Title: Chronic intermittent hypoxia alters neurotransmission from the lateral paragigantocellular nucleus to parasympathetic cardiac neurons in the brainstem

Abbreviated title: Chronic hypoxia alters REM sleep pathway to cardiac neurons

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ABSTRACT

Patients with sleep related disorders, including obstructive sleep apnea (OSA), have an increased risk of cardiovascular diseases. OSA events are more severe in rapid eye movement (REM) sleep. REM sleep further increases the risk of adverse cardiovascular events by diminishing cardioprotective parasympathetic activity. The mechanisms underlying REM sleep-related reduction in parasympathetic activity likely include activation of inhibitory input to cardiac vagal neurons (CVNs) in the brainstem originating from the lateral paragigantocellular nucleus (LPGi), a nucleus which plays a role in REM sleep control. This study tests the hypothesis that chronic intermittent hypoxia and hypercapnia (CIHH), an animal model of OSA, inhibits CVNs due to exaggeration of the GABAergic pathway from the LPGi to CVNs. GABAergic neurotransmission to CVNs evoked by electrical stimulation of the LPGi was examined using whole-cell patch-clamp recordings in an in vitro brain slice preparation in rats exposed to CIHH and control rats. GABAergic synaptic events were enhanced following 4-weeks CIHH in both male and female rats with a greater degree in males. Acute hypoxia and hypercapnia (H/H) reversibly diminished the LPGi-evoked GABAergic neurotransmission to CVNs. However, GABAergic synaptic events were enhanced post acute H/H in CIHH male animals. Orexin-A elicited a reversible inhibition of LPGi-evoked GABAergic currents in control animals but evoked no significant changes in CIHH male rats. In conclusion, exaggerated inhibitory neurotransmission from the LPGi to CVNs in CIHH animals would reduce cardioprotective parasympathetic activity and enhance the risk of adverse cardiovascular events.

Key words: cardiac vagal neurons, brainstem, REM sleep, apnea, hypoxia, hypercapnia.
INTRODUCTION

Obstructive Sleep Apnea (OSA) is a common sleep-related disorder with an incidence of approximately 24% in US adult males and 9% of US adult females (Bazzano et al. 2007; Punjabi 2008). Patients with OSA experience chronic nocturnal recurrent apneas and intermittent hypoxia, and they have an increased risk of adverse cardiovascular-related symptoms and events including sudden cardiac death, hypertension, arrhythmias, myocardial ischemia and stroke (Kato et al. 2009; Parish and Somers 2004; Punjabi 2008). Rapid eye movement (REM) sleep further increases the health risk as obstructive events occur for a longer duration of time, and with more desaturation of oxyhemoglobin occurring during REM sleep than during non-REM sleep (Arens and Marcus 2004; Charbonneau et al. 1994; Findley et al. 1985; Goh et al. 2000; Neves et al. 2010). REM sleep independently also increases sympathetic and attenuates cardiac vagal tone and is associated with cardiac arrhythmias (Berlad et al. 1993; Valladares et al. 2008; Verrier and Josephson 2009). Accordingly, the relative risk for sudden death during REM sleep is as high as 1.2 times the risk during wakefulness (Verrier et al. 1996). Despite the evidence of these prevalent REM sleep-related adverse cardiovascular events and negative long term consequences of OSA, the neurophysiological mechanisms that link OSA, REM sleep state, and cardiovascular abnormalities are poorly understood.

Chronic exposure to intermittent hypoxia (CIH) or hypoxia and hypercapnia (CIHH) during the nocturnal period in animals mimics the repetitive episodes of apneas that occur in humans with OSA. Both OSA patients and animals exposed to CIH have an altered
balance of autonomic activity with elevated sympathetic and reduced parasympathetic activity to the heart with resulting tachycardia and decreased baroreflex sensitivity (Bonsignore et al. 2006; Freet et al. 2013; Lin et al. 2007; Narkiewicz et al. 1998; Parati et al. 1997; Parish and Somers 2004; Reynolds et al. 2007). The results from recent animal studies suggest that the mechanisms for decreased baroreflex control of heart rate and diminished parasympathetic activity to the heart following CIHH likely involve central autonomic dysregulation and in particular altered function of parasympathetic cardiac vagal neurons (CVNs) in the nucleus ambiguus (NA).

CVNs receive neurotransmission that include GABAergic, glycinergic, and glutamatergic inputs (Dergacheva et al. 2013; Evans et al. 2005; Neff et al. 2004). Recent work (Dergacheva et al. 2010) has found GABAergic pathway from the REM sleep active lateral paragigantocellular nucleus (LPGi) (Sirieix et al. 2012; Verret et al. 2006) to CVNs. This pathway likely provides a neurochemical mechanism for REM sleep-related reductions in parasympathetic cardiac activity (Dergacheva et al. 2010). However, the alterations that occur with CIHH within the GABAergic pathway from the LPGi to CVNs are unknown. This study tested the hypothesis that CIHH impairs parasympathetic activity to the heart via an exaggeration of the inhibitory GABAergic pathway from the LPGi to CVNs in the NA.

The results from recent studies have demonstrated that the inhibitory neurotransmission to CVNs is very sensitive to acute hypoxia or acute hypoxia/hypercapnia (H/H) (Dergacheva et al. 2010; Neff et al. 2004). However, these central parasympathetic
responses may be altered by chronic repetitive exposures to H/H. This study tested the hypothesis that CIHH alters GABAergic neurotransmission from the LPGi to CVNs during and/or following acute H/H.

In addition to reduced parasympathetic cardiac activity (Wiklund et al. 2000), patients with OSA have significantly altered levels of hypothalamic neuropeptide orexin-A (Igarashi et al. 2003; Liao and Yu 2005; Sakurai et al. 2005). Since orexin-A is also involved in cardiovascular regulation and, in particular, in parasympathetic control of heart rate (Ciriello et al. 2003; Dergacheva et al. 2011; Dergacheva et al. 2005) it is reasonable to speculate that altered levels of orexin-A are implicated in adverse cardiovascular events associated with OSA. However, the pathophysiological mechanisms by which orexin-A may elicit the alterations in autonomic cardiovascular control associated with OSA are unknown. Accordingly, this study tested the hypothesis that orexin-A modulation of LPGi-evoked GABAergic pathway to CVNs is altered following CIHH.
MATERIALS AND METHODS

Experiments were conducted on Sprague–Dawley rats of both genders. Animals were purchased from Hilltop Lab Animals (Scottsdale, PA) and housed in the George Washington University animal care facility under standard environmental conditions. All animal procedures were performed in compliance with the institutional guidelines at George Washington University and are in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and the National Institutes of Health publication Guide for the Care and Use of Laboratory Animals.

CVN labeling

The fluorescent retrograde labeling of parasympathetic CVNs was performed as described previously (Dergacheva et al. 2013; Dergacheva et al. 2010). Rat pups (postnatal days 2–3) were anesthetized with hypothermia and received a right thoracotomy. The heart was exposed, and 0.05 ml of 1–5% rhodamine (XRITC; Molecular Probes, Eugene, OR) was injected into the pericardial sac. Specificity of the cardiac vagal labeling has been confirmed in a previous study by the absence of any labeled neurons in the brainstem when rhodamine is injected either outside the pericardial sac or within the pericardial sac if the cardiac branch of the vagus nerve is sectioned (Bouairi et al. 2004). After surgery, buprenorphine was administered, and animals were monitored for 30 minutes and every 20 minutes thereafter until ambulatory.
CIHH exposures

The animals that received CVN labeling were kept in animal research facility cages until they were 4 weeks old. At 4 weeks of age the animals, kept in their normal cages with unrestricted access to food and water, were placed in a commercial (Biospherix) chamber pod with computer controlled atmospheric gas control. Animals were kept on a cycle of 12/12 hours light/dark and exposed to CIHH during light phase. CIHH was performed by cycling between room air (20.9% oxygen, 79.1% nitrogen) and H/H (6% oxygen, 5% CO2, and 89% nitrogen). There were 4 phases to this CIHH cycle; in the first phase the room air was changed to H/H in 90 seconds, and H/H was maintained for an additional 90 seconds (second phase). In the third phase the hypoxic/hypercapnic gas mixture was returned to room air levels (over 90 seconds) and this room air mixture was maintained for 90 seconds (fourth phase). This CIHH protocol was maintained at a frequency of 10 times each hour, 8 hours each day, 4 weeks long. Control animals were age-matched to CIHH animals, and were subjected to the same handling and gas exchange conditions as CIHH rats except the gas mixture was maintained with room air.

Slice preparation and electrophysiology

After 4 weeks of CIHH or room air exposures animals were sacrificed and coronal brainstem slices were obtained for electrophysiological experiments. To obtain viable brain slices from 8 weeks old animals the methodology by Ye and colleagues (Ye et al.
2006) was adopted and glycerol-based artificial cerebrospinal fluid (aCSF) was used for cardiac perfusion and brainstem sectioning. Glycerol-based aCSF contained (in mM): 252 glycerol, 1.6 KCl, 1.2 NaH2PO4, 1.2 MgCl, 2.4 CaCl2, 26 NaHCO3, and 11 glucose. On the day of experiment, the animals were anesthetized with isoflurane, and glycerol-based aCSF (4°C) was perfused transcardially. Then the brain was carefully removed and 300-μm thickness slices of the medulla that contained the NA and LPGi were obtained using a vibratome. The obtained slices of the medulla were transferred to a solution of the following composition (in mM): 110 N-methyl-d-glucamine (NMDG), 2.5 KCl, 1.2 NaH2PO4, 25 NaHCO3, 25 glucose, 110 HCl, 0.5 CaCl2, and 10 mM MgSO4 equilibrated with 95% O2 and 5% CO2 (pH 7.4) at 34°C for 15 min. This brief protective recovery step using NMDG-based aCSF reduces neuronal swelling during rewarming and is critically important for viable brain slice preparation in animals (Zhao et al. 2011). The slices were then transferred from NMDG-based aCSF to a recording chamber, which allowed perfusion (5–10 ml/min) of aCSF at room temperature (25°C) containing (in mM): 125 NaCl, 3 KCl, 2 CaCl2, 26 NaHCO3, 5 glucose, and 5 HEPES equilibrated with 95% O2 and 5% CO2 (pH 7.4) for at least 30 minutes before experiments were conducted.

Individual CVNs were identified by the presence of the fluorescent tracer. These identified CVNs were then imaged with differential interference contrast optics, infrared illumination, and infrared-sensitive video detection cameras to gain better spatial resolution. Patch pipettes (2.5–3.5 MΩ) were filled with a solution consisting of 150 mM KCl, 2 mM MgCl2, 2 mM EGTA, 10 mM HEPES, and 2 mM Mg-ATP, pH 7.35. With
this pipette solution, the chloride current induced by activation of GABA receptors was recorded as an inward current. Voltage-clamp whole cell recordings were made at a holding potential of −80 mV with an Axopatch 200B and pClamp 8 software (Axon Instruments, Union City, CA, USA).

GABAergic inhibitory postsynaptic currents (IPSCs) were isolated by continuous focal application of strychnine (1 μM), d-2-amino-5-phosphonovalerate (50 μM), and 6-cyano-7-nitroquinoxaline-2,3-dione (50 μM) to block glycine, NMDA and AMPA/kainate receptors, respectively. Continual focal drug applications were performed using a pneumatic picopump pressure system (WPI, Sarasota, FL). Drugs were released from a patch pipette positioned within 30 μm of the patched CVN. The maximum range of drug application was determined previously to be 100–120 μm downstream from the drug pipette and was considerably less behind the drug pipette (Wang et al. 2002). At the end of experiments GABAergic neurotransmission was nearly abolished by application of the GABA receptor antagonist gabazine (25 μM). Other drugs used in this study included orexin-A (0.1 μM and 1 μM). All drugs used in this study were purchased from Sigma-Aldrich Chemical (St. Louis, MO).

In experiments that examined the role of acute H/H in modulation of the evoked responses in CVN slices were exposed to acute H/H by changing control aCSF equilibrated with 95% O2, and 5% CO2, pH 7.4, to an identical solution equilibrated with 85% N2, 6% O2, and 9% CO2, pH 7.1. Slices were exposed to acute H/H for 10 min and
then slices were reoxygenated during 20 min by returning the perfusate to the control aCSF.

The location of the LPGi was identified using stereotaxic coordinates (Paxinos and Watson 2007) in addition to the location relative to fluorescently identified CVNs in the NA. The LPGi was stimulated with electrical stimuli of 1ms duration at a frequency of 0.1 Hz using a stimulus isolator (A.M.P.I., Jerusalem, Israel). Stimulus intensity was 1.5 times of the minimum intensity that evoked a response in CVNs. There were no differences in the stimulus intensity required to evoke IPSC in CVNs between male/female or air/CIHH groups of animals.

**Data analysis and statistics**

Synaptic events were measured using pClamp 8 software (Molecular Devices, Sunnyvale, CA, USA). The responses to a series of 10 consecutive stimulations in each neuron were averaged in all series of experiments. The mean value from each neuron in the population was then averaged for the population of neurons to create a summary of results for each condition. Results are presented as mean±SE and statistically compared using GraphPad Prism 5 software and using Student’s T-test, one-way repeated measures ANOVA or two ways ANOVA, as appropriate.
RESULTS

*CIHH enhanced GABAergic neurotransmission from the LPGi to CVNs*

In agreement with previously published data obtained from neonatal rats (Dergacheva et al. 2011; Dergacheva et al. 2010) electrical stimulation of the LPGi evoked GABAergic current in CVNs in the NA in adult rats (see Fig. 1-5). The peak amplitude of GABAergic IPSC was significantly enhanced by CIHH in both female rats (unexposed animals, 76.0±6.7 pA, n=30 vs CIHH animals, 104.4±7.4 pA, n=31, p<0.01, Student’s unpaired T-test, Fig.1) and male rats (unexposed animals, 85.2±6.9 pA, n=32 vs CIHH animals, 178.4±18 pA, n=25, p<0.001, Student’s unpaired T-test, Fig. 1). However, this CIHH-elicited exaggeration of GABAergic currents occurred to a greater degree in males compared to females. Indeed, the amplitude of LPGi-evoked GABAergic IPSC was significantly greater (p<0.001, Student’s unpaired T-test) in male CIHH animals (178.4±18 pA, n=25, Fig. 1) than those in female CIHH animals (104.4±7.4 pA, n=31, Fig.1), whereas there were no differences in the amplitude of LPGi-evoked GABAergic IPSC between male and female rats unexposed to CIHH (85.2±6.9 pA, n=32 vs 76.0±6.7 pA, n=30, p>0.05, respectively, Student’s unpaired T-test, Fig. 1).

*Effect of acute H/H on GABAergic neurotransmission from the LPGi to CVNs in unexposed and CIHH animals*

Acute H/H resulted in a significant inhibition of LPGi-evoked GABAergic neurotransmission to CVNs in all groups of animals studied (see Table 1, Fig. 2, Fig. 3). Indeed, the amplitude of GABAergic IPSC was significantly reduced during acute H/H in
both unexposed female animals (from 79.8±14 pA to 16.2±4.1 pA, H/H at 4-5 min, then to 10.8±2.0 pA, H/H at 9-10 min, p<0.001, one-way repeated measures ANOVA and Dunnett's post test, n=13, Table 1 and Fig. 2) and unexposed male animals (from 75.6±5.0 pA to 26.4±5.3 pA, H/H at 4-5 min, then to 23.8±4.3 pA, H/H at 9-10 min, p<0.001, one-way repeated measures ANOVA and Dunnett's post test, n=16, Table 1 and Fig. 3). Similar to unexposed animals, in CIHH rats the amplitude of GABAergic IPSC was significantly diminished during acute H/H in both females (from 105.8±12 pA to 28.8±7.2 pA, H/H at 4-5 min, then to 18.4±3.8 pA, H/H at 9-10 min, p<0.001, one-way repeated measures ANOVA and Dunnett's post test, n=13, Table 1 and Fig. 2) and males (from 174.2±20 pA to 81.3±15 pA, H/H at 4-5 min, then to 55.0±13 pA, H/H at 9-10 min, p<0.001, one-way repeated measures ANOVA and Dunnett's post test, n=13, Table 1 and Fig. 3).

There were no significant differences in the reduction of the amplitude of LPGi-evoked GABAergic current by acute H/H between unexposed male and female animals rats (p>0.05, two-ways ANOVA, Bonferroni’s post test). However, acute H/H at 4-5 minutes elicited a greater reduction in the GABAergic amplitude in CIHH female animals when compared to CIHH male rats (p<0.05, two-ways ANOVA, Bonferroni’s post test (Table 1). There were no significant differences in the reduction the amplitude of LPGi-evoked GABAergic current by acute H/H between unexposed and CIHH female rats (p>0.05, two-ways ANOVA, Bonferroni’s post test). However, acute H/H at 4-5 minutes elicited a greater reduction in the GABAergic amplitude in unexposed male animals when
compared to CIHH male rats (p<0.01, two-ways ANOVA, Bonferroni’s posttest, Table 1 and Fig. 3).

The GABAergic neurotransmission from the LPGi to CVNs completely recovered and was not significantly different from control levels at 19-20 min post acute H/H in both unexposed females rats (79.8±14 pA, control conditions, vs 87.8±14 pA, post H/H, p>0.05, one-way repeated measures ANOVA and Dunnett's post test, n=13, Table 1 and Fig. 2) and unexposed male rats (75.6±5.0 pA, control conditions, vs 82.7±9.2 pA, post H/H, p>0.05, one-way repeated measures ANOVA and Dunnett's post test, n=16, Table 1 and Fig 3). Similarly, LPGi-evoked GABAergic current in CVNs under control conditions was not significantly different from those evoked at 19-20 min post acute H/H in CIHH female animals (105.8±12 pA, control conditions, vs 125.9±11 pA, post H/H, p>0.05, one-way repeated measures ANOVA and Dunnett's post test, n=13, Table 1 and Fig. 2). However, in CIHH male animals the amplitude of LPGi-evoked GABAergic current was significantly enhanced 19-20 min post acute H/H when compared to those under control conditions (174.2±20 pA, control conditions, vs 233.8±25 pA, post H/H, p<0.01, one-way repeated measures ANOVA and Dunnett's post test, n=13, Table 1 and Fig. 3).

Effect of orexin-A application on GABAergic neurotransmission from the LPGi to CVNs in unexposed and CIHH animals

Orexin-A, applied at a concentration of 0.1 µM, elicited a significant and reversible inhibition of LPGi-evoked GABAergic current in both unexposed female animals (from
68.8±9.4 pA to 37.2±8.9 pA, p<0.001, one-way repeated measures ANOVA and Dunnett's post test, n=9, Fig. 4) and unexposed male animals (from 98.6±12 pA to 46.6±8.1 pA, p<0.01, one-way repeated measures ANOVA and Dunnett's post test, n=8, Fig. 5). Similar to unexposed rats, orexin-A reversibly diminished LPGi-evoked GABAergic current to CVNs in CIHH female animals (from 115.4±14 pA to 69.7±7.9 pA, p<0.01, one-way repeated measures ANOVA and Dunnett's post test, n=9, Fig. 4). However, orexin-A elicited no significant changes in LPGi-evoked GABAergic IPSC in CIHH male animals (192.4±34 pA, control, vs 165.6±25 pA, orexin-A, p>0.05, one-way repeated measures ANOVA and Dunnett's post test, n=9, Fig. 5).

Similarly, application of orexin-A at a higher concentration of 1 µM resulted in an inhibition of GABAergic current in both unexposed and CIHH female rats (from 78.2±5.8 pA to 34.1±7.9 pA, p<0.01, n=8, and from 96.2±7.2 pA to 47.4±11 pA, p<0.05, n=9, respectively, one-way repeated measures ANOVA and Dunnett's post test, Fig. 6) as well as in unexposed male rats (from 91.0±22 pA to 29.8±4.7 pA, p<0.01, one-way repeated measures ANOVA and Dunnett's post test, n=8, Fig. 7). However, there was no significant alteration in LPGi-evoked GABAergic IPSC following orexin-A (1 µM) application in CIHH male animals (157.2±30 pA, control, vs 108±28 pA, orexin-A, p>0.05, one-way repeated measures ANOVA and Dunnett's post test, n=10, Fig. 7). There were no significant differences between males and females in the amount of reduction following orexin-A applications at concentrations of either 0.1 µM or 1 µM in unexposed animals (p>0.05, two-ways ANOVA, Bonferroni’s post test).
DISCUSSION

The major findings

There are three major findings in this study. (1) The GABAergic pathway from the LPGi to CVNs is enhanced following CIHH in both male and female animals, and to a greater extent in males. (2) Acute H/H reversibly diminishes GABAergic neurotransmission from the LPGi to CVNs in all animals studied, however to a lesser degree in males exposed to CIHH. In addition, in male CIHH animals the LPGi-evoked GABAergic neurotransmission is enhanced post acute H/H. (3) Orexin-A diminishes GABAergic current from the LPGi to CVNs in unexposed animals of both genders as well as in female CIHH rats. However, orexin-A elicited no significant changes in LPGi-evoked GABAergic current in CIHH male rats.

The LPGi pathway to CVNs

Consistent with the conclusions in previous studies (Dergacheva et al. 2011; Dergacheva et al. 2010) in this study, the GABAergic pathway from the LPGi to CVNs likely provides a neurophysiological mechanism for REM sleep-related reduction in parasympathetic cardiac activity. Considerable evidence indicates that LPGi neurons play an important role in REM sleep control. There is a reduction in the amount of REM sleep following lesions of the medullary reticular formation encompassing the LPGi (Holmes...
The LPGi contains a significant number of neurons with activity-dependent increases in c-Fos levels after REM sleep recovery from REM sleep deprivation (Verret et al. 2006; Verret et al. 2005). And finally, the LPGi contains neurons specifically active during REM sleep in both cats (Sakai 1988) and rats (Sirieix et al. 2012). Since about one-third of the neurons expressing Fos in the LPGi during a REM sleep also express glycine (Boissard et al. 2002) and 70% express GAD, the synthetic enzyme of GABA (Sapin et al. 2009) and many neurons in this region of the brain express both neurotransmitters (Stornetta et al. 2004), it is very likely that most if not all of neurons specifically active during REM sleep are GABAergic and/or glycinergic neurons (Sirieix et al. 2012). The results from this study indicate there is an important GABAergic pathway from the LPGi to CVNs in the NA. During REM sleep, activation of GABAergic neurons in the LPGi may exert an inhibitory action at CVNs that maintain parasympathetic activity to the heart and likely this contributes to withdrawal of parasympathetic activity that occurs during REM sleep (Valladares et al. 2008).

**The LPGi-evoked pathway to CVNs is exaggerated following CIHH**

The neurophysiological mechanisms underlying the reduced parasympathetic activity and impaired baroreflex control of the heart observed in both OSA patients and animals exposed to CIH (Gu et al. 2007; Lin et al. 2007; Narkiewicz et al. 1998; Parati et al. 1997; Reynolds et al. 2007; Yan et al. 2008) are poorly understood. The results from this study indicate that GABAergic neurotransmission to CVNs elicited by the LPGi stimulation is significantly enhanced in both male and female animals exposed to CIHH.
This enhanced neurotransmission could be a result of increased sensitivity of GABAergic receptors in CVNs following CIHH as spontaneous GABAergic IPSCs in CVNs have been shown to be increased in CIHH rats (Dyavanapalli et al. 2014). In addition, REM sleep-related activation of GABAergic neurons in the LPGi that likely project to CVNs could also be increased following CIHH since shortening of a REM sleep episodes by hypoxic stimuli would lead to increased REM sleep drive (Hamrahi et al. 2001). This enhanced inhibitory neurotransmission to CVNs would result in CVN inhibition and reduced parasympathetic activity to the heart which may contribute to increased risk of cardiovascular diseases. The comparison between male and female animals indicates that GABAergic synaptic currents are enhanced to a greater extent in males following CIHH. Interestingly, epidemiologic studies have found strong male predominance of OSA (Lam et al. 2010; O'Connor et al. 2000; Young et al. 1993). Although the reason for higher prevalence of OSA in men is poorly understood, the gender differences in central body fat distribution and larger neck dimension as well as the influence of sex hormones have been implicated (Millman et al. 1995; O'Connor et al. 2000). In addition to male predominance of OSA, men are generally at greater risk for cardiovascular disease than age-matched women (Reckelhoff 2001). Thus, greater male risk of CIHH-induced adverse cardiovascular events, suggested by this study, may be a contributing factor for the overall enhanced male risk for cardiovascular diseases.

**CIHH alters responses to acute H/H in male rats**
Acute H/H induces stress in the central nervous system and triggers important adaptive responses promoting survival (Gu et al. 2007). However, these adaptive responses could be altered by chronic exposures to repetitive episodes of H/H. Previous works have shown dramatic changes in both excitatory and inhibitory neurotransmission to CVNs elicited by acute hypoxia or H/H (Dergacheva et al. 2011; Kamendi et al. 2009; Neff et al. 2004). LPGi-evoked GABAergic current in CVNs is reversibly diminished by acute H/H in neonatal rats (Dergacheva et al. 2011). Consistently, the results from this study, conducted on adult rats, indicate that GABAergic current elicited by the LPGi stimulation is reversibly reduced by acute H/H in all animals studied. However, acute H/H elicits a lesser reduction in the GABAergic amplitude in CIHH male animals when compared to CIHH female rats. Acute H/H also evokes a lesser reduction in the GABAergic amplitude in CIHH male animals when compared to unexposed males. In addition, GABAergic current is exaggerated 20 minutes post acute H/H in CIHH male animals. This reduced response to acute H/H and exaggerated inhibitory neurotransmission to CVNs would predict inhibition of CVN activity and impairment of cardioprotective parasympathetic activity to the heart during and following acute H/H in CIHH male animals. Similar to evoked GABAergic current, acute H/H diminishes spontaneous GABAergic IPSCs in CVNs in unexposed rats (Dyavanapalli et al. 2014). However, this inhibitory effect of H/H on spontaneous IPSCs is completely abolished in CIHH animals (Dyavanapalli et al. 2014). The different effects of acute H/H on evoked and spontaneous IPSCs in rats exposed to CIHH could point to the segregation of evoked and spontaneous signaling including accommodation of the two release forms within the same synapse, maintained via a separate pool of vesicles (Kavalali et al. 2011).
It has been demonstrated that apneic events impair the stability of parasympathetic nerve function during both REM and non-REM sleep with greater impairment observed in REM sleep (Yamaguchi et al. 2014a; Yamaguchi et al. 2014b). Supporting the findings of this work, parasympathetic function during sleep has been found to be less stable in male OSA patients when compared to female OSA patients (Yamaguchi et al. 2014a). The reduced parasympathetic activity to the heart, along with sympathetic hyperactivity increases risk of tachycardia and sudden cardiac death that may occur during or post each episode of apnea, especially in men with OSA.

**CIHH alters responses to orexin-A application in male rats**

Orexin-A has been hypothesized to play a key role in the pathogenesis of OSA (Wang et al. 2013b). Some authors have demonstrated decreased levels of plasma orexin-A (Aksu et al. 2009; Busquets et al. 2004; Nishijima et al. 2003; Sakurai et al. 2004) while others have found increased levels of orexin-A in OSA patients (Igarashi et al. 2003; Liao and Yu 2005). The possible reason for the discrepancy of the results may include the difference of the studied populations. Plasma levels of orexin-A are elevated in cases of mild sleep apnea-hypopnea syndrome, and the levels are reduced in parallel with the severity of the syndrome (Nishijima et al. 2003).

The results from this study indicate that orexin-A at both concentrations of 0.1 µM and 1 µM diminishes GABAergic neurotransmission from the LPGi to CVNs in unexposed
animals as well as in female rats exposed to CIHH. These results are in a contrast to previously published data indicating that orexin-A (1 µM) facilitates GABAergic current from the LPGi to CVNs in neonatal rats (Dergacheva et al. 2011). These apparent differences in responses to orexin-A application between neonatal and adult rats could be explained by developmental changes in orexinergic modulation of CVNs (Dergacheva et al. 2012; Dergacheva et al. 2013). In contrast to unexposed rats of both genders and CIHH female rats orexin-A does not evoke any significant changes in male animals exposed to CIHH. Previous studies have demonstrated that orexin neurons are CO₂ sensitive and could be activated by CO₂/H+ (Williams et al. 2007) however acute exposure to prolonged hypercapnia suppresses orexin expression in the hypothalamus (Wang et al. 2013a). It is possible that chronic exposure to repetitive episodes of H/H results in desensitization of orexin receptors in CVNs in male rats. The sex differences were found between male and female rats in both content of orexin-A in the lateral and posterior hypothalamus and expression of orexin receptors in the hypothalamus (Johren et al. 2001; Taheri et al. 1999). In addition to the sensitivity to CO₂, orexin has been shown to increase neuronal viability and protect neurons against oxidative stress under conditions of chemical hypoxia (Sokolowska et al. 2014). Blunted responses to orexin-A would result in sustained GABAergic neurotransmission from the LPGi to CVNs which would lead to decreased parasympathetic activity of CVNs in male animals exposed to CIHH. In addition, it is likely that the protective function of orexin on CVNs would be diminished in CIHH male animals.
In conclusion, this study established a central neurophysiological mechanism underlying impaired parasympathetic activity in animals exposed to CIHH. During REM sleep, activation of GABAergic neurons in the LPGi likely exerts an inhibitory action at CVNs diminishing parasympathetic activity to the heart. The inhibitory pathway from the LPGi to CVNs is significantly enhanced following CIHH exposures in both male and female rats which would cause additional impairment of cardioprotective parasympathetic activity in CIHH animals. In addition to the exaggerated GABAergic pathway, the responses to acute H/H and orexin-A application are altered in male rats exposed to CIHH suggesting a greater male-related vulnerability to CIHH. Exaggerated inhibitory neurotransmission from the LPGi to CVNs may play a critical role in sleep-associated high risk of tachycardia, arrhythmias, and sudden cardiac death in individuals with OSA.
GRANTS
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DISCLOSURES
The author declares no competing financial interests.

ABBREVIATIONS
Artificial cerebrospinal fluid, aCSF; cardiac vagal neurons, CVNs; chronic intermittent hypoxia, CIH; chronic intermittent hypoxia and hypercapnia, CIHH; hypoxia and hypercapnia, H/H; inhibitory postsynaptic currents, IPSCs; lateral paragigantocellular nucleus LPGi; nucleus ambiguus, NA; obstructive sleep apnea, OSA; rapid eye movement, REM; 110 N-methyl-d-glucamine, NMDG.
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FIGURE LEGENDS

Figure 1. Electrical stimulation of the LPGi evoked GABAergic IPSCs in CVNs in the NA. This neurotransmission was significantly enhanced in both female and male rats exposed to CIHH (Student’s T-test). Comparison between genders revealed that the amplitude of the GABAergic IPSCs were significantly greater in male animals exposed to CIHH than in female animals exposed to CIHH (Student’s T-test). Representative traces are shown in A, and the summary data are demonstrated in B. Arrow indicates electrical stimulation in this and all subsequent figures. Asterisks indicate statistically significant differences, ** p < 0.01; *** p < 0.001; +++ indicate statistically significant difference (p < 0.001) between genders.

Figure 2. As shown in representative traces (A) and in the summary data (B), acute H/H elicited a significant and reversible decrease in the amplitude of GABAergic neurotransmission evoked by electrical stimulation of the LPGi in both unexposed and CIHH female rats (one-way repeated measures ANOVA and Dunnett's post test). Asterisks indicate statistically significant differences, *** p < 0.001.

Figure 3. Like in female animals, acute H/H reversibly diminished LPGi-evoked GABAergic neurotransmission in CVNs in unexposed male rats (one-way repeated measures ANOVA and Dunnett's post test, *** p < 0.001). However, LPGi-evoked GABAergic IPSCs were decreased during acute H/H (** p < 0.01) and increased post acute H/H (*** p < 0.001) in CIHH male animals (one-way repeated measures ANOVA).
and Dunnett's post test). In addition, acute H/H (5min) elicited a greater reduction in the
GABAergic amplitude in unexposed male animals when compared to CIHH male rats
(two-ways ANOVA and Bonferroni’s posttest, ++ p < 0.01). Representative traces are
shown in A, and the summary data are demonstrated in B.

Figure 4. Orexin-A (0.1 µM) elicited a significant and reversible inhibition of LPGi-
evoked GABAergic IPSC in both unexposed and CIHH female rats (one-way repeated
measures ANOVA and Dunnett's post test). Representative traces are shown in A and the
summary data are demonstrated in B. Asterisks indicate statistically significant
differences, ** p < 0.01; *** p < 0.001.

Figure 5. Application of orexin-A (0.1 µM) reversibly diminished GABAergic IPSCs
evoked by the LPGi stimulation in unexposed male rats (one-way repeated measures
ANOVA and Dunnett's post test). However, of orexin-A (0.1 µM) did not significantly
alter GABAergic IPSC in CIHH male animals (one-way repeated measures ANOVA and
Dunnett's post test). Representative traces are shown in A, and the summary data are
demonstrated in B. Asterisks indicate statistically significant differences, ** p < 0.01

Figure 6. Similar to a concentration of 0.1 µM, application of orexin-A at a higher
concentration of 1 µM resulted in a reversible inhibition of LPGi-evoked GABAergic
IPSC in both unexposed and CIHH female rats (one-way repeated measures ANOVA and
Dunnett's post test). Representative traces are shown in A and the summary data are
Figure 7. Orexin-A (1 µM) reversibly diminished LPGi-evoked GABAergic IPSCs in unexposed male rats (one-way repeated measures ANOVA and Dunnett's post test). However, orexin-A (1 µM) did not significantly alter GABAergic IPSCs in CIHH male animals (one-way repeated measures ANOVA and Dunnett's post test). Representative traces are shown in A, and the summary data are demonstrated in B. Asterisks indicate statistically significant differences, ** p < 0.01
Table 1: Effect of acute H/H on the amplitude (pA) of the LPGi-evoked GABAergic IPSC in CVNs in unexposed and CIHH animals of both genders.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>H/H (2 min)</th>
<th>H/H (5 min)</th>
<th>H/H (10) min</th>
<th>Recovery</th>
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<tbody>
<tr>
<td>Unexposed females</td>
<td>79.8±14</td>
<td>52.6±14</td>
<td>16.2±4.1***</td>
<td>10.8±2.0 ***</td>
<td>78.4±14</td>
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<tr>
<td>CIHH females</td>
<td>105.8±12</td>
<td>89.3±14</td>
<td>28.8±7.2***</td>
<td>18.3±3.8 ***</td>
<td>125.9±11</td>
</tr>
<tr>
<td>Unexposed males</td>
<td>75.6±5.0</td>
<td>59.3±7.1</td>
<td>26.4±5.3***</td>
<td>23.8±4.3 ***</td>
<td>82.7±9.2</td>
</tr>
<tr>
<td>CIHH males</td>
<td>174.2±20</td>
<td>148.7±21</td>
<td>81.3±15***+xx</td>
<td>55.0±13 ***</td>
<td>233.8±25 **</td>
</tr>
</tbody>
</table>

* Control vs H/H and recovery. Statistical significance was tested using one-way repeated measures ANOVA and Dunnett's post test (** p<0.01; *** p<0.001).

+ Males vs Females. Statistical significance was tested using two-ways ANOVA and Bonferroni’s posttest. Statistically significant difference († p<0.05) was found between males and females in the amount of reduction of the GABAergic IPSC by H/H (5min).

X Unexposed vs CIHH. Statistical significance was tested using two-ways ANOVA and Bonferroni’s posttest. Statistically significant difference (XX p<0.01) was found between unexposed and CIHH males in the amount of reduction of the GABAergic IPSC by H/H (5min).
Figure A: Graphs comparing peak amplitude between unexposed and CIHH animals for female and male animals.

Figure B: Graphs comparing peak amplitude between unexposed and CIHH animals for female and male animals, showing significant differences with *** and +++ symbols.