In Vivo Discharge Properties of Hypothalamic Paraventricular Nucleus Neurons With Axonal Projections to the Rostral Ventrolateral Medulla

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Chen Q-H, Toney GM. In vivo discharge properties of hypothalamic paraventricular nucleus neurons with axonal projections to the rostral ventrolateral medulla. J Neurophysiol 103: 4–15, 2010. First published November 4, 2009; doi:10.1152/jn.00094.2009. The hypothalamic paraventricular nucleus (PVN) and rostral ventrolateral medulla (RVLM) are key components of a neural network that generates and regulates sympathetic nerve activity (SNA). Although each region has been extensively studied, little is presently known about the in vivo discharge properties of individual PVN neurons that directly innervate the RVLM. Here extracellular recording was performed in anesthetized rats, and antidromic stimulation was used to identify single PVN neurons with axonal projections to the RVLM (n = 94). Neurons were divided into two groups that had either unbranched axons terminating in the RVLM (i.e., PVN-RVLM neurons, n = 65) or collateralized axons targeting both the RVLM and spinal cord [i.e., PVN-RVLM/intermediolateral cell column (IML) neurons, n = 29]. Many PVN-RVLM (32/65, 49%) and PVN-RVLM/IML (17/29, 59%) neurons were spontaneously active. The average firing frequency was not different across groups. Spike-triggered averaging revealed that spontaneous discharge of most neurons was temporally correlated with renal SNA (PVN-RVLM: 12/21, 57%; PVN-RVLM/IML: 6/9, 67%). Time histograms triggered by the electrocardiogram (ECG) R-wave indicated that discharge of most cells was also cardiac rhythmic (PVN-RVLM: 25/32, 78%; PVN-RVLM/IML: 10/17, 59%). Raising and lowering arterial blood pressure to increase and decrease arterial baroreceptor input caused a corresponding decrease and increase in firing frequency among cells of both groups (PVN-RVLM: 9/13, 69%; PVN-RVLM/IML: 4/4, 100%). These results indicate that PVN-RVLM and PVN-RVLM/IML neurons are both capable of contributing to basal sympathetic activity and its baroreflex modulation.

INTRODUCTION

Neurons of the hypothalamic paraventricular nucleus (PVN) control many important homeostatic functions, including release of pituitary hormones (Swanson 1991; Swanson and Sawchenko 1980; Vigas 1989) and regulation of ingestive behavior/metabolism (Konturek et al. 2005; Valassi et al. 2008). Over the past several years, studies have increasingly focused on the role of PVN neurons in regulating sympathetic nerve activity (SNA) (Akine et al. 2003; Allen 2002; Coote et al. 1998; Li and Pan 2007; Li et al. 2006; Lovick and Coote 1988b; Porter and Brody 1985; Stern 2004; Toney et al. 2003). Early studies showed that stimulation of the PVN reduces visceral organ blood flow and increases arterial blood pressure (ABP) (Martin and Haywood 1992; Porter and Brody 1985, 1986), whereas disinhibition of the PVN by GABA_A receptor blockade increases ABP, heart rate, and plasma norepinephrine concentration (Martin and Haywood 1993; Martin et al. 1991).

Collectively, these observations indicate that PVN neurons can increase SNA, although ongoing synaptic inhibition limits their influence on basal SNA.

In spite of receiving strong tonic inhibitory input (Chen and Toney 2003b; Chen et al. 2003; Kenney et al. 2003; Li et al. 2006; Martin et al. 1991), PVN neurons nevertheless do contribute to ongoing SNA. In anesthetized rats, for example, studies have shown that acute inhibition of PVN neuronal activity or blockade of excitatory inputs reduces ongoing renal SNA (RSNA) (Akine et al. 2003; Allen 2002; Stocker et al. 2004b, 2005), lumbar SNA (Stocker et al. 2005), and ABP (Akine et al. 2003; Allen 2002; Freeman and Brooks 2007; Stocker et al. 2004b, 2005). It is noteworthy that reductions of SNA in response to PVN inhibition are more pronounced in water-deprived (Freeman and Brooks 2007; Stocker et al. 2004b, 2005) and hypertensive (Akine et al. 2003; Allen 2002; Li and Pan 2007) rats, suggesting an increased contribution of PVN neuronal activity to the maintenance of resting SNA.

Anatomical studies have identified several groups of sympathetic-regulatory PVN neurons. One group monosynaptically targets the spinal intermediolateral cell column (IML; PVN-IML), the location of sympathetic preganglionic neurons (Saper et al. 1976; Swanson and Sawchenko 1980). Another group innervates presympathetic neurons in the rostral ventrolateral medulla (RVLM; PVN-RVLM) (Pyner and Coote 2000; Shafton et al. 1998; Stocker et al. 2006; Swanson and Sawchenko 1980). A third and more recently identified group has branched axons that innervate both the RVLM and IML (PVN-RVLM/IML) (Pyner and Coote 2000; Shafton et al. 1998; Stocker et al. 2004a).

In vivo electrophysiological studies performed to date have largely focused on the discharge behavior of PVN-IML neurons (Bains and Ferguson 1995; Bains et al. 1992; Chen and Toney 2003a; Lovick and Coote 1988a,b). They indicate that many are quiescent in anesthetized rats and those with spontaneous activity most often fire slowly (<1.5 spike/s) at rest (Bains and Ferguson 1995; Bains et al. 1992; Chen and Toney 2003a; Lovick and Coote 1988a,b). In response to local application of various neurotransmitters (Bains and Ferguson 1995; Cato and Toney 2005; Chen and Pan 2006; Chen et al. 2006; Lee et al. 2008; Li et al. 2004; Lovick and Coote 1988a) and inputs activated by circulating hormones (e.g., angiotensin II, ANP) (Bains and Ferguson 1995; Bains et al. 1992; Cato and Toney 2005; Lovick and Coote 1988a), high-frequency discharge can be evoked. There is also evidence that some spontaneously active PVN-IML neurons are targeted by inhibitory inputs from arterial (Bains and Ferguson 1995; Chen and Toney 2003a; Lovick and Coote 1988b) and cardiopulmonary baroreceptors (Lovick and Coote 1988a,b). However, these
Inhibitory inputs do not appear to account fully for the lack of tonic discharge exhibited by many PVN-IML neurons (Chen and Toney 2003a; Lovick and Coote 1988a,b).

In marked contrast to PVN-IML neurons, few data are available concerning the in vivo discharge properties of PVN neurons that target the RVLM (Barman 1990). This is surprising given that sympathoexcitatory challenges such as water deprivation (Stock et al. 2004a, 2006) and hemorrhage (Badoer and Merolli 1998; Badoer et al. 1993) have been shown to induce c-fos expression in PVN-RVLM neurons. Moreover, hemorrhage increases RSNA by stimulating ionotropic glutamate and vasopressin V1a receptors in the spinal cord (Yang and Coote 2006), possibly via recruitment of PVN-RVLM/IML neurons. Finally, PVN-RVLM neurons are largely glutamatergic (Stock et al. 2006) and activation of the PVN increases the discharge of RVLM vasomotor neurons (Yang et al. 2001). Here extracellular single-unit recordings were performed in anesthetized rats to test the hypothesis that the patterning of discharge of PVN-RVLM and PVN-RVLM/IML neurons is consistent with their contributing to both the generation of resting SNA and its reflex modulation.

Individual PVN-RVLM and PVN-RVLM/IML neurons were identified using antidromic activation. To establish a possible functional link between discharge and control of ongoing SNA, spike-triggered averaging was used to determine the temporal relationship between spontaneous firing and RSNA. To further investigate whether PVN-RVLM and PVN-RVLM/IML neurons have the capacity to regulate SNA and ABP, the temporal relationship between spontaneous discharge and the cardiac cycle was determined using electrocardiogram (ECG) R-wave-triggered time histogram analysis. Finally, a role for PVN neurons in reflex control of ABP was evaluated by recording neuronal responses to loading and unloading of arterial baroreceptors.

**Methods**

**General procedures**

Experiments were performed on 57 male Sprague-Dawley rats (350–450 g) (Charles River Laboratories) that were housed in a temperature-controlled room (22–23°C) with a 14:10 h light-dark cycle (lights on at 07:00 h). Tap water and laboratory chow (Harlan Teklad LM-485, 0.3% NaCl) were available ad libitum. Rats were anesthetized with a mixture of α-chloralose (80 mg/kg ip) and urethan (800 mg/kg ip) and catheters (PE-50 tubing) were placed in a femoral artery and vein to record ABP and administer drugs. Heart rate (HR) was obtained from an ECG (lead I). After tracheal cannulation, rats were artificially ventilated with oxygen-enriched room air and endtidal pCO2 was monitored and maintained between 40 and 50 mmHg. Rats were then paralyzed with gallamine triethiodide (5 mg/kg ip) and tidal pCO2 was monitored and maintained between 40 and 50 mmHg. A left renal nerve bundle was isolated and activity was recorded as previously described (Chen and Toney 2003a,b; Stocker et al. 2005). Nerve signals were acquired using a high-impedance head-stage connected to an AC amplifier equipped with half-amplitude frequency filters (band-pass: 30–1,000 Hz) and a 60-Hz notch filter. Signals were amplified (10,000–20,000-fold), full-wave rectified, integrated (time constant: 0.3–3.0 s), and digitized at a frequency of 1.0 kHz. Spike2 software (v5.16, Cambridge Electronic Design, Cambridge, UK) was used to display and analyze digitized RSNA.

**Extracellular single-unit recording**

With the skull leveled between bregma and lambda, a craniotomy was performed to gain access to the PVN. Microelectrodes were pulled from borosilicate glass capillaries (1.2 mm OD, 0.86 mm ID) and filled with 0.15 M NaCl containing 2% Chicago sky blue dye. Electrode resistance was measured in vivo and averaged 25–35 MΩ. Electrodes were lowered vertically into the PVN at the following stereotaxic coordinates (in mm): −1.6 to −2.3 mm from bregma, 0.2–0.8 mm lateral to midline, and 7.2–8.0 mm ventral to the brain surface (Chen and Toney 2001, 2003a,b; Chen et al. 2003). The PVN was probed for single-unit activity by advancing the recording electrode in 2-μm vertical steps with a piezoelectric micropositioner (EXFO-Burleigh, Quebec, Quebec, Canada). Recordings were made using an Axoclamp 2B amplifier in bridge mode. Signals were passed to a differential AC amplifier (Grass, P15D) and band-pass filtered between 0.3 and 3.0 kHz. Processed signals were led to an audio monitor, oscilloscope, and window discriminator. Each spike that crossed the window discriminator level (set to exclude background noise) generated a single TTL pulse. Pulses were used to construct rate-meter records and time histograms of cell discharge and to generate spike-triggered averages of RSNA. Data were digitized (1.0–5.0 kHz), and analyzed using Spike2 software (v5.16, Cambridge Electronic Design, Cambridge, UK).

**Antidromic stimulation**

To identify RVLM projecting PVN neurons in vivo, antidromic activation was used. A burr hole was made in the occipital bone and a concentric bipolar stimulating electrode (500 μm OD, tip tapered at 60°; FHC, Bowdoin, ME) was positioned in the RVLM at the following stereotaxic coordinates (in mm): −12.5 to −12.9 from bregma, 1.6–1.9 lateral to midline, and 8.5–9.2 ventral to the surface of cerebellum. The final position of the electrode was the site from which a significant increase of RSNA and a 15- to 20-mmHg increase of mean ABP (MAP) were recorded in response to microinjection of l-glutamate (0.1 nmol in 50 nl).

To identify PVN neurons with axons projecting to the spinal cord, a dorsal laminectomy was performed to expose the C2 spinal segment and an array of three monopolar electrodes, each separated by ~0.5 mm, was positioned to span the entire dorsolateral funiculus on the side ipsilateral to the PVN recording site (the indifferent pole of the electrode was attached to the trachealis muscle). The electrode assembly was inserted to a depth of 0.5–0.7 mm.

Because the focus of these experiments was on RVLM projecting PVN neurons in vivo, standard tests (Chen and Toney 2003a; Lipski 1981; Stocker and Toney 2005, 2007) were first used to assess the antidromic nature of spikes evoked by stimulating the ipsilateral RVLM. Units were considered to be antidromically activated when each stimulus pulse (0.1 ms, 1 Hz, 100–850 μA) evoked a single constant-latency spike at a discrete stimulus threshold (i.e., all-or-noresponse). Antidromicity was confirmed in many cases when a single spike was recorded for each pulse in a high-frequency stimulus train (i.e., 200–300 Hz, 3 pulses). For spontaneously active neurons, evoked spikes were tested for cancellation by collision with spontaneous action potentials (i.e., the collision test). Once an antidromic response was recorded, RVLM stimulus intensity was increased
gradually above threshold to determine if a discontinuous decrease (>1 ms) in the antidromic onset latency could be detected. Such antidromic latency “jumps” occur because of local axon branching and indicate the presence of a terminal arbor in the vicinity of the RVLM stimulating electrode.

Tests were then performed to determine if an antidromic response could be recorded from the same cell by stimulating the spinal cord. To guard against classification errors, large-amplitude stimuli (up to \( \sim 1.5 \) mA) were applied to each electrode in the spinal array to minimize the possibility that spinal axons, although present, would fail to be activated. Neurons that had an antidromic response to stimulation of the ipsilateral RVLM but not the IML were initially classified as PVN-RVLM neurons. Those that had an antidromic response to stimulation of the RVLM and spinal cord were classified as having branched axons targeting both the RVLM and IML. These were designated PVN-RVLM/IML neurons. Reciprocal collision testing (Amri et al. 1990; Klenfuss et al. 1987; Lipski 1981) was performed in some cases to confirm correct classification of PVN-RVLM/IML neurons. Neurons that had maximum reciprocal collision intervals exceeding the collision interval predicted for a single unbranched axon segment were formally classified as PVN-RVLM/IML neurons.

**Sympathetic-related discharge**

Spoke-triggered averaging was used as previously described (Barman 1990; Barman and Gebber 1982; Chen and Toney 2003a; Stocker and Toney 2005, 2007) to determine the temporal correlation between spontaneous action potentials and postganglionic RSA NA. For each recorded neuron, the spike-triggered average of simultaneously recorded RSA NA was compared with an average constructed from the same RSA NA data but triggered by randomly generated TTL pulses. The number and frequency of TTL triggers were equal to the number and frequency of spontaneous spikes used to generate each corresponding spike-triggered RSA NA average.

**Cardiac-related discharge**

To determine the temporal relationship between ongoing PVN neuronal discharge and the cardiac cycle, ECG R-wave triggered time histogram analysis was used as previously described (Chen and Toney 2003a; Stocker and Toney 2005, 2007). Correlated discharge occurred after the R-wave trigger, which was set to time (bin) zero. A post R-wave increase or decrease in activity \( \geq 20\% \) was considered to indicate cardiac-related activity.

**Barosensitivity**

To test the response of individual PVN-RVLM and PVN-RVLM/IML neurons to changes in ABP, we recorded discharge responses to increasing and decreasing ABP with phenylephrine (5–10 \( \mu \)g iv) and sodium nitroprusside (10–20 \( \mu \)g iv), respectively.

**Histology**

The location of each recorded PVN neuron was marked by iontophoretic deposition of Chicago sky blue dye (\( \sim 5 \mu A, 10 \) min), which produced a spheroid spot \( \sim 50–75 \) \( \mu \)m in diameter. After each experiment the brain was removed and placed in 4% paraformaldehyde for several days. The hypothalamus containing the PVN was then cut into 40-\( \mu \)m-thick coronal sections. The site of each unit recording was determined under brightfield microscopy and plotted on plates of a rat brain atlas (Paxinos 1998) that corresponded to the correct rostro-caudal plane.

**Data analysis**

Spontaneous firing frequency of each recorded neuron was determined from \( \geq 60 \) s of data from the rate-meter record (bin size: 1.0 s). Axonal conduction velocity (CV; \( \text{ms}^{-1} \)) was calculated as the ratio of the distance between the recording and stimulation sites and the antidromic onset latency (axonal refractory period was not taken into account). To evaluate reciprocal collision test data for PVN-RVLM/IML neurons, the collision interval predicted for an unbranched axon segment was estimated by dividing the linear distance between the RVLM and IML stimulation sites by the average axonal CV. The axon of a given neuron was deemed to collateralize and innervate the RVLM and spinal cord when collision of RVLM- and spinal-cord-evoked antidromic spikes occurred at stimulus intervals greater than that estimated for an unbranched axon.

To evaluate barosensitivity of unit discharge, firing rate achieved following each increase or decrease of ABP was compared with 30 s of baseline discharge recorded just prior to the change of ABP. Response data were compared using the paired Student’s \( t \)-test. Changes in firing rate in response to increases and decreases of ABP were compared across groups of neurons using the unpaired Student’s \( t \)-test.

To assess cardiac cycle-related activity from ECG R-wave triggered time histograms, we first constructed an interval histogram of R-wave trigger events. From this we determine the average duration (in ms) of the cardiac cycle. Spike data over the same post R-wave period in time histograms were then analyzed. For this purpose, spike data were placed in bins (10 ms), and those bins with spike values that exceeded by \( \geq 20\% \) the average spikes/bin for the entire cardiac cycle were considered to represent cardiac cycle-related activity. For statistical comparison using the paired Student’s \( t \)-test, spike data from time histograms were expressed as spikes/s.

To determine the temporal correlation between cell discharge and RSA NA, the peak amplitude of RSA NA in spike triggered averages was compared with the voltage at the same latency in randomly triggered “dummy” averages (see preceding text). Triggered SSA NA averages were constructed from RSA NA recorded from 500 ms before to 500 ms after the occurrence of each trigger event (cell discharge or random TTL pulse). The occurrence of each trigger event was defined as time 0 in the assembled average. Correlated activity was taken as the posttrigger data segment that exceeded the mean value of the corresponding dummy average by \( \geq 3\)-fold over the same posttrigger time interval. Statistical differences between these values were compared using the paired Student’s \( t \)-test.

To compare between-group (PVN-RVLM vs. PVN-RVLM/IML) differences in recorded variables and unit responses, a nonparametric Wilcoxon test was used. The proportion of PVN-RVLM and PVN-RVLM/IML neurons that exhibited sympathetic- and/or cardiac-related spontaneous discharge or a response to changes in baroreceptor input were compared using Fisher’s exact test. Mean differences were considered significant at a critical value of \( P < 0.05 \). Data are report as the means \( \pm \) SE.

**RESULTS**

**RVLM stimulation**

The location of RVLM was determined in each animal as the site from which microinjection of L-glutamate (Fig. 1A) elicited large-amplitude increases of RSA NA and ABP (middle). Increases of mean ABP (MAP) \( \geq 15 \) mmHg were considered to identify the RVLM. The microinjection pipette was withdrawn and replaced with an electrode for antidromic stimulation. From this location, graded intensities of high-frequency electrical stimulation evoked short-latency, graded increases of ABP (Fig. 1B).
Identification of PVN-RVLM neurons

PVN-RVLM neurons were identified by their antidromic response to stimulation of the ipsilateral RVLM and by their lack of antidromic response to stimulation of the ipsilateral spinal cord. An example of antidromic identification of a single PVN-RVLM neuron is shown in Fig. 2. In A, five superimposed sweeps of data are shown to illustrate that each RVLM stimulus evoked a single spike with invariant onset latency (top). This neuron also responded to high-frequency RVLM stimulation – each of three stimulus pulses separated by 3 ms evoked a single spike (bottom). B shows that even large-amplitude (≈1.5 mA) stimulation of the C2 spinal segment failed to evoke a response from this neuron. C shows the timed collision test for this neuron. Note that antidromic spikes evoked from the RVLM (left, top) and the C2 spinal segment (right, top) each underwent cancellation by spontaneous action potentials. D illustrates the reciprocal collision test for this neuron. When spinal and RVLM stimuli were separated by 39 ms, an antidromic response to each stimulus was recorded. When the interval between stimuli was reduced to 38 ms, the response to RVLM stimulation was cancelled by collision with the C2-evoked antidromic spike. This collision interval exceeded that pre-

Identification of PVN-RVLM/IML neurons

Neurons exhibiting an antidromic response to stimulation of the ipsilateral RVLM that were subsequently observed to also have an antidromic response to stimulation of the spinal cord were classified as PVN-RVLM/IML neurons. Figure 3 illustrates identification of one such neuron. In A, five superimposed data sweeps illustrate that each RVLM stimulus evoked a single spike with invariant onset latency (top). This neuron also responded to high-frequency RVLM stimulation – each of three stimulus pulses separated by 3 ms evoked a single spike (bottom). B shows that even large-amplitude (≈1.5 mA) stimulation of the C2 spinal segment failed to evoke a response from this neuron. C shows the timed collision test for this neuron. Note that the RVLM-evoked antidromic spike (top and bottom) was canceled by collision with a spontaneous action potential (middle). D illustrates the antidromic latency “jump” test. As the intensity of RVLM stimulation was gradually increased, the antidromic onset latency suddenly decreased by ≈2.5 ms. This indicates that a faster conducting (larger diameter) axon branch was activated in the vicinity of the RVLM stimulating electrode and suggests that the axon formed a terminal arbor in the RVLM.

Among 65 identified PVN-RVLM neurons, antidromic responses to RVLM stimulation occurred at an average stimulus threshold of 626 ± 70 μA. Among these neurons, 32 (49%) were spontaneously active and antidromic spikes were observed to undergo cancellation by collision with spontaneous action potentials. While gradually increasing suprathreshold stimulus intensity, antidromic latency “jumps” were observed in 23 of 44 neurons tested (52%). The average latency reduction was 2.4 ± 0.6 ms and occurred as stimulus intensity was increased over a range of 15 ± 2.6 μA.

FIG. 1. Determination of the antidromic stimulation site in rostral ventrolateral medulla (RVLM). A: data traces of renal sympathetic nerve activity (RSNA, top) and arterial blood pressure (ABP, bottom) responses to L-glutamate microinjection (0.1 nmol in 50 nl) at specific stereotaxic coordinates within the RVLM region. In this animal, the largest responses (→, middle) occurred at stereotaxic coordinates of 12.0 mm posterior to bregma (P), 1.8 mm lateral to midline (L), and 9.0 mm ventral to the brain surface (V). B: In the same animal, graded RSNA and ABP responses were evoked by graded electrical stimulation (≈100 μA, 1 ms, 50 Hz, 5 s) of RVLM at the coordinates indicated in A (middle).

Identification of PVN-RVLM/IML neurons

Neurons exhibiting an antidromic response to stimulation of the ipsilateral RVLM that were subsequently observed to also have an antidromic response to stimulation of the spinal cord were classified as PVN-RVLM/IML neurons. Figure 3 illustrates identification of one such neuron. In A, five superimposed data sweeps illustrate that each RVLM stimulus evoked a single spike with little onset latency variation. B shows timed collision tests for this neuron. Note that antidromic spikes evoked from the RVLM (left, top) and the C2 spinal segment (right, top) each underwent cancellation by collision with spontaneous action potentials. C illustrates the reciprocal collision test for this neuron. When spinal and RVLM stimuli were separated by 39 ms, an antidromic response to each stimulus was recorded. When the interval between stimuli was reduced to 38 ms, the response to RVLM stimulation was cancelled by collision with the C2-evoked antidromic spike. This collision interval exceeded that pre-
dicted for an unbranched axon (3 ms) by 35 ms. Therefore this neuron was considered to have a branched axon that targeted both the RVLM and spinal cord.

Among 29 identified PVN-RVLM/IML neurons, 17 (59%) were spontaneously active and antidromic spikes evoked by RVLM and C_2 spinal stimulation underwent timed collision with spontaneous action potentials. Reciprocal collisions were observed in 20 (~69%) PVN-RVLM/IML neurons. The maximum reciprocal collision interval averaged 21 ± 2.1 ms, which was considerably longer than the collision interval predicted for unbranched axons (12 ± 1.5 ms). The large discrepancy between predicted and actually reciprocal collision intervals suggests that axons of PVN-RVLM/IML neurons collateralized mid-way between the PVN and the RVLM/spinal C_2 segment (assuming each axon branch had a similar average CV).

The presence of axon collaterals to the RVLM and IML was affirmed in 13 of 20 neurons that underwent reciprocal collision (65%) by demonstrating an abrupt decrease in antidromic onset latency when RVLM stimulus intensity was gradually increased over a range of 32 ± 10 μA. The average latency jump was of 3.9 ± 1 ms. Recordings were lost prior to competing reciprocal collision testing in nine other putative PVN-RVLM/IML neurons. In these cells, however, an antidromic latency jump of 3.3 ± 1.1 ms was observed when RVLM stimulus intensity was increased from ~700 to 710 μA. Such “latency jumps” indicate local axon branching and are consistent with terminal arborization near the stimulation site. *, stimulus artifacts; ●, antidromic spikes; ○, spontaneous action potentials.

![FIG. 2. Antidromic identification of paraventricular nucleus (PVN)-RVLM neurons. A: 5 superimposed sweeps of cell activity showing constant latency (39 ms) antidromic spikes evoked by single pulse stimulation of RVLM (top). All-or-none responses were consistently elicited above the stimulus threshold (320 μA). Five superimposed sweeps of data show that this neuron consistently responded with a single spike of invariant onset latency to each of 3 RVLM stimuli delivered in a high-frequency train (333 Hz; bottom). B: in contrast to RVLM stimulation, even large-amplitude stimulation (≥1.5 mA) of the C_2 spinal segment failed to evoke antidromic spikes. C: for the same cell as in A, RVLM stimulation evoked an antidromic spike (top) that was cancelled (→, middle) by collision with a spontaneous action potential. Note that collision occurred when the interval between the spontaneous action potential and RVLM stimulus was reduced to ◆38 ms—the critical collision period. D: 2 superimposed sweeps of cell activity show that an abrupt decrease (2.5 ms) of the antidromic onset latency occurred (→) as RVLM stimulation intensity was gradually increased from ~700 to 710 μA. Such “latency jumps” indicate local axon branching and are consistent with terminal arborization near the stimulation site. *, stimulus artifacts; ●, antidromic spikes; ○, spontaneous action potentials.](http://jn.physiology.org/attachment.php/103/Jan2010/paper02f.jpg)
and in near equal proportions in the anterior subnucleus/dorsal cap (PVN-RVL: 41%; PVN-RVL/IML: 45%) and the medial and ventral subnuclei (PVN-RVL: 40%; PVN-RVL/IML: 34%). A smaller proportion of neurons in each group was located in the posterior subnucleus (PVN-RVL: 19%; PVN-RVL/IML: 21%). Spontaneously active and quiescent PVN-RVL and PVN-RVL/IML neurons were present in nearly equal proportions and, again, were similarly distributed (Fig. 4).

Properties of PVN-RVL and PVN-RVL/IML neurons

As noted in the preceding text, recording procedures were designed to select for neurons projecting to the RVLM. These were then further defined as having an IML projection or not. Among identified neurons, 69% (n = 65/94) had axons projecting to the RVLM, and the remaining 31% (n = 29/94) had collateral axons innervating both the RVLM and spinal cord. Axonal CV among PVN-RVL (0.34 ± 0.03 m/s) and PVN-RVL/IML (0.41 ± 0.04 m/s) neurons was similar (Fig. 5). Among PVN-RVL/IML neurons, PVN-to-RVL and PVN-to-spinal segments had nearly identical axonal CV. Collectively, these data indicate that neurons in each group had small diameter, unmyelinated axons. Consistent with this conclusion, the threshold stimulus intensity for antidromic responses evoked from the RVLM and C2 spinal segment (611 ± 46 µA) did not differ across groups. Spontaneous activity was recorded among 32 of 65 PVN-RVL neurons (49%) and 17 of 29 PVN-RVL/IML neurons (59%). The frequency of discharge did not differ across groups. Spontaneous activity was recorded among 32 of 65 PVN-RVL neurons (49%) and 17 of 29 PVN-RVL/IML neurons (59%). The frequency of discharge did not differ across groups. Spontaneous activity was recorded among 32 of 65 PVN-RVL neurons (49%) and 17 of 29 PVN-RVL/IML neurons (59%).

Cardiac- and sympathetic-related discharge

The temporal relationship between spontaneous cell firing and SNA was assessed by spike-triggered averaging of RSNA. In Fig. 6A, the correlation between the discharge of a PVN-RVL neuron (left) and a PVN-RVL/IML neuron (right) is shown. Note that in each, the peak of correlated RSNA occurred between ~150 and ~220 ms after spike occurrence (time 0). Bottom traces in each panel demonstrate that the same RSNA data showed no correlation when averages were triggered by randomly occurring events (TTL pulse → fRSNA). Overall, correlation was detected in similar proportions of PVN-RVL (12 of 21, 57%) and PVN-RVL/IML (6 of 9, 67%) neurons. The number of spikes used to construct RSNA averages for PVN-RVL neurons (784 ± 132) and PVN-RVL/IML neurons (614 ± 41) was not statistically different. Across groups of neurons, the latency-to-peak correlation (PVN-RVL: 137 ± 9 ms, PVN-RVL/IML: 143 ± 30 ms) and the duration of correlated RSNA (PVN-RVL: 293 ± 13 ms, PVN-RVL/IML: 301 ± 55 ms) were also similar. The same RSNA data as used for PVN spike triggered averages were also used to construct ECG R-wave-triggered averages. Figure 6B shows that there was a consistently strong correlation between RSNA and cardiac cycle rhythm in these anesthetized, arterial baroreceptor intact rats.

To assess whether cell firing was also cardiac rhythmic, ECG R-wave triggered time histograms of unit activity were
constructed. Figure 7A and B shows ECG (top) and ABP (middle) data across three representative cardiac cycles from two different rats. Simultaneously recorded cell discharge was used to construct R-wave-triggered time histograms of unit discharge (bottom) for a PVN-RVLM neuron (Fig. 7A) and a PVN-RVLM/IML neuron (B). Summary data indicate that discharge was correlated with the ECG R-wave in 25 of 32 (78%) PVN-RVLM neurons and 10 of 17 (59%) PVN-RVLM/IML neurons. Time histograms of discharge for cells in each group were constructed from similar numbers of R-wave events (899 ± 117) and unit discharges (593 ± 96). Collectively, these findings indicate that some PVN-RVLM and PVN-RVLM/IML neurons are capable of participating in the ongoing (i.e., tonic) control of sympathetic outflow.

Barosensitive discharge

The presence of cardiac cycle-related spontaneous firing suggests that discharge might be entrained by arterial baroreceptor inputs that are active at the prevailing level of ABP. To further assess the influence of baroreceptor input on the discharge of PVN-RVLM and PVN-RVLM/IML neurons, changes in dis-
FIG. 5. Axonal conduction velocities of PVN-RVLM and PVN-RVLM/IML neurons. Consistent with having fine diameter unmyelinated fibers, axonal conduction velocities of PVN-RVLM (■; n = 65) and PVN-RVLM/IML neurons (●; n = 29) were consistently 2.0 m/s, with most being 0.5 m/s.

FIG. 6. Sympathetic-related spontaneous discharge of PVN neurons. A: spike-triggered RSNA averages (top, unit—of RSNA) constructed from spontaneous spikes recorded from a PVN-RVLM neuron (left) and a PVN-RVLM/IML neuron (right). RSNA correlated with discharge recorded from the PVN-RVLM and PVN-RVLM/IML neurons occurred at peak latencies of 179 and 206 ms, respectively. Bottom traces (TTL pulse—of RSNA) are “dummy” RSNA averages constructed from the same RSNA data used for constructing the corresponding spike-triggered averages (top). The number and average frequency of square-wave (TTL) pulses used to construct dummy averages were equal to the number of spikes and the frequency of cell activity used for constructing the corresponding PVN-RVLM (571 spikes at 2.2 Hz) and PVN-RVLM/IML (729 spikes at 2.1 Hz) neuron spike-triggered averages (top). Note that dummy averages show no correlated RSNA, indicating that the correlation between cell discharge and RSNA in the top traces is not due to phase-locking of harmonic frequencies. B: traces show ECG R-wave-triggered RSNA averages. Note that in each case, RSNA was tightly coupled to the frequency of the cardiac cycle. RSNA data used for constructing R-wave triggered averages was the same as used for each spike-triggered RSNA average in A.
In contrast to neurons with cardiac-related discharge, those that were quiescent (PVN-RVLM, \(n = 9\); PVN-RVLM/IML, \(n = 3\)) or lacked cardiac rhythmic discharge (PVN-RVLM, \(n = 4\); PVN-RVLM/IML, \(n = 4\)) consistently failed to respond to either PE or SNP (data not shown).

**DISCUSSION**

Our goals for this study were to record the in vivo discharge of PVN neurons with axons projecting to the RVLM and to test the hypothesis that they have patterns of spontaneous activity related to cardiovascular function. Neurons were classified as having unbranched axons to the RVLM (PVN-RVLM) or branched axons targeting the RVLM and spinal IML (PVN-RVLM/IML). We determined that PVN-RVLM and PVN-RVLM/IML neurons had similar axonal conduction velocities, and a similar fraction of neurons in each group had slow spontaneous discharge that was temporally correlated with the cardiac cycle and RSNA. The response of PVN-RVLM and PVN-RVLM/IML neurons to acute increases and decreases of arterial baroreceptor input was also similar. Our findings suggest that these two neuronal populations serve similar functions related to the generation and baroreflex modulation of sympathetic vasomotor tone.

**Technical considerations**

Antidromic activation was used to RVLM-projecting PVN neurons. Because PVN neurons terminate over a large rostral-to-caudal portion of the ventrolateral medulla, including the caudal ventrolateral medulla (CVLM) (Hardy 2001; Krukoff et al. 1997), we were careful to place antidromic stimulating electrodes in the RVLM at sites from which large-amplitude increases of ABP were elicited by injection of l-glutamate (see Fig. 1A). This is important because activation of the PVN has been shown to directly influence the discharge of barosensitive neurons in both the RVLM (Yang and Coote 1998) and CVLM (Yang and Coote 1999). Graded intensities of stimulation were used to insure activation of axons in the RVLM and spinal dorsolateral funiculus. By also using a three-electrode array to stimulate the upper spinal cord, our design increased the likelihood of recording antidromic responses and thereby reduced the probability of making a classification error. Use of antidromic latency “jump” tests to determine if axons underwent terminal branching within the RVLM (Klemfuss et al. 1987; Lipski 1981) and reciprocal collision tests to identify branched axons innervating both the RVLM and spinal cord (Lipski 1981) further guarded against classification errors.

**PVN-RVLM and PVN-RVLM/IML neurons and support of sympathetic vasomotor tone**

Acute inhibition of PVN neuronal activity has been reported to reduce ongoing RSNA (Akine et al. 2003; Stocker et al. 2004b, 2005), lumbar SNA (Allen 2002; Stocker et al. 2005), and ABP (Akine et al. 2003; Allen 2002; Freeman and Brooks 2007; Stocker et al. 2004b, 2005) in anesthetized rats. Moreover, reductions of SNA in response to PVN inhibition or blockade of excitatory inputs are more pronounced in water-deprived (Freeman and Brooks 2007; Stocker et al. 2004b, 2005) and hypertensive (Allen 2002; Li and Pan 2007) rats. These findings indicate that PVN neuronal activity contributes both to the maintenance of resting SNA and to the elevation of SNA under physiologic and disease conditions.
In a previous study, we reported that two functionally distinct groups of paraventriculo-spinal (PVN-IML) neurons can be identified based on having distinct electrophysiological and discharge properties (Chen and Toney 2003a)—group I neurons have relatively fast conducting spinal axons and group II neurons have slower conducting axons like those of PVN-IML neurons described by others (Lovick and Coote 1988a,b).

Based on the patterning of their spontaneous discharge, group I neurons appear to contribute to the generation and baroreflex regulation of ongoing sympathetic vasomotor tone. In contrast, group II neurons more likely contribute to regulation of non-vasomotor components of sympathetic outflow (Chen and Toney 2003a).

In the present study, PVN-RVLM and PVN-RVLM/IML neurons both had sympathetic- and cardiac-rhythmic spontaneous discharge that was modulated by arterial baroreceptor input. These features closely resemble group I PVN-IML neurons from our previous study and suggest that RVLM-projecting PVN neurons might also contribute to the generation and regulation of vasomotor tone. However, RVLM-projecting PVN neurons also differ from group I PVN-IML neurons in that they have relatively slow axonal CV. This feature more...
closely resembles group II PVN-IML neurons from our previous study and studies by the Coote laboratory (Lovick and Coote 1988a,b). Thus RVLM-projecting PVN neurons appear to have characteristics unlike either group I or group II PVN-IML neurons.

Because anatomical studies have shown that 15–20% of RVLM-projecting PVN neurons have branched axons that also target the IML (Pyner and Coote 2000; Shafton et al. 1998; Stocker et al. 2006), one would expect that a reasonably large sample of cells antidromically activated from the spinal cord would include at least some neurons with branched axons that also innervate the RVLM. This being the case, one would expect that some group I or group II neurons from our previous study should have had properties nearly identical to those of PVN-RVLM/IML neurons in the present study. As noted in the preceding text, however, this was not observed. The reason for this apparent discrepancy is not clear at present, one possibility is that the percentage of axons in the PVN-spinal pathway that arise from branching PVN-RVLM/IML neurons may be exceeding small. If this is the case, then by chance alone our previous study may have failed to identify any PVN-RVLM/IML neurons. Another factor that could have contributed to the failure of our previous study to identify PVN-IML neurons with properties resembling PVN-RVLM/IML neurons is that our earlier work was performed using high resistance recording electrodes (30–50 MΩ) and triangular “pulses” to stimulate the spinal cord. These methods, separately or combined, might have prevented detection of neurons with behaviors resembling those in the present study.

Baroreflex modulation of SNA by RVLM-projecting PVN neurons

In earlier work, ~50% of PVN-IML neurons were reported to receive inhibitory input from arterial baroreceptors (Bains and Ferguson 1995; Chen and Toney 2003a; Lovick and Coote 1988a,b). Here baroreceptor inputs targeted a similar proportion of PVN-RVLM and PVN-RVLM/IML neurons and caused abrupt silencing of neuronal discharge. In this regard, RVLM-projecting PVN neurons resemble RVLM vasomotor neurons (Guyenet 2006). Although baroreceptor inhibition of RVLM neuronal firing is known to be mediated by GABAergic inputs from the CVLM (Guyenet 2006), the neurotransmitter(s) mediating inhibition of RVLM-projecting PVN neurons is not known. Because discharge inhibition was abrupt and spontaneous discharge of most barosensitive PVN-RVLM and PVN-RVLM/IML neurons was also cardiac rhythmic, a fast synaptic transmitter such as GABA is likely to be involved.

If GABA were the principle mediator of baroreceptor inhibition of RVLM-projecting PVN neurons, then a recent study by Stern and co-workers is of interest (Park et al. 2009). They showed that in vitro discharge of PVN-RVLM neurons is suppressed by a tonic GABA current. Whereas one would not expect a tonic GABA current to underlie cardiac rhythmic discharge (i.e., baroreceptor entrainment) or abrupt baroreceptor inhibition of discharge, a tonic GABA current could help to set the overall level of activity/excitability of PVN sympathetic-regulatory neurons. If extracellular levels of GABA in the PVN rise and fall in proportion to the level of arterial baroreceptor input, then a reduction of GABA availability could possibly contribute to the gradual and more sustained increases of discharge observed in some neurons when ABP was reduced with SNP (see Fig. 8B, right).

An alternative, but perhaps less likely, mediator of baroreceptor inhibition of RVLM-projecting PVN neurons is norepinephrine. Not only do CVLM GABAergic neurons project to the RVLM, but they also target nearby A1 noradrenergic neurons as well (Chan and Sawchenko 1998). The latter have been reported to facilitate the discharge of nearby neurosecretory neurons in the PVN (Day et al. 1984), but whether A1 neurons are uniformly inhibited by baroreceptor inputs and ascend to directly synapse on RVLM-projecting PVN neurons is not known, although an A1-to-PVN pathway has been reported (Caverson and Ciriello 1984).

Concluding remarks

The present study used in vivo electrophysiological methods to identify PVN neurons with axons projecting to the RVLM, including a population of neurons with axon collaterals that innervate the spinal cord. Consistent with evidence that the PVN is strongly inhibited by GABAergic inputs in vivo (Li et al. 2006), ~50% of PVN-RVLM and PVN-RVLM/IML neurons lacked spontaneous discharge. Remaining neurons had slow spontaneous discharge that was barosensitive and temporally correlated with the cardiac cycle and RSNA. These activity patterns suggest that RVLM-projecting PVN neurons are capable of contributing to both the generation of basal SNA as well as its baroreflex modulation.

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