Alteration of NMDA NR$_1$ Receptors Within the Paraventricular Nucleus of Hypothalamus in Rats With Heart Failure

Yi-Fan Li, Kurtis G. Cornish, Kaushik P. Patel

Abstract—One of the pathophysiological characteristics of chronic heart failure (HF) is elevated sympathetic drive, which is a major factor contributing to the morbidity and mortality of HF. Recent evidence points to a central mechanism that contributes to the sympathetic abnormality in HF. The paraventricular nucleus (PVN) of the hypothalamus is an important site that integrates sympathetic nerve activity. Studies have shown that glutamate elicits excitatory effects on neurons in the PVN through the NMDA receptor. The goal of the present study was to examine the role of NMDA receptors in the altered sympathetic nerve activation during HF. The left coronary ligation-induced heart failure model in the rat was used. In α-chloralose and urethane anesthetized rats, microinjection of NMDA into the PVN (50 to 200 pmol) produced dose-dependent increases in renal sympathetic nerve discharge (RSND), arterial blood pressure (BP), and heart rate (HR). This response to NMDA was significantly potentiated (27±7%) in HF compared with sham rats. On the other hand, microinjection of the NMDA receptor antagonist AP-5 (4 to 16 nmol) into the PVN caused significant decreases in RSND, BP, and HR only in rats with HF but very slight changes in sham rats. Furthermore, using microdialysis and HPLC in combination with electrochemical detection techniques, we found that the glutamate level in the PVN was not increased significantly in HF compared with sham rats. However, using RT-PCR, Western blot, and immunofluorescence techniques, it was found that NMDA NR$_1$ subunit mRNA expression and protein level in the PVN were significantly increased in HF compared with sham rats. These data suggest that the increased glutamatergic activity on sympathetic regulation, due to the upregulation of NMDA NR$_1$ receptor subunits within the PVN may contribute to the elevated sympathoexcitation during HF. (Circ Res. 2003;93:811-816.)

Key Words: heart failure ◼ sympathetic nerve activity ◼ NMDA receptor ◼ paraventricular nucleus

It has been well established that increased sympathetic nerve activity is a characteristic symptom of congestive heart failure (CHF). 1 The elevated sympathetic activity induces vasoconstriction and an increase in peripheral resistance that increases cardiac afterload. In addition, peripheral venoconstriction and sodium and water retention leads to increased cardiac preload. Furthermore, continued elevation of cardiac sympathetic activation results in high levels of norepinephrine (NE), which contributes to myocardial toxicity and apoptosis. 2 This results in a decrease in myocardial contractility. All of these aggravate the morbidity and raise the mortality in this disorder. The accumulating studies on the peripheral sympathetic abnormality in HF have extended our understanding of its mechanisms. Blockade of sympathetic activity by using β-adrenoceptor antagonists have been an effective clinical therapy for HF. 3 However, peripheral blockade cannot completely eliminate the state of sympathoexcitation. Some studies have shown that various cardiovascular reflex functions are impaired in HF. Recent studies have suggested that altered central mechanism(s) may be responsible for these impaired reflex regulations and may contribute to the elevated neurohumoral drive in HF. 4,14

The paraventricular nucleus (PVN) of the hypothalamus is an important central site for the integration of sympathetic nerve activity. 5 Morphological and electrophysiological studies have shown that the PVN is reciprocally connected to other areas of the central nervous system that are involved in cardiovascular function. 6 Using retrograde tracing techniques, various studies have shown that the PVN is a major source of forebrain input to the sympathetic nervous system. 6 Stimulation of PVN has been shown to elicit increased discharge of several sympathetic nerves, including renal and adrenal. 7 Specifically, studies showed that the PVN plays an essential role in the mediation of RSND under resting and reflex conditions. 8 Functional studies have also implicated an important role for the PVN in cardiovascular regulation. 8,9 Our recent studies showed increased neuronal activity, as measured by hexokinase activity, in the PVN during HF 4,10 suggesting the involvement of the PVN in the altered sympathetic activity in HF. However, the specific mechanisms
within the PVN involved in regulating sympathetic nervous outflow are still unknown.

In the PVN, a number of neurotransmitters converge to influence its neural activity. As a major excitatory neurotransmitter, glutamate has been found to regulate the modulation of sympathetic nerve activity in several brain areas, including the hypothalamus and ventrolateral medulla (VLM). It has been reported that the NMDA and AMPA receptors, the two major ionotropic glutamate receptors, exist in the PVN, suggesting that both receptors may mediate the glutamate induced excitatory action in the PVN. Functional studies have shown that glutamate receptors within the PVN are involved in cardiovascular reflexes. Our recent study has shown, for the first time, that injection of NMDA into the PVN significantly increased RSND. All of these results suggest that a glutamergic system within the PVN may play an important role in regulating sympathetic nerve activity and cardiovascular function. It is not known if the function of the glutamergic system in the PVN is altered in the HF state. We hypothesize that altered NMDA receptor activity in the PVN may be involved in sympathetic dysfunction in HF. To test this hypothesis, we examined if (1) the NMDA receptor mediated changes in RSND is enhanced in HF; (2) endogenous glutamate levels in the PVN are increased during HF; (3) NMDA receptor mRNA expression and protein levels are increased in the PVN of HF rats.

Materials and Methods
Induction of Heart Failure
Male Sprague-Dawley rats weighing 220 to 280 g were obtained from Sasco Breeding Laboratories (Omaha, Neb) and were assigned randomly to two groups ( sham-operated control and HF group). HF was produced by left coronary artery ligation, as described previously. The degree of left ventricular dysfunction and failure were determined using both hemodynamic and anatomic criteria. Rats with both left ventricular end-diastolic pressure (LVEDP) ~15 mm Hg and infarct size ~35% of total left ventricle wall were considered to be in HF.

General Surgery for Hemodynamic and RSND Measurements and Microinjection
On the day of the experiment (6 to 8 weeks after cardiac surgery), the rat was anesthetized with urethane (0.75 g/kg IP) and α-chloralose (70 mg/kg IP) and instrumented for recording blood pressure (BP) and heart rate (HR) as described previously. The rat was then placed in a stereotaxic apparatus (David Kopf Instruments), and a cannula was introduced into the PVN (1.3 mm posterior and 0.4 mm lateral to the bregma, 7.8 mm ventral to the dura). The left kidney was exposed through a left retroperitoneal flank incision and a branch of the renal nerve was isolated and prepared for recording raw discharge and integrated voltage as described previously. Efferent renal sympathetic nerve discharge (RSND) at the beginning of the experiment was defined as basal nerve activity. The nerve activity recorded at the end of the experiment after the rat was injected with high dose of hexamethonium (20 mg/kg IV) and after death was considered as background noise. During the experiment, the value of RSND was calculated by subtracting the background noise from the actual recorded value. The basal nerve activity was recorded before the administration of any drugs into the PVN. The response of RSND to the administration of drugs into the PVN during the experiment was subsequently expressed as a percentage change from the basal value.

Microdialysis and HPLC Measurement of Glutamate
An in vivo microdialysis technique was used to collect a perfusate sample from the PVN for the measurement of glutamate. Under anesthesia with urethane (0.75 g/kg) and α-chloralose (70 mg/kg, IP), a small burr hole was drilled in the skull of the rat and a microdialysis probe (0.7 mm OD and 0.5 mm ID) was lowered into the PVN using the same coordinates as that for microinjection mentioned above. Both the input and output cannula of the microdialysis probe was connected to a pump by polyethylene tubing (PE-20). Artificial cerebrospinal fluid (aCSF, composition in mmol/L: 145 NaCl, 3.5 KCl, 1.0 MgCl2, and 1.3 CaCl2, adjusted to pH 7.2) was infused into the PVN at a constant flow rate of 3 μL/min through the input cannula. The perfusate from the output cannula was collected into microcentrifuge tubes on ice and frozen at −70°C for later measurement.

Glutamate in the perfusate was measured by HPLC (BAS-PM-80 delivery system with phase II column in combination with LC-40 electrochemical detector system). The electrochemical detection signal was recorded and analyzed by PowerChrom (ADInstruments). Mobile phase consists of 36% 0.1 mol/L potassium phosphate (pH 7.0) with 200 mg/L Na2EDTA, 22% 0.1 mol/L sodium acetate (pH 4.0) with 200 mg/L Na2EDTA, and 42% acetonitrile (v/v). The flow rate was 0.5 mL/min. Glutamate in the sample was polarized with a working reagent [16.75 mg of α-phthalic dicarboxaldehyde (OPA), 12.5 mL of methanol, 14 mL of i-butylthiol, 15 mL of 1 mol/L carbonate buffer, brought up 25 mL using deionized water]. Twenty microliters of the microdialysis sample was mixed with 35 μL of the working reagent and incubated at room temperature for 10 minutes. The mixture was then loaded into the HPLC. A glutamate standard curve was made and glutamate concentrations were determined in samples using the PowerChrom system (ADInstruments).

Brain Histology
For all of the microinjection and microdialysis placements in the PVN, at the end of the experiments, monastral blue dye was injected into the brain for histological verification of the location. At the end of the experiment the brains were removed, frozen, sectioned, and processed for histology as described previously. Those injections with termination in the boundaries of the PVN were considered to be histologically targeted.

Micropunch of the PVN
After the animal was euthanized, the brain was removed and sectioned at 450-μm-thick coronal sections and the PVN or the supraoptic nucleus (SON) area was punched out using a 15-gauge needle stub (ID 1.5 mm). The punched tissue was put in 0.5 mL of TRI Reagent (MRC) and homogenized using a sonicator. The total RNA and protein in the homogenate were extracted, respectively, according to the TRI Reagent manufacturer’s instruction.

Expression of NMDA Receptor Subunits NR1 and NR2B and AMPA1 Receptors in the PVN
Semi quantitative RT-PCR assays were performed to assess relative mRNA levels. Data were expressed as the ratio of NR1 and β-actin (used as a housekeeping gene) calculated from the respective densities (for additional details, see the expanded Materials and Methods section in the online data supplement, available at http://www.circresaha.org).

Western Blot for the Measurement of NR1 Receptor Protein
The protein extraction from homogenates mentioned was used for Western blot analysis for the NMDA receptor NR1 subunit (see online data supplement).

Immunofluorescence for NMDA NR1 Subunit Detection in the PVN
Under deep anesthetization with pentobarbital (50 mg/kg), rats were perfused transcardially with heparinized 0.1 mol/L PBS followed by
ice-cold 4% paraformaldehyde in 0.1 mol/L PBS. The brain was removed and postfixed in 4% paraformaldehyde in 0.1 mol/L PBS for 12 hours at 4°C, followed by soaking the brain in 30% of sucrose for 12 hours at 4°C for cryostat protection. The brain was cut into 20-μm-thick coronal sections. Sections including the PVN area were mounted on precoated glass slides. After 30 minutes in PBS, sections were incubated with 10% of normal goat serum for 1 hour followed by incubation with primary antibody (2 μg/mL anti-NR, rabbit polyclonal antibody, Santa Cruz) overnight at 4°C. Then the sections were washed with PBS and incubated with a secondary antibody (2 μg/mL goat anti-rabbit IgG conjugated Texas Red + anti-actin, Vector) for 30 minutes at room temperature. After 3 washes, sections were mounted by Fluoromount (BDH Laboratory Supplies, London, England) and coverslipped. Slides were observed under Leica microscope with appropriate excitation/emission filters. Pictures were captured by a digital camera system.

Experimental Protocols

Microinjection of NMDA Into the PVN
In sham and HF rats, three doses of NMDA (25, 50, and 100 pmol in 25, 50, and 100 nL of 1 mmol/L of NMDA in aCSF) were injected into the PVN at intervals of 30 minutes. The responses in RSND, BP, and HR over 30 minutes were recorded after each dose of NMDA. The vehicle control was performed using 100 nL of aCSF microinjected into the PVN in each rat. To substantiate the concept that any responses of RSND, BP, and HR to NMDA were not a peripheral action, in five normal rats intravenous injection of 500 pmol of NMDA were examined.

Blockade of NMDA and Non-NMDA Receptors Within the PVN
In sham and HF rats, the specific NMDA receptor antagonist dl-2-amino-5-phosphonovaleric acid (AP-5) was injected (4, 8, and 16 nmol in 25, 50, and 100 nL) into the PVN. Additionally, in sham and HF rats, CNQX, a non-NMDA receptor antagonist, was injected (4, 8, and 16 nmol in 25, 50, and 100 nL) into the PVN. The interval of each administration was 30 minutes. The responses in RSND, BP, and HR over 30 minutes after each dose administration were recorded.

Glutamate Levels Within the PVN in HF
Microdialysis and HPLC for measurement of endogenous glutamate levels in the PVN were performed as mentioned above in HF and sham rats.

Expression of NMDA Receptor Subunits NR1 and NR2B and AMPA1 Receptors in the PVN
The punched PVN tissues from HF and sham rats were used for the detections of NR1, NR2B, and AMPA1 mRNA by using RT-PCR. Similarly, the punched SON tissues from HF and sham rats were used for the detection of the NR1 mRNA expression. The punched PVN tissues from HF and sham rats were also used for NR1 protein determination by using Western blot. In addition, several HF and sham rats were used for the immunofluorescence experiment.

Data Analysis
Responses of RSND to the various doses of drugs were expressed as percentage change over the basal value. Responses of BP and HR to drugs were expressed as the difference between the basal value and the value after each dose of drug. The normalized data of RT-PCR were expressed as the ratio of intensities of NR1 bands to β-actin bands. The values of Western blot bands were expressed in intensity units. All the group data are presented as mean ± SE. The data were subjected to two-way repeated measures ANOVA followed by comparison for individual differences using the Newman-Keuls test. A value of P<0.05 was considered to indicate statistical significance.

Results

General Data
The Table summarizes the salient morphological characteristics of sham and HF rats utilized in the present study. For all the experiments, any rats subjected to coronary artery ligation that displayed myocardial infarcts less than 30% of the left ventricular wall were excluded from the study. Accordingly, the infarction area in the chronic HF group was approximately 43% of the endocardial surface. Sham rats had no observable damage to the myocardium. The minimum ventricular thickness was significantly less in HF than in the sham rats, indicating a transmural damage to the myocardium. Heart weight was significantly greater in HF rats than in sham rats, suggesting compensatory hypertrophy of noninfarcted regions of the myocardium. Left ventricular end-diastolic pressure was significantly elevated in HF rats compared with sham rats.

The dynamic characteristics of basal BP, HR, RSND, and left ventricular end-diastolic pressure (LVEDP) are presented in the Table. The rats with LVEDP greater than 15 mm Hg were chosen for the HF group. Sham rats did not exhibit an increased LVEDP. There was a higher level of raw RSND in rats with HF compared with sham rats; however, it was not statistically significant. There were no statistically significant differences in basal BP or HR between the sham and HF groups.

<table>
<thead>
<tr>
<th>General Data</th>
<th>Sham</th>
<th>HF</th>
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<tr>
<td>Body wt, g</td>
<td>386±19</td>
<td>371±23</td>
</tr>
<tr>
<td>Heart wt, mg</td>
<td>1144±94</td>
<td>1523±107*</td>
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<tr>
<td>Heart wt/body wt, mg/g</td>
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<td>4.1±0.2*</td>
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<tr>
<td>Infarct size, % of LV-endocardial</td>
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<td>43.5±5.3*</td>
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<tr>
<td>Infarct size, % of LV-epicardial</td>
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<td>35.1±4.3*</td>
</tr>
<tr>
<td>Minimum thickness of LV, mm</td>
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<td>0.5±0.1*</td>
</tr>
<tr>
<td>MBP, mm Hg</td>
<td>93±9</td>
<td>89±7</td>
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<tr>
<td>HR, bpm</td>
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<td>327±42</td>
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<td>int.RSND, μV·s</td>
<td>43.4±9.8</td>
<td>58.2±11.8</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>2±2</td>
<td>20±4*</td>
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</table>

Values are mean±SEM; *P<0.05 vs sham rats.
Figure 1 illustrates the brain histological data. Among the 36 injections that were in the PVN area, 12 injection sites belong to microinjection of NMDA, 14 injection sites belong to microinjection of AP-5, and 8 injection sites belong to microdialysis of the PVN in sham and HF rats.

Microinjection of NMDA Into the PVN

An example of the responses in RSND, BP, and HR to administration of NMDA into the PVN in a sham rat and a HF rat is illustrated in Figure 2A. Microinjections of 25, 50, and 100 pmol of NMDA elicited increases in RSND and BP, reaching 61/1006 4% and 14/1006 5 mm Hg, respectively, at the highest dose in sham rats. These responses were significantly elevated in the HF compared with sham rats, reaching 86/1006 10% and 21/1006 7 mm Hg, respectively, at the highest dose (Figure 2B). These data indicate that the sensitivity of the NMDA receptor in the PVN in the regulation of RSND in rats with HF is increased. The vehicle control, 100 nL of aCSF microinjected into the PVN had no effect on RSND, BP, and HR both in HF or sham rats. Intravenous injection of 500 pmol of NMDA also had no effect on these parameters (data not shown).

Blockade of NMDA Receptor Within the PVN

In HF rats, after microinjection of AP-5 (4, 8, and 16 nmol) into the PVN, the responses of RSND, BP, and HR were significantly decreased, reaching −54±4%, −18±12 mm Hg, and −40±11 bpm, respectively, at the highest dose (Figure 3A). However in sham rats, responses to AP-5 were negligible. These results suggest that the endogenous NMDA receptor excitation in the PVN was elevated in HF rats. Blockade of non-NMDA receptors in the PVN with CNQX did not cause significant changes in RSND, BP, and HR (Figure 3B), suggesting that non-NMDA receptors in the PVN may not play a major role in the regulation of sympathetic nerve activity.

Endogenous Glutamate Levels in the PVN

The glutamate peaks in the perfusate samples detected by the HPLC/EC system were identified relative to the glutamate standard. Figure 4A showed the HPLC tracings with glutamate peaks of standard and microdialysis samples from the PVN in HF and sham rats. The average levels of glutamate release within the PVN in sham and HF rats are presented in Figure 4B. The basal levels of glutamate in the PVN were not significantly different between the two groups. These data suggested that endogenous glutamate release in the PVN is not markedly increased during HF.

Expression of NMDA Receptor Subunits NR1 and NR2B and AMPA1 Receptors in the PVN

Result of RT-PCR experiments indicated that NR1 mRNA expression in the punched PVN tissues from the HF rats was significantly increased compared with those from sham rats (Figure 5A). Consistently, Western blots showed that NR1 protein levels were also significantly higher in HF rats compared with sham rats (Figure 5B). Finally, as an in situ confirmation of this alteration, the immunofluorescence for NR1 receptor subunit was greater in the PVN from rats with HF compared with sham rats (Figure 6). In contrast to the PVN, RT-PCR result did not show a significant change in NR1 expression in the SON in HF rats compared with sham rats (Figure 7A). Moreover, RT-PCR result did not show a significant change in NR2B mRNA expressions within the PVN in HF rats compared with sham rats (Figure 7B). Furthermore, RT-PCR did not detect a significant alteration in AMPA1 receptor mRNA within the PVN in HF rats compared with sham rats (Figure 7C).

Discussion

In the present study, we found that stimulation of NMDA receptors within the PVN induced a greater increase in...
Figure 2. A, Segments of original recordings from an individual rat with heart failure and sham-operated rat, demonstrating the representative responses of renal sympathetic nerve discharge (RSND), integral of RSND (int.RSND), arterial blood pressure (BP), and heart rate (HR) to the microinjections of different doses of NMDA into the PVN. B, Mean data of changes in RSND, BP, and HR after microinjections of NMDA (25, 50, and 100 pmol) into the PVN in sham (open bar) and HF rats (solid bar). *P<0.05 vs group of sham.

Figure 3. Mean data of changes in RSND, BP, and HR after microinjections of AP-5 (4, 8, and 16 nmol) (A) or NQX (4, 8, and 16 nmol) (B) into the PVN in sham (open bar) and HF rats (solid bar). *P<0.05 vs group of sham; **P<0.01 vs group of sham.
sympathetic excitatory responses in HF compared with sham rats, suggesting that NMDA receptor-mediated renal sympathetic nerve activity was elevated in the HF condition. On the other hand, the blockade of NMDA receptor within the PVN induced significant decreases in RSND, BP, and HR only in the rats with HF but not in sham rats, suggesting a hyperfunction of the endogenous NMDA action on sympathetic outflow in HF. This enhanced endogenous NMDA receptor function within the PVN is unlikely due to the increase in glutamate release in the PVN, because there is no significant difference of glutamate levels in the PVN in HF compared with sham rats. Furthermore, we found markedly higher

Figure 4. Glutamate levels within the PVN in the sham and HF rats measured by microdialysis and HPLC/EC detector. A, HPLC traces showing the glutamate peaks of standard and microdialysis samples. B, Mean data of glutamate levels in the PVN in the sham (open bar) and HF (solid bar) rats.

Figure 5. A, Gene expression of NMDA receptor NR1 subunit in the samples of punched PVN tissues measured by RT-PCR. Top, Example of visualized electrophoresis bands of coamplification of NR1 and β-actin using the samples from the punched PVN tissues of sham and HF rats. Bottom, Mean data of band densities of NR1 normalized by β-actin in sham and HF. B, Western blots for NMDA receptor NR1 subunit in the samples of punched PVN tissues measured by Western blotting. Top, Example of visualized electrophoresis bands of NR1 protein in the samples from the punched PVN tissues of sham and HF rats. Bottom, Mean data of band densities of NR1 protein in sham and HF. *P<0.05 vs sham group.
expressions of NMDA NR1 receptors at both mRNA and protein levels within the PVN in HF rats compared with sham rats, suggesting a possible mechanism of the increased endogenous NMDA action in HF. These results provide, for the first time, novel evidence that altered glutamate NMDA receptors within the PVN may play an important role in altered sympathoexcitation in HF.

The PVN is one of the five major central nervous system sites that directly controls sympathetic outflow. The preautonomic neurons originating in the dorsal and lateral parvocellular nuclei and in the ventral quadrant of the medial parvocellular zone of the PVN project to the dorsal vagal complex, rostral ventrolateral medulla, and intermediolateral columns of the spinal cord. Functional studies suggest that the PVN is involved in the mediation of the neural component of cardiovascular reflexes. Combining these facts with its actions in fluid balance and vasopressin release makes the PVN a prime candidate within the forebrain for a central site responsible for mediating sympathetic outflow and regulating systemic hemodynamics. HF is characterized by elevated systemic sympathetic activity and salt and water retention, both involving the function of the PVN. Thus, the PVN may play a particularly important role in the development of HF. However, the detail mechanisms, such as neurotransmitters in this region involved in sympathetic dysfunction and fluid imbalance have not been fully established. Previously, work from this laboratory has shown that inhibitory mechanisms of sympathetic regulation within the PVN via NO and GABA were reduced in HF rats. These alterations may induce an imbalance of the inhibitory and excitatory mechanisms in this area and influence sympathetic outflow. Thus, in the present study, we concentrated on the excitatory mechanism of renal sympathetic nerve regulation within the PVN. The results reveal for the first time that the altered NMDA receptor expression as well as its action is associated with the elevated renal sympathetic nerve activity in HF.

Figure 6. Immunofluorescence photomicrographs from the sections of the PVN region stained for NMDA NR1 subunit. Number of NR1-positive neuron (arrows) is increased in the HF (right) compared with sham (left). Blue spot shows the nucleus stained by Hoechst 33258 (Molecular Probes).

Figure 7. RT-PCR measurement of gene expression of NMDA receptor NR1 subunit (A) in the samples of punched SON tissues, NMDA receptor NR2B subunit (B), or AMPA receptor in the samples of punched PVN tissues (C). Top, Example of visualized electrophoresis bands of NR1, NR2B subunit, or AMPA receptor in sham and HF rats. Bottom, Mean data of band densities of NR1, NR2B subunit, or AMPA receptor in sham and HF rats.
As a major excitatory neurotransmitter, glutamate has been found to regulate sympathetic nerve activity in several brain areas, including the hypothalamus. It has been reported that NMDA and AMPA receptors, the two major ionotropic glutamate receptors mediating the excitatory signal of glutamate, exist in the PVN, suggesting that both receptors may mediate glutamate action in the PVN. Glutamate receptors have been reported to mediate activation of the PVN neurons. In the present study, it was observed that stimulation of NMDA receptor within the PVN induced a sympathetic excitation, further confirming that NMDA receptor in the PVN mediates sympathetic outflow.

However, we also observed that in sham rats blockade of NMDA receptor in the PVN with AP-5 barely induce a decrease in RSND, suggesting that NMDA receptor may not make a major contribution to tonic sympathetic outflow. Considering the existence of NMDA receptors and a basal level of glutamate in the PVN, the faint tonic action of NMDA receptor on the sympathetic outflow in resting condition is somewhat surprising. One possibility of this is that the inhibitory mechanisms in the PVN play the predominant role in resting condition, which depress the NMDA receptor-mediated excitation action on sympathetic outflow. In a slice preparation (in vitro), NMDA depolarizes PVN neurons initially followed by induction of inhibitory postsynaptic potentials (IPSP) later in the same neuron. Conversely, blocking NO with L-NAME elicited more pronounced NMDA-induced depolarization but no accompanying increase in IPSPs. Furthermore, the inhibitory actions of NO were determined to be mediated by a GABA mechanism. In vivo, we have observed that NO via a GABA mechanism exerts a tonic inhibition on sympathetic outflow. Activation of NMDA receptors in the PVN elicits an excitatory effect on RSND followed by a prolonged inhibition. The excitatory action of NMDA can be enhanced (and the prolonged inhibition is blocked) by the inhibition of endogenous NO synthesis using L-NMMA, suggesting that NO produced during NMDA receptor activation is involved in inhibiting NMDA-mediated increases in RSND. Consistent with this, perfusion of either glutamate or NMDA into PVN induced an increase in NO release, suggesting that the activation of NMDA receptors in the PVN increases the synthesis and release of NO. This indicates a short loop inhibition by NO of excitation by NMDA receptor activation to increase RSND via the PVN. Furthermore, our previous studies have shown that inhibitory mechanisms of sympathetic regulation within the PVN via NO and GABA were reduced in HF rats. These alterations may induce an imbalance between the inhibitory and excitatory mechanisms and result in an enhanced NMDA receptor–mediated sympathetic outflow in HF.

Changes in excitatory synaptic functions can occur by presynaptic mechanisms such as altered neurotransmitter release and/or postsynaptic mechanisms. In the present study, we observed that NMDA receptor NR2 subunit was upregulated in the PVN in HF rats, which is associated with the enhanced NMDA receptor function. This may be one of the central mechanisms underlying the elevated sympathoexcitation in HF. In contrast, we were not able to detect a significant increase in glutamate release within the PVN in HF rats. The upregulation of NMDA receptor has been observed in other studies in response to different fluid balance challenges. For example, in the intact animal, intracerebroventricular application of MK801, a NMDA receptor antagonist, induced a significant reduction in drinking response and plasma vasopressin level in dehydrated rats. It was reported that the NR1 subunit was upregulated in the SON and the PVN in rats subjected to dehydration. These observations suggest that alteration of the NMDA receptor functions and expressions in the hypothalamus may play a role in adaptation of body to different homeostasis challenges.

Natural NMDA receptor is composed of an NR1 subunit in combination with one or more NR2 subunits. NR2B is one of the four major subtypes of NR2A-D. Unlike NR1, which is ubiquitous, NR2B is restricted to the forebrain. Thus in this study, we also measured NR2B subunit expression using RT-PCR. However, the result did not show a significant increase in NR2B mRNA within the PVN in HF compared with sham rats. The explanation of the differential alterations of NR1 and NR2B subunits expression within the PVN in HF remains to be explored.

AMPA receptor is another important ionotropic receptor that mediates glutamate-initiated signal. However, we did not observe a significant change in both expression and function of AMPA receptors within the PVN in HF compared with sham rats (Figure 7), suggesting that the increased expression of the NMDA receptor in the PVN of rats with HF is a specific alteration. Additionally, using RT-PCR, we also measured the NR1 expression in the SON, another important hypothalamic area. The result showed that there was no significant change in NR1 expression in the SON in HF compared with sham rats (Figure 7), suggesting that the change in NR1 expression within the PVN in HF is region-specific as well.

The cause(s) of upregulation of NR1 receptors within the PVN in HF remain to be examined. In HF state, the decreased cardiac output and increased central venous pressure can stimulate cardiopulmonary receptors and induce the increased afferent input to the autonomic centers including the PVN. Also many central and peripheral humoral factors are significantly altered in HF. All of these alterations may induce the compensatory responses in the cardiovascular and autonomic centers. The upregulation of NMDA receptor and the subsequent increase in glutamate activity within the PVN may be part of the compensatory responses. However the direct factor(s) regulating NMDA receptor expression in the PVN in HF remain to be examined. NO has been recognized as a factor eliciting a wide range regulation of gene expression. The PVN has been found to be rich in nitric oxide synthase–positive neurons. A previous study from this laboratory has shown that the numbers of NOS-positive cells in the PVN was decreased in rats with HF. These observations make NO a major candidate to regulate NMDA receptor expression within the PVN. However, further evidence remains to be obtained.
tensin II, etc. It is recognized that other central sites as well as neurotransmitters/substances (e.g., serotonin, dopamine, cytokines, angiotensin, and reactive oxygen species) within the PVN may also be involved in the altered sympathetic outflow in HF. Further investigations of the interactions among these various neurotransmitter substance and glutamatergic system remains to be examined.

In conclusion, the results of the present study indicates that altered NMDA receptor function due to the upregulation of the NMDA NR1 subunit in the PVN may contribute to the elevated sympathetic nerve activity in the HF state. Because sympathoexcitation contributes to the deterioration of the heart failure state and in light of the beneficial effects of systemically administered β-blockers a comprehensive understanding of the sympathoexcitation will help to uncover new therapies for the sympathetic dysfunction in HF.

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Expanded Material and Methods

RT-PCR for the measurements of the mRNA expressions of NMDA NR₁ or NR₂B subunits and AMPAₐ receptors in the PVN

Semiquantitative RT-PCR assays were performed to assess relative mRNA levels. Total RNA extracted from the punched tissue was subjected to reverse transcription for 40 min at 38°C in presence of 1.5 μM of random hexamers (Amersham, IL) and 400 U of MMLV reverse transcriptase (USB, Ohio) in a final volume of 25 μL. Each 2 μL of the reverse transcribed cDNA was used as a template for PCR amplification. To measure the mRNA expressions of NMDA receptor NR₁ and NR₂B subunits and AMPAₐ receptor, following primers were used: For NMDA receptor NR₁ subunit (according to the cloned rat NR₁ gene sequence GenBank accession: NM 017010), the forward primer was 5’-ATAGTGACAATCCACCAAGAGCC (1463-1485); The reverse primer was 5’-GTAGCTCGCCCATCATTCCGTT (1781-1760). For NMDA receptor NR₂B subunit (GenBank accession: NM 012574), the forward primer was 5’-CCCAGGTCAATCAGCAAGAAACC (3393-3416); The reverse primer was 5’-CTGGCCGCTTCTTTTAGACTGTCC (3630-3608). For AMPAₐ receptor (GenBank accession: NM 031608), the forward primer was 5’-CAACATTCTGACAACCCACCG (722-741); The reverse primer was TGTCACATTGGCTCCGCTCT (950-931); For β-actin, used as a PCR control gene (GenBank accession: NM 031144), the forward primer 5’-CACGGCATGTGAAACACTG (217-236), reverse primer 5’-TTCAGCTGTGCTGTAAG (616-597). The 25 μL volume of PCR reaction mixture containing 0.7 μM forward and reverse primers and 1 U of Taq DNA polymerase (Gibco-BRL). After 10 min of denaturation at 94°C, the amplification was performed with 30 thermal cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min and a final extension at 72°C for 10 min. Then 8 μL of PCR products were fractionated in a 1% agarose gel and visualized by ethidium bromide staining and UV-transillumination. The visualized DNA bands of the PCR products were captured by a Kodak 290 digital camera and loaded into a G4 Macintosh. Semiquantitative analysis of DNA bands (density of the bands) was performed with Kodak 1D image analysis software. Data were expressed as the ratio of NR₁ and β-actin (used as a housekeeping gene) calculated from the respective densities.
Western blot for the measurement of NR1 receptor protein

The protein extraction from homogenates mentioned above was used for western blot analysis for the NMDA receptor NR1 subunit (see online data supplement). The protein concentration was measured using a protein assay kit (BCA, PIERCE). Samples were adjusted to the same concentration of protein and then mixed with equal volumes of 2X 4% SDS sample buffer. The samples were boiled for 5 min and then loaded on the 7.5% SDS-PAGE gel (5 μg protein/30μL per well) to be subjected to electrophoresis using Bio-Rad mini gel apparatus at 40 mA/each gel for 45 min. Then the fractionized proteins on the gel were electrophoretically transferred onto the PVDF membrane (Millipore) at 300 mA for 90 min. The membrane was probed with primary antibody (rabbit anti rat NR1 polyclonal serum, Santa Cruz, 1:1000) and secondary anti body (Peroxidase-conjugated goat anti rabbit IgG, PIERCE, 1:5000). The membrane was treated with enhanced chemi-luminescence substrate (PIERCE) for 5 min at room temperature. The membrane was used for developing a film (NEN-DuPont). The visualized bands in the film were digitized using Kodak 290 digital camera and the intensities of the bands were analyzed using Kodak 1D image analysis software.