNA, BP, and HR, reaching $-2 \pm 2\%$, -4 ± 1 mmHg, and -4 ± 2 beats/min, respectively, at the highest dose.

Experiment 2: Effect of Bicuculline During Blockade of NMDA and Non-NMDA Receptors in the PVN on Changes in RSNA, BP, and HR

Microinjection of bicuculline into the PVN induced significant increases in RSNA, BP, and HR. These responses were significantly attenuated by the prior injection of AP-5 into the PVN. RSNA, BP, and HR responses to bicuculline or bicuculline + AP-5 are illustrated in Fig. 4. As shown in Fig. 5*A*, RSNA, BP, and HR responses to the highest dose of bicuculline reach $68 \pm 7\%$, 14 ± 3 mmHg, and 36 ± 6 beats/min, respectively. However, with prior blockade of NMDA receptors with AP-5, RSNA, BP, and HR responses to bicuculline were significantly attenuated, reaching only $10 \pm 2\%$, 5 ± 1 mmHg, and 12 ± 2 beats/min, respectively. Another $GABA_A$ receptor blocker, gabazine, which does not affect the large-conductance K^+ channels, also increased RSNA, BP, and HR. This effect of gabazine was also significantly blunted by prior application of AP-5 (Fig. 5*B*).

The role of non-NMDA receptors in the response to bicuculline injections in the PVN was examined by testing responses before and after administration of the non-NMDA receptor antagonist CNQX into the PVN. Blockade of non-NMDA receptors with CNQX also blunted the responses to bicuculline; the responses of RSNA and BP to bicuculline were significantly attenuated, reaching only $44 \pm 5\%$ and 7 ± 1 mmHg, respectively (Fig. 6*A*). The effect was qualitatively similar to that of AP-5, an NMDA receptor antagonist, but quantitatively much smaller than the effect of AP-5. There was no effect on HR. Efficacy of non-NMDA receptor blockade was also tested with injections of glutamate before and after microinjection of CNQX into the PVN. CNQX significantly reduced RSNA, BP, and HR responses to glutamate (Fig. 7). Although qualitatively the responses to glutamate blockade with CNQX were similar to responses to blockade with AP-5 (Fig. 3*A*), again the magnitude of the blockade was substantially smaller (Fig. 6*B*).

Experiment 3: Effect of Microinjection of the $GABA_A$ Receptor Antagonist Bicuculline Into the PVN on Changes in Glutamate Release

Figure 7 shows that microinjection of bicuculline into the PVN induces a dose-dependent increase in the glutamate levels

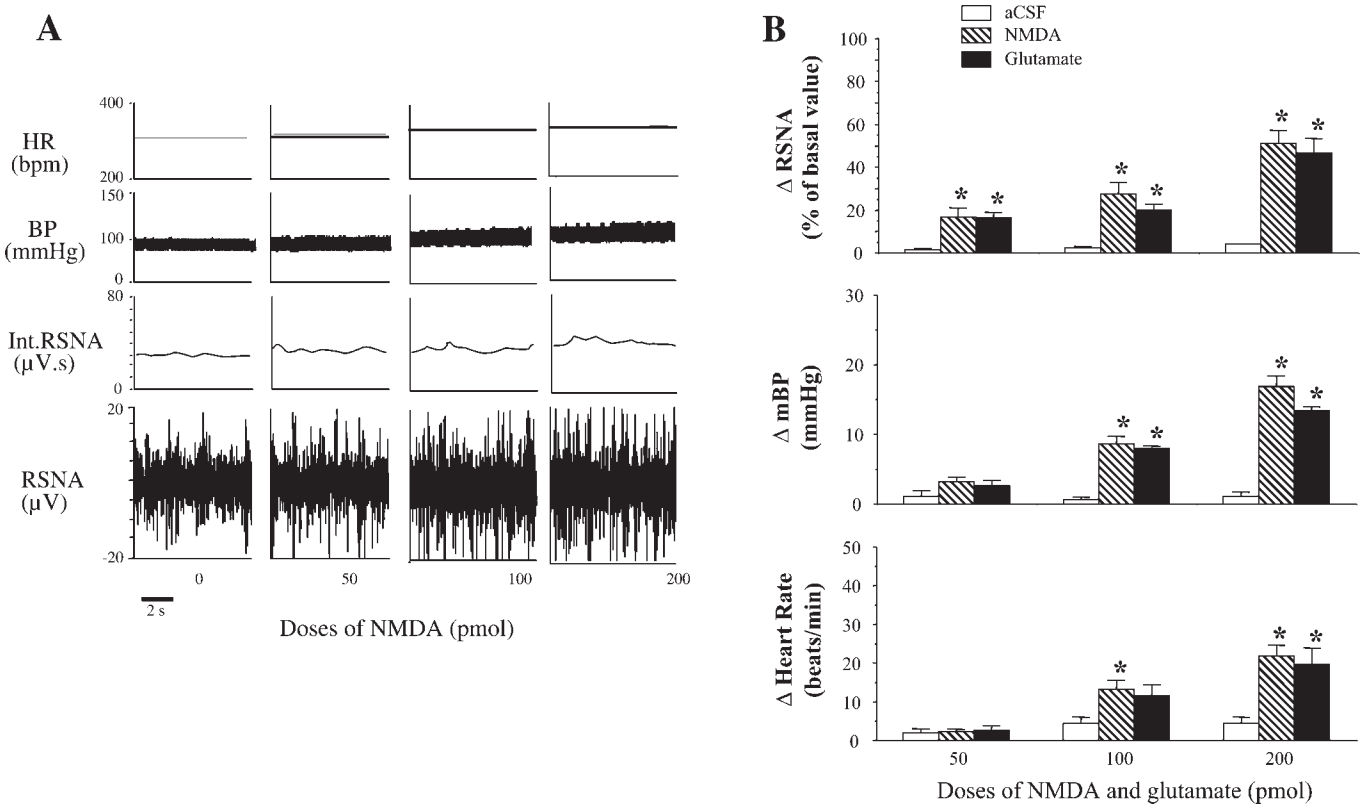


Fig. 2. *A*: segments of original recordings from individual rats demonstrating heart rate (HR), blood pressure (BP), renal sympathetic nerve activity (RSNA), and integrated (int) RSNA responses to increasing microinjected doses of NMDA (*top traces*) and bicuculline (*bottom traces*) into the PVN. *B*: RSNA, mean BP (mBP), and HR responses to microinjection of NMDA ($n = 6$), glutamate ($n = 5$), and aCSF ($n = 6$) into the PVN. Values are means ± SE. *$P < 0.05$ vs. aCSF.

in the PVN, resulting in a significantly increased concentration at the highest doses. The levels of glutamate returned to basal levels 1 h after the end of bicuculline administration. Locations of the probes within the PVN were identified by a dye injection within the PVN at the end of the experiment, as illustrated by Fig. 1.

Experiment 4: Effect of the $GABA_A$ Receptor Antagonist Bicuculline on Glutamatergic Synaptic Activity in PVN-RVLM Projecting Neurons

Figure 8 shows a representative example of GABAergic IPSCs and glutamatergic EPSCs recorded simultaneously from a retrogradely labeled PVN-RVLM projecting neuron. In addition to blocking IPSCs, bath application of bicuculline increased the frequency of EPSCs. These EPSCs were subsequently blocked by 3-hydroxy-5-methyl-4-isoxazolepropionic acid and the NMDA receptor antagonists CNQX (10 μM) and AP-5 (100 μM), which, in the presence of bicuculline, blocked all remaining spontaneous synaptic events. The group data obtained from 10 PVN-RVLM projecting neurons are summarized in Fig. 8*C*. Blockade of $GABA_A$ receptors by bicuculline significantly increased (~70%, $P < 0.001$) the mean frequency of glutamate-mediated EPSCs. On the other hand, neither the amplitude nor the decay kinetics of EPSCs were affected ($P > 0.05$ for both variables). These changes were observed in each of the tested neurons. Because of the prolonged nature of these studies, we were unable to obtain data after washout of bicuculline or CNQX + AP-5 while maintaining high-quality recording conditions. However, to rule out a potential time-dependent spontaneous increase in the frequency of EPSCs, a separate set of experiments was performed ($n = 5$) in which the frequency of spontaneous EPSCs, in the absence of bicuculline, was measured during a similar time period. In these experiments, no changes in the frequency of EPSCs were found when values obtained at 200 s were compared with values obtained at 800s (around the peak of the bicuculline-induced changes; −4.2 ± 1.2% change, $P > 0.5$, paired *t*-test). These results indicate that the basal frequency of EPSCs in our recording condition is highly stable.

DISCUSSION

In the present study, we observed that microinjection of glutamate and NMDA into the PVN induces increases in RSNA, BP, and HR. These increases are greatly attenuated by administration of the glutamate antagonist AP-5 into the PVN, suggesting that NMDA receptors in the PVN play a key role in mediating glutamatergic sympathoexcitation. However, microinjection of AP-5 alone into the PVN has little effect on RSNA, BP, and HR, suggesting that NMDA receptors mediate little tonic glutamatergic-mediated sympathetic excitation. This result was unexpected, because the PVN is known to express a variety of functional glutamate receptors and receives a substantial glutamatergic innervation, which, as shown in this study, tonically influences the activity of sympathetic-related PVN.

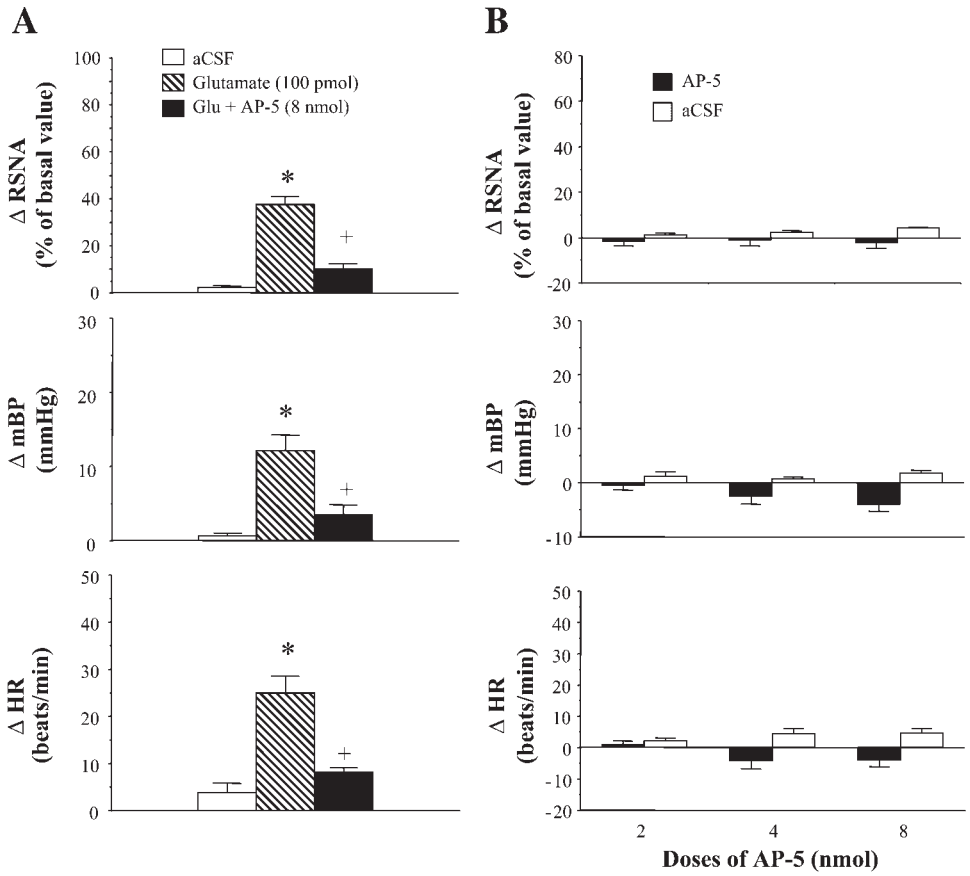


Fig. 3. *A*: RSNA, BP, and HR responses to microinjection of glutamate (100 pmol, $n = 6$), glutamate + AP-5 (8 nmol, $n = 4$), and aCSF ($n = 5$) into the PVN. Values are means ± SE. $^{*}P < 0.05$ vs. aCSF. $^{+}P < 0.05$ vs. glutamate. *B*: RSNA, BP, and HR responses to microinjection of AP-5 ($n = 6$) and aCSF ($n = 6$) into the PVN. Values are means ± SE. $^{*}P < 0.05$ vs. aCSF.

Thus possible explanations for the lack of an evident glutamate-mediated tonic sympathoexcitation are as follows: *1*) endogenous glutamatergic synaptic inputs are tonically inhibited, and/or *2*) glutamate-mediated excitation is offset by postsynaptic inhibition of the PVN sympathoexcitatory neurons. In either of these scenarios, withdrawal of the inhibition would cause a release of glutamate-mediated sympathetic excitation. As a major inhibitory neurotransmitter widely distributed in the central nervous system, GABA is also a predominant inhibitory neurotransmitter within the PVN (9, 22). Thus we tested the possibility that glutamate actions within the PVN are tonically inhibited by a GABAergic mechanism. We observed that administration of the $GABA_A$ receptor antagonist bicuculline or a more specific $GABA_A$ receptor antagonist, i.e., gabazine, into the PVN induced dramatic increases in RSNA, BP, and HR. These responses were significantly blunted by blockade of NMDA receptors in the PVN, indicating that an intact NMDA-mediated glutamatergic input is required for $GABA_A$ receptor blockade-mediated sympathoexcitation. Observation of this effect concomitantly with enhanced levels of glutamate within the PVN, as well as an increase in the frequency of glutamate-mediated EPSCs in PVN-RVLM projecting neurons, suggests that GABA, acting via $GABA_A$ receptors, exerts a tonic inhibition on NMDA receptor-mediated glutamatergic sympathoexcitation. These results are consistent with a previous observation that the nonselective iono-

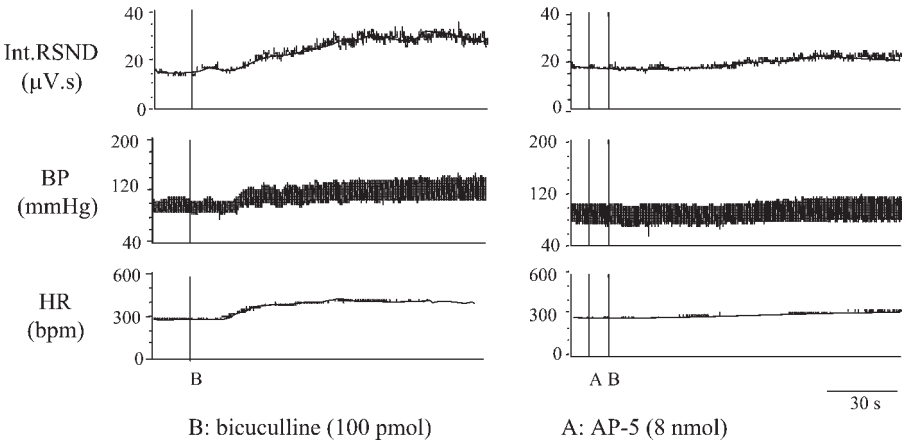


Fig. 4. Segments of original recordings from individual rats demonstrating HR, arterial BP, RSNA, and integrated renal sympathetic nerve discharge (RSND) responses to microinjection of bicuculline (100 pmol) and bicuculline + AP-5 (8 nmol) into the PVN.

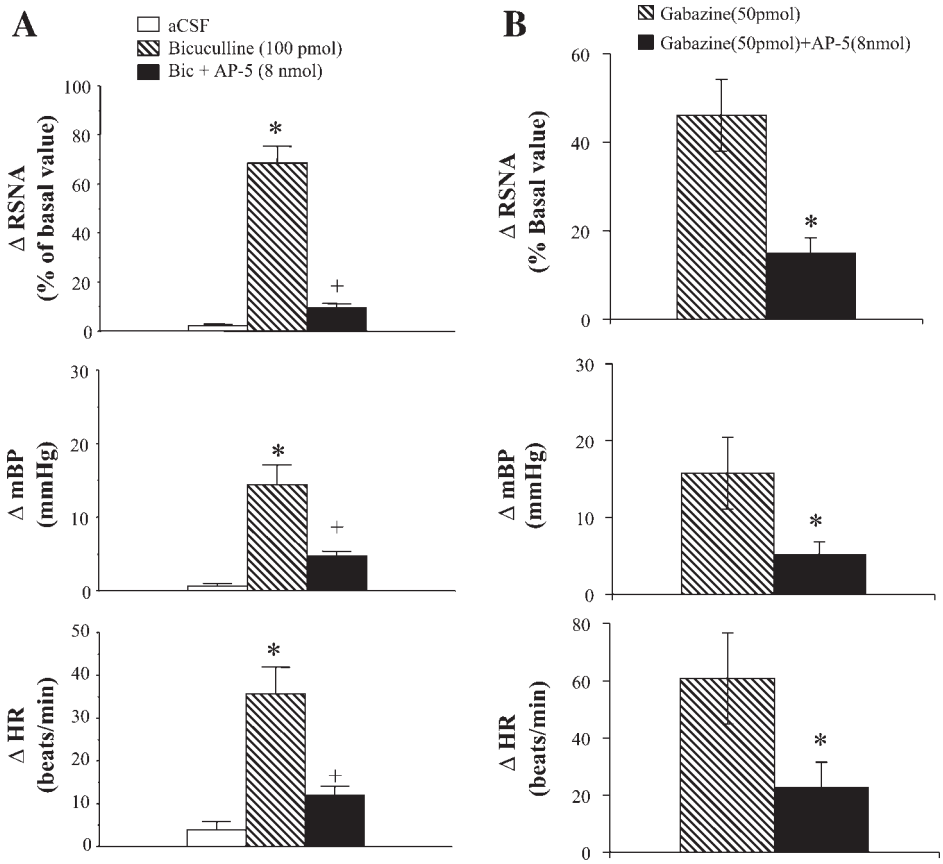


Fig. 5. *A*: RSNA, BP, and HR responses to microinjection of bicuculline (100 pmol, $n = 5$), bicuculline + AP-5 (8 nmol, $n = 6$), and aCSF ($n = 5$) into the PVN. Values are means ± SE. $^{*}P < 0.05$ vs. aCSF. $^{+}P < 0.05$ vs. bicuculline. *B*: RSNA, BP, and HR responses to microinjection of gabazine (50 pmol, $n = 5$) and gabazine + AP-5 (8 nmol, $n = 5$) into the PVN. Values are means ± SE. $^{*}P < 0.05$ vs. gabazine.

tropic excitatory amino acid antagonist kynurate was able to block the renal sympathoexcitation mediated by GABA blockade within the PVN (6).

Because NMDA and non-NMDA receptors are expressed in the PVN (3, 12, 13, 36) and nonselective ionotropic kynurate was able to block the effects of bicuculline, we also examined the effect of CNQX, a non-NMDA receptor antagonist. The results were qualitatively similar to those of AP-5 administration, demonstrating a blunting of the response to bicuculline and confirming the results previously shown with 2,3-dihyroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline, another non-NMDA receptor antagonist (6). However, the responses to blockade with AP-5 were much greater than those to blockade with CNQX. These results suggest that perhaps the non-NMDA receptors do not contribute as much as the NMDA receptors to the sympathetic responses evoked by microinjection of bicuculline in the PVN. Furthermore, CNQX was not able to block HR responses to bicuculline, but AP-5 was effective in blocking this HR response. These data suggest that HR may be specifically regulated by $GABA_A$ receptors in the PVN.

According to histological data, the dye was distributed within the PVN, suggesting that the effects of the drug administrations were due to actions within the PVN. It is recognized that although the spread of the dye and bicuculline coincide, they might not be identical. Our previous work and work by others (27) have validated this technique of microinjection and appropriate controls in terms of, for example, adjacent nonactive sites, vehicle controls, and leakage into the peripheral circulation (38). The vehicle controls in this study also suggest that any response or the absence of a response to microinjection of drugs into the PVN was not due to mechanical damage of the PVN by cannula placement or microinjection volumes.

The mechanism of the inhibitory action of GABA on the glutamatergic system remains to be fully elucidated. This action may occur at presynaptic and/or postsynaptic levels (Fig. 8). Our findings showing a bicuculline-induced increase in glutamate levels in the PVN, along with an increased frequency of glutamate-mediated EPSCs (without changes in EPSC waveform) in PVN-RVLM projecting neurons, support an enhanced release of glutamate, rather than changes in the properties and/or efficacy of postsynaptic glutamate receptors. Thus, along with a well-characterized $GABA_B$-mediated presynaptic inhibition of glutamate synaptic activity within the PVN (8, 37), our present results suggest that $GABA_A$ receptors located in glutamatergic neurons and/or terminals also contribute to a tonic, GABA-mediated restraint of excitatory inputs to PVN neurons.

GABAergic inputs are also known to inhibit in a direct manner PVN sympathoexcitatory neurons (15, 16). Thus an alternative mechanism to be considered in interpretation of the present results includes postsynaptic interactions between GABA and glutamate in the control of PVN neuronal membrane potential. In this sense, and similar to other neuronal types, resting membrane potential in PVN sympathoexcitatory neurons is likely to be dependent on the balance between glutamate excitatory and GABA inhibitory inputs. The fact that both RVLM and spinal cord projecting PVN neurons are quiescent under basal conditions supports an overall predominance of inhibitory over excitatory effects (2, 20). Still,

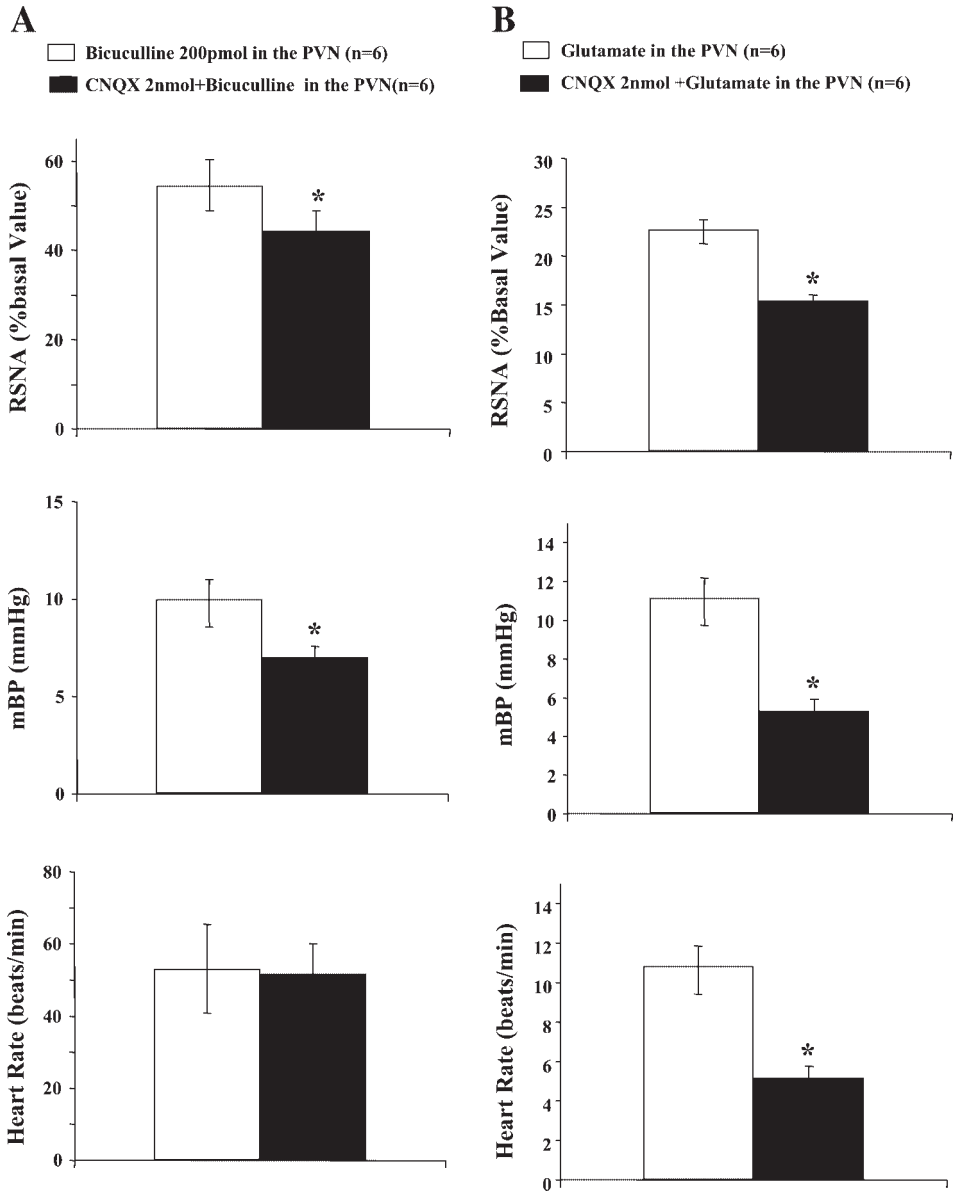


Fig. 6. *A*: RSNA, BP, and HR responses to microinjection of bicuculline (100 pmol, $n = 6$) and bicuculline + CNQX (2 nmol) into the PVN. *B*: RSNA, BP, and HR responses to microinjection of glutamate (100 pmol, $n = 6$) and glutamate + CNQX (2 nmol) into the PVN. Values are means ± SE. $*P < 0.05$ vs. aCSF.

excitatory inputs may help maintain resting membrane potential relatively close to Na^+ action potential threshold, such that blockade of a direct $GABA_A$-mediated inhibition (along with the enhanced release of glutamate) induces a depolarization that is large enough to induce firing discharge and sympathoexcitatory output from the PVN. In this scenario, blockade of glutamate excitatory inputs will result in membrane hyperpolarization, increasing in turn the voltage deflection needed to reach action potential threshold by a subsequent depolarizing event. Thus blockade of a direct $GABA_A$-mediated inhibition in this case may result in a depolarization that is not large enough to reach action potential threshold. In summary, it is likely that GABA, acting via $GABA_A$ receptors, inhibits PVN sympathoexcitatory neurons through direct and indirect (glutamate-mediated) mechanisms.

Tight interactions between GABA inhibitory and glutamate excitatory actions may also be involved in other neurosecretory functions in the PVN. For example, Cole and Sawchenko (7) observed that glutamate microinjection into the PVN elicited a surprisingly weak effect on plasma corticosterone levels. They further observed that administration of glutamate increased c-Fos expression in GABAergic neurons, suggesting that glutamate action might be inhibited by evoked GABAergic action. These findings indicate that bidirectional interactions between GABA and glutamate inputs may be a common counterbalance mechanism within the PVN. The importance of a balance between excitatory and inhibitory inputs in the integration of sympathetic function within the PVN is also underscored by clinical conditions involving altered sympathohumoral drive. Studies have shown that a deficiency or hypofunction of GABAergic mechanisms in the PVN may be related to the pathogenesis of a variety of clinical conditions characterized by altered sympathetic nerve activity, such as hypertension (10, 11) and heart failure (HF) (38). A dysfunction in the GABAergic inhibitory mechanism has been implicated in hypertension, which is characterized by increased sympathetic nerve activity.

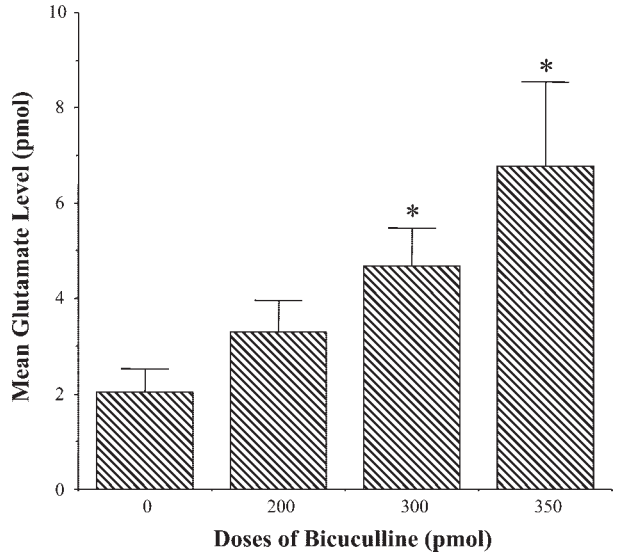


Fig. 7. Effects of microinjection of bicuculline into the PVN on glutamate levels in perfusates from the PVN ($n = 8$). Values are means ± SE. *$P < 0.05$ vs. basal value.

Hambley et al. (10) reported significant reductions in endogenous hypothalamic GABA concentrations and in density of muscimol binding to hypothalamic membranes, which is an indication of a reduced number of $GABA_A$ receptors in spontaneously hypertensive rats compared with Wistar-Kyoto rats. Because GABA is a dominant inhibitory neurotransmitter in the hypothalamus, it was hypothesized that a decreased inhibition of GABA on sympathetic outflow contributes to the pathogenesis of hypertension (28, 35).

In a recent study, we observed that microinjection of bicuculline into the PVN induced an attenuated increase in RSNA and arterial blood pressure in rats with HF compared with sham-operated control rats, suggesting that the endogenous GABA-mediated input into the PVN is less effective in suppressing sympathetic outflow in rats with HF than in sham-operated control rats (38). This altered GABAergic mechanism may contribute to the elevated sympathetic activity in HF (38). Whether a glutamatergic mechanism is involved in all these dysfunctions of the GABAergic system remains to be studied. Indeed, elevated glutamatergic actions have also been observed to be involved in some physiological and pathophysiological processes. In our recent study, we found that NMDA-mediated sympathetic outflow in the PVN was increased in HF due to NMDA receptor upregulation (18). According to the observations in our present study, we can further postulate that the imbalance between GABAergic inhibitory and glutamatergic excitatory mechanisms may play a key role in the sympathoexcitation dysfunction observed in pathophysiological processes such as hypertension and HF. However, more direct evidence is needed to show a link between these two altered mechanisms within the PVN in HF. Obviously, further investigations on the interaction of inhibitory GABAergic and excitatory glutamatergic actions in the PVN will further our understanding of the central integrating processes of sympathetic regulation and could lead to new therapeutic strategies for the disorders characterized by sympathetic outflow dysfunction, such as hypertension, HF, and diabetes.

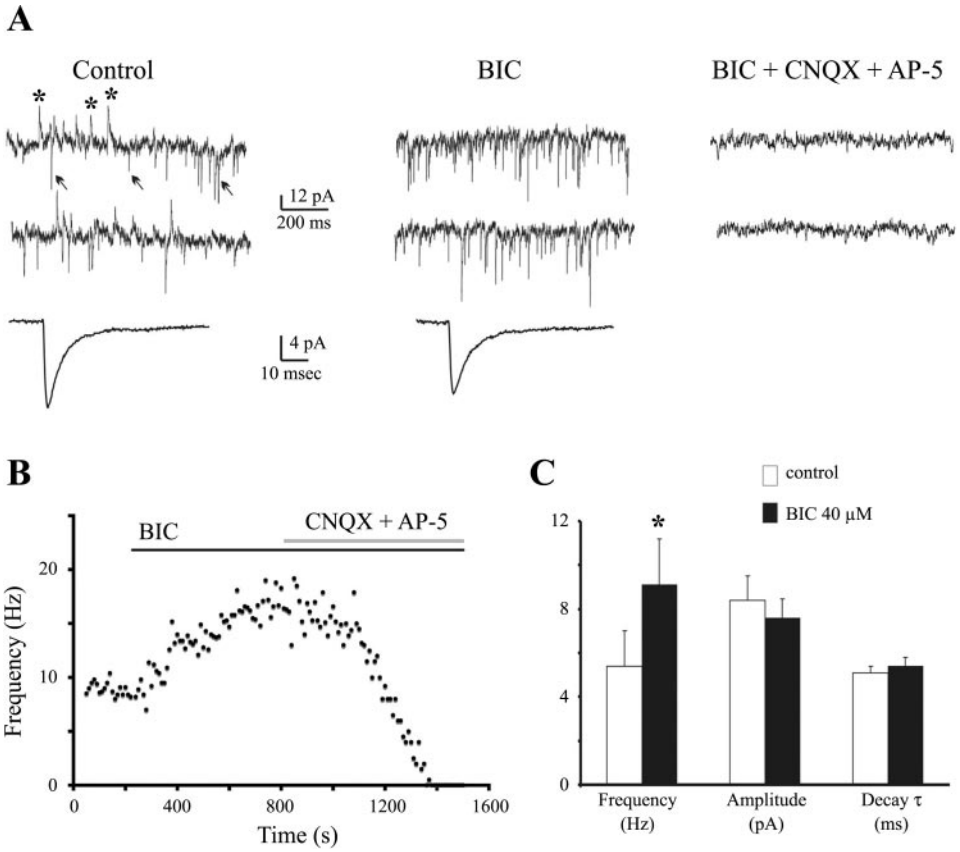


Fig. 8. Blockade of $GABA_A$ receptors increases frequency of glutamate-mediated excitatory postsynaptic currents (EPSCs) in PVN-RVLM projecting neurons. *A*: representative recordings of inhibitory postsynaptic currents (IPSCs) and EPSCs from a PVN-RVLM neuron in control aCSF, in the presence of the $GABA_A$ blocker bicuculline (BIC, 40 μM), and in the presence of BIC + 3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptor blockers CNQX (10 μM) and AP-5 (100 μM), respectively. Note presence of outward IPSCs (*) and inward EPSCs (arrows) in control. In the presence of BIC, IPSCs were blocked and frequency of EPSCs was increased; both were completely blocked by subsequent addition of CNQX and AP-5. *B* and *C*: averaged EPSC (>100 events) obtained in each condition. *B*: plot of mean frequency (10-s bin) of spontaneous EPSCs (sEPSCs) over time in a representative PVN-RVLM neuron before and during bath application of BIC (40 μM) and BIC + CNQX (10 μM) + AP-5 (100 μM). *C*: summary data obtained from 10 PVN-RVLM neurons. Values are means ± SE. *$P < 0.001$ vs. control.

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Parts of these studies have been presented in abstract form (17).

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REFERENCES

1. **Antonaccio MJ, Kerwin L, and Taylor DG.** Reductions in blood pressure, heart rate and renal sympathetic nerve discharge in cats after the central administration of muscimol, a GABA agonist. *Neuropharmacology* 17: 783–791, 1978.
2. **Bains JS and Ferguson AV.** Paraventricular nucleus neurons projecting to the spinal cord receive excitatory input from the subfornical organ. *Am J Physiol Regul Integr Comp Physiol* 268: R625–R633, 1995.
3. **Boudaba C, Szabo K, and Tasker JG.** Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus. *J Neurosci* 16: 7151–7160, 1996.
4. **Brennan TJ, Haywood JR, and Ticku MK.** GABA receptor binding and hemodynamic responses to ICV GABA in adult spontaneously hypertensive rats. *Life Sci* 33: 701–709, 1983.
5. **Butcher KS and Cethetto DF.** Receptors in lateral hypothalamic area involved in insular cortex sympathetic responses. *Am J Physiol Heart Circ Physiol* 275: H689–H696, 1998.
6. **Chen QH, Haywood JR, and Toney GM.** Sympathoexcitation by PVN-injected bicuculline requires activation of excitatory amino acid receptors. *Hypertension* 42: 725–731, 2003.
7. **Cole RL and Sawchenko PE.** Neurotransmitter regulation of cellular activation and neuropeptide gene expression in the paraventricular nucleus of the hypothalamus. *J Neurosci* 22: 959–969, 2002.
8. **Cui LN, Coderre E, and Renaud LP.** Glutamate and GABA mediate suprachiasmatic nucleus inputs to spinal-projecting paraventricular neurons. *Am J Physiol Regul Integr Comp Physiol* 281: R1283–R1289, 2001.
9. **Decavel C and Van den Pol AN.** GABA: a dominant neurotransmitter in the hypothalamus. *J Comp Neurol* 302: 1019–1037, 1990.
10. **Hambley JW, Johnston GAR, and Shaw J.** Alterations in a hypothalamic GABA system in the spontaneously hypertensive rat. *Neurochem Int* 6: 813–821, 1984.
11. **Haywood JR, Mifflin SW, Craig T, Calderon A, Hensler JG, and Hinojosa-Laborde C.** γ-Aminobutyric acid $(GABA)_A$ function and binding in the paraventricular nucleus of the hypothalamus in chronic renal-wrap hypertension. *Hypertension* 37 *Suppl*: 614–618, 2001.
12. **Herman JP, Eyigor O, Ziegler RD, and Jennes L.** Expression of ionotropic glutamate receptor subunit mRNAs in the hypothalamic paraventricular nucleus of the rat. *J Comp Neurol* 422: 352–362, 2000.
13. **Hermes MLHJ, Coderre EM, Buijs RM, and Renaud LP.** GABA and glutamate mediate rapid neurotransmission from suprachiasmatic nucleus to hypothalamic paraventricular nucleus in rat. *J Physiol* 496: 749–757, 1996.
14. **Kannan H, Hayashida Y, and Yamashita H.** Increase in sympathetic outflow by paraventricular nucleus stimulation in awake rats. *Am J Physiol Regul Integr Comp Physiol* 256: R1325–R1330, 1989.
15. **Li DP, Chen SR, Finnegan TF, and Pan HL.** Signalling pathway of nitric oxide in synaptic GABA release in the rat paraventricular nucleus. *J Physiol* 554: 100–110, 2004.
16. **Li Y, Zhang W, and Stern J.** Nitric oxide inhibits the firing activity of hypothalamic paraventricular neurons that innervate the medulla oblongata: role of GABA. *Neuroscience* 118: 585–601, 2003.
17. **Li YF, Rabeler B, and Patel KP.** Paraventricular nucleus-mediated sympathetic outflow: role of glutamate and GABA systems. In: *FASEB Summer Research Conferences-Neural Mechanisms in Cardiovascular Regulation*, edited by Chapleau M and Hay M. Bethesda, MD: FASEB, 2002.
18. **Li YF, Cornish KG, and Patel KP.** Alteration of NMDA NR1 receptors within the paraventricular nucleus of hypothalamus in rats with heart failure. *Circ Res* 93: 990–997, 2003.
19. **Li YF, Mayhan WG, and Patel KP.** NMDA-mediated increase in renal sympathetic nerve discharge within the PVN: role of nitric oxide. *Am J Physiol Heart Circ Physiol* 281: H2328–H2336, 2001.
20. **Lovick TA and Coote JH.** Electrophysiological properties of paraventriculo-spinal neurones in the rat. *Brain Res* 454: 123–130, 1988.
21. **Luiten PGM, ter Horst GJ, Karst H, and Steffens AB.** The course of paraventricular hypothalamic efferents to autonomic structures in medulla and spinal cord. *Brain Res* 329: 374–378, 1985.
22. **Martin DS, Segura T, and Haywood JR.** Cardiovascular responses to bicuculline in the paraventricular nucleus of the rat. *Hypertension* 18: 48–55, 1991.
23. **Miyawaki T, Minson J, Arnolda L, and Chalmers J.** Role of excitatory amino acid receptors in cardiorespiratory coupling in ventrolateral medulla. *Am J Physiol Regul Integr Comp Physiol* 271: R1221–R1230, 1996.
24. **Paxinos G and Watson C.** *The Rat Brain in Stereotaxic Coordinates*. Orlando, FL: Academic, 1986.
25. **Saper CB, Loewy AD, Swanson LW, and Cowan WM.** Direct hypothalamo-autonomic connections. *Brain Res* 117: 305–312, 1976.
26. **Schmidt B and DiMicco JA.** Blockade of GABA receptors in the paraventricular forebrain of anesthetized cats: effects on heart rate, arterial pressure and hindlimb vascular resistance. *Brain Res* 301: 111–119, 1984.
27. **Segura T, Martin DS, Sheridan PJ, and Haywood JR.** Measurement of the distribution of [^{3}H]bicuculline microinjected into the rat hypothalamus. *J Neurosci Methods* 41: 175–186, 1992.
28. **Shonis CA, Peano CA, Dillon GH, and Waldrop TG.** Cardiovascular responses to blockade of GABA synthesis in the hypothalamus of the spontaneously hypertensive rat. *Brain Res Bull* 31: 493–499, 1993.
29. **Stern JE.** Electrophysiological and morphological properties of pre-autonomic neurones in the rat hypothalamic paraventricular nucleus. *J Physiol* 537: 161–177, 2001.
30. **Strack AM, Sawyer WB, Platt KB, and Loewy AD.** CNS cell groups regulating the sympathetic outflow to the adrenal gland as revealed by transneuronal cell body labeling with pseudorabies virus. *Brain Res* 491: 274–296, 1989.
31. **Sun AY and Li DX.** Cardiovascular responses to intracerebroventricular injection of GABA in renovascular hypertensive rats. *Zhongguo Yao Li Xue Bao* 15: 136–138, 1994.
32. **Swanson LW and Kuypers HGJM.** The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of the projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. *J Comp Neurol* 194: 555–570, 1980.
33. **Swanson LW and Sawchenko PE.** Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. *Annu Rev Neurosci* 6: 269–324, 1983.
34. **Swanson LW and Sawchenko PE.** Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology* 31: 410–417, 1980.
35. **Takenaka K, Sasaki S, Uchida A, Fujita H, Nakamura K, Ichida T, Itoh H, Nakata T, Takeda K, and Nakagawa M.** $GABA_B$-ergic stimulation in hypothalamic pressor area induces larger sympathetic and cardiovascular depression in spontaneously hypertensive rats. *Am J Hypertens* 9: 964–972, 1996.
36. **Tasker JG, Boudaba C, and Schrader LA.** Local glutamatergic and GABAergic synaptic circuits and metabotropic glutamate receptors in the hypothalamic paraventricular and supraoptic nuclei. *Adv Exp Med Biol* 449: 117–121, 1998.
37. **Wang D, Cui LN, and Renaud LP.** Pre- and postsynaptic $GABA_B$ receptors modulate rapid neurotransmission from suprachiasmatic nucleus to parvocellular hypothalamic paraventricular nucleus neurons. *Neuroscience* 118: 49–58, 2003.
38. **Zhang K, Li YF, and Patel KP.** Reduced endogenous GABA-mediated inhibition in the PVN on renal nerve discharge in rats with heart failure. *Am J Physiol Regul Integr Comp Physiol* 282: R1006–R1015, 2002.