Chronic intermittent hypoxia alters neurotransmission from lateral paragigantocellular nucleus to parasympathetic cardiac neurons in the brain stem

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Dergacheva O. Chronic intermittent hypoxia alters neurotransmission from lateral paragigantocellular nucleus to parasympathetic cardiac neurons in the brain stem. J Neurophysiol 113: 380–389, 2015. First published October 15, 2014; doi:10.1152/jn.00302.2014.—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 brain stem originating from the lateral paragigantocellular nucleus (LPGi), a nucleus that 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 because of exaggeration of the GABAergic pathway from the LPGi to CVNs. GABAergic neurotransmission to CVNs evoked by electrical stimulation of the LPGi was examined with 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 after 4-wk CIHH in both male and female rats, to 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 after 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.

Cardiac vagal neurons; brain stem; REM sleep; apnea; hypoxia; hypercapnia.

OBSURCTIVE SLEEP APNEA (OSA) is a common sleep-related disorder with an incidence of approximately 24% in US adult men and 9% in US adult women (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 includes GABAergic, glycinergetic, 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.

Results from recent studies have demonstrated that the inhibitory neurotransmission to CVNs is very sensitive to acute hypoxia or acute hypoxia and 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 the hypothalamic neuropeptide orexin-A (Igarrashi 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. 2005, 2011), 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 after CIHH.

MATERIALS AND METHODS

Experiments were conducted on Sprague-Dawley rats of both sexes. 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 Guide for the Care and Use of Laboratory Animals. All procedures were approved by the George Washington University Institutional Animal Care and Use Committee.

CVN labeling. Fluorescent retrograde labeling of parasympathetic CVNs was performed as described previously (Dergacheva et al. 2010, 2013). 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 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 (in mM) 150 KCl, 2 MgCl2, 2 EGTA, 10 HEPES, and 2 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).

GABAergic inhibitory postsynaptic currents (IPSCs) were isolated by continuous local 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 with a pneumatic pipocap 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 CVNs, slices were exposed to acute H/H by changing control aCSF equilibrated with 95% O2-5% CO2, pH 7.4, to an identical solution equilibrated with 85% N2-6% O2-9% CO2, pH 7.1. Slices were exposed to acute H/H for 10 min, and then slices were reoxygengated over 20 min by returning the perfusate to the control aCSF.

The location of the LPGi was identified with 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 1-ms duration at a frequency of 0.1 Hz with a stimulator (A-MPI, Jerusalem, Israel). Stimulus intensity was 1.5 times 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 with pCLAMP 8 software (Molecular Devices, Sunnyvale, CA). 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 means ± SE and statistically compared with GraphPad Prism 5 software, using Student’s t-test, one-way repeated-measures ANOVA, or two-way ANOVA, as appropriate.

RESULTS

CIHH enhanced GABAergic neurotransmission from LPgi to CVNs. In agreement with previously published data obtained from neonatal rats (Dergacheva et al. 2010, 2011), electrical stimulation of the LPgi evoked GABAergic current in CVNs in the NA in adult rats (see Figs. 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 with 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 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 not exposed 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 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, 1-way repeated-measures ANOVA and Dunnett’s posttest (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, 1-way repeated-measures ANOVA and Dunnett’s posttest (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, 1-way repeated-measures ANOVA and Dunnett’s posttest (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, 1-way repeated-measures ANOVA and Dunnett’s posttest (n = 13); Table 1 and Fig. 3].
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 96.2 ± 7.9 pA to 47.4 ± 11 pA (after H/H); P < 0.05, 1-way repeated-measures ANOVA and Dunnett’s posttest (n = 9); Fig. 5]. 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, 1-way repeated-measures ANOVA and Dunnett’s posttest (n = 9); Fig. 5].
ANOVA and Dunnett’s posttest; Fig. 6] as well as in unexposed male rats [from 91.0 ± 22 pA to 29.8 ± 4.7 pA; \( P < 0.001 \)] and increased after acute H/H (**\( P < 0.01 \)) in CIHH male animals (1-way repeated-measures ANOVA and Dunnett’s posttest). In addition, acute H/H (5 min) elicited a greater reduction in the GABAergic amplitude in unexposed male animals compared with CIHH male animals (2-way ANOVA and Bonferroni’s posttest, + + \( P < 0.01 \)). Representative traces are shown in A, and the summary data are demonstrated in B.

DISCUSSION

Major findings. There are three major findings in this study. 1) The GABAergic pathway from the LPGi to CVNs is enhanced after 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 but, however, to a lesser degree in males exposed to CIHH. In addition, in male CIHH animals LPGi-evoked GABAergic neurotransmission is enhanced after acute H/H. 3) Orexin-A diminishes GABAergic current from the LPGi to CVNs in unexposed animals of both sexes as well as in female CIHH rats. However, orexin-A elicited no significant
changes in LPGi-evoked GABAergic current in male CIHH rats.

**LPGi pathway to CVNs.** Consistent with the conclusions in previous studies (Dergacheva et al. 2010, 2011), 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 after lesions of the medullary reticular formation encompassing the LPGi (Holmes and Jones 1994). 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. 2005, 2006). 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 REM sleep also express glycine (Boissard et al. 2002), 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 the 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).

LPGi-evoked pathway to CVNs is exaggerated after 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 CIH. This enhanced neurotransmission could be a result of increased sensitivity of GABAergic receptors in CVNs after CIHH, as spontaneous GABAergic IPSCs in CVNs have been shown to be increased in CIHH rats (Dya-vanapalli et al. 2014). In addition, REM sleep-related activation of GABAergic neurons in the LPGi that likely project to CVNs could also be increased after CIHH, since shortening of a REM sleep episode 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 after 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 sex 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 male CIHH animals compared with female CIHH rats. Acute H/H also evokes a lesser reduction in the GABAergic amplitude in CIHH male animals compared with unexposed males. In
addition, GABAergic current is exaggerated 20 min after 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 after 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, 2014b). Supporting the findings of this work, parasympathetic function during sleep has been found to be less stable in male OSA patients compared with 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 after 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 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 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 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, 2013). In contrast to unexposed rats of both sexes 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/HCO3- (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.

**Conclusions.** 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 after 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.

**REFERENCES**


