Nervous System

Modulation of Bulbospinal Rostral Ventral Lateral Medulla Neurons by Hypoxia/Hypercapnia but Not Medullary Respiratory Activity

Carie R. Boychuk, Amanda L. Woerman, David Mendelowitz

Abstract—Although sympathetic vasomotor discharge has respiratory modulation, the site(s) responsible for this cardiorespiratory interaction is unknown. One likely source for this coupling is the rostral ventral lateral medulla (RVLM), where preganglionic sympathetic neurons originate in close apposition to respiratory neurons. The current study tested the hypothesis that RVLM bulbospinal neurons are modulated by medullary respiratory network activity using whole-cell patch-clamp electrophysiological recordings of RVLM neurons while simultaneously recording fictive respiratory bursting activity from the hypoglossal rootlet. Additionally, we examined whether challenges to cardiorespiratory function, mainly hypoxia/hypercapnia, alter the activity of bulbospinal neurons and, secondarily, whether changes in synaptic input mediate these responses. Surprisingly, our results indicate that inspiratory-related activity did not modulate glutamatergic, γ-aminobutyric acid-ergic, or glycinegenic synaptic events or spontaneous action potential firing in these RVLM neurons. However, hypoxia/hypercapnia reversibly decreased the frequency of γ-aminobutyric acid and glycine inhibitory postsynaptic currents. Glycinergic inhibitory postsynaptic current frequency was depressed from the fifth through the 10th minute, whereas the depression of γ-aminobutyric acid-ergic events became significant only at the 10th minute of hypoxia/hypercapnia. On the basis of spontaneous firing activity, there were 2 populations of RVLM bulbospinal neurons. The firing frequency of low-discharging RVLM neurons was facilitated by hypoxia/hypercapnia, and this increase depended on reduced inhibitory neurotransmission. The firing frequency in RVLM neurons with high-discharge rates was inhibited, independent of synaptic input, by hypoxia/hypercapnia. This article demonstrates that sympathetic-respiratory coupling is not active in the neonatal brain stem slice, and reductions in inhibitory neurotransmission to low spontaneously active bulbospinal RVLM neurons are responsible for hypoxia/hypercapnia-elicited increases in activity. (Hypertension. 2012;60:1491-1497.)

Key Words: rostral ventral lateral medulla □ sympathetic □ hypoxia □ hypercapnia □ respiration □ bulbospinal □ cardiorespiratory

Neurons within the rostral ventral lateral medulla (RVLM) monosynaptically project to the intermediolateral column of the spinal cord and provide the primary tonic excitatory drive to spinal sympathetic vasomotor neurons, which regulate and maintain blood pressure. Sympathetic vasomotor neurons discharge in phase with respiration, a phenomenon known as sympathetic-respiratory coupling.1,2 Although the exact patterning varies, nearly all sympathetic discharge increases during inspiration and reaches a peak either during inspiration or the postinhibitory/early excitatory period.2,3 This coupling can become abnormal during diseases, such as sleep apnea or hypertension,4 and this altered activity may contribute to cardiorespiratory disease and sympathetic dysfuction. However, the neural mechanism(s) responsible for the entrainment of sympathetic activity with respiration remains contentious. Although peripheral pulmonary stretch receptor afferents may contribute, sympathetic-respiratory-related activity persists in lung transplant patients5 and after vagotomy.6,7 An alternative hypothesis suggests that sympathetic-respiratory coupling occurs via central respiratory modulation of brain stem bulbospinal RVLM neurons. In support of a brain stem–mediated mechanism, the pressor-related neurons of the RVLM themselves possess respiratory-related modulation8 and are in close proximity to medullary respiratory groups.9,10 Therefore, it is possible that RVLM bulbospinal neurons receive respiratory-related synaptic inputs that serve to entrain sympathetic vasomotor neurons with respiration.11

In addition to normal respiratory alterations, challenges to the cardiorespiratory system, such as hypoxia and hypercapnia, evoke large increases in sympathetic activity. Hypoxia and hypercapnia reversibly increase the firing frequency in RVLM bulbospinal neurons after a brief exposure (20–40 s)12; however, the mechanisms responsible for this increase are unknown. Previous work has demonstrated that the respiratory modulation of parasympathetic cardioinhibitory neurons is mediated by increases in inhibitory neurotransmission to cardiac vagal...
neurons within the nucleus ambiguus. Furthermore, these synaptic pathways are biphase transitionally modulated during hypoxia and combined hypoxia/hypercapnia and likely mediate the biphase changes in heart rate that occur during these cardiopulmonary challenges. The goal of this study was to test whether similar brain stem respiratory network pathways to those that drive activity in cardiac vagal neurons can generate respiratory-related coupling in RVLM bulbospinal neurons. This study also tested the hypothesis that bulbospinal RVLM neurons alter their activity in response to hypoxia/hypercapnia and tested whether synaptic neurotransmission mediates these responses to this challenge.

**Material and Methods**

Experiments were performed on male Sprague Dawley rats (Hilltop Lab Animals Inc, Scottsdale, PA) housed in the George Washington University animal care facility. All procedures were approved by the George Washington University Institutional Animal Care and Use Committee. Rats were maintained under standard conditions (12:12 hours light:dark cycle) with free access to food and water.

Pups at postnatal days 3 to 5 were anesthetized with hypothermia. The upper thoracic spinal cord was exposed. The retrograde tracer cholera toxin subunit B conjugated with an Alexa Fluor 555 (C22843; Invitrogen, Carlsbad, CA) was biilaterally injected (50–60 nL per injection; 1% in artificial cerebrospinal fluid) into the spinal cord ≈0.25 mm from the dorsal surface. After surgery, buprenorphine was administered, and pups were monitored for 30 minutes and every 20 minutes thereafter until ambulatory.

After 2 to 4 days of recovery, the animals were overdose with isoflurane and euthanized by cervical dislocation. Tissue was obtained from the end of the spinal cord, and combined hypoxia/hypercapnia and likely mediate the synaptic pathways are biphasically modulated during hypoxia/hypercapnia. Tukeys multiple comparisons post hoc tests were used to determine statistically significant differences (P<0.05). Student t tests were used to determine the effect of inhibitory blockade on spontaneous firing frequencies. Repeated-measures 1-way ANOVAs were used to determine statistical significance for the overall effect of hypoxia/hypercapnia. Tukeys multiple comparisons post hoc were used when appropriate to determine statistically significant differences (P<0.05).

**Results**

Bulbospinal neurons retrogradely labeled from the spinal cord were localized to a discrete region of the RVLM. Colocalization of these neurons with tyrosine hydroxylase, as shown in Figure S1, illustrates that these neurons are located in the C1 region, and most, but not all, of the bulbospinal neurons are tyrosine hydroxylase positive, consistent with the work of others.16,17 Recordings were limited to those bulbospinal neurons in this discrete, spatially limited region of the RVLM.

**RVLM Bulbospinal Neurons Did Not Receive Inspiratory-Related Synaptic Neurotransmission**

Although bulbospinal RVLM neurons received spontaneous GABAergic, glycinergic, and glutamatergic synaptic events, none of the synaptic inputs in bulbospinal RVLM neurons (glutamatergic [n=7], GABAergic [n=7], or glycinergic [n=7]) possessed any significant inspiratory-related activity (Figure 1). Additionally, during hypoxic/hypercapnic challenge, no significant respiratory-related synaptic input to bulbospinal neurons was observed, regardless of the neurotransmitter (glutamatergic, GABAergic, or glycinergic).

Examination of synaptic inputs for the duration of the hypoxic/hypercapnic exposure revealed a significant decrease in the frequency of inhibitory (Figure 2), but not excitatory, neurotransmission. Glutamatergic inputs to bulbospinal neurons of the RVLM did not change at any time during hypoxia/hypercapnia in either frequency (P>0.9) or amplitude (P=0.2). However, inhibitory postsynaptic currents (IPSCs) were selectively diminished, and the time courses of the evoked changes in GABAergic and glycinergic neurotransmission were different. More specifically, hypoxia/hypercapnia significantly (P<0.05) depressed GABAergic neurotransmission to bulbospinal RVLM neurons at the 10th minute of exposure (1±0.3 Hz) when compared with preperiod conditions (2.7±0.8 Hz), but amplitude was unchanged at any time during the exposure (P=0.3).

Glycinergic neurotransmission to bulbospinal RVLM neurons was also depressed by hypoxia/hypercapnia; however, this decrease occurred sooner than the changes in GABAergic input (n=6; Figure 2, bottom trace). Unlike GABAergic...
IPSCs, glycinergic IPSC frequency was significantly (P<0.05) decreased by more than half compared with preperiod conditions (1.0±0.3 versus 2.2±0.4 Hz, respectively) by the fifth minute of hypoxia/hypercapnia. Glycinergic IPSC frequency remained significantly attenuated for the remainder of the hypoxic/hypercapnic exposure, and by minute 10 the mean glycinergic IPSC frequency was 0.5±0.2 Hz. Glycinergic IPSC amplitude was not altered after hypoxia/hypercapnia (P>0.3).

Both glycinergic and GABAergic IPSCs recovered during the postperiod and were not significantly different from preperiod levels (P>0.05).

Two Populations of RVLM Bulbospinal Neurons Were Distinguished by Firing Patterns but Neither Possessed Inspiratory-Like Modulation of Firing

Previous works from other groups have identified 2 populations of bulbospinal neurons within the RVLM distinguished by their bimodal spontaneous firing rates. Consistent with previous work, we identified a fast-firing group with an average firing rate of 6.5±0.4 (n=12) and a separate slow-firing group that discharged at 2.8±0.3 Hz (n=14). On the basis of previous results from this study showing that synaptic neurotransmission to these neurons is not inspiratory related, it was not surprising that neither slow (n=16) nor fast-firing (n=10) bulbospinal RVLM neurons possessed any significant inspiratory-related changes in action potential firing (Figure S2). The challenge of hypoxia/hypercapnia also did not evoke any inspiratory-related firing in either the slow (n=7) or the fast (n=6) populations of RVLM neurons.

Hypoxia/Hypercapnia Alters the Spontaneous Firing Rates of RVLM Bulbospinal Neurons

Hypoxia/hypercapnia elicited divergent responses in these 2 subpopulations of bulbospinal RVLM neurons (Figure 3). The slow-firing bulbospinal neurons (n=7) significantly increased their firing rates only at the fifth minute (4.8±0.9 Hz) and the 10th minute (4.9±1.0) of exposure to hypoxia/hypercapnia. Unlike synaptic IPSCs, the firing frequency in slowly firing neurons did not return to preperiod conditions but rather stayed significantly elevated during postperiod conditions compared with preperiod (2.8±0.3 versus 5.0±0.9 Hz, respectively; P<0.05). Similar to the slow-firing neurons, the fast-firing bulbospinal neurons demonstrated no significant difference in firing at the beginning of hypoxia/hypercapnia. However, unlike their slow-firing counterparts, the firing rate in the fast-firing neurons was significantly depressed by the 10th minute of hypoxia/hypercapnia (4.8±1.1 versus 7.4±0.9 Hz, respectively). During postperiod, fast-firing neurons were not significantly different from preperiod baseline conditions (P>0.05).

To test whether this response was dependent on hypoxia/hypercapnia-evoked changes in inhibitory synaptic inputs to these neurons, additional experiments were performed in which each population of bulbospinal RVLM neurons was exposed to hypoxia/hypercapnia, while GABAergic and glycinergic neurotransmission was blocked by the presence of gabazine and strychnine, respectively (Figure 4). As previously demonstrated, application of gabazine and strychnine did not significantly increase the baseline firing frequency of either fast (6.5±0.4 versus 6.8±1.3 Hz; P=0.71) or slow RVLM
neurons (2.7±1.0 versus 3.5±0.7 Hz; P=0.22); however, the hypoxia-/hypercapnia-evoked increase in firing frequency in slow-firing bulbospinal neurons was prevented by blocking inhibitory neurotransmission (Figure 4; P>0.05).

Discussion

There are 4 major conclusions from this study. First, RVLM bulbospinal neurons, recorded in an in vitro brain stem slice, do not possess inspiratory-like patterns of spontaneous firing or synaptic inputs unlike other cardiovascular neurons within this preparation. Second, hypoxia/hypercapnia reversibly depressed the frequency of inhibitory glycinergic and GABAergic neurotransmission to bulbospinal RVLM neurons; and the changes in glycinergic IPSCs occurred before the changes in GABAergic neurotransmission. Third, slow-firing RVLM bulbospinal neurons increased their firing activity in response to hypoxia/hypercapnia, which was dependent on the depression of inhibitory neurotransmission. Fourth, hypoxia/hypercapnia decreased the activity of fast-firing bulbospinal neurons, and this depression was independent of changes in synaptic neurotransmission.

Surprisingly, the results of this study failed to identify inspiratory-like coupling within the bulbospinal neurons of the RVLM in vitro. We, therefore, suggest that sympathetic-respiratory coupling is mediated by the following: (1) peripheral mechanisms related to pulmonary stretch receptors, (2) higher brain centers not contained within our in vitro preparation, and (3) medullary centers not active in this neonatal slice of tissue with fictive respiratory bursting activity. Pulmonary stretch receptors have been shown to modulate respiratory-related brain stem regions, and neurons in the pons are thought to play an important role in respiration, particularly postinspiratory activity. Indeed, previous work has shown transection of the pontomedullary region significantly attenuated the respiratory modulation of sympathetic discharge. Possible

Figure 2. During hypoxia/hypercapnia, spontaneous inhibitory postsynaptic current (IPSC) frequencies were decreased in rostral ventral lateral medulla (RVLM) bulbospinal neurons. \(\gamma\)-Aminobutyric acid (GABA)-ergic frequencies (n=7) became significantly depressed from control (preperiod) conditions only by the 10th minute of hypoxia/hypercapnia (top panel; P<0.05). Glycinergic frequencies (n=7) became significantly lower from preperiod at the fifth minute, and this depression was maintained through the 10th minute of hypoxia/hypercapnia (top panel; P<0.05). * Significant differences (P<0.05) from preperiod.

Figure 3. Rostral ventral lateral medulla (RVLM) bulbospinal neurons demonstrated opposing responses to hypoxia/hypercapnia depending on their spontaneous action potential (AP) frequencies. Slow-firing RVLM neurons (n=7) significantly increased AP frequencies at the fifth and 10th minutes of hypoxia/hypercapnia (top panel; P<0.05), whereas the fast-firing subtype (n=6) significantly decreased their AP frequencies only at the 10th minute of hypoxia/hypercapnia (bottom panel; P<0.05). *Significant differences (P<0.05) from preperiod.
pontine structures involved include the Kölliker-Fuse, which sends glutamatergic projections to RVLM neurons and are known to regulate breathing and the parabrachial nucleus, which has been shown to regulate sympathetic-respiratory coupling. It is unlikely the lack of coupling in this study was age dependent, because sympathetic-respiratory coupling has been shown in rats in this age range.

The present study did, however, demonstrate that hypoxia/hypercapnia evokes a significant increase in the firing activity of a subpopulation of RVLM bulbospinal neurons. The increase in firing of presympathetic RVLM neurons would mediate an increase in blood pressure, and, as predicted, hypercapnic hypoxia increases blood pressure. This pressor response after chemoreceptor activation is related to elevations in both renal and cardiac sympathetic nerve activity, which is consistent with an increase in the activity of RVLM neurons in vivo and during shorter durations of exposure in vitro. However, unlike previous reports using short exposures (40 s), the increase in firing frequency in this study was maintained during the postperiod, which may be responsible for the elevated sympathetic tone seen during chronic diseases associated with hypoxia/hypercapnia, such as sleep apnea.

In contrast, the activity of fast-firing neurons decreased their firing during hypoxia/hypercapnia. Opposing responses from these 2 subsets of RVLM bulbospinal neurons have been previously demonstrated. For example, RVLM neurons were either depolarized or hyperpolarized after the induction of hypoxia. Application of angiotensin II modulated the firing rate of slow firing, but not fast firing, RVLM neurons. Although further studies using both in vitro and in vivo techniques are needed to determine the role of these subtypes in the overall generation of vasomotor output, the current results suggest that slowly firing, but not fast firing, RVLM bulbospinal neurons play a significant role in the initiation and sustained increase in vasomotor activity during chemoreflex activation.

Our data indicate the increase in firing that occurs in slow-firing neurons in response to hypoxia/hypercapnia is caused by the hypoxia/hypercapnia elicited reduction in inhibitory neurotransmission to these neurons, that is, the increase in firing is attributed to disinhibition via the withdrawal of inhibitory inputs. When this inhibitory neurotransmission was blocked, the increase in firing no longer occurred. The significant role of decreased inhibitory neurotransmission in the activation of presympathetic neurons during hypoxia/hypercapnia is supported by previous work demonstrating that glutamate blockade does not attenuate the central sympathetic chemoreflex. Additionally, the presence of high H+ concentrations significantly reduced exogenously applied GABA and glycine signaling in bulbospinal RVLM neurons. The time course of the changes (5–10 minutes) in the present study suggests an activation of long latency cellular signals, such as oxidative stress, that may serve as the initial trigger for this disinhibition. Reactive oxygen species generated in the RVLM can increase blood pressure and attenuate GABAergic neurotransmission to the RVLM. In addition, the increase in firing frequency lasted well after the synaptic inputs had returned to baseline conditions, suggesting that the altered synaptic signal may also change long-lasting cellular signaling pathways. Because the response of presympathetic neurons in the RVLM to cyanide application is dependent on calcium, it is interesting to speculate that altered Ca2+-dependent intracellular signaling events could play a role in these longer-term changes, and challenges, such as hypoxia, may alter the types and density of receptors expressed on these neurons, providing long-lasting changes that act to sustain a response.

The lack of inspiratory-like activity, but robust response to hypoxia/hypercapnia in the present study, reinforces the proposed parallel pathway theory of sympathetic reflex control. Previous studies demonstrated a similar discoupling of central respiratory modulation and the chemoreflex in vivo by micro-injecting muscimol into the pre-Bötzinger complex, which eliminated respiratory oscillations in sympathetic discharge but did not change the sympathetic response to chemoreceptor stimulation. Taken together, this study and previous studies suggest that there are 2 divergent pathways to drive RVLM bulbospinal neurons that maintain respiratory coupling and regulate chemosensation independent of respiratory phase/
pattern. Moreover, the respiratory-independent modulation of the RVLM during central chemosensitive stimuli is probably mediated through inhibitory neurotransmission, because no change was seen in glutamate activity during hypoxia/hypercapnia, which is consistent with previous research demonstrating that glutamate blockade does not attenuate the centrally mediated sympathetic chemoreflex.53

The source(s) of inhibitory neurotransmission to RVLM neurons is unknown. It is possible that inhibitory neurons within the caudal ventral lateral medulla that receive significant inspiratory modulation themselves are part of the disinhibition seen in the present study.40 In addition, the nucleus of the solitary tract may be responsible for respiratory-independent activation of bulbospinal neurons. Both the nucleus of the solitary tract and caudal ventral lateral medulla contain an extensive network of interneurons41 and send robust projections to the RVLM.6,24 Because at least a large number of neurons within the nucleus of the solitary tract with peripheral chemoreceptor inputs do not demonstrate central respiratory modulation,44 the nucleus of the solitary tract may also play an important role in the parallel pathway for RVLM activation. Therefore, future investigations are needed to examine the key nuclei responsible for the disinhibition and subsequent increase in firing frequency from the slow-firing RVLM neurons.

In the present study, glycine was significantly decreased during hypoxic/hypercapnic exposure 5 minutes before any significant changes in GABAergic IPSC frequency. This decline was temporally associated with the first significant increase in firing frequency in slow-firing RVLM neurons, suggesting that the disinhibition of glycinergic neurotransmission was responsible for the initial increase in RVLM activity evoked by hypoxia/hypercapnia. The subsequent decline in GABAergic IPSC frequency was temporally matched with the second increase in action potential generation at 10 minutes. This suggests that these 2 neurotransmitters are controlled by different populations of neurons. For example, perhaps the majority of GABAergic activity comes from projections originating within the caudal ventral lateral medulla, whereas glycinergic inputs are largely from another brain region. Indeed, glycinergic neurotransmission dictates a significant component of respiratory motor output,45,46 and previous studies have demonstrated that glycinergic blockade application completely abolishes respiratory bursting, whereas antagonizing GABA only attenuates the amplitude.47 Therefore, given their importance in overall respiratory function, it is possible that the glycinergic projections to slow-firing RVLM neurons are more sensitive to hypoxia/hypercapnia. In addition, although previous studies suggest that caudal ventral lateral medulla stimulation results in the activation of both GABAergic and glycinergic receptors in the RVLM, the pathways mediating the release of these neurotransmitters may be a direct connection for GABA but indirect for glycine.48 The different time courses of glycinergic and GABAergic withdrawal may be explained by different pathways to the RVLM that are modulated differential by hypoxia/hypercapnia. Therefore, if the pathway attenuating glycinergic activity is more sensitive to central chemosensation stimuli, than as seen in the present study, glycinergic neurotransmission would begin to decrease before GABA. Overall, these different sensitivities are probably dictated by either intrinsic sensitivity or different overall pathways where glycinergic neurons are modulated more quickly by central chemosensation.

The present study is one of the first to find that glycine is likely partly responsible for the increase in RVLM activity evoked by hypoxia/hypercapnia. Similar to the present study, the application of the glycinergic antagonist, strychnine, did not elicit a significant change in baseline firing frequency in RVLM vasomotor neurons.16 However, RVLM neurons responsible for cardiovascular regulation have been shown to have glycinergic receptors48 that are likely involved in the vasoconstrictor responses to carotid body occlusion.49

**Perspectives**

In summary, sympathetic-respiratory coupling is not likely mediated by a medullary mechanism active in the present slice preparation but rather is generated by peripheral afferents, a pontine network connection and a brain stem region not active in this preparation. However, RVLM bulbospinal neurons with spontaneous low-firing activity increase their firing in response to hypoxia/hypercapnia and likely play a role in the central chemosensitive activation of vasomotor activity. This increase in frequency is caused by reductions in inhibitory neurotransmission. It is possible that the increase in the sympathetic tone that occurs with prolonged hypoxia/hypercapnia, as seen with diseases such as sleep apnea, may be related to increased spontaneous activity of slow-firing neurons elicited, at least in part, by diminished inhibitory neurotransmission.

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**Disclosures**

None.

**References**


Novelty and Significance

What Is New?

- Hypoxia/hypercapnia increased firing in slow rostral ventral lateral medulla neurons even after reoxygenation.
- Inhibitory receptor antagonists abolished this increase.

What Is Relevant?

- Hypoxia/hypercapnia may be related to increased sympathetic drive during hyperventilation.
- Because the rostral ventral lateral medulla generates sympathetic drive, this study provides a mechanism for the maintenance of elevated activity.

Summary

- Hypoxia/hypercapnia increased the spontaneous firing of slow-firing rostral ventral lateral medulla neurons, which are dependent on reduced inhibitory neurotransmission. It is possible that increased sympathetic tone after hypoxia/hypercapnia, as seen with sleep apnea, is related to this increased activity elicited by diminished inhibitory neurotransmission to slow-firing neurons.
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Supplemental Figure S1: Representative images of cholera-toxin subunit B (CTB) fluorescently labeled neurons localized in the rostral ventral lateral medulla (RVLM). (A) A schematic tracing from Paxinos and Watson illustrating the region where neurons were selected from. (B) A merged confocal fluorescence image of CTB-positive (red) and tyrosine hydroxylase (green) positive neurons within the RVLM. Line represents 30 µM. (4V; fourth ventricle; Amb: nucleus ambiguus; icp: inferior cerebellar pedundle; IO; inferior olive; NTS: nucleus of the solitary tract; pr: pyramidal complex, SP5: spinal tegeminal tract, SP5l: spinal nucleus).
Supplemental Figure S2: Bulbospinal neurons in the RVLM did not demonstrate spontaneous inspiratory-like action potential (AP) frequency in either the slow firing subtype (n=16) or fast firing subtype (n=10). Inspiratory-like bursting activity was recorded from the hypoglossal (XII) rootlet and electronically integrated (fXII). Fluorescently labeled RVLM neurons were patch clamped in the whole cell configuration and spontaneous APs were recorded. Arrows indicates where the inspiratory-like burst occurs.