Intermittent hypoxia-induced sensitization of central chemoreceptors contributes to sympathetic nerve activity during late expiration in rats

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Molkov YI, Zoccal DB, Moraes DJ, Paton JF, Machado BH, Rybak IA. Intermittent hypoxia-induced sensitization of central chemoreceptors contributes to sympathetic nerve activity during late expiration in rats. J Neurophysiol 105: 3080–3091, 2011. First published April 6, 2011; doi:10.1152/jn.00070.2011.—Hypertension elicited by chronic intermittent hypoxia (CIH) is associated with elevated activity of the thoracic sympathetic nerve (tSN) that exhibits an enhanced respiratory modulation reflecting a strengthened interaction between respiratory and sympathetic networks within the brain stem.Expiration is a passive process except for special metabolic conditions such as hyperventilation, when it becomes active through phasic excitation of abdominal motor nerves (AbN) in late expiration. An increase in CO2 evokes late-expiratory (late-E) discharges phase-locked to phrenic bursts with the frequency increasing quantally as hypercapnia increases. In rats exposed to CIH, the late-E discharges synchronized in AbN and tSN emerge in normocapnia. To elucidate the possible neural mechanisms underlying these phenomena, we extended our computational model of the brain stem respiratory network by incorporating a population of presympathetic neurons in the rostral ventrolateral medulla that received inputs from the pons, medullary respiratory compartments, and retrotropezoid nucleus/parafacial respiratory group (RTN/pFRG). Our simulations proposed that CIH conditioning increases the CO2 sensitivity of RTN/pFRG neurons, causing a reduction in both the CO2 threshold for emerging the late-E activity in AbN and tSN and the hypoxic apneic threshold for apnea. Using the in situ rat preparation, we have confirmed that CIH-conditioned rats under normal conditions exhibit synchronized late-E discharges in AbN and tSN similar to those observed in control rats during hypercapnia. Moreover, the hypocapnic threshold for apnea was significantly lowered in CIH-conditioned rats relative to that in control rats. We conclude that CIH may sensitize central chemoreception and that this significantly contributes to the neural impetus for generation of sympathetic activity and hypertension.

chronic intermittent hypoxia; hypertension; modeling; respiration

RECURRENT EPISODES OF HYPOXIA, such as those observed in obstructive sleep apnea (OSA), are a risk factor for the development of cardiovascular diseases, including hypertension (Caples et al. 2005; Dempsey et al. 2010). In rats, it was demonstrated that exposure to chronic intermittent hypoxia (CIH) produced a sustained increase in arterial pressure (Fletcher 2003; Fletcher et al. 1992a, 1992b; Lesske et al. 1997). The fact that coexposure to intermittent hypoxia and hypercapnia produced no additional increment on arterial pressure of rats (Lesske et al. 1997) indicates that intermittent hypoxia is the main factor contributing to the development of arterial hypertension. Previous studies in vivo have reported that CIH-induced hypertension is associated with elevation of the sympathetic vasomotor tone (Zoccal et al. 2007, 2009a), indicating that the sympathetic nervous system plays a major role in the etiology of CIH-induced hypertension. Moreover, the elevated sympathetic nerve activity of CIH-conditioned rats was shown to exhibit an enhanced respiratory modulation that was not dependent on the afferent inputs from lungs or peripheral chemoreceptors (Zoccal et al. 2008). This raises the possibility that central coupling between brain stem respiratory and sympathetic neurons provides a significant contribution to the development of hypertension in CIH-conditioned animals (Zoccal et al. 2009b).

Central coupling of respiratory and sympathetic neurons may occur at the level of the ventrolateral medulla, where many of the neurons involved in the generation of respiratory and sympathetic activities are located (Barman and Gebber 1980; Dampney 1994; Guyenet 2006; Höbler et al. 1994; Haselton and Guyenet 1989; Koshiya and Guyenet 1996; McAllen 1987; Richter and Spyer 1990; Zhong et al. 1997). Specifically in this region, the inspiratory and expiratory neurons of the ventral respiratory column (VRC) interact with the presympathetic neurons of rostral ventrolateral medulla (RVLM) as well as with inhibitory interneurons of caudal ventrolateral medulla (CVLM) (Guyenet 2006; Haselton and Guyenet 1989; Mandel and Schreihofer 2006; Richter and Spyer 1990; Sun et al. 1997). The pons was also shown to play a role in the respiratory modulation of sympathetic nerve activity, since pontine transection significantly attenuated this modulation (Baekey et al. 2008, 2010). In addition, the activities of many medullary and pontine neurons involved in sympatho-respiratory functions are modulated by central chemoreceptors located in the retetropezoid nucleus/parafacial respiratory group (RTN/pFRG) (Abdala et al. 2009b; Guyenet 2006; Guyenet et al. 2008, 2009; Moreira et al. 2006; Nattie and Li 2009).

We have previously found that hypercapnia evokes late-expiratory (late-E) discharges in the abdominal nerve (AbN) that occur just before phrenic bursts (Abdala et al. 2009a; Molkov et al. 2010). With increasing CO2, the frequency of these AbN late-E discharges is quantally increased until reaching a 1:1 ratio to the phrenic burst frequency (Abdala et al.
under normoxic conditions (control, \(n = 7\)). All rats were obtained from the Animal Care of the University of São Paulo at Ribeirão Preto, Brazil. Our experimental approaches were approved by the Ethical Committee on Animal Experimentation of the School of Medicine of Ribeirão Preto, University of São Paulo (protocol 019/2006).

**Chronic Intermittent Hypoxia**

CIH and control rats were housed in Plexiglas chambers (volume: 210 liters) equipped with gas injectors as well as sensors of \(O_2\), \(CO_2\), humidity, and temperature. The CIH group was exposed to a protocol of 5 min of normoxia [fraction of inspired \(O_2 (Fr_{O_2})\) of 20.8%] followed by 4 min of pure \(N_2\) injection to reduce \(Fr_{O_2}\) from 20.8 to 6%, remaining at this level for 40 s. After this hypoxic period, pure \(O_2\) was injected to return \(Fr_{O_2}\) back to 20.8%. This 9-min cycle was repeated 8 h per day (from 9:30 AM to 5:30 PM) for 10 days. During the remaining 16 h, the animals were maintained at a \(Fr_{O_2}\) of 20.8%. The injections of \(N_2\) and \(O_2\) (White Martins, Sertãozinho, Brazil) into the chambers were regulated by a solenoid valve system whose opening-closing control was operated by a computerized system (Oxycycler; Biospherix, Lacona, New York). In an identical chamber in the same room, the control group of rats was exposed to a \(Fr_{O_2}\) of 20.8% for 24 h per day for 10 days. The control rats were also exposed to a similar valve noise due to the frequent injection of \(O_2\) to maintain the \(Fr_{O_2}\) at 20.8%. In both CIH and control chambers, the gas injections were performed at the upper level of the chamber to avoid direct jets of gas impacting on the animals, which could cause stress.

**In Situ Arterially Perfused Preparation**

Working heart-brain stem preparations (as per Paton 1996) from control and CIH-conditioned rats were made on the 11th day of the experimental protocol, as previously described (Paton 1996; Zoccal et al. 2008). Briefly, rats were deeply anesthetized with halothane (AstraZeneca, Cotia, SP, Brazil), transected caudal to the diaphragm, submerging in cooled Ringer solution, and decerebrated at the precollicular level to be made insentient. Preparations were then transferred to a recording chamber, and the descending aorta was cannulated and perfused retrogradely with a Ringer solution (in mM: 125 NaCl, 24 NaHCO\(_3\), 5 KCl, 2.5 CaCl\(_2\), 1.25 MgSO\(_4\), 1.25 KH\(_2\)PO\(_4\), 10 dextrose, and 2 lactate) containing 1.25% polyethylene glycol 20,000 (an oncoitic agent; Sigma, St. Louis, MO) and a neuromuscular blocker (vecuronium bromide, 3–4 \(\mu\)g/ml; Cristália Produtos Químicos Farmacêuticos, São Paulo, SP, Brazil) using a roller pump (Watson-Marlow 502s; Falmouth, Cornwall, UK) via a double-lumen cannula. The perfusion pressure was maintained in the range 50–70 mmHg by adjusting the flow between 21 and 25 ml/min and adding vasopressin (0.6–1.2 nM; Sigma) to the perfusate, as previously described (Picking and Paton 2006; Zoccal et al. 2008). The perfusate was continuously gassed with 5% \(CO_2\) and 95% \(O_2\) (White Martins), warmed to 31–32°C (temperature measured at the point of entry into the aorta), and filtered using a nylon mesh (pore size: 25 \(\mu\)m; Millipore, Billerica, MA).

**Nerve Recordings**

Cardiorespiratory motor nerves were isolated and recorded simultaneously using glass suction electrodes held in three-dimensional micromanipulators (Narisighe, Tokyo, Japan). Left phrenic nerve (PN) was recorded from its central end, and its rhythmic ramping activity was used as a continuous physiological index of preparation viability. The efferent activity of the left tSN was recorded from the sympathetic chain at the T8–T12 level. The AbN was isolated from the abdominal muscles on the right at lumbar level, cut distally, and its efferent activity recorded. PN, tSN, and AbN nerve activities were recorded using bipolar glass suction electrodes. All signals were
amplified, band-pass filtered (0.5–5 kHz), and acquired in an analog-to-digital converter (CED micro 1401; Cambridge Electronic Design, Cambridge, UK) to a computer using Spike2 software (Cambridge Electronic Design).

Data Analyses

The phrenic burst and the late-E AbN burst frequencies were determined by the time interval between consecutive respective integrated bursts (expressed in Hz). Peak amplitude of phrenic bursts was also calculated as the difference value between baseline (noise level determined at the end of experiments 10–20 min after the perfusion ceased) and maximal activities. Thoracic sympathetic and abdominal nerve activities were evaluated using their mean values. The magnitude of alterations in phrenic amplitude, thoracic sympathetic, and abdominal activities in response to hyper- and hypopcapnia were determined as the percentage of change in relation to the respective baseline activity (5% CO₂). All the analyses were carried out on rectified and integrated signals (time constant of 50 ms) and were performed off-line using Spike2 software with custom-written scripts.

Hyper- and Hypocapnic Stimuli

Using a gas mixer device (GF3/MP gas mixing flowmeter; Cameron Instrument, Port Aransas, TX), the proportion of the gases in the perfusate was altered to raise or lower CO₂. For hypercapnic stimuli, the concentrations were 7% CO₂-93% O₂ and 10% CO₂-90% O₂ whereas for hypocapnic stimuli, the concentrations were 3% CO₂-97% O₂ and 1% CO₂-99% O₂. The time duration of exposure for each stimulus was at least 20 min.

Statistical Analyses

Results are means ± SE and were compared, depending on the experimental design, using one-way or two-way ANOVA, followed by the Newman-Keuls or Bonferroni posttest, respectively. The comparisons were carried out on GraphPad Prism software (version 4; GraphPad Software), and differences were considered significant at P < 0.05.

Modeling and Simulations

The model was developed as a consolidation of two previous models proposed by Molkov et al. (2010) and Baekkey et al. (2010). Both models were based on the model proposed by Smith et al. (2007). All neurons were modeled in the Hodgkin-Huxley style (single-compartment models) and incorporated known biophysical properties and channel kinetics characterized in respiratory neurons in vitro. Specifically, the kinetics of the fast sodium and the persistent (slowly inactivating) sodium channels were described using the experimental data obtained in studies of neurons from the rat RVLM (Rybak et al. 2003a); the kinetics of high-voltage-activated calcium current was described based on the study of calcium currents in rat medullary neurons in vitro (Elsen and Ramirez 1998); the intracellular calcium dynamics were described using data by Freermann et al. (1999). The descriptions of other ion channels, e.g., the potassium rectifier and calcium-dependent potassium ones, and all other cellular parameters were derived from our previous models (Rybak et al. 1997a, 1997b, 2003b, 2004a, 2004b). Each neuronal type was represented by a population of 20–50 neurons. Heterogeneity of neurons within each population was set by a random distribution of some parameters and the initial conditions for values of membrane potential, calcium concentrations, and channel conductances. Each neuron of a target population received synaptic inputs from all neurons of a source population and/or a corresponding source of excitatory drive in accordance with the network architecture of the model. A full description of the modeling approach and major model parameters can be found in our previous publications (Molkov et al. 2010; Smith et al. 2007).

All simulations were performed with the simulation package NSM 3.0, developed at Drexel University by S. N. Markin, I. A. Rybak, and N. A. Shevtsova and ported to multiprocessor parallel computing systems by Y. I. Molkov using the OpenMPI environment. Differential equations were solved using the exponential Euler integration method with a step of 0.1 ms. Specific details of the model and model parameters can be found in Table 1.

This study utilized the high-performance computational capabilities of the Biowulf Linux cluster at the National Institutes of Health (Bethesda, MD; http://biowulf.nih.gov).

RESULTS

Respiratory and Sympathetic Activities in Control and CIH-Conditioned Rats In Situ Under Normal Conditions and During Hypercapnia

Control rats. Typical patterns of respiratory and sympathetic activities from a representative control rat (1 of n = 7) are shown in Fig. 1, A1–A5. Under normal conditions (5% CO₂) (see Abdala et al. 2009a; Molkov et al. 2010), the integrated burst of activity in the PN had an augmenting profile, the AbN exhibited a low-amplitude activity, and the integrated activity in the tSN expressed an augmenting inspiratory modulation with the activity profile slowly increasing during inspiration, reaching a peak at the inspiration-to-expiration transition, and rapidly falling at the beginning of expiration (Fig. 1A4). As described previously (Abdala et al. 2009a; Iizuka and Fregosi 2007; Molkov et al. 2010; Rubin et al. 2010), hypercapnia (an increase in CO₂ level) evoked high-amplitude late-E AbN discharges in control preparations (Fig. 1, A2, A3, and A5). The emerging AbN discharges were always phase-locked to PN bursts. With progressive development of hypercapnia (increase in CO₂ in the perfusate from 5 to 10%), the ratio of frequency of AbN late-E discharges to frequency of PN bursts increased stepwise from about 1:4/1:3 to 1:2 (see Fig. 1A2 at 7% CO₂, where approximately each second respiratory cycle skipped an AbN discharge) and, finally, to 1:1 as the CO₂ level was elevated to 10% (Fig. 1A3). Importantly, although the frequency of the late-E bursts increased with increasing hypercapnia, they were always coupled (phase-locked) to the PN bursts, evidencing the “quantal acceleration” as we described previously (Molkov et al. 2010; Rubin et al. 2010).

Hypercapnia-evoked late-E activity and its quantal acceleration with increasing CO₂ were also observed in tSN of control preparations (Fig. 1, A2, A3, and A5). The tSN late-E discharges always coincided with AbN late-E bursts, and a skipping of late-E activity in the tSN (e.g., at 7% CO₂) always coincided with its skipping in the AbN, suggesting a common excitatory source of late-E discharges in AbN and tSN. To make sure that the late-E bursts in AbN did not reflect the activity of sympathetic fibers contained in this nerve, in a subset of animals (n = 3) we performed activation of baroreceptors by increases in perfusion pressure. These stimuli produced a marked attenuation of tSN but not AbN late-E activity (data not shown), indicating that the late-E bursts observed in AbN were produced by separate motoneurons controlling AbN activity. The sympathoexcitatory response to hypercapnia in control rats (ΔtSN in Fig. 1C1) was associated with an increase in the average tSN activity during expiration, which was mainly because of the evoked late-E discharges but also be-
cause of an increase in activity during postinspiration (Fig. 1A). In relation to the PN activity, hypercapnia produced an increase in both PN frequency (0.35 ± 0.03 Hz at 7% CO₂, P < 0.05, and 0.40 ± 0.03 Hz at 10% CO₂, P < 0.05; Fig. 1C2) and amplitude (9 ± 3% at 7% CO₂, P < 0.05, and 23 ± 3% at 10% CO₂, P < 0.05; Fig. 1, A1–A5).

CIH rats. The pattern of baseline sympathetic and respiratory activities from a representative CIH preparation (1 of n = 5) is shown in Fig. 1B1. The baseline frequency of PN bursts in CIH-conditioned rats (0.29 ± 0.02 Hz) was not significantly different from that in the control rat preparations (0.33 ± 0.04 Hz) (Fig. 1C2). Similar to that in rats of the control group, the PN frequency in CIH rats increased with the increase in CO₂ level in the perfusate (0.32 ± 0.03 Hz at 7% CO₂ and 0.37 ± 0.02 Hz at 10% CO₂; see Fig. 1C2).

In contrast to control rats, the CIH-treated preparations expressed coincident late-E discharges in AbN and tSN with a frequency ratio to PN of between 1:3 and 1:2 in normocapnic conditions (5% CO₂; see Fig. 1, B1, B4, and C3). At 7% CO₂ this ratio was 1:1 (Fig. 1, B2 and C3) and unlike that seen in control rats (where frequent skipping was observed; Fig. 1A2).

The tSN profile in CIH-conditioned rats in normocapnia differed from the tSN profile in control preparations. Specifically, after CIH conditioning, tSN activity was elevated during the late-E interval and maintained throughout the PN burst but not during postinspiration (Fig. 1, B4 and B5).

### Table 1. Weights of synaptic connections in the network

<table>
<thead>
<tr>
<th>Target Population (Location)</th>
<th>Excitatory Drive [Weight of Synaptic Input] or Source Population [Weight of Synaptic Input From Single Neuron]</th>
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</thead>
<tbody>
<tr>
<td>Bs-E (cVRG)</td>
<td>Early-I(2) [−2]; late-E [0.02]</td>
</tr>
<tr>
<td>Ramp-I (rVRG)</td>
<td>Drive(pons) [2.0]; pre-I/I [0.12];* post-I [−2.0]; aug-E [−0.1]</td>
</tr>
<tr>
<td>Early-I(2) (rVRG)</td>
<td>Drive(pons) [2.5]; post-I [−0.5];* aug-E [−0.25]; late-E [0.1]</td>
</tr>
<tr>
<td>Pre-I (pre-BötC)</td>
<td>Drive(pons) [0.22];* drive(RTN/pFRG) [0.65];* drive(raphé) [0.3]; pre-I/I [0.03]; post-I [−0.16]; aug-E [−0.06]; late-E [0.02]</td>
</tr>
<tr>
<td>Early-I(1) (pre-BötC)</td>
<td>Drive(pons) [1.0];* drive(RTN/pFRG) [1.1];*</td>
</tr>
<tr>
<td>Post-I (BötC)</td>
<td>Drive(pons) [1.7];*</td>
</tr>
<tr>
<td>Post-I(e) (BötC)</td>
<td>Drive(pons) [1.7];*</td>
</tr>
<tr>
<td>Aug-E (BötC)</td>
<td>Drive(pons) [2.7];*</td>
</tr>
<tr>
<td>Late-E (RTN/pFRG)</td>
<td>Drive(RTN/pFRG) [0.33];*</td>
</tr>
<tr>
<td>RVLM*</td>
<td>Drive(VLM) [1]*</td>
</tr>
<tr>
<td>CVLM*</td>
<td>Drive(VLM) [1]*</td>
</tr>
<tr>
<td>IE* (pons)</td>
<td>Ramp-I [0.2];* post-I(e) [0.35]*</td>
</tr>
</tbody>
</table>

Values in brackets represent average relative weights of synaptic inputs from the corresponding source populations or drives. Neural populations: bs-E, bulbospinal E; ramp-I, ramp inspiratory; early-I, early inspiratory; pre-I/I, preinspiratory/inspiratory; post-I, postinspiratory; post-I(e), postinspiratory (excitatory); aug-E, augmenting expiratory; late-E, late expiratory; IE, phase-spanning inspiratory-expiratory. Locations: cVRG, caudal ventral respiratory group; rVRG, rostral ventral respiratory group; pre-BötC, pre-Bötzinger complex; BötC, Bötzinger complex; RTN/pFRG, retrotapezoid nucleus/parafacial respiratory group; RVLM, rostral ventrolateral medulla; CVLM, caudal ventrolateral medulla. *Populations not present in the model of Molkov et al. (2010) and weights of connections adjusted in the present model relative to that model. #After CIH conditioning.

### Modeling and Simulations

Model description. The experimental findings described above suggest that synchronized late-E discharges observed in the AbN and tSN after CIH conditioning have a common source. On the basis of this suggestion, we combined two previous models, the one describing the origin of the late-E abdominal activity (Molkov et al. 2010) and the other simulating respiratory-sympathetic coupling (Baekey et al. 2010), and developed a united computational model. The resultant model (Fig. 2A) has the same core circuitry of the respiratory network as both of the previous models (originally proposed by Smith et al. 2007). This circuitry includes the excitatory pre-inspiratory/inspiratory (pre-I/I) and inhibitory early-inspiratory [early-I(1)] populations of the pre-Bötzinger complex (pre-BötC) and the inhibitory augmenting-expiratory (aug-E) and postinspiratory (post-I) [and excitatory premotor post-I(e)] populations of the BötC (see Fig. 2A). The united model also includes a ramp-inhibitory (ramp-I) population of premotor bulbospinal inspiratory neurons and an inhibitory early-inspiratory [early-I(2)] population, which are both located in the rostral ventral respiratory group (rVRG; Fig. 2A) (Smith et al. 2007).

Similar to the model of Molkov et al. (2010), a late-E population of neurons with the intrinsic bursting properties has been included in the RTN/pFRG. This population represents a putative source of late-E oscillations and projects to the bulbospinal premotor expiratory (bulbospinal E, or bs-E) popula-
tion located in the caudal ventral respiratory group (cVRG), which drives AbN motor output (Fig. 2A).

Following the model of Baekey et al. (2010), we have incorporated excitatory RVLM and inhibitory CVLM populations of the ventrolateral medulla (Barman and Gebber 1980; Dampney 1994; Guyenet 2000, 2006), as well as a phase-spanning inspiratory-expiratory (IE) population in the pons (Cohen and Shaw 2004; Dick et al. 1994, 2008; Mörschel and Dutschmann 2009; Segers et al. 2008). The latter was suggested to project to RVLM, hence providing a pontine-dependent inspiratory modulation of tSN (Baekey et al. 2010) (see Fig. 2A).

In our model, both the pre-I/I population of pre-BötC and the late-E population of RTN/pFRG consist of neurons with the persistent (slowly inactivating) sodium current ($I_{NaP}$; defining endogenous bursting properties of neurons that can be expressed under certain conditions) and mutual excitatory interactions within the corresponding population (Molkov et al. 2010; Rybak et al. 2007; Smith et al. 2007). The neurons in the post-I, post-I(e), aug-E, early-I(1), and early-I(2) populations are characterized by adapting firing properties defined by high-voltage-activated calcium ($I_{CaL}$) and calcium-activated potassium ($I_{K,Ca}$) currents. The remaining neurons (bs-E, ramp-I, IE, RVLM, and CVLM) have only a minimal set of ionic currents (fast sodium, $I_{Na}$; potassium rectifier, $I_{K}$; and leakage, $I_{L}$) necessary for generating spiking activity.

The behavior of respiratory and sympathetic networks and their interactions depend on a variety of external inputs (or drives) to all neural populations involved in network oper-
ations. These inputs allow the system to maintain the appropriate homeostatic levels of O2 and CO2 and adaptively respond to various metabolic demands (Fortuna et al. 2008; Guyenet 2006; Guyenet et al. 2005, 2008, 2009; Moreira et al. 2006; Mulkey et al. 2007; Nattie 1999; Nattie and Li 2009; Richerson 2004). These drives have been conditionally modeled as excitatory “tonic drives” from multiple sources distributed within the brain stem (pons, RTN, raphé; see Fig. 2A). Although currently undefined, these drives seem to have specific mapping on the spatial organization of the brain stem networks (note that only some connection from drive sources in the pons and RTN/pFRG are shown in Fig. 2A; all connections with their weights are specified in Table 1).

Among the drive sources mentioned above, the RTN/pFRG is considered to be a major central chemoreceptor site whose drive is sensitive to CO2 (Guyenet 2006; Guyenet et al. 2005, 2008, 2009; Mulkey et al. 2007). To explicitly simulate this property, the RTN tonic drive was considered not constant, as other tonic drives in the model, but dependent on the CO2 level. The CO2 dependence of the RTN drive was modeled as a sharply saturating function:

\[ f(\text{CO}_2) = 0.3 + 0.85 \sigma \left( \frac{\text{CO}_2 + \alpha}{\beta} \right), \]

where \( \text{CO}_2 \) is the CO2 content in perfusate (in %), \( \sigma(x) = \tanh (x + x^3) \), \( \beta = 9.5\% \) is the saturation level, and \( \alpha \) is the CIH sensitization parameter: \( \alpha = 0 \) for the control case and \( \alpha = 2\% \) after CIH conditioning. This hypothetical dependence of RTN/pFRG tonic drive on CO2 is shown in Fig. 2B (black curve). As hypothesized above, the CO2 sensitivity of RTN/pFRG increases due to CIH conditioning. This was simulated by the horizontal shift of the curve representing CO2-dependent RTN drive by \( \alpha = 2\% \) CO2 to the direction of lower CO2 values (i.e., to the left; see red curve in Fig. 2B). In addition, we suggest that all synaptic weights of inhibitory inputs to the RVLM population are reduced as a result of CIH conditioning (see Table 1). The implementation of this suggestion in the model allowed us to reproduce some specific changes in the tSN activity profile in CIH-conditioned preparations described below.

Regarding the activity of the pre-I/I and late-E populations, the previous theoretical studies have demonstrated that such neural populations (i.e., populations comprising neurons with
$I_{\text{NaP}}$-dependent bursting properties and mutual excitatory interconnections) can be silent (at a low level of neuronal excitability), operate in a population-bursting mode (at the increased excitability or external drive, exceeding some threshold), or switch to a sustained asynchronous activity (when the excitability or drive is additionally increased, exceeding a higher threshold) so that with the increasing neuronal excitability or external drive, the firing behavior of the population switches sequentially from a silent state to bursting and then to a sustained firing (Butera et al. 1999; Rybak et al. 2003b). Importantly, although the pre-I/I and late-E populations are almost identical in the model, their behavior is different because of the different levels of basal excitability. Specifically, under normal conditions, the pre-I/I population receives a strong total excitatory drive from the pons, RTN/pFRG and raphe that keeps this population in the operation regime not critically dependent on $I_{\text{NaP}}$ (Rybak et al. 2003b, 2007; Smith et al. 2007). In contrast, the late-E population during normocapnia is silent and can be switch to the bursting regime either by hypercapnia (Abdala et al. 2009a; Molkov et al. 2010) or as a result of sensitization of RTN/pFRG during CIH conditioning as hypothesized in this study.

Simulations and Modeling Predictions

Control Conditions. Figure 3, A1 and A2, shows the integrated activity of the PN, AbN, and tSN outputs in the model (A1) and the corresponding burst profiles (A2) in control conditions, when CO2 was stepwise increasing from 1% CO2 (hypocapnia) through 5% CO2 (normocapnia) to 10% CO2 (hypercapnia), as shown in Fig. 3B. Progressive hypercapnia led to the emergence and quantal acceleration of late-E bursts in both AbN and tSN (Fig. 3A1), which was consistent with our experimental data described above. The late-E discharges appeared at about 7% CO2 and reached a 1:1 ratio to the PN bursts at 9% CO2. The profiles of PN and tSN activity with and without preceding AbN late-E burst in the model (Fig. 3A2) closely reproduced the profiles of activity obtained from corresponding experimental recordings (Fig. 1, A4 and A5). Figure 3A1 also shows that a reduction of CO2 below 3% (an apneic threshold for hypocapnia in the control conditions) caused apnea (lack of PN activity), a phenomenon well known from previous experimental investigations (e.g., Boden et al. 1998).

CIH Conditioning. As described above, we hypothesize that CIH conditioning augments the CO2 sensitivity of central chemoreceptors. To simulate this phenomenon, the curve reflecting the CO2 dependence of RTN drive was shifted to the left (to lower values of CO2) by 2% (see red curve in Fig. 2B). In addition, weights of inhibitory synaptic connections to RVLM were reduced (see above and Table 1).

The results of the corresponding simulation of the effects of CIH conditioning are shown in Fig. 3, C1 and C2. In this simulation, CO2 was again increased stepwise from 1% CO2 (hypocapnia) through 5% CO2 (normocapnia) to 10% CO2 (hypercapnia; Fig. 3B). However, in contrast to the control scenario (Fig. 3A1), the late-E bursts in AbN and tSN emerged at 3% CO2, and at the normocapnic state (5% CO2) they
showed a stable 1:2 ratio to the PN bursts (see Fig. 3C1), which was consistent with our experimental observations (Fig. 1B1). At 7% CO₂, this ratio reached 1:1 (Fig. 3C1), a ratio not seen until 10% CO₂ in control conditions (Figs. 1B2 and 3A1). Note also that after CIH, the profiles of PN and tSN activity with and without preceding AbN late-E burst in the model (Fig. 3C2) closely reproduced the profiles of activity obtained from the corresponding experimental recordings (Fig. 1, B4 and B5).

The second observation from the above simulation was that CIH conditioning reduced the apneic threshold for hypocapnia in the model by at least 2% CO₂, since the PN bursts were still generated even at 1% CO₂ (see Fig. 3C1). This modeling prediction was tested experimentally (see below).

**DISCUSSION**

In agreement with previous in vivo observations (Guyenet 2006; Guyenet et al. 2008, 2009; Nattie 1999), we demonstrated in situ that stimulation of central chemoreceptors with high CO₂ produced increased sympathetic and respiratory activities. In our experiments, the increase in sympathetic activity during hypercapnia occurred preferentially within the expiratory phase and was associated with the simultaneous emergence of late-E bursts in the AbN and tSN (Fig. 1, A2 and A3). The neural mechanisms underlying the CO₂-dependent sympathoexcitatory response during late expiration seem to contribute to the enhanced baseline sympathetic activity of CIH rats, because the reduction of CO₂ to 3% eliminated late-E activities in AbN and tSN (Fig. 4). In addition, decreasing CO₂ to 1% abolished PN activity in control but not in CIH rats (Fig. 4), supporting the hypothesis that central chemoreceptors may be sensitized after CIH exposure and contribute to the develop-

![Image](https://via.placeholder.com/150)

**Fig. 4.** Changes in the sympathetic and respiratory activities in response to hypocapnia in control and CIH-conditioned rats. Raw and integrated recordings are shown of tSN, AbN, and PN activities of representative preparations from control (A) and CIH groups (B) in normocapnic (5% CO₂) and hypocapnic conditions (3 and 1% CO₂). An expressed late-E activity in AbN and tSN was observed in CIH-conditioned preparations at 5% CO₂ and was abolished with reduction of CO₂ to 3%. Hypocapnia at 1% CO₂ eliminated PN activity in control but not in CIH-conditioned preparations.
opment of active expiratory pattern as well as an augmented sympathetic activity observed in CIH rats in normoxic/normocapnic conditions.

**Preparation Specificity**

Like other experimental preparations, such as anesthetized and decerebrate animals, the arterially perfused in situ preparation used in this study has its own technical limitations. However, accumulating evidence demonstrates that sympathetic and respiratory activities (either baseline or evoked) observed in this preparation are, in most cases, similar to those observed in vivo. A recent example is the study by Marína et al. (2010) showing that inhibition of the Phox2b-expressing neurons in the ventrolateral brain stem including the RTN produced a comparable attenuation of the hypercapnia-induced abdominal late-E activity in the in situ rat preparations and in the anesthetized and conscious rats. Moreover, in our previous studies (Zoccal et al. 2009), we demonstrated that hypertensive CIH-treated conscious rats exhibited an increased respiratory modulation of arterial pressure similar to that originally demonstrated in the in situ preparations (Zoccal et al. 2008). Therefore, our current (and previous) data obtained in situ do not seem to be exclusive to, or critically dependent on, the preparation used.

One specific technical aspect of our studies in situ concerns the evaluation of CO₂ content, which in our study is conditionally considered equal to the amount of gas bubbled in the perfusate. This value is not the same as the percentage of CO₂ measured at end-expiration as generally used in vivo. Also, the amount of CO₂ in the preparation is likely to be greater in the blood vessels because of the additional CO₂ generated by the metabolism. This can partly explain the difference in the threshold for hypocapnic apnea between our preparation and the in vivo data. Specifically, we found that this threshold was achieved in situ at lower levels of CO₂ (1% CO₂; Fig. 4A) than in the previous experiments performed in vivo (3–4% CO₂) (see Moreira et al. 2006; Takakura et al. 2008).

**Abdominal and Sympathetic Activities Evoked by Hypercapnia**

It has been proposed that central chemoreception involves a cluster of Phox2b-expressing neurons located in RTN/pFRG, which play a dominant role in respiratory and sympathetic responses to central chemoreceptor activation (Guyenet 2006; Guyenet et al. 2005, 2008, 2009; Marina et al. 2010; Stornetta et al. 2006). It was demonstrated that the RTN chemosensitive neurons are predominantly glutamatergic, since they express the glutamatergic vesicular transporter, and establish connections with respiratory neurons of the VRC, parabrachial, and Kölliker-Fuse nuclei in the pons and dorsal respiratory column (Rosin et al. 2006). Through these connections, excitatory inputs from RTN/pFRG to respiratory neurons possibly mediate the respiratory response involved in central chemoreception. Our experimental study demonstrated that hypercapnia induced increases in both inspiratory and expiratory activities. In relation to phrenic inspiratory activity, both frequency and amplitude of phrenic bursts of control preparations increased with the incrementing of CO₂ in the perfusate (Fig. 1), a response that may involve connections between RTN/pFRG and inspiratory neurons of pre-BötC (e.g., pre-I/I; see Fig. 2A) as suggested by our model (Abdala et al. 2009a; Molkov et al. 2010).

With respect to the expiratory response, we confirmed that high levels of CO₂ result in the emergence of late-E bursts in AbN, which was not seen in control rats in basal conditions (5% CO₂, Fig. 1, A1–A3). Recent studies have demonstrated that RTN/pFRG neurons are essential for the expression of abdominal late-E bursts, because pharmacological inhibition of RTN (Abdala et al. 2009a; Molkov et al. 2010) or inhibition of predominantly Phox2b-expressing neurons in this region (Marína et al. 2010) abolished the AbN late-E activity in response to hypercapnia without interfering with the activity of BötC expiratory neurons (Abdala et al. 2009a). Therefore, during hypercapnia the RTN/pFRG appears to be an important source of excitation to bulbospinal expiratory neurons located in cVRC that relay excitatory drive to the lumbar abdominal motoneurons that drive late-E bursting in the AbN (Abdala et al. 2009a; Molkov et al. 2010).

The sympathoexcitatory response to hypercapnia suggests excitatory connections from CO₂-sensitive neurons of RTN/pFRG to the presympathetic RVLM neurons (Moreira et al. 2006; Takakura et al. 2011). In the present study, we demonstrated that the CO₂-induced increase in sympathetic activity in control rats occurred, at least in part, due to the emerging late-E activity. The source of this activity was suggested to be in RTN/pFRG, which contains neurons that are silent during normocapnia (5% CO₂) and activated during hypercapnia, exhibiting a pattern of activity that is strongly correlated with AbN late-E activity (Abdala et al. 2009a; Molkov et al. 2010). These late-E neurons of RTN/pFRG could be an excitatory source of excitation not only to cVRC bulbospinal expiratory neurons but also to presympathetic RVLM neurons, culminating in an increase of sympathetic activity correlated with late-E bursts in abdominal motor activity. The synchronous activation of abdominal and sympathetic late-E activities was more evident at 7% CO₂, when sympathetic late-E activity skipped in the respiratory cycles in which abdominal late-E activity was also absent (Fig. 1, A2 and B1).

**Central Chemoreceptor Sensitization and Sympathetic Nerve Activity After CIH Exposure**

According to previous studies (Abdala et al. 2009a; Janczewski and Feldman 2006; Janczewski et al. 2002; Molkov et al. 2010), late-E activity originates in RTN/pFRG and can be evoked by hypercapnia (Abdala et al. 2009a; Molkov et al. 2010). In the present study, we have demonstrated that this hypercapnia-evoked late-E activity can substantially contribute to the sympathoexcitatory response. We have also provided evidence for a previous conclusion (Zoccal et al. 2008) that CIH-treated rats exhibit late-E activity in AbN and tSN under normocapnic conditions (5% CO₂; see Figs. 1B1 and 4B). These observations provide important insights into possible mechanisms involved in the elevation of sympathetic activity and development of arterial hypertension observed after CIH conditioning.

We hypothesize that the excitability and hence CO₂ sensitivity of a subset of RTN/pFRG neurons increases after CIH conditioning. This CIH-evoked increase in the neuronal excitability or CO₂ sensitivity results in a lowering of the CO₂
threshold for generation of late-E activity within the RTN/pFRG. This hypothesis was explicitly incorporated in our computational model.

**Computational Model**

The model developed in the present study represents our first attempt to integrate sympatho-respiratory circuits. This model was developed based on direct combination of two previous models describing the emergence of late-E activity in the RTN/pFRG (Molkov et al. 2010) and the sympathetic-respiratory coupling (Baekey et al. 2010). The integrated model could reproduce all the experimental data that each of the two basic models reproduced. In addition, we explicitly incorporated CO₂ concentration as a model variable to simulate the CO₂-dependent central chemoreceptor drive in the model.

The model supports the important role of chemosensitive RTN/pFRG neurons for activation of sympatho-neurons in the RVLM. It hypothesizes specific interaction between the pons (IE population), respiratory populations of VRC, central chemoreceptors located in RTN/pFRG (late-E population), and RVLM and CVLM populations (Fig. 2A). These interactions and connectivities predicted by the model await further experimental testing. However, the role of CVLM in these interactions in the present model is, so far, minimal and does not account for the different respiratory-modulated patterns observed in CVLM neurons (Mandel and Schreihofer 2006). Clarification of the respiratory network interactions with the RVLM and CVLM are the focus of our future experimental and modeling studies.

The model reproduces the experimentally observed effects of hypercapnia and CIH conditioning on AbN and tSN motor activities. The central assumption (hypothesis) of our model is that CIH-conditioning increases the excitability of RTN/pFRG (late-E) neurons or their CO₂ sensitivity, leading to the emergence of late-E activity at a normal level of CO₂ (after CIH conditioning) and resulting in elevation of the average tSN activity contributing to the CIH-evoked hypertension. In the model, this was reproduced by the simulated shift of the curve representing CO₂-dependent RTN drive by 2% CO₂ to the direction of lower CO₂ values (Fig. 2B). The mechanism(s) for this shift and the nature of the plastic changes in the excitability or CO₂ sensitivity of RTN/pFRG neurons after CIH conditioning have not been considered so far and remain to be determined. One possible explanation for this may be based on a reduction of inhibitory inputs from the BötC post-I neurons to the RTN/pFRG late-E neurons (see Fig. 2A), which could increase the excitability in the latter neurons (see Molkov et al. 2010). The other mechanism could be based on the increased excitatory drive from peripheral chemoreceptors (mediated by corresponding neurons in the nucleus of solitary tract) to central chemoreceptors (late-E neurons) (Guyenet et al. 2009; Takakura et al. 2006) and/or on the peripheral chemoreceptor control of the gain of central chemoreceptors (Blain et al. 2010). However, the suggestion of the involvement of peripheral chemoreceptor drive would contradict our previous data showing that the carotid body denervation after CIH exposure did not eliminate the late-E discharges from sympathetic activity (Zoccal et al. 2008). Nevertheless, peripheral-central chemoreceptor interaction may be involved in the development of plastic changes in the excitability of central chemoreceptors after CIH conditioning via activation of neuromodulators that enhance the activity of RTN chemosensitive neurons, such as serotonin (Mulkey et al. 2007), ATP (Mulkey et al. 2006), or locally produced oxidative stress (Jurado-Gomez et al. 2011). These or other, currently unknown mechanisms may be involved in the observed CIH-evoked sensitization of RTN/pFRG chemoreceptors, which requires additional studies.

The important prediction of our model is a CIH-dependent lowering of the threshold for hypocapnic apnea (Fig. 3). This prediction has been confirmed experimentally in the in situ preparations of CIH-treated rats exposed to hypocapnia (Fig. 4). The latter confirms the plausibility of the model per se, as well as the plausibility of the neural mechanisms and interactions suggested and incorporated in the model.

Our model also predicts a CIH-evoked reduction in the synaptic inhibition to the RVLM. Incorporation of this feature allowed the model to reproduce the experimentally observed changes in the tSN burst profile induced by CIH conditioning. This prediction is consistent with raised sympathetic activity and reflex sympathetic responses in CIH rats (Braga et al. 2006) and also awaits experimental testing.

In conclusion, our combined experimental and modeling studies support an important role of late-E oscillations originated in the RTN/pFRG in the generation of forced expiration and their significant contribution to the elevated sympathetic activity after CIH treatment. The neurons responsible for these effects may provide a novel target for correcting the hypertension in conditions of CIH and perhaps sleep apneic patients.

At the same time, our study does not dismiss or diminish a possible role of other mechanisms in the CIH-evoked hypertension not necessarily connected with the increasing respiratory-sympathetic interactions and/or emerging late-E activity. For example, the Prabhakar group (Peng and Prabhakar 2004; Peng et al. 2003) reported an increase in the sensitivity of peripheral chemoreceptors in CIH treated rats, which can also be the case in OSA patients (Narkiewicz et al. 1998). Other hypertension mechanisms independent of peripheral or central chemoreceptor sensitization may also be involved (Xing and Pilowsky 2010). Therefore, our finding that respiratory-sympathetic interactions and sympathetic late-E activity may significantly contribute to the development of hypertension in CIH-conditioned animals does not exclude a possibility that there are other factors involved in CIH-evoked hypertension.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).
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