One Breath at a Time: Stability in Respiratory Neural Networks

By

Joel Green Weltman

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Comparative Biomedical Sciences)

at the

UNIVERSITY OF WISCONSIN-MADISON

2017

Date of final oral examination: November 22, 2017

The dissertation is approved by the following members of the Final Oral Committee:

Tracy L. Baker, Associate Professor, Department of Comparative Biosciences
Jyoti J. Watters, Associate Professor, Department of Comparative Biosciences
Rebecca A. Johnson, Associate Professor, Department of Surgical Sciences
Nadine P. Connor, Associate Professor, Department of Surgery
John Svaren, Associate Professor, Department of Comparative Biosciences

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ACKNOWLEDGEMENTS

First and foremost I would like to acknowledge the trust and support that Dr. Tracy Baker has placed into me by taking me under her wing and guiding me through my graduate studies. Your mentorship has and always will be invaluable in my development as a scientist. Thanks are also due to Dr. Gordon Mitchell for accepting me into his lab and supporting me in my initial work as a researcher. I would also like to thank Becky Johnson for her enthusiastic support for my acceptance and continuation in my graduate programs and also for guidance in my research and clinical work-I look forward to continuing to collaborate with you in the future. In addition I would like to thank Drs Watters, Connor and Svaren for their ideas and support as members of my thesis committee and as collaborators to the Baker laboratory. Angela Navarette-Opazo was instrumental in setting the foundation for these studies as well as providing my initial instruction on surgical techniques and performance of plethysmography. For these reasons I owe her very much thanks. Considerable appreciation is due Jonathan Ouellette and Sam Lines for their tireless work on gas exposure systems. Finally, I owe considerable thanks to my friends and family for the personal and emotional support through a challenging but very gratifying part of my career development. In particular I owe thanks to Latoya Allen, Nichole Nicols, Ibis Agosto, Franzo Law II, Ryan Lockwood and my parents.

ABSTRACT

In order to maintain physiologically appropriate levels of oxygen and carbon dioxide, neural networks controlling breathing must be consistent and precise at all times, from birth to death. Under certain conditions, such as sleep apnea syndrome, these complex systems may falter leading to life threatening loss of ventilation. Processes underlying disturbances in respiratory neural activity are poorly understood and reflect the complexity of the system; however, the work presented here begins to explore the cellular basis for this phenomenon. Recent work from our lab has identified a novel form of compensation activated by respiratory disturbances called inactivity-induced respiratory motor facilitation, in which reductions in respiratory neural activity are sensed within spinal motor systems and trigger rebound enhancements in inspiratory motor output to maintain appropriate drive to inspiratory muscles. Here, I demonstrate that chronic intermittent hypoxia, as experienced with sleep apnea, limits expression of this compensatory mechanism and may propagate sleep disordered breathing. Further, I demonstrate that chronic intermittent hypoxia results in irregular breathing patterns in spontaneously breathing rodents when breathing stability is disrupted by high fat diet. Cells responsible for sensing respiratory neural activity have yet to be determined, however, I explore the role of astrocytes in controlling breathing and surprisingly found that astrocytes are necessary to restrain the strength of neural responses to respiratory disturbances. Finally, while chronic intermittent hypoxia may destabilize breathing, I demonstrate that low dose intermittent hypoxia recruits inspiratory activity in non-diaphragm muscles of breathing, providing the potential to harness neuroplastic processes to improve ventilation in disorders in which ventilation is compromised.

CHAPTER I

Introduction

Maintaining concise and consistent levels of breathing is critical at all times from birth to death. By initiating and coordinating the activity of the diaphragm and thoracic wall muscles, the central and peripheral nervous systems function to provide oxygen to and eliminate carbon dioxide from the body. These processes involve complex coordination between specialized cells capable of sensing oxygen and carbon dioxide, a central integration network, neuroplasticity to adjust system properties to meet the demands of an ever-changing organism, and precise activation of muscles of breathing. While these processes are robust and well regulated in health, under certain conditions, their regulation breaks down and may lead to catastrophic losses in ventilatory capabilities. The overarching focus of this document is on these periods wherein central control of breathing breaks down as well as mechanisms to combat the instability of breathing that ensues.

One relevant example when these processes are lost is that of sleep apnea syndrome, which is the recurrent reduction or complete absence of ventilation during sleep. While normal individuals may experience infrequent failures of ventilation during sleep, the frequency and severity of these events are what define sleep apnea syndrome. Aspects of the sleep apnea syndrome can be modeled in rodents. Indeed, normal rodents demonstrate apnea both spontaneously and following deep breaths as we have demonstrated by whole-body plethysmography (figure 1). Infrequent disruptions in breathing like this are common in humans as well; however, when these events become excessive, intermittent episodes consisting of systemic hypoxemia and sleep disruption lead to the clinical syndromes that afflict individuals with sleep

disordered breathing. Sleep apnea can result generally from two different mechanisms: 1) obstructive sleep apnea (OSA), which is the consequence of repetitive collapse of the upper airways preventing normal ventilation despite continued respiratory neural activity and 2) central sleep apnea (CSA), which is the intermittent loss of respiratory neural activity during sleep (Eckert et al 2007, Javaheri & Dempsey 2013). The etiology of sleep apnea is poorly understood, but a hallmark feature of both CSA and OSA is an unstable neuromuscular control alongside frequent, repetitive episodes of intermittent hypoxia (Salloum et al 2010, Xie et al 2013). Repeated exposure to hypoxia is thought to contribute to the destabilization of breathing, wherein chronic exposure to moderate intermittent hypoxia (CIH) (Younes 2001), leads to an increase in respiratory pattern variability in both rats and humans primarily through an increase in the number of apneas and hypopneas during sleep (Deacon & Catcheside 2015, Edge et al 2012, Mateika & Syed 2013). This suggests a self-perpetuating cycle of intermittent hypoxia and apnea sets the stage for progression of disease in individuals afflicted with either form of sleep apnea by influencing the neural network controlling breathing.

Precise control of breathing occurs on several levels

In order to dictate the appropriate the level of ventilation necessary to maintain ideal concentrations of oxygen and carbon dioxide in the blood, the neural systems controlling breathing utilize a negative feedback mechanism consisting of both peripheral and central nervous system components. Traditionally, oxygen tension is thought to be sensed within the carotid and, in some species, the aortic bodies of the

peripheral nervous system (Funk 2010, Sapru 1996); however, as discussed in chapter IV, recent investigations support the presence of a central oxygen sensing mechanism (Funk 2010, Gourine & Funk 2017). Signals regarding blood oxygen levels are transmitted by the glossophyrngeal nerve to the brainstem. Systemic carbon dioxide is thought to be sensed predominantly within the raphe nucleus, nucleus tractus solitarus, locus coeruleus and retrotrapezoid nucleus regions of the brainstem; however the exact mechanism by which the level of carbon dioxide is monitored and defended is a matter of debate (Guyenet & Bayliss 2015, Guyenet et al 2008, Nattie 1999). Likely complex connections between these regions and brainstem centers generating respiratory motor output are responsible for passing integral information on deviations in blood CO2 levels to trigger necessary changes in the level of ventilation. While the cells responsible for central nervous system chemosensation have been historically thought to be neurons, recent work argues that astrocytes likely fill this role, at least in part. Astrocytes in culture have been shown to be sensitive to elevated carbon dioxide and reduced oxygen levels (Angelova et al 2015, Funk 2010, Gourine & Funk 2017). Further, inhibition of this mechanism reduces ventilation in response to high carbon dioxide *in vivo* (Huckstepp et al 2010). Given that astrocytes are capable to responding to much smaller deviations in gas tension than neurons in culture (Angelova et al 2015), it is feasible that under normal physiologic settings, these cells play a predominant role. Chapter IV of this document attempts to address the role of astrocytes in regulating breathing in fully intact mice with morphologic disorders to their astrocytes.

Motor processes dictating breathing activity and rhythm originate in the medulla; however, they are capable of modifications within the spinal cord. The ventral respiratory column (VRC) is a column of respiratory nuclei in the ventolateral medulla. A critical nucleus for respiratory rhythm generation is the pre-Bötzinger complex within the VRC (Syed et al 1990). Rostral to the pre-Bötzinger complex is the Bötzinger complex which, along with the retrotrapezoid nucleus and parafacial respiratory groups (RTN/pFRG), is critical for maintaining expiratory phase of breathing by inhibiting inspiratory motor pools. These motor pools undergo mutual constraint with the pre-Bötzinger complex (Feldman et al 2013) to maintain both inspiratory and expiratory rhythm. The ventral respiratory group (VRG) is located within the caudal VRC, and is subdivided into the rostral and caudal VRG (rVRG, cVRG). The rVRG receives excitatory input from the pre-Bötzinger complex during inspiration, and inhibitory input from the Bötzinger complex during expiration (McCrimmon et al 1995). The cVRG receives excitatory input from the RTN/pFRG during expiration (Syed et al 1990). Descending bulbospinal projections from the ventral respiratory group (VRG) innervate phrenic motor neurons in the cervical spinal cord (C3-C6) through primarily glutamatergic monosynaptic connections, providing the main inspiratory drive to breathe in rats (Ellenberger & Feldman 1988, Greer et al 1992, McCrimmon et al 1989). The phrenic nerve innervates the diaphragm, which is the primary muscle involved in respiration. The VRG projects bilaterally to phrenic motor neurons, with most crossed-spinal pathways decussating in the brainstem (Edgerton et al 2001, Lipski et al 1994, Winslow & Rozovsky 2003). There are also “silent,” ineffective, contralateral projections from the VRG that decussate within the spinal cord, known as crossed-phrenic

pathways (Goshgarian et al 1991). These latent pathways can be strengthened, and transmit respiratory information to the contralateral spinal cord when respiration is disturbed (Fuller et al 2006, Fuller et al 2003, Golder & Mitchell 2005, Goshgarian 1979, Goshgarian 2003).

The spinal cord has additional means of modulating these patterns of breathing through neuroplastic processes, the most thoroughly studied of which include long-term facilitation and inactivity induced phrenic motor facilitation (LTF and iPMF, respectively). While these unique forms of neuroplasticity share some common aspects, i.e. a spinally mediated augmentation of inspiratory discharge from the phrenic nerve (Baker-Herman & Mitchell 2002, Streeter & Baker-Herman 2014), they diverge in the events leading to their initiation and maintenance. LTF is triggered by exposure to intermittent hypoxia, which leads to a PKC-theta dependent enhancement of phrenic output (Devinney et al 2015). In contrast, iPMF is initiated by a reduction in respiratory neural activity, which leads to a rebound enhancement of phrenic inspiratory activity via PKC-zeta (Baertsch & Baker-Herman 2015, Strey et al 2012). By local infusion of either local anesthetic or pharmacologic inhibition of cellular signaling pathways, these neuroplastic events have been definitively identified to be spinal in origin, and in the case of iPMF, represents a phenomenon both sensed and responded to locally (Baker-Herman & Mitchell 2002, Streeter & Baker-Herman 2014). Surprisingly, recent work has demonstrated that acute exposure to concurrent intermittent hypoxia and respiratory neural inactivity, as may be experienced during sleep apnea, obliterates plasticity altogether (Fields et al, in

preparation; Appendix B). Further work specifically investigating the influence of co-initiation of neuroplastic events is warranted, given these findings.

The role of spinal neuroplasticity in maintaining appropriate levels of ventilation is less clear. Previous work from our lab has demonstrated that in addition to enhancing inspiratory motor output, reductions in respiratory neural activity trigger a long-lasting reduction in the carbon dioxide level at which spontaneous ventilation ceases (apneic threshold, AT). In other words, reductions in respiratory neural activity trigger neuroplastic responses that make it harder to experience apnea again by 1) enhancing inspiratory motor output, and 2) lowering the threshold for apnea (Baertsch & Baker 2017). Further work is necessary to fully understand these findings and the implications they may have in disorders characterized by respiratory motor output instability; however, we have developed a working hypothesis that iPMF represents a form of compensatory neural plasticity in which local networks sense reductions in respiratory neural activity and activate mechanisms of plasticity that enhance synaptic strength to maintain a set-point of inspiratory neural output. Similar homeostatic forms of plasticity have been demonstrated and are discussed at great length elsewhere (Lee et al 2014, Turrigiano 2008).

While mechanisms of spinal neuroplasticity have been well established in healthy rats, ongoing studies suggest the presence of disease may influence these processes. For instance, CIH, as experienced during sleep apnea, has been recently shown to influence the expression of LTF. Gonzalez-Rothi and colleagues demonstrated that exposure to CIH leads to a complete loss of phrenic LTF expression (Gonzalez-Rothi et

al 2017). Following a similar paradigm of CIH, the expression of iPMF is also abolished; however, with seven days recovery, iPMF activity is restored (Chapter II, Weltman, Braegelmann, et. al. in preparation). Further investigation into these phenomena may provide information regarding the destabilizing effects of intermittent hypoxia on breathing; however, some hypotheses may be proposed based upon previous works.

Metaplasticity in neural circuits

Previous investigations have demonstrated a remarkable flexibility in the expression of functional plasticity (Abraham 2008, Grau et al 2014). This phenomenon, termed metaplasticity, is generally thought to display 3 characteristics: 1) the altered phenotype persists after the inciting experience resolves, 2) the impact is on the capacity of a neural network to enhance or depress function, and 3) the change is reversible (Abraham 2008, Grau et al 2014). This phenomenon was initially demonstrated in hippocampal slices wherein the expression of LTP and LTD may be influenced by various treatments, such as environmental enrichment, dark rearing, or conditioning (Abraham 2008). Similarly, some evidence exists supporting the potential for spinally mediated metaplastic responses. Following T12 spinal transection, rats can learn to maintain a flexed leg position by applying electric stimulation to the hind paw when the leg is in extension, an effect that is sustained when electroshock is no longer applied (Grau et al 2014). If the contralateral limb subsequently undergoes a similar paradigm, the time required to learn to maintain flexion is shortened, which would constitute an alteration in the capability to change neural behavior, or metaplasticity (Crown & Grau 2001).

Metaplastic responses in ventilation have also been documented. After administration of a CIH paradigm similar to that used in this study, McGuire, et al. were able to demonstrate an enhancement in the augmented ventilation triggered by subsequent exposure to acute intermittent hypoxia (ventilatory long-term facilation; vLTF) as compared to control, normoxia treated rats (McGuire et al 2003); however, reports since that time have demonstrated a blunted vLTF following CIH (Edge & O'Halloran 2015). The discordant findings in these papers as well as those shown by Gonzalez-Rothi, et al are likely a consequence of differences in the dose and methods of administering intermittent hypoxia, as discussed further in the following section. As you will see, we documented a reduction to complete loss in iPMF expression following seven days of CIH, a process which was reversed following seven days recovery. Given the criteria listed above, we have demonstrated the capability for metaplasticity in iPMF; however, in the context of stability of breathing, the loss of a compensatory plastic response would be considered undesirable.

Maladaptive plasticity is a concept that has emerged to describe the situations in which neural responses fail to respond appropriately to environmental demands (Elbert & Heim 2001). A classic example of this would be the musician’s cramp, or focal dystonia, wherein individuals experiencing intense, stereotyped motor behaviors, such as playing piano, develop a debilitating movement disorder related to reorganization of cortical neural systems (Altenmuller & Furuya 2016). Maladaptive responses have also been demonstrated in the context of metaplasticity. Utilizing the electric stimulation preparation described above, Crown, et al showed that if the electric shock is initially

delivered independent of the position of the limb, rats are no longer able to learn to flex their leg when electric shock is applied only during leg extension, an effect that lasts 48 hours (Crown et al 2002). In this work, we present data demonstrating maladaptive metaplastic responses may exist in the context of CIH influences on iPMF. If our hypotheses are correct, this represents the first documented form of maladaptive metaplasticity in spinal respiratory motor systems and may represent a critical step in the development of pathological conditions, such as sleep apnea, and mitigating these undesirable changes may improve therapeutic outcomes.

Intermittent hypoxia, it's a matter of dose

At this point it is relevant to discuss the systemic effects of intermittent hypoxia. As discussed above, intermittent hypoxia, as experienced with sleep apnea, is associated with several undesirable sequelae, including exacerbation in the frequency of respiratory disturbances (Deacon & Catcheside 2015, Edge et al 2012, Edge & O'Halloran 2015, Navarrete-Opazo & Mitchell 2014b). Given these findings, it may be surprising that hypoxia also elicits enhancements in inspiratory motor output (i.e., LTF), which presumably is to the benefit of neural motor control. These divergences in outcomes following intermittent hypoxia can be justified with a discussion on severity, duration, and repetition of hypoxic episodes. In general, severe hypoxia and/or high frequency of hypoxia exposure is associated with pathology, whereas mild hypoxia and/or low frequency of hypoxia exposure tends to enhance respiratory motor activity; the precise levels that determine the balance between pathological versus beneficial are unknown.

Often when discussing the beneficial effects of hypoxia, researchers describe the experience as acute intermittent hypoxia (AIH) (Dale et al 2014, Gonzalez-Rothi et al 2017, Navarrete-Opazo & Mitchell 2014b). Doses widely vary across the literature but AIH is generally described as an individual exposure to intermittent hypoxia at frequency of approximately six per hour and extending no longer than a couple of hours, and this dose has been demonstrated to initiate LTF (Dale et al 2014, Navarrete-Opazo & Mitchell 2014b). “Low dose” CIH is similar to AIH, but is repeated across several days. Intermittent hypoxia in this context has been demonstrated to reduce blood pressure in hypertensive individuals (Lyamina et al 2011, Serebrovskaya et al 2008, Shatilo et al 2008), stimulate red blood cell production (Brugniaux et al 2011, Martinez-Bello et al 2011), enhance innate immunity (Serebrovskaya et al 2011), improve metabolic state by reducing body weight, cholesterol, and blood glucose (Ling et al 2008, Morton et al 2006), enhance learning and memory (Leuenberger et al 2005, Lu et al 2009, Martin et al 2010, Shao et al 2006, Zhang et al 2005) and strengthen spinal respiratory plasticity (Fuller et al 2003; Lovett-Barr et al., 2012; Navarrete-Opazo & Mitchell 2014a). In contrast, “high dose” CIH typically is experienced over several days with exchange frequencies varying from 20-120 per hour and often lasting 8-12 hours per day. These studies are often designed to mimic the experience of individuals with sleep apnea. These high doses of intermittent hypoxia are believed to lead to systemic and central nervous system inflammation, pulmonary and systemic hypertension, insomnia, depression, daytime sleepiness, learning deficits, and hippocampal apoptosis (Brooks et al 1997, Kofler et al 2005, Lavie 2003, Lesske et al 1997, Perry et al 2008,

Row et al 2007, Smith et al 2013, Tagaito et al 2001, Tahawi et al 2001, Veasey et al 2004).

Utilizing neuroplastic processes to improve the strength of respiratory neural circuits

As stated previously, the work presented here largely represents investigations into the means by which breathing pathways lose their relative stability during periods of disease or distress. While determining the processes underlying disease is critically important, equally important is finding means by which we can combat catastrophic loss of ventilation. Harnessing neuroplasticity to improve outcomes in disease has been a matter of intense investigation over the past several years. In particular, processes to improve both diaphragm and non-diaphragm inspiratory activities have shown considerable promise. We have previously demonstrated an enhancement in diaphragm activity following exposure to acute intermittent hypoxia, presumably by LTF-like mechanisms (Navarrete-Opazo & Mitchell 2014a, Terada & Mitchell 2011). Further, intriguing new work has demonstrated the capacity for intermittent hypoxia induced enhancement of non-diaphragm muscles of breathing. Navarette-Opazo and colleagues investigated the second intercostal EMG activity prior to and following intermittent hypoxia and demonstrated an enhancement of activity which surpassed that seen in the diaphragm following treatment (Navarrete-Opazo & Mitchell 2014a, Navarrete-Opazo et al 2015). Previous investigators have demonstrated an increase in key mediators required for the expression of LTF following intermittent hypoxia within the region of the phrenic motor pool (Satriotomo et al 2012, Wilkerson & Mitchell 2009) as well as non-

phrenic regions of the spinal cord (Satriotomo et al 2016) suggesting these findings all share characteristics with LTF. Chapter V of this document expands upon this knowledge, investigating means of utilizing established neuroplastic principles to enhance the potential output of muscles involved in breathing. Further work in disease models is indicated to assess to utility of these methods in clinical conditions.

Summary of aims

The goal of this dissertation was to investigate unstable breathing patterns in disease, particularly in regards to situations in which the inherent activity of respiratory neural networks is altered. In chapter two, experiments were designed to investigate the function of iPMF in a model of sleep apnea in rats. By administration of chronic intermittent hypoxia (of the pathological type), we test the hypotheses that 1) CIH induced metaplasticity reversibly constrains iPMF expression 2) in a PKC-theta dependent manner and that 3) iPMF-like mechanisms underlie spontaneous recovery of CIH induced increases in apnea and hypopnea. We demonstrate that CIH impairs the ability to express iPMF, and that the ability to express iPMF recovers 1 week following mitigation of CIH. Contrary to literature reports (Deacon & Catcheside 2015, Edge et al 2012, Yokhana et al 2012), we did not observe that CIH exposure increased apnea/hypopnea frequency; thus, we were unable to test the hypothesis that iPMF-like mechanisms underlie spontaneous recovery of CIH-induced increases in apnea/hypopnea frequency. However, in a small subset of rats CIH exposure resulted in an increase in apnea/hypopnea frequency in the absence of any other interventional

treatments, documenting we are capable of recapitulating the findings found in previous studies.

With the knowledge that compensatory spinal neuroplasticity is inhibited with CIH, we sought to investigate the impact this treatment has on a population predisposed to apnea, diet induced obesity. Here we hypothesized that diet induced obesity leads to increases in respiratory instability and central nervous system inflammation, which is exacerbated following CIH. While we did not find that high fat diets altered the stability of breathing or levels of inflammation, CIH induced an increase in inter-breath frequency irregularity when presented alongside high fat diet. In addition, CIH induced an increase in inflammation in the brainstem. These findings suggest that high fat diets worsen phenotype of CIH induced respiratory disturbances; however, our future studies will utilize longer duration diet modifications and CIH to determine if this worsens phenotype. In addition, future studies investigating the influence of high fructose corn syrup, which is necessary to induce metabolic syndrome in rodents (Della Vedova et al 2016), are ongoing.

In Chapter IV we sought to investigate how glial cells may influence breathing stability utilizing a mouse model of Alexander disease, which afflicts predominantly astroglial cells. We hypothesized that 1) mice with abnormal astrocytes demonstrate reduced eupneic ventilation and 2) reduced responses to enhanced chemosensory drive. Instead, mutant mice demonstrated only reduced respiratory frequency and an augmented tidal volume at eupnea. In addition, Alexander disease mice demonstrated an enhanced response to chemosensory inputs. These findings would suggest a

destabilizing effect in the presence of abnormal astrocytes. Future studies should investigate what level of respiratory control is afflicted in this condition as well as how this influences the stability of breathing in disease settings.

Chapter V seeks to investigate how spinal neuroplastic principles may be utilized to help coordinate breathing events across various muscle groups. Here we sought to investigate the hypotheses that 1) non-diaphragm muscles of breathing have minimal eupneic activity but are able to be recruited during times of increased need and 2) therapeutic intermittent hypoxia can be used to recruit inspiratory activity in these muscle groups at all levels of ventilation. In this study we demonstrated that the scalene medius and serratus dorsalis show increased inspiratory events with increased chemosensory drive and also following low dose CIH, presumably by LTF-like mechanisms. Future studies designed to investigate the roles of LTF in this process are warranted.

In appendix A, we present preliminary data to support the role of compensatory neuroplasticity in recovery following spinal injury. Here we begin to test the hypothesis that acute recovery following cervical spinal injury requires the activity of phrenic PKC-zeta. While the data support our hypothesis, some modifications to the study design are discussed to reduce variability and improve the impact of the findings. Further studies on the role of iPMF in spinal injury are ongoing. And finally, in appendix B we explore the consequences of exposure to both apnea and hypoxia simultaneously in an acute setting, demonstrating a mutual constraint imposed by two unique forms of spinal neuroplasticity. This provides the basis for many of the studies presented in this

document and provides further mechanistic basis for the role of neuroplasticity in instigating instability in sleep disordered breathing.

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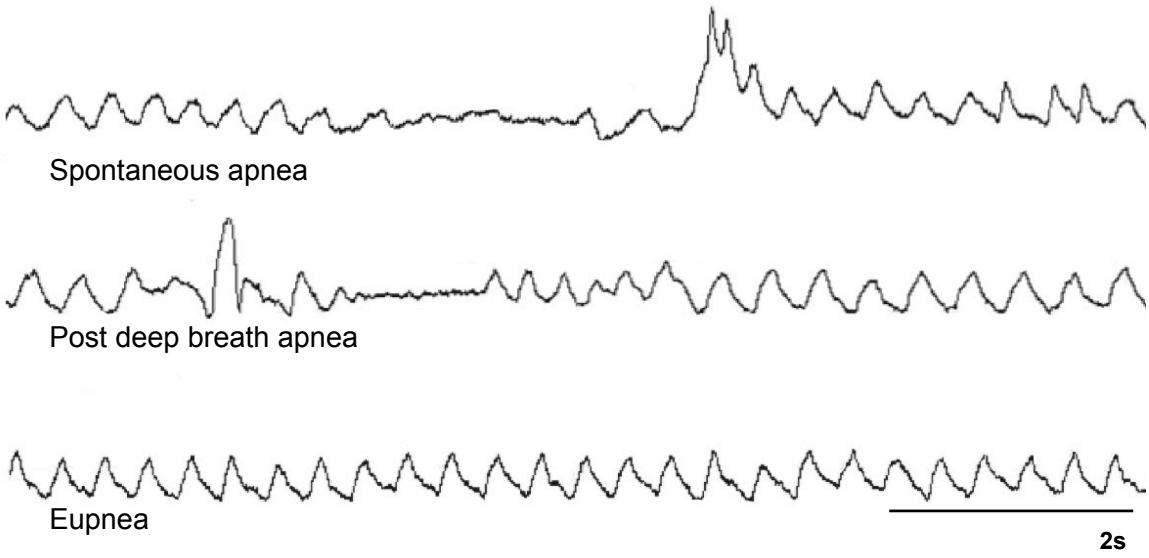

Figure 1. Representative whole-body plethysmography traces from rats. Here are demonstrations of the appearance of a spontaneous apnea, post deep breath apnea and eupnea in a flow-time tracing of plethysmography in a rat.

CHAPTER II

Chronic intermittent hypoxia induces a recoverable impairment in the capacity to elicit inactivity-induced phrenic motor facilitation (iPMF) following intermittent neural apnea

J.G. Weltman, K.M. Braegelmann, T.L. Baker

ABSTRACT

Neural circuits underlying breathing are exquisitely sensitive to reduced respiratory neural activity, even in the absence of chemoreflex feedback. Intermittent reductions in respiratory neural activity (neural apnea) trigger a spinal protein kinase C-zeta (PKCζ) dependent increase in phrenic burst amplitude, a form of compensatory plasticity known as inactivity-induced phrenic motor facilitation (iPMF). IPMF is thought to be a compensatory form of plasticity that prevents subsequent neural apnea, and we recently demonstrated that iPMF is constrained by concurrent moderate or severe hypoxia. Intermittent hypoxia triggers a distinct form of protein kinase C-theta (PKCϴ) dependent plasticity known as phrenic long-term facilitation (pLTF). Since many individuals with central sleep apnea have concomitant reductions in respiratory neural activity with hypoxia, we tested the hypotheses that: 1) chronic intermittent hypoxia (CIH) impairs the capacity to elicit iPMF, 2) the capacity to elicit iPMF is restored within one week after termination of CIH, 3) CIH induced restraint on iPMF expression is PKCϴ dependent, and 4) CIH leads to an increase in apnea and hypopnea, the recovery of which is PKCζ dependent. To test these hypotheses rats were exposed to 7 days of CIH (8 hrs/day, 2 min 10.5% O_2 separated by 2 min 21% O_2) or an equivalent duration of normoxia. In rats exposed to 7 days of normoxia, intermittent neural apnea triggered a significant increase in phrenic burst amplitude for up to 60 min post-neural apnea, (49±6 %baseline; $p<0.05$), indicating iPMF. Consistent with our hypotheses, rats exposed to CIH did not express increased phrenic burst amplitude following intermittent neural apnea (0±7, %baseline, $p>0.05$), indicating CIH impairs iPMF. However,

following 7 days recovery from CIH, increased phrenic burst amplitude following intermittent neural apnea was again apparent (56±18, %baseline, $p<0.05$). To specifically reduce PKCϴ expression within the phrenic motor nucleus, we administered small, interfering RNA targeting PKCϴ (siPKCϴ) to the intrapleural space (100pg/side) daily throughout CIH. In CIH rats receiving siPKCϴ, intermittent neural apnea triggered increased phrenic burst amplitude (34 ± 4 % baseline; $p=0<0.05$), suggesting that CIH-induced activation of pLTF constrains the ability to elicit iPMF. Despite previous reports demonstrating an augmentation in apnea and hypopnea frequency and duration with CIH, freely breathing animals failed to failed to recapitualate these findings in our hands, regardless of the level of PKCζ activity. These data demonstrate that CIH constrains the expression of iPMF, but mechanisms of iPMF spontaneously recover over time once hypoxia is resolved; however, as CIH did not induce increases in apnea or hypopnea in this population, conclusions on the role of iPMF in propagation of apneic and hypopneic states cannot be made. Further studies in freely breathing animals in the absence of siRNA or daily anesthesia should be pursued to investigate the role of these phenomena in normally behaving animals.

INTRODUCTION

The respiratory control system does a remarkable job of integrating rhythmic signals generated in the brainstem and faithfully transmitting that signal to respiratory motor neuron pools to execute a coordinated breath. In healthy individuals, respiratory control maintains adequate ventilation not just during eupneic breathing, but also is very adept at responding to our ever-changing metabolic demands and the challenges posed by the environment in which we breathe. Such exquisite control and responsiveness is conquered through a compliment of attributes. Respiratory plasticity, or the system's capacity to alter its properties in response to certain stimuli, is one of the features that afford the respiratory control system such robust adaptability in response to the demands it faces (Braegelmann et al 2017, Dale-Nagle et al 2010, Mitchell & Johnson 2003, Strey et al 2013).

Respiratory plasticity is often initiated by signals of respiratory failure. For example, reductions in respiratory neural activity and hypoxia are each potent stimuli for plasticity in respiratory motor control, and both manifest as a long-lasting increases in phrenic burst amplitude that could serve to prevent such failures from occurring again (Braegelmann et al 2017, Dale-Nagle et al 2010, Devinney et al 2013, Strey et al 2013). Data support the hypothesis that mechanisms local to phrenic motor neurons sense and respond to reductions in synaptic drive by activating a compensatory form of plasticity termed inactivity-induced phrenic motor facilitation (iPMF; Baertsch & Baker 2017, Baertsch & Baker-Herman 2013, Braegelmann et al 2017, Streeter & Baker-Herman 2014a). Similarly, acute, intermittent exposure to hypoxia stimulates the release of

serotonin in the phrenic motor pool to activate a distinct form of plasticity termed phrenic long-term facilitation (pLTF; Baker-Herman et al 2004, Dale-Nagle et al 2010, Devinney et al 2013). Over the course of many years, meticulous investigations into the signaling pathways that underlie iPMF and pLTF have uncovered distinct cellular cascades. Particularly, pLTF diverges from iPMF in its requirement for protein kinase C-theta (PKCθ; Devinney et al 2015); whereas iPMF requires protein kinase C-zeta activity (PKCζ; Strey et al 2012). Even though the respiratory control system is equipped with these robust, mechanistically distinct forms of plasticity that are both well-situated to respond to a lack of adequate ventilation, circumstances remain in which the system fails to appropriately respond and adapt to challenges. A prime example, central sleep apnea, is characterized by repetitive, concurrent reductions in respiratory neural activity and hypoxia, which would be expected to activate these endogenous plastic mechanisms and restore adequate ventilation. Instead, the condition perpetuates and individuals who experience sleep apnea often experience worsening of both intermittent hypoxia and apnea (Papa et al 2014, Punjabi et al 2009, Young et al 2002). Why these endogenous mechanisms fail to stabilize breathing for individuals that suffer from sleep apnea is of great interest as the number of afflicted individuals continues to rise and the serious comorbidities associated with sleep apnea continue to be unveiled (Young et al 2002).

While both iPMF and pLTF have been studied extensively, the interaction between these forms of plasticity has rarely been investigated. Research is required to understand the complexity of the interactions between distinct forms of respiratory

plasticity and their role in stabilizing breathing *in vivo*. Recent observations from our lab illustrate an acute interaction between mechanisms initiated by respiratory neural activity deprivation and hypoxia that occludes the expression of respiratory plasticity altogether. Indeed, concurrent exposure to hypoxia and reduced respiratory activity fail to elicit phrenic motor facilitation, indicating neither form of neuroplasticity is expressed in response to the physiologically relevant exposure to both neural apnea and hypoxia. However, blocking spinal NMDA receptor activation restores the capacity for intermittent neural apnea to elicit iPMF, even in the presence of hypoxia (Appendix B, in preparation). These observations led us to consider the interaction of plasticity initiated by chronic intermittent hypoxia (CIH), representative of the nightly exposure to intermittent hypoxia experienced by individuals afflicted with sleep apnea, with mechanisms of iPMF.

CIH induces a multitude of changes throughout the respiratory control system that include: altered chemosensory responses, induction of inflammatory gene expression and oxidative stress in crucial respiratory regions, increased frequency of apneic events, disruption of neural respiratory signal transduction, and neuronal apoptosis (Deacon & Catcheside 2015, Edge et al 2012, Garcia et al 2016, Kiernan et al 2016, Mateika & Syed 2013, Rey et al 2004, Smith et al 2013, Souza et al 2015, Xu et al 2004). Together, these CIH-induced alterations in the respiratory control system can precipitate either the enhancement or depression of pLTF (Gonzalez-Rothi 2017, Julien et al 2008, Ling et al 2001, McGuire et al 2003, McGuire et al 2004, Reeves et al 2006), indicating CIH may alter the capacity for the neural circuit to express plasticity, a

phenomenon termed metaplasticity (Devinney et al 2013, Fields & Mitchell 2015). Little is known about the metaplastic events that influence the expression of spinal respiratory plasticity. In particular, we were interested in whether prior exposure to CIH occludes phrenic motor plasticity initiated by intermittent activity deprivation.

Here we tested the hypothesis that CIH impairs the capacity to elicit iPMF via CIH-induced activation of pLTF-like mechanisms. We demonstrate that one week of CIH impairs the capacity to elicit iPMF, but this recovers within one week of termination of CIH. Further, impairing PKCθ-dependent signaling in phrenic motor neurons throughout CIH exposure alleviates the impairment of iPMF. Collectively these data suggest that CIH-induced activation of pLTF results in transient metaplastic changes to phrenic motor neurons and impairs their ability to respond to neural apnea, which rapidly recovers when hypoxia is mitigated. Given that a reduction in phrenic PKCθ expression abrogates CIH-induced constraint on iPMF, spinal pLTF-like mechanisms likely contribute to the abolishment of iPMF. Despite previous reports, freely breathing animals failed to display respiratory disturbances following CIH, regardless of the level of PKCζ activity, and, therefore, conclusions on the role of iPMF in propagation of apneic and hypopneic states cannot be made. Further studies in freely breathing animals in the absence of siRNA or daily anesthesia should be pursued to investigate the role of these phenomena in normally behaving animals.

METHODS

Animals

All experiments were performed on 8-16 week-old, male Sprague-Dawley rats (colonies 206 and 217, Harlan/Envigo, Indianapolis, IN). Animals were co-housed in temperature and humidity controlled environment with 12:12 hour light/dark cycle. The Animal Care and Use Committee of the School of Veterinary Medicine, University of Wisconsin approved all experimental procedures used in this study.

Surgical Preparation

Isoflurane anesthesia was induced (2.5-3.5%) in 50% O_2:N_2 balance. Rat body temperature was monitored using a rectal probe (Physitemp, Model 700 1H) and maintained within a degree of 37.0ºC with a customized, heated table. Rats were tracheostomized and pump ventilated ~70 breaths/min, 2-3mL; VentElite Small Animal Ventilator, Harvard Apparatus, Holliston, Massachusetts). End-tidal CO_2 ($ETCO_2$) was monitored from the expired line of ventilator circuit with a flow-through capnograph (Capnogard; Respironics), and inspired tracheal pressure was monitored. Rats were slightly hyperventilated so a small amount of CO_2 was added to the inspired gas mix to maintain an $ETCO_2$ of ~45mmHG; inspired CO_2 and/or ventilator rate were adjusted throughout the surgery to ensure continuous generation of respiratory efforts and to prevent unintended neural apnea. The vagus nerve was isolated and severed bilaterally at the cervical level to prevent entrainment with the ventilator. Catheters were placed into the tail vein and femoral artery for delivery of fluids (lactated Ringers solution, ~20% sodium bicarbonate) and to monitor blood pressure and sample blood-gas and pH (ABL800; Radiometer, Copenhagen, Denmark). Rats were converted to urethane anesthesia (1.7-1.8g/kg I.V. infused at 6.5mL/hour) while isoflurane was

simultaneously withdrawn. A minimum of one hour was allowed following completion of the anesthetic conversion to enable adequate washout of isoflurane prior to the experimental protocols. Appropriate depth of anesthesia was verified by testing the blood pressure response to paw pad-pinch. The left phrenic nerve was dissected via a dorsal approach, cut distally, desheathed, and recorded using a bipolar silver recording electrode submerged in mineral oil. Following surgery, rats were paralyzed with pancronium bromide (1mg/kg, i.v.) and phrenic nerve activity was monitored and allowed to stabilize during the isoflurane washout period prior to the experimental protocol.

Electrophysiological Recordings

Baseline phrenic nerve activity was established after a minimum of 20 min of stable phrenic burst amplitude and frequency under isocapnic conditions, and arterial blood gas was sampled to obtain baseline $PaCO_2$, PaO_2, and pH measurements. Following the establishment of baseline conditions, intermittent neural apnea was delivered by rapidly reducing inspired CO_2 until rhythmic phrenic burst activity ceased (i.e. CO_2 apneic threshold). Upon neural apnea, inspired CO_2 was immediately returned to the mix of inspired gas and restored to baseline levels as determined by $ETCO_2$. Nerve activity was maintained for a recovery period of 5 min. This apneic protocol was repeated for a total of 5, ~1 min apneas interspersed by 5 min of stable phrenic burst activity. In all experiments, mechanical ventilation ensured PaO_2 was maintained >200mmHg throughout the protocol (including during neural apnea). Arterial blood was sampled 15, 30, and 60 min following the last neural apnea

to ensure baseline levels of PaO_2, $PaCO_2$, pH, and SBE were maintained. $PaCO_2$ was maintained within 1.5 mmHG of baseline levels by adjusting inspired CO_2 as necessary. A subset of rats underwent the same surgical preparation and were recorded for a similar duration, but were not exposed to intermittent apnea to serve as time controls.

Chronic intermittent hypoxia protocol

Rats were randomly divided into experimental and control groups. Rats in the experimental group were exposed to chronic intermittent hypoxia (CIH), which consisted of cyclic exposures from normoxia to hypoxia (240, 2 minute episodes of 10.5% O_2 interspersed with 2 minute episodes of normoxia 21% O_2; 8 hours daily) for seven consecutive days. Gas concentrations were tightly controlled under custom Plexiglas cage inserts (2 rat per cage), and oxygen and carbon dioxide levels were continuously measured within the chambers (CWE, Gemini model) to ensure consistent and precise hypoxia exposures. Target gas concentrations were achieved by mixing nitrogen and oxygen and were controlled with a custom-made computer-controlled system. Gas flow rates were 15 liters per minute per cage. 90% of the reduction in O2 levels was obtained within the first 45 s and 90% of the increase in O2 levels was obtained within the first 15 s of transition between oxygen concentrations. Carbon dioxide levels were maintained < 0.5% at all times. Control rats were administered continuous normoxia/normocapnia for seven consecutive days (NX). Rats that were allowed to recover from CIH for 1 week were returned to room air for the duration of the recovery period.

RNA interference in Respiratory Motor Neurons

Small interfering RNAs (siRNA) were administered to the intrapleural space to selectively impair PKCθ (siPKCθ) and PKCζ (siPKCζ) expression in phrenic motor neurons. Intrapleural siRNAs have been demonstrated to retrogradely transport to phrenic motor neurons and specifically knockdown target mRNA to ultimately diminish targeted protein levels within phrenic motor neurons (Devinney et al 2015, Mantilla et al 2009, Nakajima et al 2012). Under isoflurane anesthesia, bilateral intrapleural injections of siRNA (100 picomoles, Accell SMARTpool, Dharmacon; 30ul 1X siRNA buffer in RNAse free water; 30ul/side) into the 5th intercostal space were performed daily for 3 days prior to, during, and throughout the recovery period following CIH until the day prior to terminal measurements. Rats serving as control received non-targeting siRNA (NTsi) under the same dosing strategy.

Whole Body Plethysmography

A subset of rats was utilized to investigate spontaneous, freely behaving breathing characteristics. At the onset of the study these rats were randomly assigned to siPKCζ or NTsi, as discussed above. These groups went on to be further subdivided to receive either normoxia or chronic intermittent hypoxia treatment. Final group compositions were NTsi + NX (n=7), siPKCζ + NX (n=8), NTsi + CIH (n=8), and siPKCζ + CIH (n=8).

Rats were individually placed in whole body plethysmography chambers (4 liters, Data Sciences International model 601-1425-001, St. Paul, MN). Pressurized air

continuously flowed through the chamber at 4 liters per minute, allowing precise control of inspired gas concentration and limiting expiratory gas accumulation. Respiratory flow signals were sent to a data exchange matrix (model ACQ-7700; Data Sciences International, St. Paul, MN) and collected through purposely-designed software (PONEMAH Physiology Platform, Data Sciences International, St. Paul, MN). For the purpose of analyses, body temperature was measured immediately prior to and following data acquisition. Body temperature was assumed to follow a linear change over time. Data was gathered for six consecutive hours at pretreatment and 16 hours following the final CIH or NX exposure.

Data analysis

Plethysmography data were averaged over 50 ms and periods of quiet breathing suitable for analysis were manually identified. A purpose written MatLab script was utilized to assess for tidal volume (TV), respiratory frequency (RR), minute ventilation (MV) and hourly rates and duration of apnea and hypopnea utilizing correction for pressure and temperature (Drorbaugh & Fenn 1955, Flatau et al 1992). The irregularity score of breathing frequency (ISBF) was calculated as previously described (Zanella et al 2014). A period of absent inspiratory flow for the average length of two or more breaths was defined as an apnea. If TV of any of five preceding breaths was equal to or greater than 125% of the mean tidal volume, the apnea was classified as post-deep breath; the remaining apneas were classified as spontaneous apnea (SA). Hypopnea was defined as a minimum of three successive breaths with individual TV less than 30% of mean TV of the tracing. All data are expressed as mean and standard error.

In electrophysiological preparations, phrenic inspiratory activity was amplified (x10k), band-passed filtered (0.3-10 kHz; AM Systems), integrated (time constant of 50ms), and rectified. The signal was then digitized and analyzed with PowerLab (AD Instruments; Lab Chart 8.0 Software). Integrated phrenic burst amplitude and frequency were averaged in 60-breath bins immediately preceding blood gas samples at baseline and 15, 30, and 60 min following intermittent neural apnea. Phrenic burst amplitude was expressed as a percent change from baseline (% baseline) to prevent any misinterpretation of the data from experimental differences from rat to rat. As burst frequency is not altered through the dissection and nerve placement on the bipolar electrode, burst frequency was expressed as an absolute change from baseline (Δbaseline). A two-way repeated measures ANOVA measured statistical differences between groups and Holm-Sidak post-hoc analyses were used to determine differences at individual time points at a significance level of $p<0.05$ when the ANOVA determined there was a significant treatment or time effect between groups (Prism 7, GraphPad Software). $ETCO_2$ values were collected at the point at which phrenic burst activity ceased for each neural apnea, referred to as the apneic threshold (AT) in each experimental protocol. Changes in the AT were calculated as an absolute change from the initial AT. Mean changes in the AT over the course of the five neural apneas were compared between treatment groups using a two-way ANOVA and Holm-Sidak post hoc analyses at a significance level of $p<0.05$. At the end of each protocol, rats were exposed to a maximal inspired CO_2 challenge to verify the preparation had not deteriorated and to investigate whether CIH treatment groups exhibited a change in sensitivity to CO_2. Phrenic amplitude was expressed as a percent change from

baseline (% baseline) at the maximal amplitude during hypercapnia. Statistical differences between treatment groups were determined using one-way ANOVA and Holm-Sidak post-hoc test at a significance level $p<0.05$ (Prism 7, GraphPad Software). Data are expressed as a mean $\pm$ standard error. Physiological parameters collected through blood gas measurements were also analyzed with a two-way repeated measures ANOVA and Holm-Sidak post-hoc analyses at a significance level of $p<0.05$. A ROUT outlier test of the data set identified one statistical outlier in the magnitude of phrenic burst amplitude from the 1d post CIH group (60min: 85 % baseline) and was removed from further analysis. Further analyses revealed that there was no significant difference in the phrenic burst amplitude between time control rats regardless of siRNA treatment or exposure paradigm ($P>0.05$). Therefore, time control rats exposed to normoxia treated with or without siRNA (n=2) and rats exposed to CIH with (n=6) or without (n=7) siRNA were combined (n=15) and used as a collective time control group in all analyses. Further, there was no statistical difference in phrenic burst amplitude following intermittent neural apnea in rats treated with Nx (n=8) vs Nx exposure with NTsi injection (n=8) at 1 or 7 days post Nx exposure, nor was there a difference in rats treated with CIH (n=8) vs CIH with NTsi injections (n=4) at 1 day or CIH (n=8) vs CIH with NTsi (n=4) at 7 days post exposure ($p>0.05$). Therefore, the groups were combined to create a collective Normoxia (n=16), 1 day post CIH (n=12), and 7 day post CIH group (12) to maximize our statistical power and minimize rats. There were three additional groups that were not combined with any other: 7 day post CIH + siPKCζ (n=7), 1 day post Nx + siPKCθ (n=8), 1 day post CIH + siPKCθ (n=10).

RESULTS

Chronic intermittent hypoxia transiently impairs iPMF

Recent evidence suggests that plasticity induced by reduced respiratory neural activity (iPMF) and plasticity induced by intermittent hypoxia (pLTF) are co-occluded when neural apnea and hypoxia are presented simultaneously (Appendix B, in preparation). Thus, we tested the hypothesis that CIH also interferes with the ability to elicit iPMF. The response to intermittent neural apneas the day following a one-week CIH exposure or one week normoxic exposure is shown in Figures 1A - D. Figure 1A presents representative compressed phrenic neurograms illustrating phrenic burst amplitude at baseline, during, and for 60 min following intermittent neural apnea or a similar duration in a time control rat given the same surgery but no neural apnea. Time control rats exposed to CIH did not express significant changes in phrenic burst amplitude from baseline at any time point (15 min: 10 ± 3, 30 min: 9 ± 2, 60 min: 7 ± 2; %baseline; $p>0.05$; Figure 1A-C), indicating that CIH did not affect baseline phrenic burst stability over the recording period. As expected, intermittent neural apnea elicited a significant increase in phrenic burst amplitude in NX rats when compared to time controls (58 ± 7; % baseline, $P<0.0001$; Figure 1A-C), indicating iPMF. By contrast, phrenic burst amplitude in rats exposed to intermittent neural apnea 12-24 hours following the CIH protocol was not significantly different from time controls at 60 min (6 ± 7; % baseline; $p=0.9682$) and was significantly lower than NX rats ($p<0.0001$; Figure 1A-C), indicating that CIH impaired the ability to elicit of iPMF.

To test the hypothesis that CIH-induced impairments in the ability to elicit iPMF recover when hypoxia is mitigated, a cohort of rats were allowed to recover for 1 week following CIH exposure before testing for the expression of iPMF (Figure 1A-C). In rats that recovered in normoxia for 1 week following CIH exposure (7d post CIH), intermittent neural apnea triggered significant increases in phrenic burst amplitude (60 min: 54 ± 12; % baseline; $p=0.0004$ from time controls and $p=0.0006$ from 1 day post-CIH rats), indicating that the capacity to elicit iPMF spontaneously recovers from CIH-induced impairment upon return to normoxic conditions.

To verify that the return of neural apnea induced phrenic motor facilitation following CIH still proceeded by iPMF-like mechanisms, PKCζ expression was impaired with siRNA prior to intermittent neural apnea exposure (Strey et al 2012; Baertch et al., in preparation). Control NTsi-treated rats exhibited a significant increase in phrenic burst amplitude following intermittent neural apnea seven days following CIH, indicating normal iPMF expression. By contrast, in rats that were treated with siPKCζ, intermittent neural apnea did not trigger a significant increase in phrenic burst amplitude (60 min: 17 ± 5; % baseline; $p=0.9101$ from time controls; Figure 1A-C). Further, phrenic burst amplitude 60 min following intermittent apnea was significantly lower in siPKCζ treated recovery rats compared with 7 day post CIH recovery rats treated with NTsi ($p<0.0392$), indicating that PKCζ-dependent mechanisms are required to recover intermittent neural apnea induced plasticity following CIH.

As a transient expression of frequency plasticity is characteristic of iPMF (Baertsch & Baker 2017, Baertsch & Baker-Herman 2013, Baertsch & Baker-Herman

2015), we compared the change in phrenic burst frequency from baseline across groups over the 60 min recording period (Figure 1D). Intermittent neural apnea triggered a transient increase in average burst frequency in NX control rats (5 ± 1; change from baseline, p=0.0075) and 7 day post-CIH recovery (7 ± 1; change from baseline, p=0.0002) compared with time controls (1 ± 1; change from baseline). By contrast intermittent neural apnea did not trigger a significant increase in burst frequency 1 day following CIH (1 ± 1; change from baseline; p=0.6412). Intermittent neural apnea also did not trigger significant increases in burst frequency in siPKCζ treated rats 7 day following CIH (3 ± 1; change from baseline; p=0.9389). There were no significant differences in average burst frequency from time controls at 30 or 60 min following intermittent apnea in any group (P>0.05). Together these data demonstrate a transient (~15min) increase in phrenic burst frequency following intermittent apnea in rats that expressed iPMF (NX-treated controls and 7-day post CIH recovery rats), but not in rats that did not express iPMF (1 day post CIH, 7 day post CIH + siPKCζ groups).

PKCθ is necessary for CIH to impair the capacity to elicit iPMF

Next, we sought to uncover mechanisms whereby CIH undermines iPMF. To test the hypothesis that CIH-induced activation of pLTF constrains the ability to activate iPMF we impaired the expression of PKCθ, a kinase required for normal expression of pLTF (Devinney et al 2015), within phrenic motor neurons throughout CIH exposure and investigated iPMF the next day (Figure 2A – D). Representative compressed phrenic neurograms depicting phrenic burst amplitude at baseline, during, and for 60 min

following intermittent apnea as well as a time control rat recorded for a similar duration are presented in Figure 2A. Average changes in phrenic burst amplitude (Figure 2B) and a scatter plot of the data (Figure 2C) at 60 min post intermittent apnea are presented to illustrate the group means as well as variation in the physiological response. As expected, NX control rats expressed a significant increase in phrenic burst amplitude following intermittent apnea compared with time controls (60 min: 58 ± 7; % baseline, $P<0.0001$; Figure 2A-C), indicating iPMF. Similarly, intermittent neural apnea elicited significant increases in phrenic burst amplitude in NX rats treated with siPKCθ (53 ± 10; % baseline, $p<0.0001$), indicating that the level of PKCθ activity does not impact the ability to elicit iPMF amplitude. As reported above, intermittent neural apnea 12-24 hours following CIH exposure in NTsi treated rats did not induce an increase in phrenic burst amplitude (60 min: 6 ± 7; % baseline; $p=0.9633$), indicating that CIH impairs the ability to elicit iPMF. However, in rats treated with siPKCθ to reduce PKCθ activity throughout the CIH exposure, intermittent neural apnea elicited a significant increase in phrenic burst amplitude compared with time control (60 min: 34 ± 4; % baseline; $p=0<0.05$) and compared with CIH + NTsi treatment ($p<0.05$), together suggesting that PKCθ activity contributes to CIH-induced impairment of iPMF. Although CIH + siPKCθ phrenic burst amplitude tended to be reduced from NX treated rats following intermittent neural apnea, expression of iPMF in these groups was not statistically different ($p=0.08$). Mean phrenic burst frequency of NX + siPKCθ and CIH + siPKCθ was not statistically different from each other, time controls, nor NX burst frequency at any time point ($p>0.05$, data not shown).

CIH mitigates neural apnea-induced reductions in apneic threshold

In addition to increasing phrenic burst amplitude, intermittent apnea also induces a progressive reduction in the CO_2 level at which spontaneous ventilation ceases (apneic threshold; AT, Baertsch & Baker 2016). Therefore, we tested the hypothesis that 1) CIH impairs the ability for intermittent neural apnea to reduce the AT and 2) the ability for intermittent neural apnea to reduce the AT will be restored upon recovery from CIH. The mean change in $ETCO_2$ at which phrenic inspiratory activity ceased from the first apnea was compared over the course of the subsequent four apneas for each group of interest (Figure 3). In groups that expressed iPMF (NX, CIH + 7 day recovery, CIH + siPKCθ) intermittent neural apnea elicited a significant decrease in AT by the 3rd apnea ($p<0.05$). However, in rats that did not express iPMF (1 day post-CIH and CIH + siPKCζ), intermittent neural apnea did not elicit a significant decrease in AT until the 4th apnea ($p<0.05$). These data suggest that although all groups responded to intermittent neural apnea by lowering the AT, CIH and PKCζ inhibition impair the ability for intermittent neural apnea to lower the AT. Further, the NX group expressed a significantly greater reduction in AT than the 1 day post CIH group at the 5th apnea ($p<0.05$). Together, these data suggest there is impairment in the reduction of the AT in rats conditioned with CIH.

Physiological parameters

Average arterial blood gas levels of CO_2, O_2, pH, and mean arterial pressure (MAP) at baseline and 60min following the intermittent apnea protocol are presented in

table 1. Throughout the recording PaO_2 was maintained above 200mmHg and $PaCO_2$ was maintained within 1.5mmHg of baseline following neural apnea. Although others report an increase in MAP following CIH (Souza et al 2015, Tkacova et al 2014), we did not observe any significant differences in baseline MAP between normoxia and CIH exposed rats. This deviation from the literature may be a consequence of the duration of exposure, the intermittent hypoxia paradigm, anesthesia, or other surgical related alterations. There were no time dependent changes in $PaCO_2$, PaO_2, nor pH, indicating that the observed changes in phrenic nerve activity cannot be attributed to alterations in chemoreceptor feedback. There was a small, but significant decrease in MAP over the course of the protocol, but was inconsistent and within the normal physiological range, suggesting these changes are not likely to contribute to the observed results. There were no consistent group differences in any of the parameters measured.

Chronic intermittent hypoxia fails to induce respiratory disturbances

With the data presented above documenting constraint of iPMF expression following CIH, we sought to investigate the influence this loss of iPMF has on the incidence and severity of respiratory disturbances. Given that seven days of intermittent hypoxia has been demonstrated to lead to an increase in apnea frequency and duration (Yokohama 2012), breathing was measured in unanesthetized rats by plethysmography at one day prior to and the day following seven days of intermittent hypoxia as described above. For two days prior to plethysmography and throughout intermittent hypoxia, rats received intrapleural siPKCζ or NTsi in order to investigate if iPMF is responsible for any recovery in apnea or hypopnea phenotype following CIH.

The average duration of apnea was no different between groups or following treatment (1.6 +/- 0.07 vs 1.6 +/- 0.04 s Nx + NTsi, 1.6 +/- 0.07 vs 1.5 +/- 0.05 s Nx + siPKCζ, 1.6 +/- 0.04 vs 1.5 +/- 0.03 s CIH + NTsi, 1.6 +/- 0.04 vs 1.6 +/- 0.05 s CIH + siPKCζ, data not shown). Additionally there was no change in frequency of spontaneous apnea (figure 4A), post deep breath apnea (figure 4B), or hypopnea (figure 4C) over time in any group (figure 4). The spontaneous apnea duration (figure 4D) and post deep breath apnea duration (figure 4E) also did not change following CIH as compared to values gathered prior to treatment. The irregularity score of both volume and frequency were calculated continuously and subsequently averaged within each individual. Neither irregularity score of volume or frequency showed a change over time in any group (figure 5).

DISCUSSION

A suite of endogenous mechanisms of respiratory plasticity may preserve breathing in response to respiratory challenges (e.g. neural apnea, hypoxia exposure). However, conditions characterized by intermittent experience of neural apnea and concomitant hypoxia, such as sleep apnea, persist and their prevalence is on the rise (Maspero et al 2015, Punjabi et al 2009, Young et al 2002). While often studied in isolation, identifying interactions between different forms of plasticity elicited by respiratory challenges is critical in determining their influence under normal breathing conditions as well as in disease.

Here we present evidence that chronic exposure to intermittent hypoxia induces changes in the respiratory neural network that prevent the subsequent expression of

compensatory plasticity in response to neural apnea. There is evidence that CIH alters the capacity for future expression of pLTF (Gonzalez-Rothi 2017, Ling et al 2001, McGuire et al 2003), but this is the first demonstration that CIH transiently impairs the expression of iPMF through a PKCθ dependent mechanism. While both CIH and intermittent neural apnea are characteristic of central sleep apnea, CIH-induced impairment of iPMF suggests compensatory mechanisms that are thought to ensure adequate ventilation during sleep are obstructed through repetitive exposure to hypoxia, leaving individuals increasingly vulnerable to future ventilatory instability. Advancing our understanding of mechanisms that underlie CIH-induced impairment of iPMF may provide opportunities for intervention to restore endogenous compensatory mechanisms to stabilize breathing in individuals co-expressing central apnea and intermittent hypoxia.

Mechanistic interaction between CIH-induced metaplasticity and spinal motor plasticity

Metaplasticity is a persistent change in system properties that alters the capacity of future neural function in a reversible manner (Abraham & Bear 1996, Fields & Mitchell 2015, Grau et al 2014). Prior to this study, investigation into CIH-induced spinal metaplasticity was limited to how CIH alters the future expression of various forms of long-term facilitation (LTF). CIH exposure has divergent effects on LTF depending on the protocol. Low cycle frequency of CIH has been shown to enhance both phrenic and hypoglossal LTF (Ling et al 2001, Peng & Prabhakar 2003, Zabka et al 2003) and the ventilatory response to acute intermittent hypoxia (McGuire et al 2003). By contrast, a higher cycle frequency of CIH abolishes the expression of pLTF (Gonzalez-Rothi 2017),

suggesting that the “dose” of CIH is critical in determining the impact of CIH on LTF expression (Navarrete-Opazo & Mitchell 2014). While recovery of pLTF was not investigated, we demonstrate that PKCζ-dependent iPMF spontaneously recovers by 7 days following a relatively high frequency, repetitive CIH exposure, similar to that which occludes pLTF expression (Figure 1). Although pLTF and iPMF are divergent forms of spinal neuroplasticity, previous investigations have demonstrated mutual inhibition between the two phenomena during acute, concurrent hypoxic and apneic exposure (Appendix B, in preparation). It is possible similar constraints are placed upon pLTF and iPMF following CIH. Indeed, our data demonstrating that spinal PKCθ inhibition prevents CIH-induced constraint of iPMF is consistent with this hypothesis. Alternatively there may be a common mechanism of CIH induced inhibition, such as inflammation (Huxtable et al 2015, Huxtable et al 2013), that neuroplastic responses following CIH.

Here we employed the use of siRNA targeting PKCθ delivered to the intrapleural space, which has been shown to impair PKCθ expression in phrenic motor neurons (Devinney et al 2015, Mantilla et al 2009, Nakajima et al 2012), during CIH to test the hypothesis that CIH induction of pLTF abolishes the ability to trigger iPMF. Our results demonstrate that impairing PKCθ results in a conservation in the ability to elicit iPMF following CIH when expression of plasticity following intermittent neural apnea would otherwise have been completely abolished (Figure 2); however, a trend for reduced magnitude of iPMF expression was observed following CIH + siPKCθ which may be significant with larger sample size. We hypothesize that this trend is due to 1) alterations of the respiratory control system that are not spinal (discussed in the next

section) or 2) through a signaling pathway in the phrenic motor pool that is independent of PKCθ. Indeed, recent evidence suggests that mild doses of CIH "flip" alternative pathways that normally constrain pLTF, rendering them now capable of inducing pLTF instead of impairing it (Fields & Mitchell 2015). Applying that logic here, we hypothesize that a CIH-induced, PKCθ-independent signaling cascade mediates CIH-induced changes in phrenic motor neurons, which may maintain partial constraint of iPMF.

CIH induces plasticity throughout respiratory control

Selective blockade of descending pathways innervating the phrenic motor pool (without altering chemosensory input or activity of other respiratory centers) elicits iPMF, providing robust evidence that spinal mechanisms sense and respond to reduced neural activity to elicit iPMF (Streeter & Baker-Herman 2014a). Therefore, it is reasonable to hypothesize that CIH induced changes in the phrenic motor pool underlie the impairment of iPMF. Indeed, CIH strengthens phrenic synapses following spinal injury (Fuller et al 2003) and acute exposure to intermittent hypoxia can recruit additional cervical spinal interneurons to the phrenic neural network (Streeter et al 2017). Further, the expression of critical proteins known to underlie pLTF such as serotonin receptors, BDNF, and ERK (McGuire et al 2004, Wilkerson & Mitchell 2009) is enhanced following mild dose intermittent hypoxia. In addition, NMDA receptors, required for pLTF (McGuire et al 2008, McGuire et al 2005) and a known inhibitor of iPMF (Streeter & Baker-Herman 2014b; Appendix B, in preparation) undergo phosphorylation in the region of the phrenic motor pool following CIH, in a similar paradigm to that used here (Appendix B, in preparation). Inflammatory gene expression

(Kiernan et al 2016, Smith et al 2013) is also upregulated in the region of the phrenic motor pool following chronic, high dose intermittent hypoxia. Together, these data provide evidence that intermittent hypoxia modifies the activity of molecules known to influence signal transduction and the response to future stimuli in the phrenic motor pool. However, CIH induces plasticity throughout many components of the cardio-respiratory control system.

While we do not directly test the contributions of central versus peripheral mechanisms in CIH mediated iPMF inhibition, Accell SMARTpool siRNA is designed to be taken up by available nerve terminals and is not believed to cross synapses (Nakajima et al 2012). Since phrenic terminals dominate the intrapleural space, the targeted protein is primarily reduced in phrenic motor neurons (Devinney et al 2015, Mantilla et al 2009). Using this technique we were able to restore iPMF with intrapleural siPKCθ in CIH treated rats when it otherwise would not be expressed (Figure 2). These findings support the hypothesis that at least a component of the disruption in iPMF expression following CIH is a spinally mediated.

The carotid bodies, responsible for peripheral oxygen sensing, have increased basal discharge after exposure to CIH (Peng & Prabhakar 2004, Rey et al 2004), and express further enhanced discharge for up to 60 minutes following acute intermittent hypoxia, which is not observed in rats not exposed to CIH (Peng et al 2001, Peng et al 2003, Peng & Prabhakar 2004). In addition to potential changes in oxygen sensing through carotid body plasticity, the phrenic response to carotid nerve stimulation (thereby bypassing the carotid bodies) is also amplified, suggesting that carotid

plasticity following CIH extends beyond chemosensitivity (Ling et al 2001). In support of this hypothesis, modifications in neurotransmission within the nucleus of the solitary tract, which is critical for sensory integration, have been identified following CIH (Almado et al 2012, de Paula et al 2007, Kline et al 2009, Kline et al 2007). Together, these changes contribute to a sensitization of the ventilatory response to acute hypoxia (Del Rio et al 2010, Peng et al 2003, Peng & Prabhakar 2004, Rey et al 2004), but it is difficult to speculate how these changes affect the expression of iPMF.

As previously demonstrated (Baertsch & Baker 2016), we observed a progressive reduction in the CO_2 level at which inspiratory activity ceases (apneic threshold: AT) with successive exposure to apnea in all groups (Figure 3). This reduction in apneic threshold takes longer to develop and the magnitude of reduction is attenuated in rats 1 day following CIH compared with control rats (Figure 3), suggesting CIH impairs mechanisms that modify AT following neural apnea. Despite complete recovery of iPMF, the reduction in AT in the 7 day post CIH group and 1 day post CIH + siPKCθ group was not statistically different from 1 day post CIH rats (Figure 3); however the trend for reduced AT in these groups leaves the possibility that spinal mechanisms contribute to this reduction in AT. We speculate that iPMF related reductions in AT protect the system from apnea during future swings that drop arterial CO_2, and that the constraint of these mechanisms by CIH contributes to unstable breathing patterns following CIH (Deacon & Catcheside 2015, Edge et al 2012).

Beyond the potential enhancement in strength of synaptic activity, neuroplastic processes have demonstrated the potential to alter the stability of the respiratory

network by influencing the ‘gain’ of the system, or the change in output for a given current input by influencing the controller and/or plant gain. The controller gain is the strength by which the central nervous system centers of breathing respond to alterations in blood gas levels as detected by peripheral and central chemoreceptors (Cherniack & Longobardo 2006, Javaheri & Dempsey 2013). Plant gain refers to the magnitude of change in blood gas concentration within the lung circulation after a given change in ventilation (Javaheri & Dempsey 2013). An increase of either value leads to unstable breathing due to an excessive response to the magnitude of disturbance. As discussed above, alongside the initiation of iPMF, we observe a long-lasting reduction in AT. Assuming that eupneic CO_2 remains stable, the overall effect of reducing AT would be to reduce controller gain and widen the CO_2 reserve, leading to stabilization of breathing. In the setting of sleep apnea; however, data presented here would suggest this stabilizing quality of iPMF is also lost. As discussed previously, CIH has influences on the activity of the carotid bodies and the nucleus of the solitary tract, both of which may have an influence on the gain of the system. In fact, as discussed previously, the previous experience of CIH leads to an augmentation to the ventilatory response to acute hypoxia, suggesting a high loop gain. These qualities of breathing following CIH may underlie increased apnea frequency noted in the literature (Deacon & Catcheside 2015, Edge et al 2012); however, as we were unable to elicit similar respiratory disturbances, additional studies will be necessary to determine the role of iPMF in stabilizing post-CIH breathing. It is possible that our study design influenced these results, as discussed below.

Deterioration of breathing stability and development of sleep apnea?

Sleep apnea is defined by a recurrent reduction or complete absence of airflow during sleep, which can result from different initiating mechanisms. Obstructive sleep apnea (OSA) is characterized by the repetitive collapse of the upper airways that prevents normal ventilation despite continued respiratory efforts. On the other hand, loss of wakefulness drive to breathe during sleep can precipitate variable breathing patterns in otherwise healthy individuals and can lead to recurrent reductions or complete loss of respiratory motor activity termed central sleep apnea (CSA; Eckert et al 2007, Javaheri & Dempsey 2013). The development of CSA involves complex interactions between sleep state transition, arousal threshold, ventilatory response to arousal, and apneic threshold. While CSA is not as prevalent as OSA, certain conditions, such as heart failure (Levy et al 2013), neurologic disease (Deak & Kirsch 2014) and long-term opioid use (Wang & Teichtahl 2007) are associated with a high incidence of CSA. Although often diagnosed as separate conditions, it is increasingly recognized that patients can express both CSA and OSA concurrently in mixed and complex sleep apnea and that CSA can often lead to OSA (Dernaika et al 2007, Javaheri & Dempsey 2013, Morgenthaler et al 2006, Xie et al 2013b).

The etiology of sleep apnea is poorly understood, but a hallmark feature of both CSA and mixed CSA/OSA is unstable breathing control and frequent, repetitive episodes of intermittent hypoxia (Salloum et al 2010, Xie et al 2013a). Repeated exposure to hypoxia is thought to contribute to the destabilization of breathing control; chronic exposure to moderate intermittent hypoxia (CIH), as experienced by individuals

with CSA or OSA (Younes et al 2001), leads to an increase in respiratory pattern variability in both rats and humans primarily through an increase in the number of apneas and hypopneas during sleep (Deacon & Catcheside 2015, Edge et al 2012, Mateika & Syed 2013, Souza et al 2015).

Some evidence provides correlative evidence that CIH propagates disordered breathing. Individuals with mixed or complex sleep apnea undergoing treatment for OSA that mitigates intermittent hypoxia often also gradually and spontaneously recover from CSA through mechanisms that are unknown (Khan & Franco 2014). This observation suggests that obstructive apneic events impede self-corrective mechanisms, and after alleviation of OSA the neural system resets, possibly through the recovery of endogenous compensatory mechanisms. Further, mild levels of concurrent hypoxia do not interfere with the expression of iPMF, but moderate levels that elicit hypoxia induced plasticity do interfere with this compensatory plasticity (Fields et al., in preparation). These data support the hypothesis that individuals with healthy breathing who experience brief, very mild apneas in a night of sleep (<10; Sateia 2014) self-recover through the expression of iPMF. However, more severe apneas marked by moderate hypoxic exposure may prevent iPMF and render the system vulnerable to subsequent reductions in respiratory drive and repeated exposure to hypoxia. Over many nights the chronic exposure to intermittent hypoxia may wipe out iPMF entirely and explain the propagation of disordered breathing.

Currently, there are no specific therapies for the treatment of CSA. One highly successful management strategy for CSA is increased inspired carbon dioxide;

however, due to concerns for patient safety, this treatment has not been widely applied clinically (Badr et al 1994, Lorenzi-Filho et al 1999, Steens et al 1994, Xie et al 1997). Pharmaceuticals to increase central drive have been developed; however, long term use of medications such as theophylline and acetezolamide have yet to be assessed (Eckert et al 2007). This leaves a critical need for treatment targeting centrally mediated respiratory instability. We propose targets that promote the recovery of iPMF such as impairing CIH-induced metaplasticity or initiating iPMF exogenously will restore endogenous mechanisms that stabilize breathing in respiratory control.

While our findings reported here are supported by statistical testing and mean group data demonstrate robust differences in iPMF magnitude following our treatment paradigms (Figure 1B, 2B), we intentionally included dot plots of the data to highlight the physiological variability in the response to CIH and the propensity for iPMF recovery. In a group of 13 rats that were exposed to intermittent neural apnea 16-hours following exposure to CIH, three (one removed from analysis as a statistical outlier) rats maintained the ability to express iPMF (Figure 1). Also against the norm, iPMF typically recovered 7 days following CIH (10/12 rats), but one rat hyper-recovered and expressed an abnormally large increase in phrenic burst amplitude and two rats did not recover the ability to express iPMF in that week time frame (Figure 1). The mechanisms protecting rats from CIH-induced occlusion of iPMF, predisposing them for robust recovery, and preventing the recovery of iPMF 7 days following the exposure are entirely unknown, but may have interesting parallels to human populations in which certain individuals have a higher disposition for the development of central sleep apnea than others.

Understanding the mechanisms that underlie the disparity of iPMF impairment may identify potential interventions to reveal this compensatory plasticity.

Certain limitations to the study design may have contributed to our failure to worsen apnea phenotype in this study population. Small interfering RNAs were utilized to specifically reduce gene expression PKC isoforms involved in expression of pLTF and iPMF (Devinney et al 2015, Mantilla et al 2009, Morris 2005, Nakajima et al 2012). While the targeted as well as non-targeted siRNA are generally thought to demonstrate predictable activity, off-target effects have been reported (Fedorov et al 2006, Jackson & Linsley , Singh et al 2011). In addition to this, administration of intrapleural injections was performed daily under general anesthesia. General anesthesia by isoflurane has been shown to influence neural activity, and in particular leads to altered excitablity (Caraiscos et al 2004, Joksovic & Todorovic 2010, Sandstrom 2004). Finally, while every attempt was made to maintain sterility during injections, the potential for contamination of the pleural space with commensal skin microorganisms remains. Inflammation has been demonstrated to influence the expression of several forms of neuroplasticity, including pLTF (Huxtable et al 2013). Given that the same protocol of siRNA administration was utilized prior to electrophysiologic recordings, it is not likely this would explain our findings; however, measures in unanesthetized animals may differ from those under urethane anesthesia. In future investigations, modification of neuroplastic expression should be achieved in the absence of siRNA, daily anesthesia or intrapleural injection to assure these confounding factors do not influence induction of increased apnea/hypopnea frequency in our hands.

Both neural apnea and hypoxia are commonly experienced by individuals with sleep apnea and other breathing disorders. Even though the respiratory control system is equipped with robust forms of plasticity triggered by neural apnea and hypoxia, disordered breathing persists. While respiratory plasticity is thought to confer adaptability and promote functionality, the data presented in this series of studies demonstrates that plasticity initiated by CIH transiently impairs the expression iPMF. We hypothesize that CIH-induced metaplasticity is maladaptive in that its occlusion of compensatory plasticity induced by neural apnea/hypopnea leaves the system vulnerable to subsequent challenge and therefore perpetuates pathology in the control of breathing. Work is underway to test the hypotheses that 1) CIH-induced breathing instability is in part due to CIH-induced plasticity that constrains the self-corrective role of iPMF, and that 2) iPMF contributes to the recovery of breathing stability following CIH in awake and freely moving rats and may provide additional evidence for the role of iPMF in promoting breathing stability. Data presented here provides the basis for novel intervention strategies that promote the recovery of endogenous compensatory mechanisms to stabilize breathing in the treatment of sleep apnea and other respiratory control disorders.

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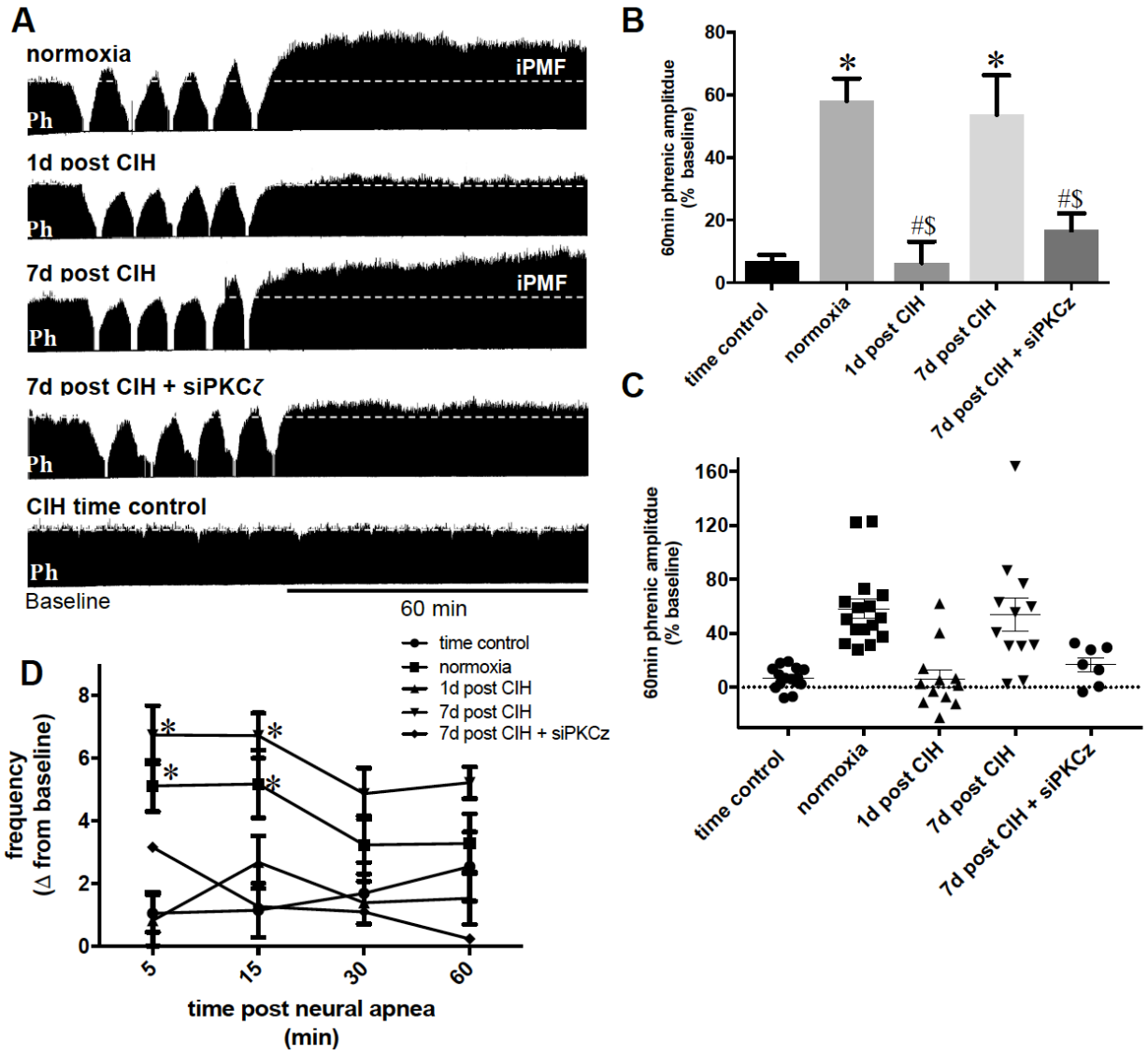

Figure 1. Chronic intermittent hypoxia transiently impairs the expression of PKCζ-dependent iPMF. **A.** Representative, compressed phrenic neurograms depicting integrated phrenic burst amplitude at baseline, during, and for 60 min following intermittent neural apneas in rats exposed to NX, 1 day post CIH, 7 days post CIH, siPKCζ treated rat 7 days post CIH and an equivalent duration in a CIH exposed time control rat. **B.** Mean changes in phrenic burst amplitude at 60 min following intermittent apneas in rats exposed to NX, 1 and 7 days following CIH, and 7 days post CIH +

siPKCζ, and **C.** dot plot at 60 min for each experimental group. Together indicating CIH exposure induces a recoverable impairment in PKCζ-dependent iPMF. **D.** Mean changes in phrenic burst frequency 5, 15, 30, and 60 min following intermittent apnea in treated rats. *significantly different from time control. # significantly different from normoxia. $significantly different from 7 days post CIH.

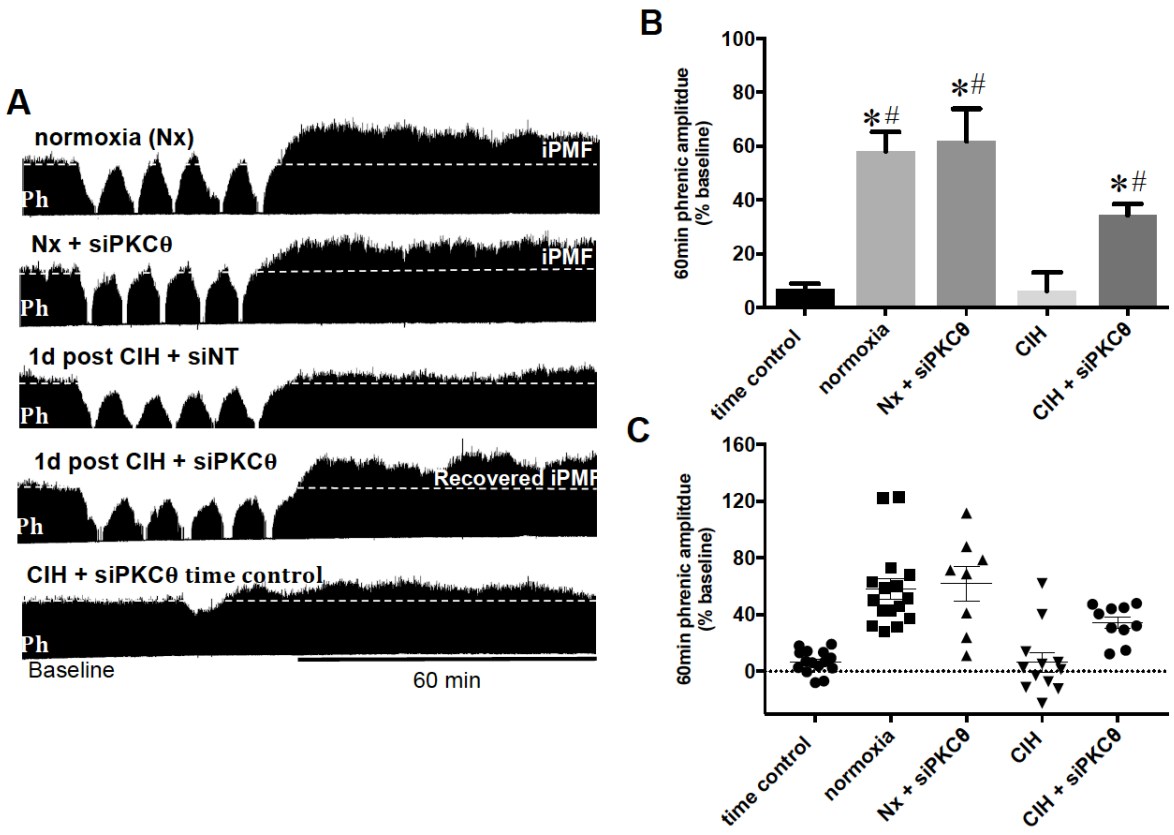

Figure 2. Knockdown of PKCθ in phrenic motor neurons partially restores iPMF 1 day following CIH. **A.** Representative, compressed phrenic neurograms illustrating integrated phrenic burst amplitude at baseline, during, and for 60 min following intermittent neural apneas in Normoxia (NX) or chronic intermittent hypoxia (CIH) exposed and siRNA treated rats and an equivalent duration in a CIH exposed, siPKCθ treated time control rat. **B.** Mean changes in phrenic burst amplitude at 60 min post the intermittent apnea and **C.** a dot plot presenting individual data points at 60 min for each treatment group to depict the physiological variability in the response. Together these data indicate impairing mechanisms of hypoxia induced plasticity partially relieves the CIH-induced impairment of iPMF 1 day following CIH exposure. *significantly different from time control. #significantly different from CIH.

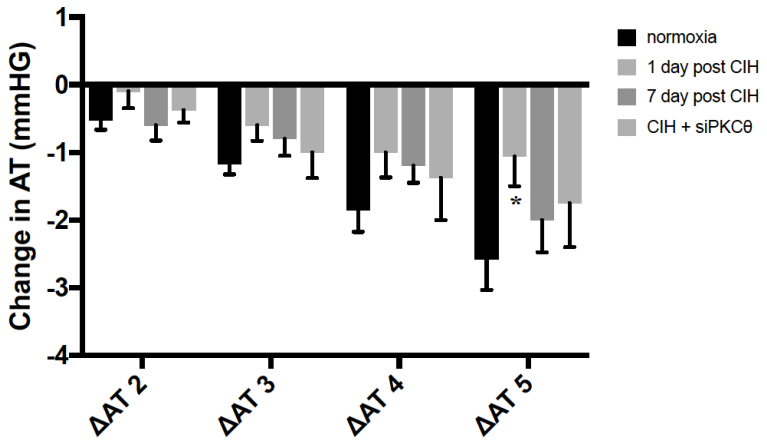

Figure 3. Intermittent neural apnea reduces the apneic threshold for breathing. Mean changes in AT during successive intermittent neural apneas across treatment groups. Data are presented as means ± SE. The reduction in AT observed in rats at 1 day following CIH is significantly less than that observed in all other groups. Rats normally expressing iPMF (NX, CIH + 7 day recovery, CIH + siPKCθ) demonstrate a significant decrease in AT by the 3rd apnea ($p<0.05$). However, in rats that did not express iPMF (1 day post-CIH and CIH + siPKCζ), a significant reduction in AT is not observed until the 4th apnea ($p<0.05$). Significance indications were not included for these findings to emphasize the partial block of AT reduction at 1 day post CIH. *significantly different from normoxia; $p<0.05$.

Treatment	Time	PaCO2 (mmHg)	PaO2 (mmHg)	pH	MAP (mmHg)
Normoxia (Nx) (n=16)	Baseline	47.4 ± 0.7	283.1 ± 3.3	7.36 ± 0.01	136.8 ± 4.0
	60 min	47.9 ± 0.8	277.8 ± 3.3	7.35 ± 0.01	135.4 ± 4.3
1 day post CIH (n=12)	Baseline	46.8 ± 1.0	283.9 ± 3.9	7.36 ± 0.01	141.8 ± 3.2
	60 min	46.6 ± 1.2	276.5 ± 4.7	7.35 ± 0.01	135.0 ± 3.8*
7 days post CIH (n=12)	Baseline	48.2 ± 0.6	296.2 ± 3.7	7.37 ± 0.01	147.1 ± 3.8
	60 min	47.9 ± 0.5	290.8 ± 3.8	7.35 ± 0.01	141.1 ± 4.7
7 days post CIH + siPKCz (n=7)	Baseline	50.2 ± 1.5	294.9 ± 5.1	7.35 ± 0.01	148.1 ± 3.8
	60 min	49.7 ± 1.3	294.4 ± 5.5	7.34 ± 0.01	148.6 ± 3.9
1 day post Nx + siPKCθ (n=8)	Baseline	50.9 ± 0.9	292.0 ± 3.5	7.33 ± 0.01	152.8 ± 2.6
	60 min	51.2 ± 1.0a	288.4 ± 3.1	7.32 ± 0.01	156.1 ± 3.7a
1 day post CIH + siPKCθ (n=10)	Baseline	48.7 ± 0.7	274.9 ± 4.1	7.35 ± 0.01	150.7 ± 3.9
	60 min	48.2 ± 0.7	273.5 ± 4.0	7.33 ± 0.01	156.5 ± 3.6a
Time Controls (n=15)	Baseline	47.5 ± 0.6	284.7 ± 5.4	7.35 ± 0.01	142.3 ± 4.2
	60 min	47.4 ± 0.6	274.5 ± 10	7.33 ± 0.01	135.8 ± 5.2*

Table 1. Arterial $PaCO_2$ (mmHG), PaO_2 (mmHG), pH, and mean arterial pressure (MAP, mmHG) at baseline and 60 min post intermittent apnea. Data are presented as mean ± SE. (*) significantly different from baseline and (a) significantly different between groups at that time point are denoted within the table; $p<0.05$. CIH did not significantly alter baseline physiological parameters.

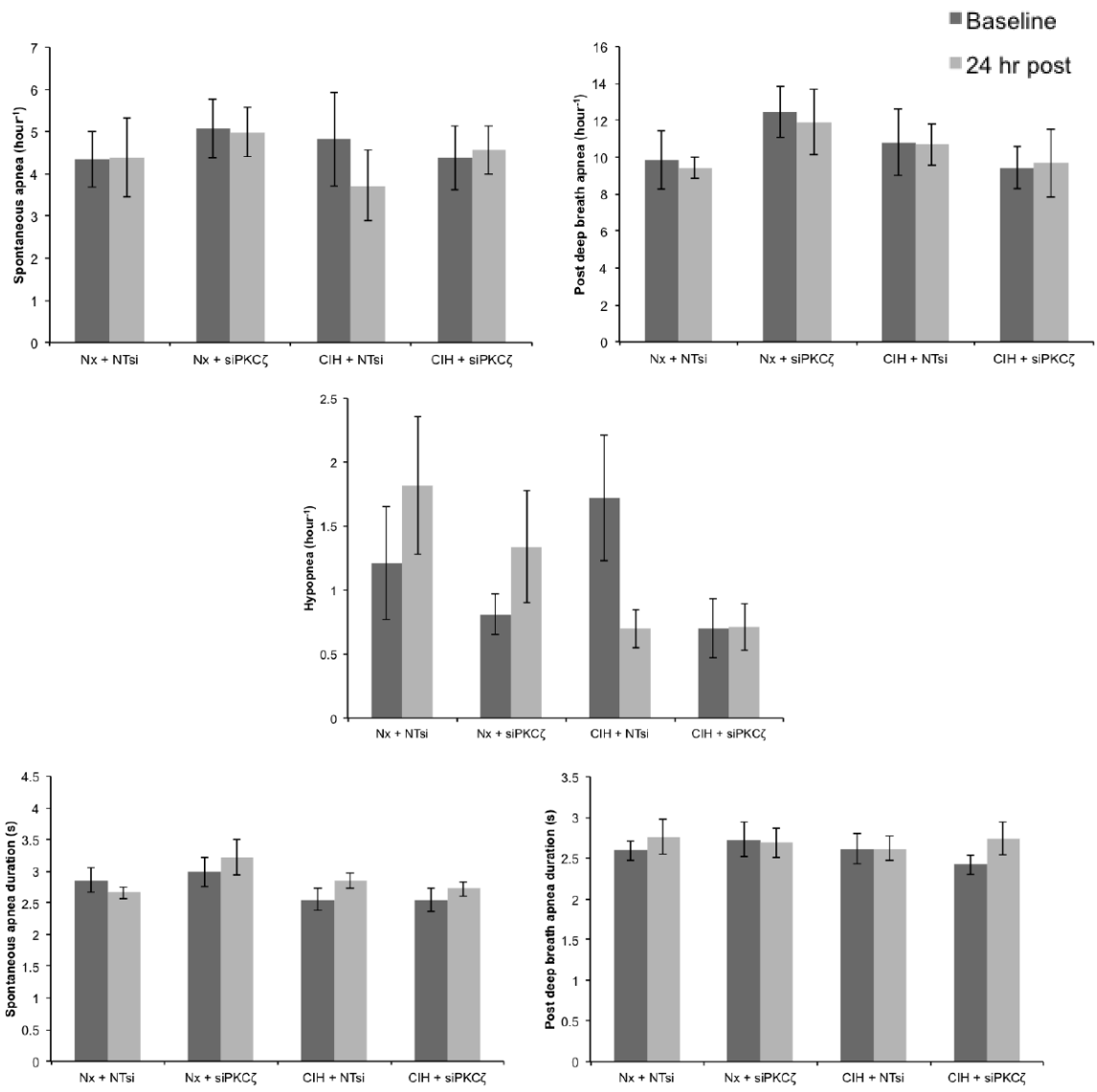

Figure 4. CIH fails to increase frequency or duration of breathing disturbances. **A/B/C.** Seven days of chronic intermittent hypoxia fails to increase the frequency of spontaneous apnea (4.35 +/- 0.67 hour^{-1} vs 4.38 +/- 0.93 hour^{-1} Nx + NTsi, 5.06 +/- 0.7 hour^{-1} vs 4.99 +/- 0.59 hour^{-1} Nx + siPKCζ, 4.81 +/- 1.1 hour^{-1} vs 3.72 +/- 0.83 hour^{-1} CIH + NTsi, 4.37 +/- 0.75 hour^{-1} vs 4.56 +/- 0.57 hour^{-1} CIH + siPKCζ), post deep breath apnea (9.87 +/- 1.6 hour^{-1} vs 9.43 +/- 0.57 hour^{-1} Nx + NTsi, 12.44 +/- 1.37 hour^{-1} vs

11.9 +/- 1.75 hour^{-1} Nx + siPKCζ, 10.81 +/- 1.8 hour^{-1} vs 10.69 +/- 1.15 hour^{-1} CIH + NTsi, 9.42 +/- 1.13 hour^{-1} vs 9.7 +/- 1.85 hour^{-1} CIH + siPKCζ), or hypopnea (1.21 +/- 0.44 hour^{-1} vs 1.82 +/- 0.54 hour^{-1} Nx + NTsi, 0.81 +/- 0.16 hour^{-1} vs 1.34 +/- 0.44 hour^{-1} Nx + siPKCζ, 1.72 +/- 0.49 hour^{-1} vs 0.7 +/- 0.15 hour^{-1} CIH + NTsi, 0.7 +/- 0.23 hour^{-1} vs 0.71 +/- 0.18 hour^{-1} CIH + siPKCζ). c/d) In addition, the administration of daily intermittent hypoxia for seven days leads to no change in the duration of spontaneous apnea (2.86 +/- 0.2 s vs 2.66 +/- 0.1 s Nx + NTsi, 2.99 +/- 0.23 s vs 3.22 +/- 2.8 s Nx + siPKCζ, 2.55 +/- 0.17 s vs 2.85 +/- 0.12 s CIH + NTsi, 2.54 +/- 0.18 s vs 2.72 +/- 0.12 s CIH + siPKCζ) or post deep breath apena (2.59 +/- 0.12 s vs 2.76 +/- 0.21 s Nx + NTsi, 2.73 +/- 0.21 s vs 2.69 +/- 0.18 s Nx + siPKCζ, 2.62 +/- 0.19 s vs 2.62 +/- 0.15s CIH + NTsi, 2.42 +/- 0.12 vs 2.74 +/- 0.2 s CIH + siPKCζ).

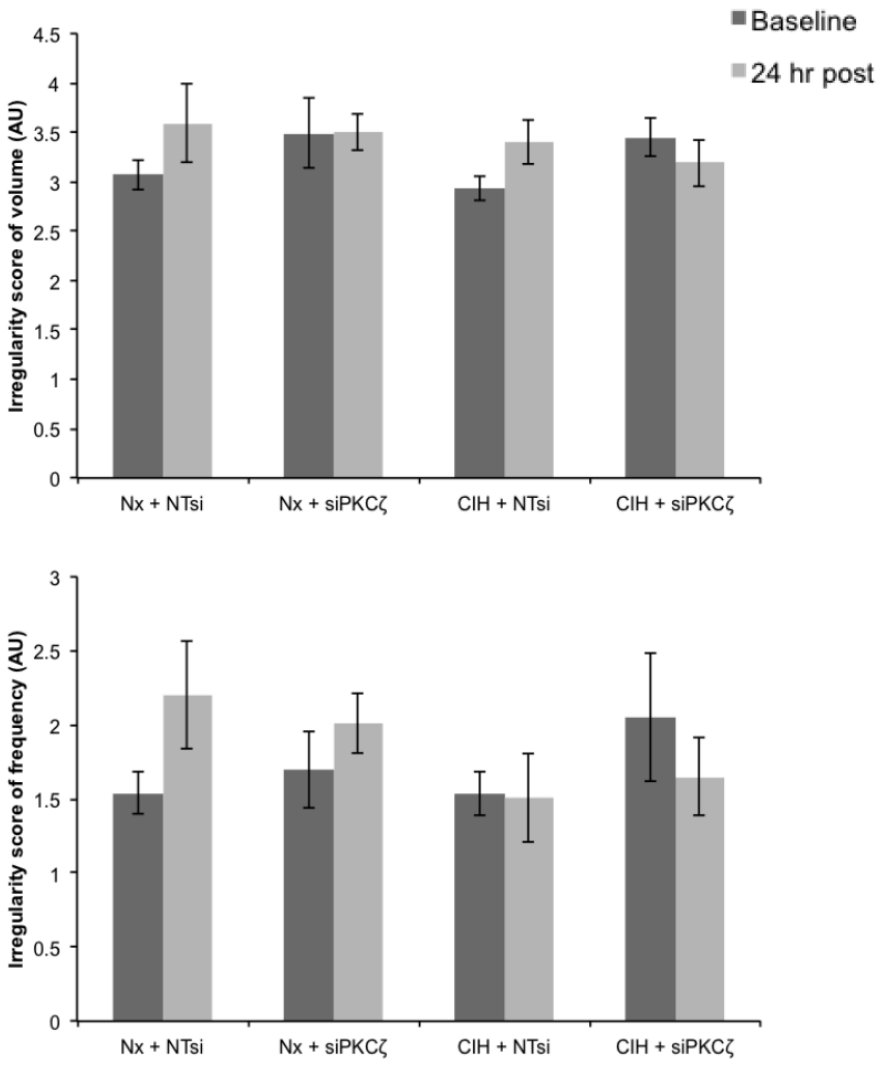

Figure 5. CIH fails to induce irregularity of frequency or volume. **A/B.** No change is seen in the mean irregularity score of tidal volume (3.07 +/- 0.15 vs 3.59 +/- 0.4 Nx + NTsi, 3.49 +/- 0.35 vs 3.5 +/- 0.19 Nx + siPKCζ, 2.93 +/- 0.12 vs 3.4 +/- 0.22 CIH + NTsi, 3.45 +/- 0.2 vs 3.19 +/- 0.24 CIH + siPKCζ) or frequency (1.54 +/- 0.14 vs 2.2 +/- 0.36 Nx + NTsi, 1.7 +/- 0.26 vs 2.01 +/- 0.2 Nx + siPKCζ, 1.54 +/- 0.15 vs 1.51 +/- 0.3 CIH + NTsi, 2.05 +/- 0.43 vs 1.65 +/- 0.26 CIH + siPKCζ) following chronic intermittent hypoxia.

CHAPTER III

High fat diet along with chronic intermittent hypoxia leads to irregular breathing and central nervous system inflammation

J.G. Weltman, A. Ewald, I.T. McSweeny, J.J. Watters, T.L. Baker

ABSTRACT

An increasing prevalence in both sleep disordered breathing and obesity has been documented throughout the world. As a means of better understanding these phenomena, extensive investigations into mechanisms leading to irregular breathing have been performed and research supports that both diet and the experience of hypoxia in sleep apnea play a role in propagation of disease. Diets high in saturated fat (HFD) have been demonstrated to lead to a clinical condition called metabolic syndrome, which has been associated with the diagnosis of sleep apnea syndrome (SAS). SAS leads to intermittent reductions in breathing throughout sleep alongside intermittent reductions in systemic oxygen tension (chronic intermittent hypoxia; CIH). Sources are conflicted on correlations between these two states, with some stating CIH worsens metabolic state and others demonstrating worsened apneic phenotype with HFD. Here we tested the hypothesis that high fat diet (HFD) leads to an increase in breathing disturbances, a finding that is exacerbated by the experience of chronic intermittent hypoxia (CIH). Further, we examined the consequences of HFD and CIH on the development of central nervous system inflammation. Following six weeks of continuous high fat diet or normal chow, mice were exposed to either normoxia or CIH (90 sec 10.5% O_2, 90 sec 21% O_2, 12 hours per day) for 14 days. Plethysmography was measured immediately prior to and following CIH or normoxia treatment and analyzed for apnea, hypopnea and irregularity in frequency and volume. At the completion of the study, brainstem and spinal cord were collected for assessment for inflammatory gene expression. While we were able to demonstrate mild but significant increases in irregularity of frequency alongside increases in brainstem inflammation, apnea and

hypopnea were no different across groups. These findings support the need for further research with more severe HFD and/or CIH experiences and may have implications on the management of individuals with sleep apnea syndrome.

INTRODUCTION

Approximately 9% of women and 24% of men worldwide experience some form of sleep disordered breathing (Young et al 2002). Intermittent losses of ventilation during sleep are broadly categorized as obstructive when due to obstruction of the upper airway (obstructive sleep apnea; OSA) and central in the setting of a loss of neural signal driving breathing (central sleep apnea; CSA, (Javaheri & Dempsey 2013). Regardless of type of disease, sleep apnea leads to intermittent reductions in the arterial oxygen content, and this chronic intermittent hypoxia (CIH) has been thought to contribute to signs related to sleep apnea, including increased daytime sleepiness, mood alterations, and cardiovascular disease (Eckert et al 2007). CIH has been modeled extensively in rodents in the hopes of better understanding the consequences of sleep apnea syndrome (SAS); however true obstruction of the airways is challenging to replicate in animal models. Furthermore, people with SAS often experience concurrent conditions including obesity and cardiovascular disease, and these comorbidities likely play a role in disease state and progression.

While obesity has been long recognized as a predisposing factor in the development of SAS, recent work has shown that diet composition also plays a role, independent of body weight. High fat foods are prevalent in developed countries, and represent 2.3-27.3% of total daily energy intake in a survey of 113 different countries (Micha et al 2014). On average adults consume >10% of daily calories in saturated fat (Micha et al 2014). Animal models commonly use diets high in saturated fat (HFD) to induce a metabolic state similar to that seen in humans with insulin resistance and type 2 diabetes mellitus (Collins et al 2004, Mosser et al 2015, Sato et al 2010). High fat diet

(HFD) is associated with an increase in daytime sleepiness and number of apnea events during sleep in previously undiagnosed people with SAS (Cao et al 2016). In mice, feeding a HFD for only one week reduces wakefulness regardless of body weight (Perron et al 2015), suggesting disturbances in sleep are independent of body fat composition. Feeding a diet high in saturated fats to rats for three weeks increases the frequency of apnea observed alongside a trend to hyperinsulinemia (Ramadan et al 2007). The incidence of apnea also appears to be influenced by the experience of intermittent hypoxia alone. Reports in the literature suggest chronic intermittent hypoxia (CIH), such as may be experienced by some patients with CSA or OSA, leads to an increase in respiratory pattern variability. CIH has been shown to increase the number of spontaneous neural apneas as well as induce an irregular breathing pattern in both humans and rats (Deacon & Catcheside 2015, Edge et al 2012, Mateika & Syed 2013, Younes 2001). We hypothesize that the simultaneous experiences of high fat diet and intermittent hypoxia set up a self-propagating cycle allowing for progression of SAS in people.

Another unifying factor between intermittent hypoxia, sleep apnea, and high fat diet is that of inflammation. Both CIH and HFD have been shown to increase inflammatory cytokine expression in the brainstem (Guillemot-Legris et al 2016, Guillemot-Legris & Muccioli 2017, Smith et al 2013). The presence of systemic and central nervous inflammation has been shown to influence breathing, in particular, the expression of normal neuroplasticity in respiratory neural circuits (Hocker et al 2017). One form of neuroplasticity in respiratory motor systems, inactivity-induced phrenic motor facilitation (iPMF), leads to an augmentation of the phrenic discharge amplitude

following intermittent periods of inactivity by enhancing the strength of synapses within the phrenic motor pool (Fuller et al 2000, Fuller et al 2001, Navarrete-Opazo & Mitchell 2014, Olson et al 2001). It has been proposed that this enhanced phrenic activity serves to stabilize breathing in periods of reduced central respiratory drive, representing the first documented form of homeostatic neuroplasticity in spinal respiratory neural circuits (Baertsch & Baker-Herman 2013, Baertsch & Baker-Herman 2015, Strey et al 2012). Recently we have demonstrated that CIH constrains the expression of iPMF, indicating the potential for worsened breathing stability in the presence of SAS (Braegelmann and Weltman, Chapter II). While there is some indication this may be related to the co-expression of multiple forms of spinal neuroplasticity, i.e. iPMF and hypoxia induced neuroplasticity, the definitive underlying cause of constraint has not been fully elucidated, though inflammation may play a role.

The purpose of this study was to investigate the consequences of concurrent HFD and CIH in the mouse. We hypothesized that high fat diet leads to an increase in central nervous system inflammation alongside an increase in apnea frequency and irregularity of breathing during sleep. Further, the addition of CIH-induced constraint of iPMF mechanisms would be expected to exacerbate the consequences of HFD. While we do report an increase in inflammation with CIH and increased irregularly of breathing frequency with concurrent CIH and HFD; HFD alone did not destabilize breathing patterns in mice and no condition led to exacerbation of apnea frequency or duration.

METHODS

Animals and spinal cord collection.

Male C57BL/6J and 380050 mice, 6-12 weeks old, were housed on a 12-hour light-dark cycle in polycarbonate cages and were given water and food *ad libitum*. Half of the mice were fed high fat chow (HFC; diet number D12492, Research Diets, New Brunswick, NJ), while the other half received normal chow (NC; Teklad 7002, Envigo, Madison, WI). Following six weeks of their assigned diet, baseline measures of plethysmography were collected and mice went on to be divided between chronic intermittent hypoxia (CIH) or normoxia (NX) groups (see below). Final group assignments were NX + NC (n=7), NX + HFD (n=8), CIH + NC (n=11) and CIH + HFD (n=10). Sixteen hours following the final CIH exposure, post-treatment plethysmography was collected. Animals going on for microglial cell isolation (n=6 per group) were euthanized and perfused with intracardiac, ice-cold 1 X phosphate buffered saline solution. Brain stem and spinal cord (C3-6) were collected in Hank's buffered saline solution on ice for downstream microglia isolation. The Animal Care and Use Committee of the School of Veterinary Medicine, University of Wisconsin, approved all experimental procedures used in this study.

Whole body plethysmography

Mice were individually placed in whole body plethysmography chambers (1.5 liters, Data Sciences International model 601-1425-001, St. Paul, MN). Pressurized air continuously flowed through the camber at 1.5 liters per minute, allowing precise control of inspired gas concentration and limiting expiratory gas accumulation. Respiratory flow signals were sent to a data exchange matrix (model ACQ-7700; Data Sciences International, St. Paul, MN) and collected through purposely-designed software (PONEMAH Physiology Platform, Data Sciences International, St. Paul, MN). For the

purpose of analyses, body temperature was measured immediately prior to and following data acquisition. Body temperature was assumed to follow a linear change over time. Data were gathered for six consecutive hours at baseline and 16 hours following the final intermittent hypoxia or normoxia exposure (see below).

Chronic intermittent hypoxia protocol

Desired inhaled gas concentrations during normoxic (21% O2) and hypoxic (10.5% O2) conditions were tightly controlled by specialized computer controlled custom acrylic cage inserts (4 mice per cage). Computer software connected to an O_2 and a CO_2 gas analyzer (Gemini, CWE, Inc., Ardmore, PA) controlled chamber gas concentrations by regulating the flow of O_2, N_2 and room air into the chamber at a constant flow rate of 9 L/min. For CIH, animals within the chamber were exposed to cycling conditions of 21% and 6.5% O_2, alternating every 90 seconds during the 12-hour light period. During the 12-hour dark period, O_2 within the chamber was maintained at a constant 21% by injecting room air. CO_2 concentrations were maintained below 0.3% at all times. CIH + HFD and CIH + NC mice underwent daily CIH for 14 consecutive days (480, 1.5 minute episodes of 6.5% O2 interspersed with 1.5 minute episodes of 21% O2; total 12 hour daily). NX + HFD and NX + NC mice were administered continuous normoxia during the same time period.

Microglia isolation

CD11b+ cells were immunomagnetically isolated from the brain stem and spinal cords (C3-6) of 6 individual animals for each treatment group. Tissues were collected in Hank's balanced saline solution on ice and finely minced with a razor blade. A small aliquot of the minced tissue was taken from each sample for homogenate tissue

analysis, the remainder of the tissue was then enzymatically dissociated (Papain, Sigma Aldrich, St. Louis, MO; DNAse, Worthington, Biochemicals, Lakewood, NJ) and passed through a 40 µm mesh filter. Myelin was removed by resuspending dissociated samples in an isotonic solution of 26% Percoll in 0.1M phosphate buffered saline (GE Healthcare, Pittsburgh, PA), and centrifuging at 1050g for 15 minutes. Samples were then tagged with an anti-CD11b+ antibody conjugated to a magnetic bead (Miltenyi, Germany). The magnetically-tagged CD11b positive cells were passed through a magnetic column according to Miltenyi MACS protocol. The CD11b positive cells were collected in Trizol (Invitrogen, Carlsbad, CA) and subsequently used for RNA isolation and complimentary deoxyribonucleic acid (cDNA) synthesis.

Quantitative Polymerase Chain Reaction

All quantitative polymerase chain reaction (qPCR) studies were performed cDNA using Power SYBR Green using the ABI 7500 Fast system (Applied Biosystems) as previously described (Smith et al 2013). The National Center for Biotechnology Information Basic Local Alignment Search Tool was used to design primers and confirm their gene specificity (table 1). Serial dilutions were used to test primer efficiency. Dissociation curves for each reaction had a single peak and an observed Tm consistent with amplicon length for each gene. Each sample was run in duplicate and closure time (C_T) values from duplicate reactions were averaged and normalized to the expression of ribosomal RNA (18s). Relative gene expression levels were determined by the Δ/ΔCt method.

Data analysis

Plethysmography data were averaged over 50 ms and periods of quiet breathing suitable for analysis were manually identified. A purpose written MatLab script was utilized to assess for tidal volume (TV) respiratory frequency (RR), minute ventilation (MV) and hourly rates and duration of apnea and hypopnea utilizing correction for pressure and temperature (Drorbaugh & Fenn 1955, Flatau et al 1992). The irregularity scores of breathing frequency and tidal volume were calculated as previously described (Zanella et al 2014). A period absent of inspiratory flow extending beyond the frequency of two average breath durations was defined as an apnea. The defined period of apnea was compared between groups and showed no differences (Table 2). If TV of any of five preceding breaths was ≥125% the mean TV, the apnea was classified as post-deep breath and not included in analyses. The remaining breaths were classified as spontaneous apnea (SA). Hypopnea was defined as three consequent breaths with individual TV less than 30% of mean TV of the tracing.

All data are expressed as mean and standard error unless otherwise stated. Prior to analysis, data were assessed for any outlying values by the maximum normed residual test and significant values were removed from analysis. Data was assessed for normality utilizing the Shapiro-Wilk test. Baseline measures of weight, ventilation, irregularity, apnea and hypopnea were compared by ANOVA and post-exposure values were compared to preexposure values by paired Student's T-test. qPCR data were normalized to gene expression for 18s and compared by Kruskall Wallis and subsequently assessed for significant interactions by Mann Whitney U with Bonferroni correction. All qPCR data is expressed as fold change from NX + NC. A p value of less than .05 was deemed significant.

RESULTS

High fat diet increases body weight and reduces tidal volume while intermittent hypoxia reduces body weight

In previous publications, the experience of six weeks high fat diet induced respiratory disturbances in rats with no differences observed in body weight (Ramadan et al 2007). In addition, diets associated with obesity are correlated with the symptoms and incidence of sleep apnea in humans (Cao et al 2016). In order to investigate the consequence of high fat diet, mice underwent measures of weight and plethysmography prior to gas exposure. At baseline the mean weight was higher in mice fed HFD as compared to NC (32.7 +/- 1.0 NX + HFD and 34.2 +/- 0.9 CIH + HFD vs 28.7 +/- 1.2 NX +NC and 27.8 +/- 0.5 CIH + NC g, $p < .01$, table 3) indicating that six weeks high fat diet was adequate to induce weight gain in our population, a finding consistent with previous reports (Mosser et al 2015, Sato et al 2010).

Next, to investigate the consequence of high fat diet on respiratory parameters, which has yet to be reported in mice, we performed whole-body plethysmography following six weeks of high fat diet. Mice going on to normoxia treatment and fed HFD express a lower tidal volume than those fed a low fat diet (9.5 +/- 0.6 NX + NC and 14.5 +/- 2.8 CIH + NC vs 5.6 +/- 0.4 NX + HFD uL/g CIH + NC, $p < .01$, table 3). Further, this difference is observed regardless of correction for body weight (0.27 +/- 0.01 NX + NC and 0.31 +/- 0.03 CIH + NC vs 0.18 +/- 0.02 X mL $p<.01$, table 3). Given that the correction for body weight remains reduced following high fat diet, it is not likely that body size plays a role in the differences found between these groups. Those going on to CIH treatment and fed HFD express a higher baseline respiratory frequency than those

fed NC (156.1 +/- 3.9 NX + NC and 160.1 +/- 3.6 CIH + NC vs 177.9 +/- 5.0 CIH + HFD min^{-1}, p <.005 , table 3). An increase in this setting is not likely to be an affect of data acquisition, i.e. lack of acclimiation to the testing device, given the length of acquisition (i.e. approximately 3 hours of analyzed data) and instead likely represents a true finding. In the absence of a difference in tidal volume, the significance of this finding is unclear and appears to be lost following treatment with CIH. In addition, the rats going on to NX treatment and fed HFD show a trend to increase in ventilation (172.2 +/- 5.6 min^{-1}) which suggests that HFD alone has an influence on central respiratory patterning, a phenomenon that has not been previously reported. Further studies utilizing measure of discharge frequency at various levels of the respiratory control network would be of benefit in investigating this finding further.

To determine the affect of additional two weeks of HFD as well as CIH on weight gain, body weight was compared following CIH or normoxia treatment and compared across groups as well as to measures taken prior to CIH or NX treatment. After either gas exposure, no significant differences were noted across groups regardless of diet or gas intervention despite noted differences prior to CIH treatment (table 3). In investigating the consequence of gas treatment, a significant reduction in body weight was noted in mice administered CIH, regardless of diet administration (26.2 +/- 0.5 g vs 28.7 +/- 1.2 g CIH + NC and 29.9 +/- 1.1 g vs 34.23 +/- 0.9 g CIH + HFD, p<0.001, table 3). This phenomenon has been previously demonstrated (Drager et al 2011, Jun et al 2008), and in previous investigations has been shown to be correlated with a reduction in food intake (Jun et al 2008, Li et al 2007). Here we chose to cohouse animals to minimize stress, which prevented us from determining the exact amount of food eaten

per subject. Future studies should include accurate estimations in the quantity of food ingested in study populations.

Chronic intermittent hypoxia with high fat diet alters breathing regularity

To test the hypothesis that HFD and CIH disturb the stability of breathing, periods of quiet breathing were assessed on a breath-by-breath basis to establish tidal volume and frequency as well as investigate the frequency of apnea and hypopnea. A minimum of 50.5 minutes of data was analyzed per mouse at each time point. As there was a significant difference in the breathing rate at baseline, as discussed above, we chose to define the duration of apnea as the time representative of two missed breaths for each individual mouse, to avoid the influence of different basal rate on the apnea duration. In comparing the duration of apnea, no differences were observed between treatment groups at any time point (table 2). Further, spontaneous apnea and hypopnea showed no difference in regards to rate or duration between any treatment group at any time point (Table 4). This is in contrast to previous reports investigating the frequency of apnea in high fat diet or with chronic intermittent hypoxia (Ramadan et al 2007, Souza et al 2015, Yokhana et al 2012). The differences in this study may be attributable to dose of intermittent hypoxia, the severity of metabolic dysfunction elicited by the diet administered, and/or species difference (i.e. rats vs mice).

The irregularity score was established in previous reports as a means of looking at alterations in the regularity of phasic physiologic behaviors (Zanella et al 2014), and is calculated by subtracting a value of interest, i.e instantaneous respiratory rate, from the value determined on the previous observation and dividing by the value of the previous observation. A large value would represent a large difference between

observations and irregular pattern. Given that respiratory patterns are generally thought to be fairly regular, we sought to utilize this calculation to further investigate the consequence of HFD and CIH on breathing regularity. Prior to any gas exposure, no difference was observed between any treatment group (figure 1). However, administration of HFD in addition to CIH for two weeks led to an increase in the irregularity of respiratory frequency as evidenced by the irregularity equation (median 1.7 vs median 1.2, z-value—2.073, $p < 0.05$, figure 1A). No differences were observed in irregularity score of frequency in any other group investigated (figure 1A). Measures investigating the irregularity of tidal volume showed no difference (figure 1B). While the physiologic implications of respiratory frequency irregularity are not entirely clear at this time, it may be that this represents a low level of instability that, with higher doses of HFD and CIH, may unveil overt apneas which were not observed in this population.

Chronic intermittent hypoxia induces an increase in brainstem inflammation

Previous investigations have demonstrated an increase in inflammation within the brainstem as a result of chronic intermittent hypoxia exposure (Smith et al 2013). Further brainstem inflammation has been shown to lead to increased irregularity in breathing frequency (Jacono et al 2011). Here we sought to investigate whether increases in inflammation were observed alongside altered breathing regularity in mice exposed to HFD and CIH. In a subset of mice, brainstem and spinal cord were collected to assess for inflammatory gene expression. Here we chose to investigate TNFα, IL1β, and COX2 as their expression was increased in microglia and CNS homogenate following CIH in rats (Smith et al 2013). When mice are grouped by diet, CIH led to a significant increase in the level of TNFα gene transcript in brainstem microglia (1 +/-

0.17 NX + NC, 0.8 +/- 0.13 NX + HFD, 1.09 +/- 0.15 CIH + NC, 1.55 +/- 0.32 CIH + HFD, p=0.03; figure 2A); however, no differences were seen in brainstem homogenate (figure 2B). No differences were seen in spinal cord TNFα or in gene expression of IL1β or COX2 in any sample investigated (data not shown). While we are able to demonstrate that the increase inflammation has a correlation with chronic intermittent hypoxia, we did not show that diet alone influenced the magnitude of inflammation nor augmented the inflammation seen with chronic intermittent hypoxia. Any correlation between these findings would be presumptive and future work is necessary to determine if inflammation plays a role in developing abnormal breathing patterns in mice exposed to CIH.

DISCUSSION

In the present study we sought to investigate the relationship between a diet high in saturated fat and chronic, intermittent hypoxia in the development of unstable breathing patterns and inflammation in mice. Here we found six weeks of HFD led to the development of increased body weight and reduced tidal volume as compared to control mice. Further, two weeks of CIH leads to a significant reduction in body weight regardless of diet. After two weeks of CIH, those mice ingesting HFD experienced a significant increase of their irregularity score of frequency, a measure of difference in breathing rate between breaths. While HFD failed to influence inflammatory gene expression, CIH led to a significant increase in TNFα gene expression in brainstem microglia. These data suggest a synergistic effect of inflammation, CIH and HFD, which lead to disruptions in the regularity of respiratory frequency.

The association between metabolic state and sleep disordered breathing has been previously demonstrated. In particular, much work has been focused on the association between obstructive sleep apnea (OSA) and metabolic syndrome, a complex of coincident conditions including hypertension, obesity, insulin resistance (IR), hyperglycemia, and dyslipidemia (Jehle 2002). Given the complexity of both syndromes and overlap in predisposing conditions, a disagreement in causality and correlation is not surprising; however, most publications focus on the potential for development of IR as a consequence of OSA despite evidence available to the contrary. Here we present data to support a synergistic relationship between the induction of insulin resistance and intermittent hypoxia, a key component of SAS, in the development of unstable breathing patterns in mice.

Both HFD and CIH lead to metabolic derangements in mice

The influences of diet upon metabolic homeostasis have been a matter of considerable investigation, particularly as they may lead to lifelong debilitating conditions in people. Several dietary manipulations in mice are utilized to mimic the human condition of metabolic syndrome. A thorough review of literature regarding feeding protocols utilized to influence metabolic state is beyond the scope of this paper. Here we utilized a diet high in saturated fat, which represents a common diet in both adults and children. Similar diets have been shown to lead to pathologies common to the human condition including obesity, insulin resistance and hypertension. Data suggests that prolonged feeding of high fat diet results in hyperinsulinemia similar to type 2 diabetes whereas hyperglycemia occurs much earlier in feeding (Mosser et al 2015, Sato et al 2010). While many strain differences exist, C57Bl/6 mice exhibit a

progression to hyperglycemia, hyperinsulinemia, and insulin resistance similar to people when fed a diet high in saturated fats (Collins et al 2004). In people, there is a positive association between high saturated fat diet and increase in insulin regardless of body weight (Marshall et al 1997, Parker et al 1993).

Chronic intermittent hypoxia also seems to play a role in both establishing metabolic disorders and also the progression of severity. Mice fed HFD and exposed to 4 weeks of CIH experience an exacerbation of diet induced metabolic syndrome with an increase in fasting blood glucose and insulin and a coincident development of glucose intolerance (Drager & Polotsky 2011), suggesting simply the experience of intermittent hypoxia leads to altered blood glucose homeostasis in predisposed individuals. While the underlying cause of these findings has yet to be investigated, intermittent hypoxemia may lead to the accumulation of metabolic byproducts that, upon the return of normal oxygen levels, enhance oxidative stress in tissues due to altered balance of reactive oxygen species. These reactive oxygen species could lead to a cascade of events culminating in inflammation, cellular damage and apoptosis (Alam et al 2007). Of particular relevance to IR, activation of NFkB, activator protein 1 and hypoxia inducible factor 1 lead to increased transcription of pro-inflammatory cytokines such as IL-6 and TNFα (Alam et al 2007).

Pro-inflammatory states, hypoxia, and sleep fragmentation have been linked to altered production of adipokines such as leptin and adiponectin (Alam et al 2007, Lam et al 2015). Adiponectin is generally thought to sensitize the tissues to insulin by enhancing free fatty acid transport, reducing circulating triglycerides, and lowering circulating glucose both by enhanced uptake and reduced production, and in people

experiencing SAS, a relative hypoadiponectinemia has been documented (Lam et al 2015, Yadav et al 2013). Leptin appears to be at least partially regulated by sleep and shortened sleep times have been associated with elevations in leptin levels in people with OSA (Phillips et al 2000). Elevated leptin levels have also been associated with increased insulin resistance (Lam et al 2015). In OSA, arousal events initiated by hypoxemia lead to ann increase in sympathetic neural activity, which has been shown to be mediated by peripheral chemoreceptor activity, endothelin-1 release, and consequential baroreflex responses (Kohler et al 2010, Narkiewicz & Somers 2003, Somers et al 1995). The metabolic response to increased sympathetic activity is predominantly to increase available substrate for energy by stimulating glycogenolysis, gluconeogenesis and lipolysis leading to increased glucose and reduced insulin-mediated glucose uptake, or insulin resistance (Delarue & Magnan 2007, Punjabi & Polotsky 2005). While this relationship between IR and CIH has been well documented, we did not specifically investigate metabolic disease in this study. Future studies would be required to investigate the relationships between the severity of metabolic disease and exposure to CIH; however, data presented here suggest a more complex and reciprocal relationship between the two conditions.

Metabolic disease disrupts breathing stability

Despite the large body of evidence correlating the presence of OSA or CIH to the initiation of IR in animal models as well as humans, some studies suggest an individual role of IR in the development of unstable breathing patterns. Following induction of IR by high fat diet, non-obese Spraque-Dawley rats develop an increase in apneas that is reversed by administration of the oral hypoglycemic agent metformin, an AMPK-

activating drug (Ramadan et al 2007). These data suggest that insulin-glucose dynamics have the capability of altering ventilation. While speculation exists on the roles of insulin and glucose in control of breathing, some profound data support a carotid body mediated enhancement in respiratory neural activity in response to increased insulin receptor signaling (Conde et al 2014). In cultured carotid body, hypoglycemic or aglycemic perfusate led to increased neurotransmitter release, inhibition of potassium currents, and calcium entry into cells, in a similar manner to hypoxemic perfusate (Conde et al 2014, Pardal & Lopez-Barneo 2002). Further, the carotid body has been shown to contain insulin receptors that become phosphorylated in the presence of insulin. Insulin infusion to the intact carotid body leads to increased ventilation, an effect ameliorated by resection of the carotid sinus nerve (Ribeiro et al 2013). Given that insulin resistance is associated with both an overall reduction in insulin receptors and a loss of autophosphorylating capability in the insulin receptor (Olatunbosun `2015), it stands to reason that some chemosensitive responses of the carotid body to enhance central respiratory drive may be lost in IR leading to a loss of central mediated respiratory drive. Studies investigating the carotid body response to sustained hyperglycemia and in insulin resistant states as would be experienced in type 2 diabetes in humans have yet to be performed but are warranted considering the aforementioned associations. While we were able demonstrate an increase in irregularity in breathing, specific investigation into insulin receptor expression, phosphorylation and sensitivity within the carotid body in mice experiencing HFD and CIH will need to be investigated in future studies.

Disrupting the stability of breathing

Here we demonstrated that the combination of HFD and CIH lead to irregular breathing patterns in mice. While it is challenging to speculate on mechanisms leading to this finding, a brief discussion on potential sources of irregularity is warranted. As mentioned previously, the expression of iPMF has recently been shown be inhibited following a CIH paradigm similar to that utilized in this study (Braegalmann and Weltman, chapter II). This form of neuroplasticity is initiated following reduction in the neural signal driving breathing as sensed within the phrenic motor pool (Baertsch & Baker-Herman 2013, Braegelmann et al 2017, Mahamed et al 2011, Streeter & Baker-Herman 2014). This phenomenon represents the only known mechanism in which the cells within the spinal cord locally sense and respond to the loss of input to the phrenic pool, enhancing the synaptic strength to bring the system back to a normal functional level. Alongside this augmentation to the phrenic motor output, we have demonstrated a progressive, long-lasting reduction in the CO_2 level at which breathing ceases (Baertsch & Baker 2017) (apneic threshold; AT). The difference between the CO_2 at eupnea and the AT is referred to as the CO_2 reserve and is one of a constellation of characteristics defining the stability of breathing (Javaheri & Dempsey 2013). While no studies have been performed investigating the eupneic CO_2 following neural apnea, presuming that there is no change in basal ventilation, a reduction in AT would broaden the CO_2 reserve and serve to stabilize breathing. If, in the presence of CIH, iPMF fails to maintain a lowered AT, unstable breathing patterns may develop. Further investigations into the role iPMF plays in the instigation of breathing irregularities with HFD and CIH are warranted.

Beyond the disruption in neuroplastic processes, another potential mechanism leading to irregularities in breathing with SAS, HFD and CIH is central nervous system inflammation. In humans, the C-reactive protein, an inflammatory biomarker, reduces with treatment of OSA by CPAP by 20% (Gottlieb et al 2014). While this resolution in inflammation may be multifactorial as treating OSA alleviates not only CIH, but also sleep fragmentation and hypercapnia, rodent studies corroborate these findings in humans. Previous investigations into inflammation following CIH have shown increases in systemic TNFα and IL-6 expression following CIH administration in rodents (Ciftci et al 2004, Svensson et al 2012). Further administration of 2 weeks of CIH leads to an increase in microgial IL-1β, IL-6, TNFα, and COX2 gene expression in rats (Smith et al 2013). In regards to HFD, increased saturated and mono-unsaturated fatty acids activate a TLR4 dependent NFkB production in cell culture (Button et al 2014, Wang et al 2012a, Wang et al 2012b). *In vitro*, HFD results in increases in IL-1β, TNFα and MCP-1 expression in the brainstem after just 1 week and 16 weeks of CIH (Guillemot-Legris et al 2016). While there is only scant literature investigating breath-by-breath stability in breathing, some work has found correlations between altered breathing stability and central nervous system inflammation. Following induction of acute lung injury, an increase in brainstem expression of IL-1β is correlated with altered breathing patterns (Jacono et al 2011). Increased brainstem expression of TNFα in this report may underlie altered respiratory irregularity; however, this study was not designed to specifically investigate alterations in brainstem respiratory signals as a consequence of inflammation, and future studies looking at both consequences of inflammation in the

brainstem and physiologic changes following anti-inflammatory administration are warranted.

In summary, the coincident administration of HFD and CIH leads to an increase in inter-breath irregularity and level of brainstem microglial inflammation. These data suggest individuals experiencing both high fat diet and sleep disordered breathing may develop a self-propagating cycle of irregular breathing and inflammation during sleep brought upon by the experience of intermittent hypoxia. While the changes in ventilation seen here were mild, it is possible with longer duration of HFD or CIH may compound these effects and further studies are indicated.

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Table 1. qPCR Primer sequences

Name		Sequence
18s	F	CGGGTGCTCTTAGCTGAGTGTCCCG
	R	CTCGGGCCTGCTTTGAACAC
M IL-1b	F	TGTGCAAGTGTCTGAAGCAGC
	R	TGGAAGCAGCCCTTCATCTT
mCox2	F	CAGGTCATTGGTGGAGAGGTGTAT
	R	CCAGGCACCAGACCAAAGACTT
TNFα	F	TGTAGCCCACGTCGTAGCAA
	R	AGGTACAACCCATCGGCTGG

Table 1. Primers for amplification of inflammatory gene transcript in polymerase chain reaction.

	Apnea definition		
	Baseline	*24 hr post*	*p-value*
NX + NC	0.81 +/- 0.02	0.81 +/- 0.03	0.93
NX + HFD	0.79 +/- 0.02	0.81 +/- 0.01	0.39
CIH + NC	0.72 +/- 0.02	0.73 +/- 0.02	0.77
CIH + HFD	0.7 +/- 0.02	0.74 +/- 0.01	0.05

Table 2. Neither intermittent hypoxia nor high fat diet leads to changes in the definition of apnea. The definition of apnea showed no difference between groups either before or after administration of CIH, nor were changes observed within groups following CIH treatment.

Table 2. Baseline and post-gas exposure variables per group

Measure	Baseline				Post-exposure			
	Nx + NC	*Nx + HFD*	*CIH + NC*	*CIH + HFD*	*Nx + NC*	*Nx + HFD*	*CIH + NC*	*CIH + HFD*
Weight (g)	28.7 +/- 1.2	32.7 +/- 1.0[2]	27.8 +/- 0.5	34.2 +/- 0.9[2]	29.1 +/- 0.6	33.8 +/- 0.9	26.2 +/- 0.5[a]	29.9 +/- 1.1[a]
Tidal volume (mL)	0.27 +/- 0.01	0.18 +/- 0.02[1]	0.31 +/- 0.03	0.25 +/- 0.02	0.28 +/- 0.03	0.21 +/- 0.01	0.24 +/- 0.02	0.31 +/- 0.03
Tidal volume (µL/g)	9.5 +/- 0.6	5.6 +/- 0.4[1]	14.5 +/- 2.8	8.9 +/- 0.7	9.7 +/- 0.9	6.9 +/- 0.6	11.5 +/- 0.9	8.3 +/- 0.8
Frequency (min^{-1})	156.1 +/- 3.9	172.2 +/- 5.6	160.1 +/- 3.6	177.9 +/- 5.0[3]	154.8 +/- 6.2	170.1 +/- 3.8	155.7 +/- 2.8	167.5 +/- 2.0

[1] Mean differs significantly from CIH + NC at baseline, $p < 0.01$

[2] Mean differs significantly NC at baseline, $p < 0.005$

[3] Mean differs significantly from Nx + NC at baseline, $p < 0.005$

[a] Mean differs significantly to baseline measure per group, $p < 0.001$

Table 3. Mean baseline and post-exposure ventilation and weight measures. The mean baseline weight was higher in mice fed HFD as compared to NC, and mice treated with CIH show a significant weight loss as compared to baseline measures. Mice going on to normoxia treatment and fed HFD express a lower tidal volume than those fed a low fat diet and going on to CIH treatment. Mice treated with CIH and fed HFD express a higher respiratory frequency than those fed NC at baseline.

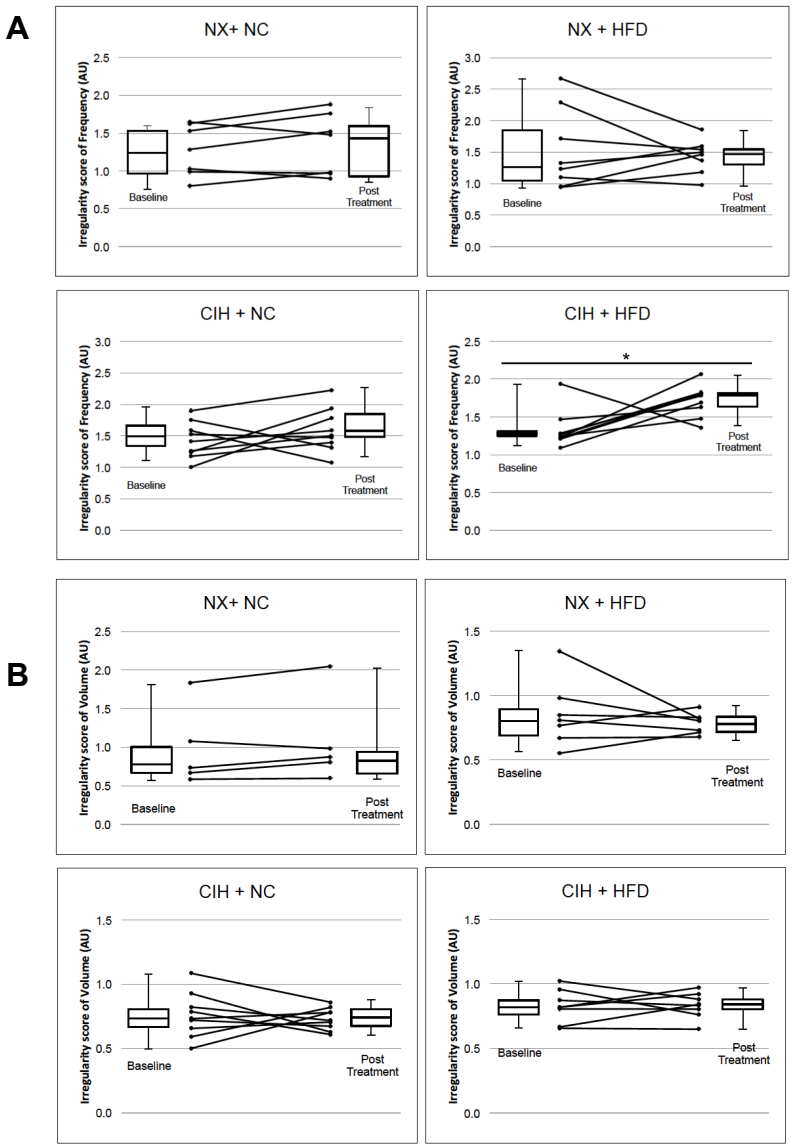

Figure 1. Administration of HFD in addition to CIH for two weeks leads to an increase in the irregularity of respiratory frequency. **A.** Irregularity score for frequency is depicted here both as individual change and median with ranges for each treatment group. Only mice administered both HFD and CIH show a significant increase in the respiratory frequency irregularity as compared to baseline measures: (NX + NC 1.28 (0.8-1.64) vs 1.47 (0.9-1.88), U=11, p=0.83; NX + HFD 1.28 (0.94-2.67) vs 1.48 (0.98-1.85), U=28,

p=0.71; CIH + NC 1.41 (1.0-1.9) vs 1.5 (1.07-2.22), U=48, p=0.65; CIH + HFD 1.25 (1.09-1.94) vs 1.78 (1.36-2.07), U=20, p=0.02). No other group shows a change in irregularity of frequency over time. **B.** Irregularity of tidal volume represented similarly to that in A. Tidal volume failed to show any change in irregularity over time in any group examined: (NX + NC 0.79 (0.59-1.84) vs 0.84 (0.6-2.05), U=12, p=1.0; NX + HFD 0.79 (0.55-1.34) vs 0.77 (0.64-0.91), U=30, p=0.87; CIH + NC 0.74 (0.5-1.08) vs 0.75 (0.61-0.89), U=60, p=1.0; CIH + HFD 0.82 (0.66-1.02) vs 0.84 (0.65-0.97), U=48, P=0.91).

	Hypopnea/hr			Hypopnea duration		
	Baseline	*24 hr post*	*p-value*	*Baseline*	*24 hr post*	*p-value*
NX + NC	3.99 +/- 0.91	3.48 +/- 0.84	0.74	0.61 +/- 0.04	0.58 +/- 0.02	0.51
NX + HFD	5.32 +/- 1.98	3.57 +/- 0.51	0.29	0.59 +/- 0.04	0.57 +/- 0.03	0.84
CIH + NC	7.89 +/- 1.62	6.98 +/- 1.49	0.38	0.67 +/- 0.04	0.62 +/- 0.02	0.4
CIH + HFD	5 +/- 1.58	5.1 +/- 1.11	0.96	0.56 +/- 0.02	0.57 +/- 0.02	0.75
	Spontaneous apnea/hr			Spontaneous apnea duration		
	Baseline	*24 hr post*	*p-value*	*Baseline*	*24 hr post*	*p-value*
NX + NC	2.25 +/- 0.97	0.30 +/- 0.18	0.11	0.93 +/- 0.06	0.93 +/- 0.09	0.85
NX + HFD	3.32 +/- 1.6	2.37 +/- 0.94	0.4	0.78 +/- 0.05	0.82 +/- 0.05	0.28
CIH + NC	2.28 +/- 0.61	2.05 +/- 0.56	0.59	0.86 +/- 0.03	0.91 +/- 0.04	0.23
CIH + HFD	2.05 +/- 0.63	2.28 +/- 0.31	0.72	0.81 +/- 0.03	0.84 +/- 0.03	0.54

Table 4.

Spontaneous apnea and hypopnea frequency and duration do not change with either high fat diet nor intermittent hypoxia: Spontaneous apnea and hypopnea showed no difference in regards to rate or duration between any treatment group either before or following treatment.

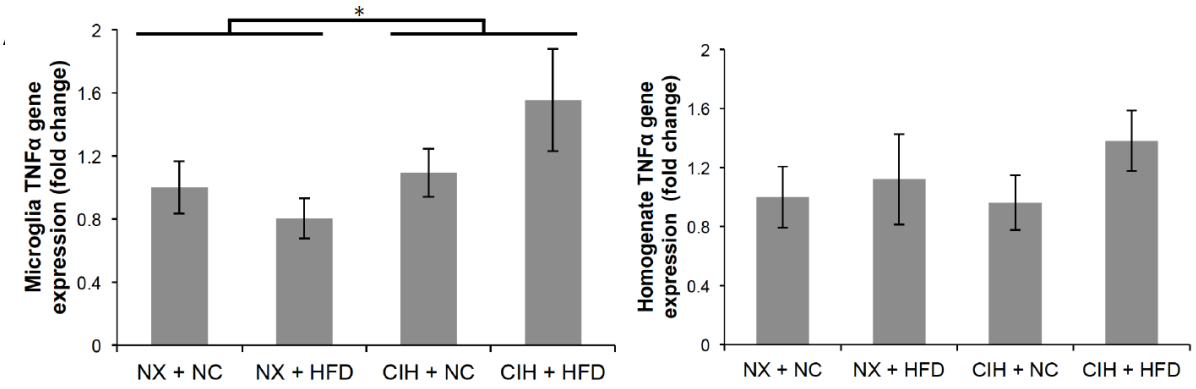

Figure 2. CIH increases microbial inflammatory gene expression in the brainstem. **A.** When grouped by diet, CIH leads to an increase in the level of TNFα gene transcript in brainstem microglia (1 +/- 0.17 NX + NC, 0.8 +/- 0.13 NX + HFD, 1.09 +/- 0.15 CIH + NC, 1.55 +/- 0.32 CIH + HFD, p=0.03). **B.** No difference is seen in TNFα gene expression in any condition in brainstem homogenate (1 +/- 0.21 NX + NC, 1.12 +/- 0.3 NX +HFD, 0.96 +/- 0.19 CIH + NC, 1.38 +/- 0.2 CIH + HFD, p=0.014).

CHAPTER IV

Evaluation of ventilation and chemosensory responses in a model of astrocyte dysfunction

J.G. Weltman, T. L. Hagemann, A. Messing, T.L. Baker

ABSTRACT

Despite considerable investigation into the neural control of breathing, previous investigations have only begun to uncover the complexity of neural respiratory network. Recent evidence has shed light onto the specific mechanisms by which the central nervous system senses and responds to alterations in the level of ventilation. Intriguingly astrocytes have been shown mount a calcium dependent ATP release in the presence of reduced oxygen and increased carbon dioxide, leading to modifications in the level of ventilation (Angelova et al 2015, Funk 2010, Gourine & Kasparov 2011, Gourine et al 2010, Huckstepp et al 2010). While astrocyte dysfunction is seen as a part of several diseases of the CNS, no investigation to date has examined breathing in a disease model predominantly afflicting astrocytes. Here we seek to investigate breathing in a mouse model of Alexander disease ($GFAP^{+/R236}$; +/R236H), a gain of function mutation leading to altered astrocyte morphology and function (Messing et al 2012). We hypothesized that astrocyte dysfunction in mice would result in a reduction in ventilation as well as responses to chemosensory input. Whole-body plethysmography was utilized to gather tidal volume and respiratory frequency, which was compared between groups as well as between normoxia and hypoxia, hypercapnia and the combination of the two. Mutant mice expressed a significant reduction in respiratory frequency (162.7 +/- 9.8 wild type vs 135.1 +/- 1.57 $hour^{-1}$ +/R236H) alongside an elevation in tidal volume (6.72 +/- 0.17 wild type vs 9.18 +/- 0.46 ug/mL +/R236H) at normoxia. When compared to normoxic values, mutant mice demonstrate augmented frequency responses to all levels of chemosensory drive as compared to wild type

control (hypoxia: 1.66 +/- 0.07 wt vs 2.17 +/- 0.05 +/R236H, hypercapnia: 1.81 +/- 0.45 wt vs 2.72 +/- 0.09 +/R236H, hypoxia + hypercapnia: 1.78 +/- 0.07 wt vs 2.67 +/- 0.08 +/R236H). This data provides evidence that alterations in astrocytes are associated with changes in breathing at normoxia and under high ventilatory drive. Further work to elucidate the site and mechanism of this dysfunction may shed light on the clinical significance as well as potential therapeutic interventions to astrocyte dysfunction in relation to breathing in neurodegenerative and neurodevelopmental conditions.

INTRODUCTION

The respiratory control centers are quite remarkable in their capacity to maintain precise and physiologically appropriate levels of both carbon dioxide and oxygen at all times. In addition, the flexibility this network provides in appropriately responding to perturbations including disease, exercise, pregnancy and development is reflected in the complexity of the system. Control of the respiratory network is dictated by the activity of both peripheral and central sensors capable of detecting small changes in oxygen, carbon dioxide and hydrogen ion levels (Guyenet et al 2008, Nattie 1999, Sapru 1996, Smith et al 1991). The peripheral sensors are predominantly located within the carotid body; however, in some species the aortic bodies are also capable of mounting a response to respiratory disturbances. Despite a fairly thorough understanding of the peripheral systems controlling breathing, the location and cell type of the central chemoreceptors remains uncertain (Funk 2010).

Recent evidence has demonstrated the potential for glial cells, particularly astrocytes, to sense and respond to alterations in oxygen, carbon dioxide and hydrogen ion levels. Astrocytes in culture have been shown to respond to reduced oxygen and increased carbon dioxide by increasing intracellular calcium and releasing ATP, a gliotransmitter (Angelova et al 2015, Funk 2010, Gourine & Kasparov 2011, Gourine et al 2010, Huckstepp et al 2010). Astrocytic ATP release leads to an enhanced output from the neural cells driving breathing *in vivo*, and a loss of astrocytes at an early age leads to a reduction in ventilation (Young et al 2005). Astrocytes are widely distributed throughout the central nervous system and, in particular, are ideally located adjacent to

blood vessels with extensions around nearly every neuron in the central nervous system (Bernardinelli et al 2014, Gourine & Kasparov 2011, Rusakov et al 2014). Given these properties, astrocytes have been put forth as a potential source of central sensory information within respiratory control networks.

Many central nervous system pathologies possess some degree of astrocyte dysfunction, including Rett syndrome, fragile X syndrome, multiple sclerosis, Alzheimer's, amyotrophic lateral sclerosis, and Huntington's disease (Molofsky & Deneen 2015). Animal models for many of these diseases have been developed; however, disease models displaying solely astrocyte dysfunction or apoptosis with no effects on other cell types do not exist. Alexander disease is a rare condition in humans arising as a result of a mutation leading to over expression of glial fibrillary acidic protein (GFAP) (Johnson 2002, Olabarria & Goldman 2017, Quinlan et al 2007, Rodriguez 2013, Sawaishi 2009, Yoshida & Nakagawa 2012). This protein is expressed predominantly in astrocytes, however, may also be found within oligodendrocytes (Eng et al 2000, Herpers & Budka 1984, Ogawa et al 1985). Humans afflicted with this condition develop protein aggregates within astrocytes called Rosenthal fibers along with an abnormal astrocytic morphology and altered surface protein levels (Johnson 2002, Olabarria & Goldman 2017, Quinlan et al 2007, Rodriguez 2013, Sawaishi 2009, Tian et al 2010, Yoshida & Nakagawa 2012). Animal models overexpressing human GFAP have been developed as a means of investigating pathologies related to Alexander disease.

Our goals with this study were to investigate ventilation in the presence of astrocyte abnormalities utilizing a mouse model of Alexander disease. In this investigation we first established the baseline respiratory parameters under normal air breathing followed by the response with increased chemosensory drive. We hypothesized that a disruption in astrocyte physiology, as seen with GFAP overexpression, leads to reduced ventilation at both normoxia and under increased chemosensory input; however, surprisingly here we demonstrated an enhanced chemosensory response with altered distribution of ventilation between volume and frequency at normoxia. Results from this study provide the basis for further investigation into the site and function of astrocytic influences in the control of breathing.

MATERIALS AND METHODS

Animals

Plethysmography studies were performed on ten-month-old male $GFAP^{+/R236}$ mice (+/R236H, n=10). Age-matched wild-type (wt, n=8) male littermates served as controls. All animals were housed on a 12-hour light-dark cycle in polycarbonate cages and were given water and food *ad libitum*. The Animal Care and Use Committee of the School of Veterinary Medicine, University of Wisconsin, approved all experimental procedures used in this study.

Whole body plethysmography

Mice were individually placed in whole body plethysmography chambers (1.5 liters, Data Sciences International model 601-1425-001, St. Paul, MN). Pressurized air

continuously flowed through the camber at 1.5 liters per minute, allowing precise control of inspired gas concentration and limiting expiratory gas accumulation. Respiratory flow signals were sent to a data exchange matrix (model ACQ-7700; Data Sciences International, St. Paul, MN) and collected through purposely-designed software (PONEMAH Physiology Platform, Data Sciences International, St. Paul, MN). For the purpose of analyses, body temperature was measured immediately prior to and following data acquisition. Body temperature was assumed to follow a linear change over time. Normoxia (FiO2 21%, FiCO2 0%; Nx), hypoxia (FiO2 10.5%, FiCO2 0%; Hx), hypercapnia (FiO2 21%, FiCO2 7%; CO_2) and maximum chemoreceptor stimulation (FiO2 10.5%, FiCO2 7%; MCS) conditions were established by mixing O_2, N_2, and CO_2 via a custom-made, computer controlled system of mass flow controllers to obtain desired gas concentrations as assessed by gas analyzer (CWE, model Gemini). Data was gathered for a minimum of 10 minutes during each condition (PONEMAH Physiology Platform; DSI, St. Paul, MN).

Data analyses

Data gathered at all states were averaged over 50 ms and a purpose written MatLab script was utilized to assess for respiratory frequency (RR) at all levels of chemoreceptor stimulation. Purpose designed software (PONEMA Physiology Platform; DSI, St. Paul, MN) was utilized to gather tidal volume (TV) data for comparisons. Data were averaged over five minutes of manually identified quiet breathing periods for analyses (figure 1). Data from Hx, CO_2, and MCS were normalized to normoxia values for comparisons to eliminate any influence of baseline differences (Hx/Nx, CO_2/Nx, and

MCS/Nx, respectively). Data were assessed for any outlying values by the maximum normed residual test and significant values were removed from analysis. Data were assessed for normality utilizing the Shapiro-Wilk test and determined to be parametric. Comparison of ventilation parameters at all levels of chemosensory input were performed by an independent Student's T-test. All data are expressed as mean and standard error and a p-value of less than .05 was deemed significant.

RESULTS

GFAP overexpression influences both tidal volume and frequency in normoxia

Based upon previous studies demonstrating the potential for astrocytic chemoresponsiveness (Angelova et al 2015, Funk 2010, Gourine & Kasparov 2011, Gourine et al 2010), we sought to investigate breathing in a mouse model of Alexander disease, a disease which affects predominantly astrocytes. Given only few studies have looked at breathing in the presence of astrocyte dysfunction, we first hoped to establish ventilation parameters under normal, eupneic breathing. By utilizing plethysmography, we were able to determine the tidal volume and respiratory frequency in +/R236H mice as well as age matched wt controls. We found mice overexpressing GFAP demonstrate a significantly higher tidal volume as compared to control (6.72 +/- 0.17 uL/g wt vs 9.18 +/- 0.46 uL/g +/R236H, p=.0001, figure 2A). Further, respiratory frequency was significantly lower with GFAP overexpression as compared to control (162.7 +/- 9.8 $hour^{-1}$ wt vs 135.1 +/- 1.57 $hour^{-1}$ +/R236H, p=.01, figure 2B). To assist in determining of overall ventilation was influenced by GFAP overexpression, we compared minute

ventilation between groups (i.e. the product of respiratory frequency and tidal volume). No differences were noted in minute ventilation between groups (1001.6 +/- 68.1 vs 1228.3 +/- 63 uL/g*s, p>.05, figure 2C), suggesting that while the distribution of ventilation between frequency and volume are altered with GFAP overexpression, the phenotype of ventilation is unaltered. Since we are measuring the entire system and not individual components within the respiratory neural network, it is not possible with this study to determine if the primary effect of GFAP mutation is predominantly on volume or frequency (i.e., and the other measure compensated to maintain normal minute ventilation).

GFAP overexpression results in enhanced responses to chemosensory inputs

Since mice overexpressing GFAP demonstrated altered distribution of ventilation between volume and frequency as compared to wild type, we sought to investigate how these groups compare in their response to system disturbances. In order to assess the responsiveness of the respiratory neural network, mice were exposed to reduced fractional inspired oxygen, increased fractional inspired carbon dioxide, and the two in combination. Tidal volume, minute ventilation and frequency data were normalized to normoxia values and are presented in figure 3. Raw ventilation parameters for chemosensory stimulation can be found in table 1. We hypothesized that mice expressing abnormal astrocytic phenotype would have reduced responses to chemosensory input. To our surprise, however, wild type mice expressed a decline in tidal volume in response to hypoxia, which was not demonstrated in GFAP overexpression (0.87 +/- 0.02 wt vs 1 +/- 0.05 +/R236H, p=.04, figure 3A). No

differences were seen between groups in tidal volume response to hypercapnia alone or in combination with hypoxia (figure 3A). Further, we found that mice overexpressing GFAP demonstrate an enhanced frequency responses to hypoxia (1.66 +/- 0.07 wt vs 2.17 +/- 0.05 +/R236H, p=.0001), hypercapnia (1.81 +/- 0.45 wt vs 2.72 +/- 0.09 +/R236H, p=.0003), and the combination of hypoxia and hypercapnia (1.78 +/- 0.07 wt vs 2.67 +/- 0.08 +/R236H, p<.00009, figure 3B) as compared to their wild type counterparts. No differences were noted in the minute ventilation responses to any chemosensory input between groups (figure 3C). In comparing raw data, measures of tidal volume, frequency and minute ventilation during hypoxia, hypercapnia, and the combination of the two were significantly higher in the GFAP overexpressing mice as compared to wild type (table 1). Given differences demonstrated prior to chemosensory stimulation, the reduced frequency observed in GFAP overexpression under normoxia is not a consequence of a reduced capacity, as these mice are capable of increasing their frequency to a level beyond that demonstrated in wild type mice. In addition, our failure to achieve significance in the normalized tidal volume and minute ventilation chemosensory responses between groups reflects the elevated tidal volume and minute ventilation observed in GFAP overexpression under normoxic conditions. Collectively these data suggest that the abnormalities expressed in astrocytes in this model more drastically influence respiratory frequency, and particularly so with increased chemosensory input; however, the increased tidal volumes observed under normoxia in GFAP overexpression are carried over into the tidal volume demonstrated under high chemosensory drive but do not lead to augmented tidal volume responses to hypercapnea or MCS in mutant mice.

DISCUSSION

Data presented here support the notion that healthy astrocytes are necessary to maintain normal breathing at eupnea as well as under increased chemosensory input. Here we demonstrated that mice overexpressing GFAP display a reduced respiratory frequency and increased tidal volume. Further, these mice expressed augmented responses to chemosensory input including high carbon dioxide and low oxygen breathing. This is the first investigation into the consequences on breathing in aged animals expressing predominantly abnormal astrocyte morphology and function in the absence of primary neuronal disease; however, because GFAP overexpression may affect non-astrocytic cell types as well (Eng et al 2000), this study does not prove conditions affecting astrocytes alone cause changes in breathing. Instead this study provides evidence that the pathology associated with GFAP overexpression influences ventilation and provides impetus for additional investigation into the role of astrocyte modifications in the abnormal breathing patterns seen with neurodegenerative and neurodevelopmental diseases.

Astrocytes serve diverse functions within the central nervous system

Rudolf Virchow originally described the non-neuronal cells of the central nervous system to be predominantly supportive to neurons and for many years astrocytes and other glial cells were believed to play minimal roles in central nervous system physiology (Allen 2014, Molofsky & Deneen 2015, Seth & Koul 2008). As a result, the study of the CNS became very neuron-centric; however, over the past 50 years it has

become clear that astrocytes play far more complex roles. Astrocytes have been shown to have diverse roles including formation and maintenance of the blood brain barrier, participation in synaptogenesis, neurotransmission, metabolic regulation and neurotransmission (Molofsky & Deneen 2015). Additionally, astrocytes express receptors for several neurotransmitters including glutamate, GABA, adenosine, norepinephrine, acetylcholine and endocanabinoids (Allen 2014, Volterra et al 2014). It is believed that neurotransmitters signal to astrocytes via g-protein coupled receptor mediated release of intracellular calcium stores, which leads to the release gliotransmitters including ATP, adenosine, glutamate and d-serine (Allen 2014). The roles of astrocytes seem to vary depending on an individual's life stage and the cell's location within the CNS. In cortical development, astrocytes play a role in the formation of synapses, but in adulthood their roles shift to that of neurotransmitter handling, potassium buffering and structural support to the neurons and blood brain barrier (Allaman et al 2011, Clarke & Barres 2013). Further, in the brainstem, astrocytes have been shown to contribute to formation of normal ventilation and response to chemosensory inputs (Gourine & Kasparov 2011).

Astrocytes and breathing

In the past several years, fascinating research has demonstrated a unique capability of astrocytes to sense and respond to alterations in chemosensory stimuli. It has been long recognized that specialized cells exist within the central nervous system with the capability to respond to alterations in systemic carbon dioxide by either enhancing or reducing the level of ventilation (Guyenet & Bayliss 2015, Guyenet et al

2008, Guyenet et al 2010, Nattie 1999). In line with many investigations into CNS physiology, researchers have long focused on determining which neurons provide this chemosensory activity, and have predominantly focused on the cells of the raphe nucleus, nucleus tractus solitarius, locus coeruleus, and retrotrapezoid nucleus (Guyenet & Bayliss 2015, Guyenet et al 2008, Nattie 1999). Similar to neurons, astrocytes cultured from these key brainstem regions have demonstrated similar responses to altered carbon dioxide and hydrogen ion levels, suggesting their role in modulating breathing. Gourine and colleagues documented a pH dependent release of ATP associated with an increase of intracellular calcium levels in astrocyte culture (Gourine et al 2010). Further, by inserting channelrhodopsin channels with relative specificity to astrocytes, investigators demonstrated astrocyte activation alone is capable of enhancing phrenic nerve activity (Gourine et al 2010). Huckstepp and colleagues investigated the consequences of altering levels of carbon dioxide applied to brainstem slices at the levels of both the medullary raphe and retrotrapezoid nuclei and found a linear correlation between the level of carbon dioxide and ATP release, a phenomenon that was inhibited by connexin-26 antagonism (Huckstepp et al 2010). While the connexin-26 antagonist utilized may have off target affects, these data suggest that astrocytes have receptors sensitive to pH and carbon dioxide and are able to respond to both hypercapnia and acidemia through neurotransmitter release. Limited information is available *in vivo*; however one study investigated breathing under normoxia and high carbon dioxide following administration of methionine sulfoximine, an astrocytic toxin, in neonatal rats. With this toxin, they were able to demonstrate a reduction in respiratory frequency and a reduced response to hypercapnia (Young et al

2005). To date ours is the only study looking at breathing in a fully intact, adult model of astrocyte dysfunction and, while we showed similar reductions in respiratory frequency to previous studies utilizing toxic disruption of astrocyte function, mice in our study showed enhanced chemosensory responses to hypercapnia. The toxin utilized by Young and colleagues, methionine sulfoxamine, functions by disrupting the activity of glutamine synthetase, leading to glycogen accumulation within astrocytes and subsequent altered morphology (Pena-Ortega et al 2016, Ronzio et al 1969). Since this toxin disrupts the normal glutamate/glutamine metabolism and has been shown to alter both excitatory and inhibitory transmission (Liang et al 2006, Pena-Ortega et al 2016), it is challenging to compare the findings in our study to that of this previous study; however, it is likely that the function of the neurorespiratory network in the two models is not similarly altered.

For many years there has been relative agreement that the CNS is not capable of responding to reductions in systemic oxygen tension, particularly in humans. This notion has been based upon studies performed in carotid denervated people, most of which had underlying disease (Gourine & Funk 2017). While only limited studies have been performed investigating hypoxic ventilatory response in normal people in the absence of carotid body input, information gathered from animal models conflicts these findings in people. Studies performed both *in vivo* and *in vitro* following carotid body denervation show both cellular responses as well as enhanced ventilation in hypoxia, and in particular these studies suggest a predominantly astrocytic role. Angelova and colleagues investigated the consequence of reduced inspired oxygen in rats and

demonstrated astrocytic ATP release following very small changes in oxygen tension, approximately 3 mmHg below normal values (Angelova et al 2015). Further, they demonstrated an increase in intracellular calcium in astrocytes in response to hypoxia both in culture and *in vivo* (Angelova et al 2015). Tadmouri, et al utilized continuous hypoxia for various time periods and showed an increase in levels of astrocyte activation as determined by GFAP expression at one and six hours of hypoxia within the nucleus tractus solitarius, but by 24 hours astrocyte activity returned to that seen under normoxic conditions (Tadmouri et al 2014).

While these previous studies make it clear that astrocytes have the capability of sensing and responding to alterations in oxygen, carbon dioxide, and hydrogen ion concentrations, it is not clear what the influence they play on breathing *in vivo*. While the design of the current investigation did not allow for differentiation on the primary disturbance in ventilation, it is clear that GFAP overexpression disturbs the system's capability of shaping normal breathing on a continuous basis. Following increased chemosensory input, we demonstrate an augmentation of the response of respiratory frequency under all conditions studied. In contrast, tidal volume responses to chemosensory input were only different under hypoxic conditions. Given that greater differences were seen in frequency response, it may be that cells in the region of the Pre-Bötzinger complex, the region of the brainstem responsible for rhythm generation (Syed et al 1990), are affected to a larger degree than those in regions responsible for governing tidal volume, i.e. the ventral respiratory group and the phrenic motor nucleus (Dale et al 2014, Golder & Mitchell 2005, McCrimmon et al 1995, Syed et al 1990).

Given the complexity and redundancy of central neural network controlling breathing, future studies isolating key regions within the central nervous system, such as those listed above, are warranted.

As mentioned previously, astrocytes respond to reduced oxygen and elevated carbon dioxide, which, presumably, enhances ventilation by influencing the level of respiratory neural activity. Given the responses to hypoxia and hypercapnia reported here, it seems unlikely that overexpression of GFAP leads to loss of these chemosensory capabilities; however it is possible that enhanced response to chemosensory input in +/R632H is a result of augmented astrocytic responses to hypoxia and hypercapnia, though other potential explanations should be considered. While GFAP overexpression may influence neuron and oligodendrocyte physiology (discussed below), additional aspects of astrocytes merit discussion first.

Additional mechanisms by which astrocytes may modify neural signaling

The astrocytic modulation of respiratory signals may be achieved by several mechanisms, and the time scale for onset and resolution may vary. As noted previously, astrocytes have the capability of responding to as well as secreting compounds involved in neural and glial excitability and function. ATP excreted by astrocytes has the potential to, via purinergic receptors (Wenker et al 2013), alter neuron firing, and it is likely this gliotransmitter serves to modify the neuronal activity in response to altered gas concentration levels as sensed by connexin channels in astrocytes (Angelova et al 2015, Funk 2010, Gourine & Kasparov 2011, Gourine et al 2010, Huckstepp et al 2010,

Tadmouri et al 2014). As mentioned above, however, it seems unlikely that a loss of these functions is seen in this disease model, given the reported augmentation in chemosensory drive. Therefore additional attributes of astrocytes that may influence neuronal activity warrant discussion.

Over the course of repolarization in neurons, intracellular potassium moves to the extracellular space (Bellot-Saez et al 2017, Seifert et al 2016); however, if this were to remain extracellular it would raise resting membrane potential and promote depolarization events. Astrocytes have well-established roles in potassium buffering and are capable of passively absorbing extracellular potassium (Kuffler 1967) presumably through the K_{ir} 4.1 channel. Knockout in this channel has been shown to lead to a reduction in intracellular astrocytic potassium and also glutamate uptake due to the requirement of potassium for glutamate uptake transporters (Djukic et al 2007, Sibille et al 2014). Glutamate uptake transporters (GLT-1 and GLAST) are also highly expressed on astrocytes and are believed to be the primary means of clearing excess glutamate in the synaptic cleft (Lopez-Bayghen & Ortega 2011). If glutamate clearance is mitigated by GLT-1 antagonism, synaptic activity is prolonged (Marcaggi & Attwell 2004) and complete knockout of GLT-1 leads to a severe epileptic phenotype and early death in mice (Tanaka et al 1997). Immunohistochemical studies have demonstrated that GFAP overexpression leads to an overall reduction in GLAST and GLT-1 expression in the mouse and pig brain tissue (Cho & Messing 2009, Sullivan et al 2007, Tian et al 2010). While the brainstem astrocytic transporter density was not specifically investigated, it

stands to reason this alone or in combination of loss of potassium buffering may be responsible for enhanced chemoresponses in this study.

As a relatively abundant cell type within the central nervous system, astrocytes have the capability of providing both a physical barrier and physiologic link between other cell types within the CNS. The high proportion of astrocytes in the cellular population of the CNS along with their position near synapses allows them to physically prevent neurotransmitter spillover and excessive excitatory or inhibitory signaling (Allen 2014). These astrocytic projections have also been postulated to play a role in modifying neuronal function beyond their roles in synaptic compartmentalization. Projections from astrocytes are dynamic and may extend or retract in response to local levels of activity (Haber et al 2006, Hirrlinger et al 2004, Nishida & Okabe 2007), and it has been suggested this is a means of allowing for new synapse formation, one potential mechanism of neuroplasticity beyond the developmental period (Theodosis et al 2008). Even in the mature brain, astrocytes have been shown to have the capability to modulate expression of neuroplasticity. Following release of ATP from astrocytes, adenosine is formed in the extracellular space, which is capable of decreasing presynaptic release probability by A1 receptors as well as increasing release probability by A2A receptors (Bonansco et al 2011, Panatier et al 2011, Pascual et al 2005). Mouse models of GFAP overexpression have demonstrated alterations in the astrocyte morphology and in particular there is a loss of astrocytic projections and overall enlargement of the cell (Eng et al 1998, Olabarria & Goldman 2017). These changes in the morphology of astrocytes could allow for neurotransmitter spillover across many

synapses and also may enhance release probability from neurons controlling breathing due to a loss of their modulatory effects of ATP on neuronal activity.

While the expression of GFAP is highest in astrocytes, GFAP is also expressed in other cell types including Schwann cells, Kupffer cells of the liver, pineal cells, pituicytes, immature oligodendrocytes, and various neoplastic cells (Eng et al 2000). While oligodendrocytes have not been shown to have an influence on the control of breathing, similar to astrocytes, they serve roles in potassium buffering (Menichella et al 2006) (Kofuji & Newman 2004, Olsen & Sontheimer 2008) and as such disturbances in this cell type may have influenced the results of this study. Further, previous studies have demonstrated non-astrocytic pathology including dysmyelination, microglial activation, neuronal cell loss, and synaptic elimination in this model (Messing et al 2001, Olabarria & Goldman 2017, Quinlan et al 2007, Sawaishi 2009). While this complicates the assumption that astrocyte dysfunction is the sole contributing factor to the altered ventilation seen, it is worthy to point out that these pathologies are common in many degenerative diseases, including Alexander disease, and authors propose these findings are direct consequence of abnormal astrocytic function and morphology (Johnson 2002, Messing et al 2012, Messing et al 2001, Olabarria & Goldman 2017). Given this, the use of GFAP knock-in still provides relevant information regarding breathing in neurodegenerative and neurodevelopmental diseases. Future studies utilizing a model expressing a purely astrocytic dysfunction are warranted.

Here we present the first investigation into ventilation in a mouse model of Alexander disease, a pathology predominantly afflicting the astrocytic cells of the central

nervous system. Data presented in this report suggests the presence of astrocyte disease is associated with altered tidal volume and respiratory frequency alongside enhanced responses to chemosensory inputs. This state may predispose the development of unstable breathing in neurodegenerative conditions affecting astrocyte morphology and function. These findings provide a foundation for additional work investigating the site and function of abnormal breathing in diseases effecting central chemoreception. These future studies may provide information to better understand how unstable breathing develops in conditions such as ALS and Rett syndrome as well as provide strategies to intervene in these conditions.

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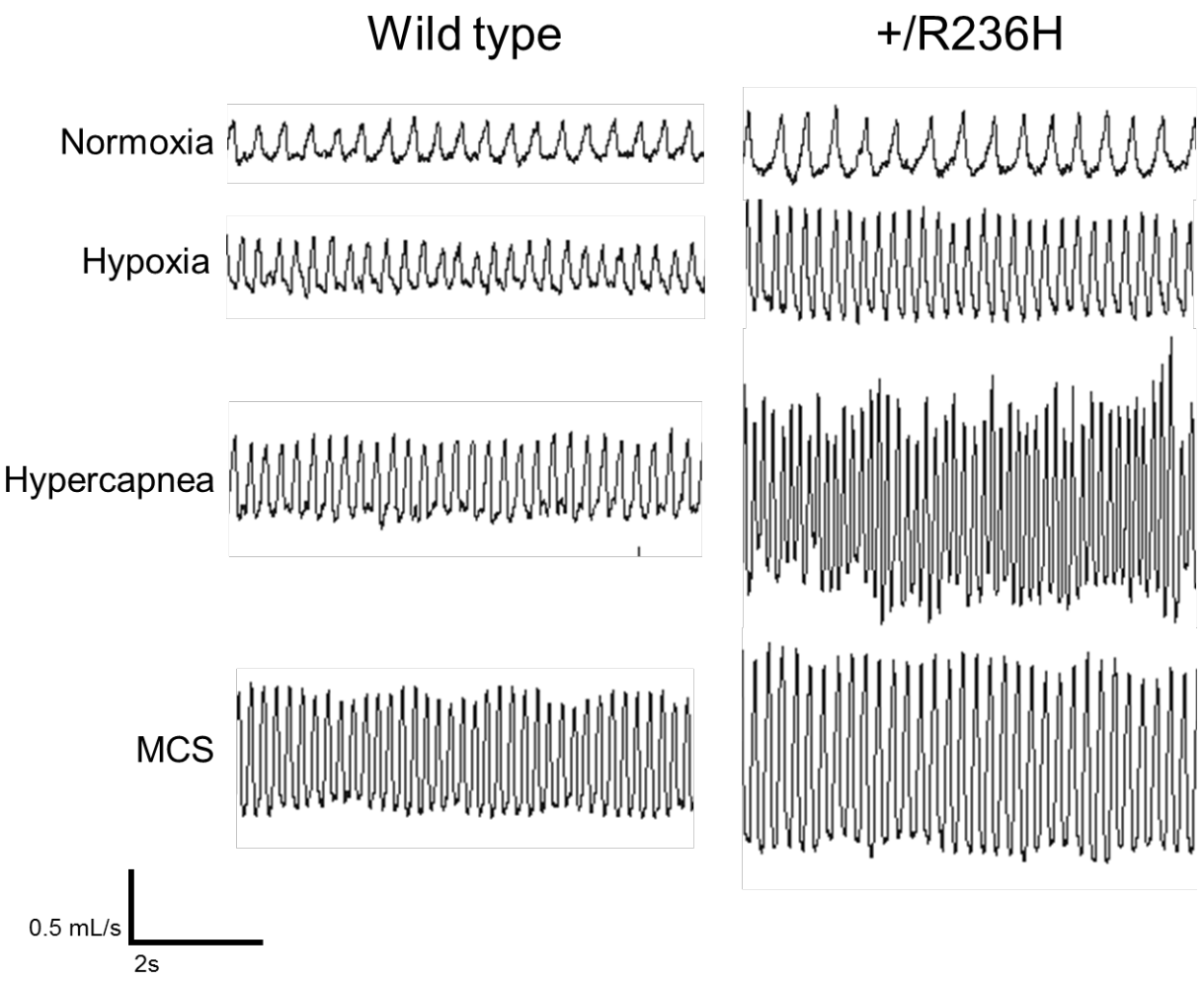

Figure 1. Representative whole-body plethysmography traces. Representative flow-time trace gathered by plethysmography in a wild type mouse and from a mouse demonstrating GFAP overexpression under each level of chemosensory input studied.

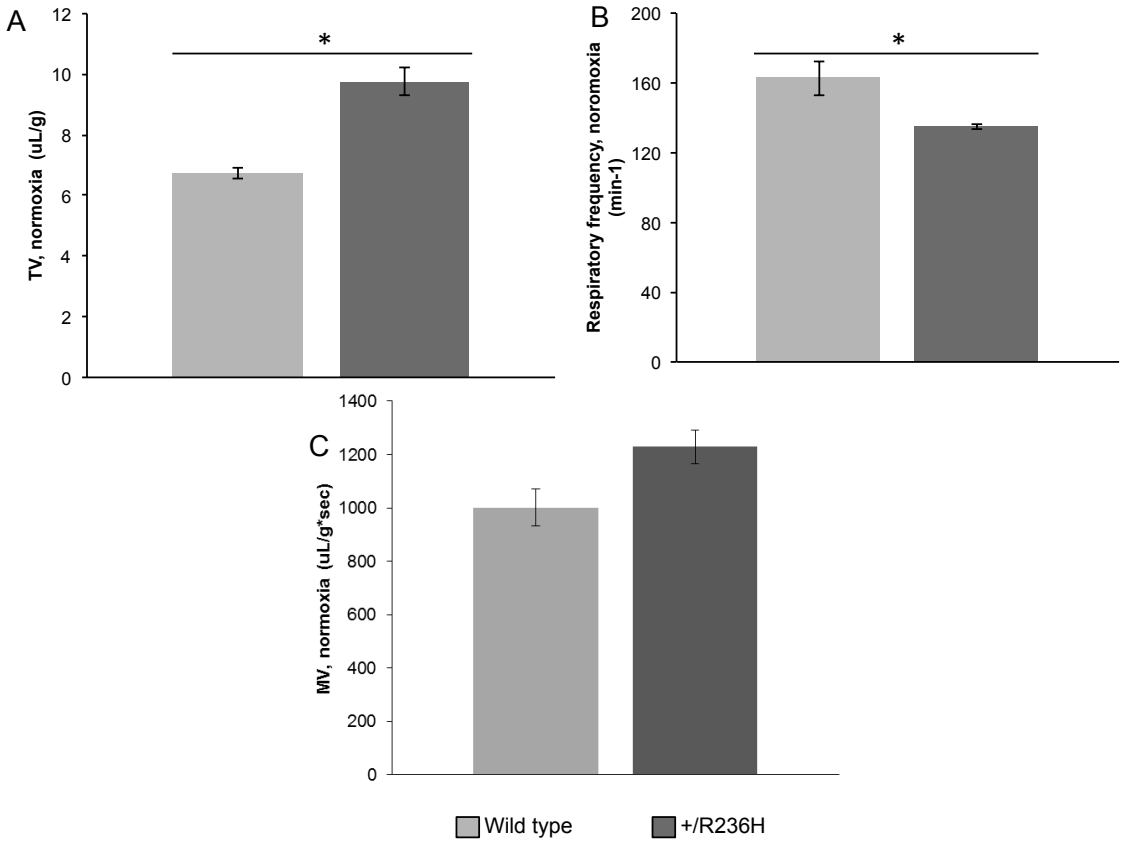

Figure 2. Mice overexpressing GFAP demonstrate alterations in ventilation parameters under eupneic breathing. **A**. +/R236H mice breathe at a significantly higher mean tidal volume as compared to wile type control (6.72 +/- 0.17 wt vs 9.18 +/- 0.46 uL/g +/R236H, p=0.0001). **B.** Alongside a significant increase in TV, +/R236H mice display a significant reduction in respiratory frequency as compared to wild type (162.7 +/- 9.8 wt vs 135.1 +/- 1.57 hour^{-1} +/R236H, p=.01). **C.** No difference is observed in the minute ventilation between groups (1001.6 +/- 68.1 vs 1228.3 +/- 63 uL/g*s, p>.05).

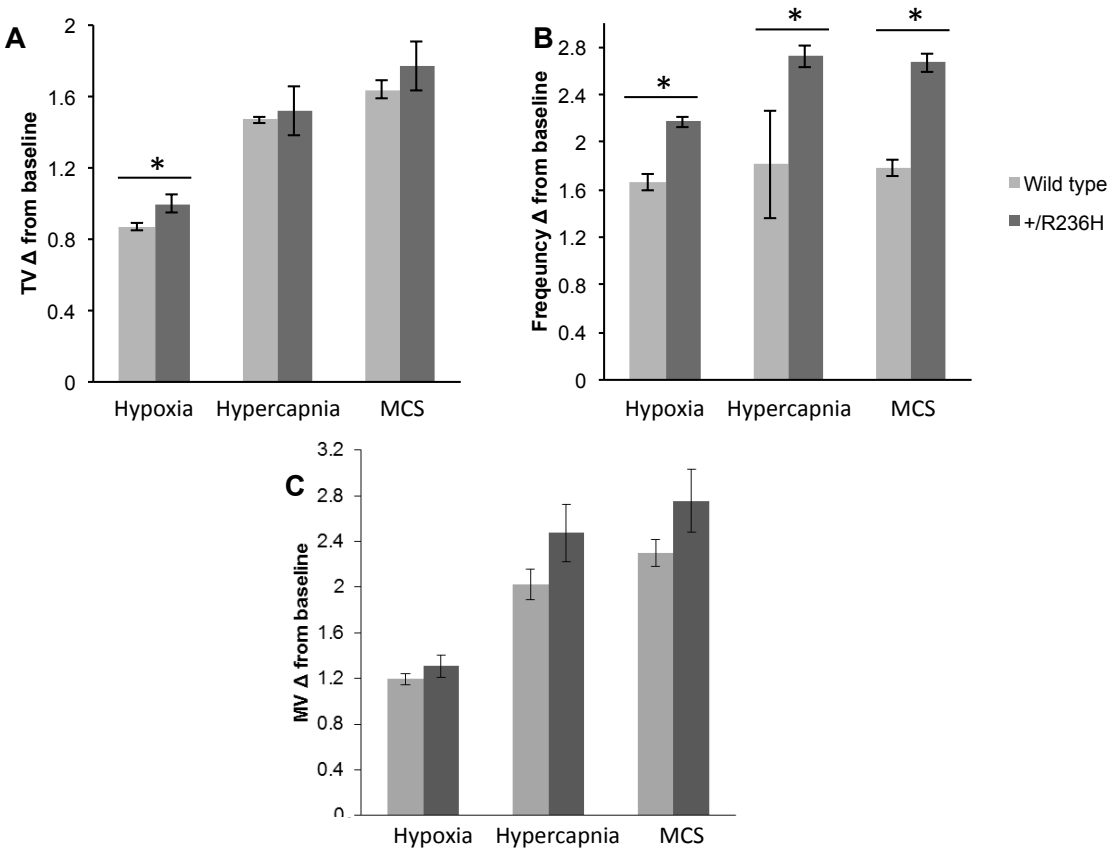

Figure 3. GFAP overexpression leads to augmented responses to chemosensory input, which is predominantly seen in frequency. **A.** Under hypoxic conditions (10.5% FiO2) mice overexpressing GFAP show a significant increase in the proportionate change in tidal volume as compared to the reduced tidal volume observed in wild type mice (0.87 +/- 0.02 wt vs 1 +/- 0.05 +/R236H, p=.04). No differences were seen in tidal volume in the remaining chemosensory stimuli (CO_2/Nx 1.47 +/- .02 wt vs 1.52 +/- 0.14 +/R236H, p=.76; MCS/Nx 1.64 +/-1 0.05 wt vs 1.77 +/- 0.14 +/R236H p=.45). **B.** GFAP overexpression additionally leads to an enhanced frequency response to all chemosensory states investigated as compared to wild type control (Hx/Nx: 1.66 +/-

0.07 wt vs 2.17 +/- 0.05 +/R236H, p=.0001; CO2/Nx: 1.81 +/- 0.45 wt vs 2.72 +/- 0.09 +/R236H p=.0003; MCS/Nx: 1.78 +/- 0.07 wt vs 2.67 +/- 0.08 +/R236H p<.00009). **C.** No differences were noted between groups in regards to minute ventilation response to any chemosensory input (Hx/Nx: 1.19 +/- 0.05 wt vs 1.31 +/- 0.1 +/R236H; CO_2/Nx 2.02 +/- 0.14 wt vs 2.48 +/- 0.25 +/R236H; MCS/Nx 2.3 +/- 0.12 wt vs 2.76 +/- 0.28 +/R236H, p>.05)

	Tidal Volume (uL/g)			Frequency (min^{-1})			Minute ventilation (uL/g*s)		
	wt	*+/R236H*	*p-value*	*wt*	*+/R236H*	*p-value*	*wt*	*+/R236H*	*p-value*
Hypoxia	5.84 +/- 0.15	9.83 +/- 0.47	<0.00001	265.8 +/- 6.3	300.1 +/- 8.0	0.0050	1225.0 +/- 87.5	1665.0 +/- 82.5	0.0018
Hypercapnia	9.87 +/- 0.25	14.86 +/- 0.71	0.000038	279.1 +/- 21.8	365.6 +/- 11.4	0.0013	2079.4 +/- 148.6	3152.0 +/- 156.2	0.0002
MCS	11.02 +/- 0.28	17.3 +/- 0.82	0.000018	299.5 +/- 18.7	361.4 +/- 8.2	0.0044	2367.7 +/- 169.2	3507.9 +/- 173.8	0.000316

Table 1. Raw, non-normalized chemosensory responses. In comparing the raw values for tidal volume, respiratory frequency and minute ventilation between groups, GFAP overexpressing mice demonstrate significantly higher parameters under all levels of chemosensory stimulation.

CHAPTER V

Therapeutic daily acute, intermittent hypoxia recruits coordinated inspiratory activity in non-diaphragm muscles of breathing

J.G. Weltman, F. F. Law, G.S. Mitchell

ABSTRACT

At rest, the predominant force driving breathing is generated by contraction of the diaphragm; however, there are additional muscles that have the capacity to contribute to ventilation, particularly so under increased ventilatory drive. Relatively little work has been done to investigate recruitment of non-diaphragm muscles of breathing; however, some work has demonstrated graded increases in activity with increased need. Acute intermittent hypoxia (AIH; 5 min 10.5% O_2/5 min 21% O_2; 10 cycles) elicits a long-lasting enhancement of diaphragm and external intercostal muscle activity of awake, freely behaving rats, referred to as long-term facilitation. Here we tested the hypotheses that 1) ribcage muscles in rats have the capacity to increase their activity under high ventilatory drive and 2) daily AIH reveals inspiratory activity in three accessory breathing muscles not normally active in awake, freely behaving rats. Sprague Dawley rats (Harlan colony 211, 11-16 wks. old) were chronically instrumented with electromyographic (EMG) radiotelemeter electrodes in the sternomastoid, scalene medius, serratus dorsalis, and diaphragm muscles. EMG activity was recorded with room air breathing (FIO_2/$FICO_2$=0.21/0.0) and with maximal chemoreceptor stimulation (FIO_2/$FICO_2$= 0.105/0.07, MCS), after which they were exposed to daily AIH or continuous normoxia for the same duration as described above. Our results showed that there was minimal inspiratory activity during room air breathing, but that both MCS and daily AIH led to coordinated inspiratory activity in scalene medius and serratus dorsalis muscles. Recruitment and coordination of non-diaphragm muscles of breathing may be beneficial in states of weakened, paralyzed or otherwise impaired diaphragm activity.

INTRODUCTION

Breathing represents one of the most crucial life-sustaining physiologic processes that is constituently active at all times, from birth to death. Surprisingly, the bulk of work involved in generating inspiratory events is provided by just one muscle: the diaphragm (Josenhans 1969, Mognoni et al 1969, Sant'Ambrogio et al 1966). Although the diaphragm generates the largest contribution to tidal volume, the relative contribution of several non-diaphragm muscles of breathing has been reported previously in rats, horses, cats, hamsters, non-human primates, dogs, and humans (De Troyer & Farkas 1994, De Troyer et al 1985, De Troyer & Wilson 2002, Fournier & Lewis 1996, Hall et al 1991, Navarrete-Opazo & Mitchell 2014, Taylor 1960, Wilson & De Troyer 2004). Many of these previous investigations focused on activity under normal breathing conditions; information regarding consequences of increased chemosensory inputs in non-diaphragm inspiratory muscles is lacking. Further, few studies have focused on strategies to recruit and coordinate non-diaphragm breathing muscles during inspiratory events. In the present study we sought to investigate the ability of select non-diaphragm muscles of breathing to contribute to breathing in normal conditions and under increased chemosensory drive.

The ribcage muscles have the capacity to contribute to inspiratory events by expanding the ribcage and further reduce intrathoracic pressure, beyond that achieved by diaphragm contraction alone. Whereas several muscles have the theoretical capacity to expand the thorax, most reported findings have focused on the parasternal intercostal muscles, as these muscles demonstrate consistent inspiratory activity in many species (De Troyer & Decramer 1985, De Troyer et al 1985, Hudson et al 2007, Legrand et al 2003, Raper et al 1966). In addition, variable inspiratory related phasic activity has been found in the scalene medius and sternomastoid muscles (Campbell 1955, Hudson et al 2007, Legrand et al 2003) with the scalene medius demonstrating more consistent activity during eupnea; however, some

interspecies differences exist (Hudson et al 2007, Raper et al 1966). Another muscle group, the serratus muscles, has received considerably less attention, despite having the capacity to expand the ribcage. Although the is no evidence that humans use this muscle in breathing (Cannon et al 2007, Loukas et al 2008, Vilensky et al 2001), no studies in non-humans have been done at this point.

Given the relative importance of maintaining consistent and appropriate levels of ventilation, it is not surprising that mechanisms exist to modify or enhance activity in periods of increased need. *Neuroplasticity* is the persistent alteration in the output of a neural system beyond the resolution of the inciting event. Neuroplasticity in respiratory motor systems has been well documented as the enhancement of phrenic burst amplitude following a variety of stimuli. In particular, application of hypoxia in an intermittent pattern leads to a form of respiratory neuroplasticity referred to as long-term facilitation (LTF). In normal rats, acute intermittent hypoxia (AIH; 5 min 10.5% O_2/5 min 21% O_2; 10 cycles) initiates an augmentation in the peak amplitude of compound action potentials of the diaphragm and second external intercostal muscle (Navarrete-Opazo & Mitchell 2014, Terada & Mitchell 2011). Similar treatments repeated daily (dAIH) have been shown to increase diaphragm electromyographic activity, as well as increase ventilation, as determined by whole-body plethysmography (Gonzalez-Rothi et al 2017, McGuire et al 2003, Navarrete-Opazo et al 2015). In these previous studies, only the diaphragm and the second external intercostal muscle were capable of enhancing their activity following a single dose of intermittent hypoxia; however, some evidence suggests that neuroplasticity itself has the capability to undergo a change in phenotype. *Metaplasticity* is the term applied to the modification in neuroplastic expression. Indeed, LTF has been demonstrated to undergo modifications in expression following prior treatment with daily intermittent hypoxia (Gonzalez-Rothi et al 2017, McGuire et al 2003, Navarrete-Opazo et

al 2015). Therefore, repetitive intermittent hypoxia may have the capacity to unveil neuroplasticity in neuromotor systems in which an individual exposure failed to elicit changes.

In the experiment presented here, we sought to determine the capability of three thoracic cage muscles to coordinate inspiratory events. We hypothesized that the sternomastoid, scalene medius, and serratus dorsalis muscles express inconsistent or minimal inspiratory activity under normoxic conditions; however, with enhanced chemosensory input, these muscles might be recruited to participate in inspiratory events. In addition, we tested the whether the application of dAIH leads to coordinated inspiratory events in these muscles, in the absence of enhanced chemosensory input. Although we confirmed an enhancement of inspiratory-related activity in the scalene medius and serratus dorsalis under increased chemosensory input and following daily, intermittent hypoxia, there was no significant increase of coordinated inspiratory discharges of the sternomastoid under any condition studied. These data support the use of daily, acute intermittent hypoxia to improve respiratory activity in non-diaphragm muscles; however, further work in disease models is indicated.

MATERIALS AND METHODS

Animals

All experiments were performed on 12-16 week-old, male Sprague-Dawley rats (*N*=10, colony 211, Harlan, Indianapolis, IN). Animals were co-housed in a temperature- and humidity-controlled environment with 12:12 hour light/dark cycles. The Animal Care and Use Committee of the School of Veterinary Medicine, University of Wisconsin, approved all experimental procedures used in this study.

Surgical procedure

Surgical implantation of electromyographic leads was performed a minimum of one week before initial data collection. Radiotelemeter unit battery voltage was determined to be adequate prior to implantation. If voltage was measured as low, battery units were replaced. Prior to surgery, the surgical suite and surgical tools were all disinfected or sterilized. Approximately 2 mm. of silicone insulation was removed from the distal end of each biopotential lead of radiotelemetry units (4ET-S1/2; Data Sciences International, St. Paul, MN). Telemeter transmitter body, biopotential leads and battery unit were sterilized. All surgeries were performed under isoflurane anesthesia in 100% inspired oxygen. Rats were treated pre-operatively with buprenorphine (0.03 mg/kg), carprofen (5 mg/kg) and enrofloxicin (10 mg/kg) subcutaneously to treat pain and minimize post-operative infections. Body temperature was maintained at 36-37.5°C with external heat support. An orotracheal tube was utilized for positive pressure ventilation (Tidal volume 6 mL/kg; Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA) with 1.5-3% isoflurane in 100% oxygen. Adequate anesthetic depth was determined by pedal withdrawal and corneal reflex. Oxygen saturation was monitored by pulse oximetry (model 8600; Nonin Medical Plymouth, MN) during surgical procedures. Following midline celiotomy, paired biopotential leads were placed into the right, mid-costal diaphragm, guided by a 23g needle within the body of the muscle at an orientation perpendicular to the direction of muscle fibers, as observed grossly. Both leads were sutured to the body of the muscle (6-0 Ethilon; Ethicon, Cornelia, GA) and adhered with tissue adhesive (Vetbond 1469SB; 3M Animal Care Product, St. Paul, MN). Accessory muscle dissection was achieved as follows: 1) the left sternomastoid (StM) muscle was identified midway between the sternum and mandible, 2) the left scalene medius (ScM) was exposed over the third rib, and 3) the left serratus dorsalis (SeD) was approached over the level of the eleventh rib. All paired biopotential leads were placed by needle guided technique as described above. Leads were subcutaneously tunneled from the celiotomy incision to their muscle destination. The telemetry

unit and battery were placed into the peritoneal cavity, the linea alba was opposed with a simple interrupted pattern (3-0 Polysorb; Covidien Ltd., Dublin, Ireland) and the skin was opposed by wound clips (3M Precise disposable skin stapler; 3M Animal Care Products, St. Paul, MN). Post-operative pain was managed with once-daily carprofen and twice-daily buprenorphine at the same dose as preoperatively. Rats were visually monitored and weighed daily. At the completion of the experiment, radiotelemetry biopontential leads were dissected to their insertion sites within the muscles of interest to assure that all leads remained in place throughout the period of study.

Telemetry signal acquisition

Rats were placed in custom Plexiglas® four-liter chambers (12 x 4 in inner diameter; one-rat-per-chamber with a radiotelemetry receiver unit (model RPC-2; Data Sciences International, St. Paul, MN) placed below the chamber floor. Radiotelemetric signals were sent to a data exchange matrix (model ACQ-7700; Data Sciences International, St. Paul, MN) and collected through purposely-designed software (PONEMAH Physiology Platform, Data Sciences International, St. Paul, MN). Normoxia and MCS conditions were established by mixing O_2, N_2, and CO_2 via a custom-made, computer controlled system of mass flow controllers to obtain desired gas concentrations, as assessed by gas analyzer (CWE, model Gemini). Data were gathered during each condition for 20 minutes and analyzed in one-minute intervals during periods in which rats were in a sternal posture with neck extended (PONEMAH Physiology Platform; DSI, St. Paul, MN). All data were collected prior to any gas treatment, as well as 60 minutes following the final intermittent hypoxia exposure (see below).

Acute intermittent hypoxia protocol

Desired inhaled gas concentrations during normoxic (21% O_2) and hypoxic (10.5% O_2) conditions were tightly controlled in the Plexiglas® chambers. Oxygen and carbon dioxide levels were continuously measured within the chambers (CWE, Gemini model). Target gas concentrations were achieved by mixing nitrogen and oxygen and were controlled with a custom-made computer-controlled system. Gas-flow rates were four liters per-minute, per-chamber. 95% of the change in O_2 levels was obtained within the first 25s (+/-5) and carbon dioxide levels were maintained at a level of < 0.5% at all times. On the day prior to the first exposure, rats were acclimated to the chambers for 2 hours under normoxic conditions. On each day of exposure, rats were acclimated to the chambers for 1 hour prior to hypoxia exposure. Half of the rats were administered daily AIH (10 doses, 5-minute episodes of 10.5% O_2 interspersed with 5 minute episodes of 21% O_2; total 95 minutes). The remaining half of the rats were administered continuous normoxia. This was repeated for seven consecutive days and EMG recordings were gathered as described above.

Data analysis

Following acquisition, signals were averaged over 50 ms and full-wave rectified in Neuroscore software (Data Sciences International, St. Paul, MN). Diaphragm EMG signal was plotted alongside EMG gathered from each non-diaphragm muscle at each time period. The compound action potentials seen in the diaphragm signal were compared to those of the StM, ScM, and SeD, and any simultaneous discharges were manually counted in one-minute epochs. Respiratory frequency was identified by counting the frequency of compound action potentials of the diaphragm.

In order to investigate the consequence of maximum chemoreceptor stimulation on diaphragm discharge frequency, we applied a Wilcoxon Signed-Ranks test (nonparametric

equivalent of a paired *t*-test). To identify significant changes in breathing patterns of the muscles of interest, we employed mixed-effects logistic regression modeling because 1) logistic regression allows for a dichotomous dependent variable and 2) we were able to capitalize on repeated measures from a small sample pool. From 10 rats, over 4,300 measurements were collected; by including Subject as a random effect in all mixed-effects models, we were able to control for subject-level dependencies inherent in a repeated-measures experimental design. In addition, mixed-effects models allow for an unbalanced design. That is, having the same number of samples per-subject is not a requirement. All statistical analyses were performed in R (v3.4.2), using the lme4 package (v1.1-14; (Bates et al 2015). For further discussion on mixed effects logistic regression analysis, see Mirman, 2014.

RESULTS

Non-diaphragm muscles of breathing exhibit minimal activity at baseline

In order to measure whether accessory muscles of interest were utilized as a part of respiration, electromyographic activity was compared to that gathered from the diaphragm. Any phasic compound action potential activity that occurred simultaneously with diaphragm activity was considered to be a coordinated inspiratory burst. Figure 1 provides representative traces. To summarize, a median rate of 84 inspirations per minute was observed across subjects, as measured by diaphragm discharge frequency. Non-diaphragm muscles showed variable activity during normoxic conditions. On average, 48% of observed inspirations involved recruitment of one or more of the muscles in question (StM: 11%, ScM: 30%, SeD: 27%, figure 2).

Hypercapnia and hypoxia enhances frequency and recruits non-diaphragm muscles of breathing

Administration of MCS led to significant differences in breathing patterns when compared to control conditions. Specifically, a Wilcoxon Signed-Ranks test on the pairwise breathing rates confirmed that there was a marked increase diaphragm discharge frequency following MCS, with a median rate of 156 per minute, compared to 84 per minute under normoxic conditions. [N = 10, T+= 10, z = -2.89, p < .002, figure 2]

To determine whether there were changes in activity of non-diaphragm muscles following MCS, a mixed-effects logistics regression model was used to compute the log-odds that a discharge was detected in at least one of the muscles of interest, given a simultaneous action potential observed in the diaphragm and whether introduction of the enhanced chemoreceptor stimulation significantly increased the log odds of coordinated discharge. The administration of MCS was used as an independent variable to predict whether one or more additional muscles were recruited for inspiration. Results confirmed a significant increase in coordinated non-diaphragm muscle activity following MCS administration [β_{mcs} = 1.08, SE = 0.10, z = 10.40, p < .001, figure 3]. That is, the model estimated that non-diaphragm muscle recruitment occurred during ~46% of inspirations in normal conditions; however, non-diaphragm muscle recruitment was estimated to have occurred during ~71% of breaths in combined hypoxia and hypercapnia.

Next, it was of interest to explore whether there were differing levels of recruitment for the three muscles of interest. Given that subjects exhibited different patterns of muscle activity (e.g., some subjects exhibited only tonic activity for certain

muscles during either condition), it was not possible to calculate separate models using individual muscles as a dependent variable. Thus, an omnibus logistics mixed effects regression model was employed, including all three muscles as independent variables and baseline vs. introduction of chemoreceptor stimulation as the dependent variable. The intercept of this model represents the log-odds that muscle activity was observed in the diaphragm only and each independent variable represents the log-odds of activity in each of the muscles of interest. Significant associations between the activities of individual muscles after administration of the chemoreceptor were observed. Specifically, when either the scalene or the serratus dorsalis muscle was active, this was significantly more likely to have occurred during an inspiratory event measured during enhanced chemoreceptor activity [β_{ScM} = 0.25, SE = 0.13, z = 1.96, $p < .05$; β_{SeD} = 1.91, SE = 0.14, z = 13.73, $p < .001$, figure 3].

In exploring individual differences among subjects, seven of the ten participants exhibited an increase in scalene muscle activity post-chemoreceptor stimulation, with three of the seven exhibiting phasic activity in the scalene only during the post-chemoreceptor condition. Two participants exhibited a marginal reduction of scalene activity following MCS. Finally, one subject exhibited no phasic activity of the scalene muscle in either condition. Regarding the serratus dorsalis muscle, 9 out of 10 subjects exhibited increased coordinated phasic activity after administration of MCS. Four of the nine exhibited serratus dorsalis activity solely post introduction of chemoreceptor stimulation. One subject exhibited no serratus dorsalis activity for ether condition.

In exploring activity patterns for the sternomastoid muscle, a result contrary to our hypothesis was found; there was a significant negative association between phasic

activity of the sternomastoid and observed inspiratory events following MCS as compared to normoxia [β_{StM} = –0.50, *SE* = 0.17, *z* = –3.00, *p* < .003, figure 3]. However, upon further investigation of individual subjects, 5 of the 10 exhibited no sternomastoid activity in either condition. One subject exhibited activity solely in the post-chemoreceptor condition and two subjects demonstrated roughly equal observed coordinated discharge rates. Two other subjects did exhibit a decrease in sternomastoid discharge frequency during the post-receptor condition. However, given that half of the subjects exhibited no sternomastoid activity in either condition, and that averaging across those that yielded a marginal difference (23% vs. 28% post-chemo), it was presumed that the significant negative association was spurious.

Administration of daily, acute intermittent hypoxia leads to enhanced coordination between the diaphragm and the scalene medius and serratus dorsalis

Differences in inspiratory patterns between rats exposed to dAIH were compared to those observed in normoxia treated rats. A decrease in diaphragm discharge rate was observed in both groups following treatment. However, there was no appreciable difference in discharge frequency between groups (pre-treatment: control ~90 min^{-1}, dAIH ~81 min^{-1}; post-treatment: control ~69 min^{-1}, dAIH: ~66 min^{-1}).

To investigate whether there was an overall increase in muscle recruitment in rats exposed to dAIH, a mixed-effect logistic regression model was calculated with treatment group, pre-treatment vs. post-treatment, and treatment group × pre-treatment vs. post-treatment as independent variables. Whether or not at least one muscle was recruited alongside the diaphragm was used as the dependent measure. The results confirmed that there was a significant increase in non-diaphragm muscle activity for the

treatment group following dAIH [$\beta_{Post\text{-}test \times Treatment\ Group}$ = 1.15, *SE* = 0.23, *z* = 5.00, *p* < .001, figure 4]. No significant difference was found between groups prior to treatment [$\beta_{Intercept}$ = –0.27, *SE* = 0.34, *z* = –0.78, *p* = *ns*, figure 4], nor was any difference observed for the control group following normoxia as compared to baseline measures [$\beta_{Treatment\ Group}$ = 0.31, *SE* = 0.49, *z* = 0.66, *p* = *ns*, figure 4].

In order to explore which muscles were used most often by subjects in the treatment group following treatment, a separate mixed-effects logistic regression model was employed, including only the pre-dAIH and post-dAIH observations. Again, because subjects exhibited different patterns, the presence/absence of activity for each of the three muscles were included as independent variables. The categorization of a discharge as being observed prior to or following dAIH was used as the dependent variable.

The results revealed a significant increase in phasic, coordinated activity for the serratus dorsalis [β_{SeD} = 0.96, *SE* = 0.30, *z* = 3.24, *p* < .002, figure 5] and scalene medius [β_{ScM} = 2.52, *SE* = 0.23, *z* = 11.17, *p* < .001, figure 5] muscles following dAIH, as compared to pre-treatment. There was no observable difference in the rate of sternomastoid activity between measurements [β_{SM} = –0.12, *SE* = 0.26, *z* = –0.48, *p* = *ns,* figure 5]. The significant intercept also indicated a marked increase in muscle recruitment post-treatment, in that an observation in which solely the diaphragm was active was strongly associated with measurements made at pre-treatment [$\beta_{Intercept}$ = –1.61, *SE* = 0.24, *z* = –6.68, *p* < .001].

In exploring individual rat differences in muscle activity patterns post-treatment, all except one of the experimental subjects showed an increased rate of coordinated

discharges for both the scalene and serratus dorsalis muscles. The fifth subject exhibited no phasic activity of either the sternomastoid or serratus dorsalis muscles and a slight decrease in scalene medius activity was measured following dAIH. Regarding the sternomastoid muscle, three of the five subjects did exhibit an increase in activity following treatment; however, one subject only exhibited sternomastoid activity at baseline and (as previously mentioned) another subject exhibited no activity at either observation periods.

DISCUSSION

The results of this study provide three major findings: 1) under normoxic conditions, minimal activity was seen in the ribcage muscles studied, 2) increased chemostimulation recruits inspiratory activity in the scalene medius and serratus dorsalis but not the sternomastoid, and 3) one-week daily AIH enhances inspiratory activity in the scalene medius and serratus dorsalis under eupneic conditions. These data demonstrate one mechanism of coordinating inspiratory events in ribcage muscles not generally utilized in ventilation in normal rats.

The lungs are surrounded by a potential space, the pleural space, which under normal conditions is absent of contents, save for a small amount of fluid. To generate inspiration, the contraction of the diaphragm displaces the contents of the abdominal cavity and creates pressure in the pleural space that is negative relative to environmental pressure (Costanzo 2013, Hall & Guyton 2011, West 2014). In addition to displacement of the diaphragm, negative pressure can be generated in the pleural space by expansion of the rib cage. Therefore, some muscles of the ribcage have the potential to contribute to inspiratory activity if their contractions lead to expansion of the thoracic wall. Previous studies have shown the external intercostal

muscles, particularly that between the second and third ribs, have a consistent contribution to breathing in rats (De Troyer et al 1985, Navarrete-Opazo & Mitchell 2014, Reid et al 1976, Saboisky et al 2007, Taylor 1960). Here, we sought to expand the knowledge regarding the muscles often referred to as accessory muscles of breathing.

Ribcage muscle activity shows differences across species

There are considerable differences in the activity of ribcage muscles across species. For instance, in humans the scalene medius and parasternal intercostal muscles have received the most attention and are generally believed to be constituently active during quiet breathing with a pattern matching inspiration; however, if the person is asked to breathe with just the diaphragm, ribcage muscle activity diminishes (De Troyer & Decramer 1985, De Troyer et al 1985, Hudson et al 2007, Legrand et al 2003, Raper et al 1966). There is a more variable pattern of inspiratory activity reported in the scalene medius with consistent inspiratory activity observed only during elevated ventilation (Campbell 1955, Hudson et al 2007, Raper et al 1966). It is likely that posture plays a role in discordant findings in the contribution of scalenes to inspiration, as discussed below. The sternomastoid shows less consistent activity in inspiration, only showing potential for contribution in inspiration during maximal lung inflation and appears to be dependent upon arm and head position, as well as level of ventilatory activation (Campbell 1955, Hudson et al 2007, Legrand et al 2003). When compared to each other, the scalene medius shows inspiratory related activity at lower levels of ventilation, as compared to the sternomastoid muscle (Hudson et al 2007, Raper et al 1966).

Dogs have been shown to have phasic, respiratory-related activity in the parasternal intercostal muscles under quiet breathing conditions, similar to that observed in humans (De Troyer 1991a, De Troyer 1991b, De Troyer et al 1985, Taylor 1960, Whitelaw & Feroah 1989).

In dogs, the scalene and sternomastoid muscles have not been shown to contribute to eupneic breathing; however, when airway resistance or chemosensory input are increased, the scalene is recruited during inspiration (De Troyer 1991a, De Troyer & Kelly 1982, De Troyer et al 1985). Also, in the baboon, the neck muscles in general and the scalene medius in particular are silent during inspiration regardless of body positioning (De Troyer & Farkas 1994). Hall, et al. investigated the intercostal, serratus ventralis, internal abdominal oblique and rectus abdominus in conscious and anesthetized horses, and found phasic activity in the intercostal muscles at the end of inspiration as well as expiration, but no activity in the other muscles studied (Hall et al 1991). The scalene muscle of hamsters demonstrates some inspiratory activity but only at high chemosensory drive (Fournier & Lewis 1996). The differences seen in muscles across species likely is related to the relative use of the forelimb for locomotion, the anatomy of the thoracic cage, and tonic muscle activity in establishing and maintaining posture. Limited information is available regarding these muscles in rats; however, data presented here demonstrates only limited activity of the sternomastoid muscle in all conditions evaluated. The scalene medius appears to have some inspiratory activity in eupnea, but undergoes considerable augmentation in electrical activity following MCS.

The serratus muscle group has attachments to the proximal portion of the caudal rib bones along with attachments to either the scapulae or thoracic vertebrae, depending on the muscle of interest. This positioning gives the muscle group the anatomic potential to expand the ribcage and lead to reduction in intrathoracic pressures (Reid et al 1976). Whereas phasic muscle activity has been identified in serratus muscle groups (Reid et al 1976), few studies have investigated this muscle in breathing and all are based solely on observations in humans. In individuals with chronic obstructive pulmonary disease (COPD), no appreciable differences have been observed in serratus posterior superior or inferior muscle activity as compared to

normal individuals (Loukas et al 2008). No activity of the serratus anterior has been observed during graded levels of exercise, despite alleviation of any role of these muscles for arm positioning (Cannon et al 2007). Given the anatomy of these muscles, the authors suggested that they may play a dual role in posture, and that arm position may influence the activity of serratus muscles under increased need of breathing. It has been proposed that previous studies failing to demonstrate respiratory activity in serratus muscle groups were flawed in that the investigators did not alleviate any role of posture and future work looking at these muscles in a more relaxed position is necessary (Vilensky et al 2001). As mentioned above, the anatomy of the ribcage varies across species and serratus muscle groups may have more potential for inspiratory activity in non-human animals; however, no study investigating this muscle group has been performed in non-human species to date. In the present study, we demonstrate a high level of inspiratory activity in the serratus dorsalis, the surrogate muscle too the serratus posterior in humans. Further, based upon the findings presented above, rats are capable of enhancing the level of activity of this muscle to respond to increased demand.

Recruiting the activity of non-diaphragm muscles of breathing

Previous work has demonstrated the potential for enhancing respiratory motor activity by spinal neuroplastic manipulation. Long-term facilitation (LTF) is a long-lasting increase in the spinal output of motor systems in response to intermittent hypoxia. Much of the work in LTF has been in reduced preparations wherein phrenic nerve activity is measured prior to, during and following the induction of LTF by reduced inspired oxygen in an intermittent pattern. (Dale et al 2014, Fuller et al 2000, Navarrete-Opazo & Mitchell 2014). Briefly, immediately following the final hypoxic episode, there is a gradual increase in the amplitude of phrenic compound action potential, which is sustained to at least 60 minutes following hypoxia episodes (Dale et al 2014, Fuller et al 2000, Fuller et al 2001), due to spinal modulation of respiratory signals from the

brainstem (Baker-Herman et al 2004). Because the phrenic burst amplitude has been shown to be representative of tidal volume (Eldridge 1971), LTF has been hypothesized to be a means of enhancing ventilation. However, in the setting described above, rats were artificially ventilated, neuromuscularly blocked and anesthetized to allow for signal acquisition. Similar findings have been demonstrated in intact rats by diaphragm EMG. In utilizing electrodes measuring diaphragm electrical activity, we were able to demonstrate enhancement of diaphragm activity following 10 five-minute hypoxia episodes in rats, which is long-lasting to 60 minutes following exposure (Navarrete-Opazo & Mitchell 2014, Terada & Mitchell 2011). Although work has yet to be pursued to establish whether these processes are similar to those observed in the reduced preparation described above, both forms of neuroplasticity are initiated by intermittent hypoxia.

Whereas the capacity for the phrenic motor pool to express plasticity following intermittent hypoxia has been thoroughly documented, non-diaphragm muscles have received comparatively less attention; however, studies to date provide a basis for further investigation into LTF in ribcage muscles. Fregosi and Mitchell investigated the activity of the inspiratory intercostal nerve following stimulation of carotid sinus, which simulates the consequences of intermittent hypoxia. They demonstrated a profound enhancement: 225% of baseline activity, at 90 minutes following stimulation (Fregosi & Mitchell 1994); however in a subsequent study in spontaneously ventilating animals, Jansen and Fregosi failed to show enhancement of inspiratory intercostal muscle activity following only three episodes of intermittent hypoxia (Janssen & Fregosi 2000). Previous work from our lab has demonstrated an augmentation of activity in the second external intercostal muscle following ten episodes of intermittent hypoxia; however, the remainder of the intercostal muscles failed to show enhancement following treatment (Navarrete-Opazo & Mitchell 2014), suggesting a higher dose of intermittent hypoxia is necessary to achieve LTF in awake animals. Whereas the activity of the muscles under

investigation was not evaluated immediately following a single, acute exposure to intermittent hypoxia, our data suggest a long lasting alteration in respiratory related activity of two additional ribcage muscles following repetitive doses of intermittent hypoxia. Future studies looking at serratus dorsalis and scalene medius utilizing an acute exposure to ten episodes of hypoxia might shed further light on how accessory muscle LTF may be elicited in spontaneously ventilating animals.

Metaplasticity in respiratory motor output

There is a remarkable flexibility in the expression of functional plasticity (Abraham 2008, Grau et al 2014), called metaplasticity. This phenomenon is generally thought to display three characteristics: 1) the altered phenotype persists after the inciting experience resolves, 2) the impact is upon the capacity to change neural function, and 3) the change is reversible (Abraham 2008, Grau et al 2014). The discussion above focused on the consequences of acute exposure to intermittent hypoxia; however, repetitive doses of intermittent hypoxia have been demonstrated to enhance the expression of neuroplastic processes, which constitutes LTF metaplasticity. Animals pretreated with dAIH demonstrate an augmentation in the magnitude of LTF expression up to 75 minutes following intermittent hypoxia--an effect that is sustained for at least 3 days following treatment (McGuire et al 2003). Spinally injured rats demonstrate an enhancement in chemosensory responses in the phrenic nerve discharge amplitude if pretreated with dAIH (Lovett-Barr et al 2012). Similarly, rats show enhancement of the diaphragm and the second external intercostal muscle following daily administration of intermittent hypoxia (Navarrete-Opazo et al 2015). Although we did not measure the acute responses to intermittent hypoxia in the scalene medius or serratus dorsalis, daily administration of intermittent hypoxia did lead to enhanced respiratory-related activity, similar to that seen by McGuire and Navarette-Opazo. It is therefore plausible that metaplastic mechanisms underlie

the findings in these studies. Additional work comparing the magnitude of activity in these muscles following an individual dose of hypoxia to that seen following repetitive doses would help to clarify whether this is a metaplastic response to intermittent hypoxia or a simple neuroplastic finding.

Harnessing non-diaphragm respiratory plasticity in clinical conditions

Certain conditions have been shown to be associated with a reduction in the potential for diaphragm to reduce intrapleural pressures. Chronic obstructive pulmonary disease (COPD) is very common in people with an estimated 7.6-11.7% of the global population afflicted by 2010 (Adeloye et al 2015, Halbert et al 2006). A complete review of COPD is beyond the scope of this discussion; however, as a part of the pathology associated with the condition, air is trapped within the lungs leading to hyperinflation and flattening of the diaphragm (Rochester et al 1979). Given that the contraction of the diaphragm similarly flattens the muscle, this alteration in the mechanics leads to a reduced potential for the diaphragm to contribute to inspiratory events. Counterintuitively, there is also reduced activity of the scalene medius and inconsistent inspiratory activity in the sternocliedomastoid during REM sleep in individuals with COPD (Johnson & Remmers 1984), despite decreased diaphragm muscle activity. Improving the coordination of inspiratory efforts in COPD may improve ventilation in end stage disease; however, given the complexity of oxygen and carbon dioxide dynamics in this condition, further work investigating inspiratory effort in COPD prior to application in clinical settings is necessary.

Amyotrophic lateral sclerosis (ALS) results from suite of potential mutatations culminating in the degeneration of upper and lower motor neurons alongside considerable muscle loss, leading to both neural and muscular functional abnormalities (Hardiman et al 2017, Nichols et al 2015, Nichols et al 2013). Respiratory

compromise has been well documented and is a consequence of paresis of muscles controlling the pharynx, larynx, as well as the muscles generating inspiration (Nichols et al 2013). Ribcage muscles, including the scalene medius, demonstrate a neuronal dependent activation in ALS models; however, as disease progresses, the level of ribcage muscle activity declines alongside ventilatory decline (Romer et al 2017). Given that ventilatory failure represents the most common cause of death in amyotrophic lateral sclerosis, improving the fidelity of inspiratory events in non-diaphragm muscles may improve outcomes, and further studies into the use of AIH in this setting are indicated.

Disruption of the descending neuro-respiratory tracts in spinal injury may lead to catastrophic loss of the capacity of the diaphragm activity to affect inspiration. In addition, dependent upon the level and severity of injury, there may also be reduced activity of chest wall muscles following injury (Navarrete-Opazo et al 2015). Following a lateral cervical spinal injury, daily AIH leads to enhancement of the contralateral diaphragm and second intercostal muscle activity alongside an augmented tidal volume (Navarrete-Opazo et al 2015). Future studies investigating scalene medius and serratus dorsalis in this condition may reveal additional means of improving ventilation in spinally injured individuals.

Studies investigating accessory muscle activity in the complete absence of diaphragm activity are scant. Reversible diaphragm paralysis has been utilized to look at intercostal muscle activity, demonstrating an inverse relationship between diaphragm activity and the external intercostal muscle activity at the second intercostal space (Nochomovitz et al 1981). Further, the parasternal intercostal muscles have the capability of altering their level of activity acutely after the loss of diaphragm activity due to diaphragm paralysis (De Troyer & Farkas 1989). Remarkably, following either unilateral or bilateral diaphragm paralysis, dogs show little to no reduction in parameters of ventilation during slow wave sleep or quiet wakefulness alongside a

notable increase in the degree of ribcage expansion. In REM sleep, however, dogs display a significant reduction in tidal volume and inspiratory time alongside an increase in $PaCO_2$, indicating the loss of accessory muscle activity in REM sleep destabilizes the ventilatory pattern (Stradling et al 1987). Given these findings, investigations into the activity of the scalene medius and serratus dorsalis in the absence of diaphragm activity are warranted. Enhancement of non-diaphragm muscles of breathing may show utility in conditions where diaphragm paresis or paralysis may be anticipated, such as cardiac and abdominal surgery or long-term mechanical ventilation (Halbert et al 2006, Medrinal et al 2016, Simonneau et al 1983, Supinski & Callahan 2013).

Some limitations to the interpretation of this data warrant discussion. Direct application of this information to non-quadruped animals may be problematic given the differences in the relative use of these muscles to maintain or affect posture. Early studies in humans established that people differentially utilize the ribcage muscles for breathing dependent upon their posture (Druz & Sharp 1981, Gandevia et al 1990, Goldman et al 1985, Haas et al 1982, Koulouris et al 1989, Reid et al 1976). In particular, seated or supine postures while resting the arms have been shown to enhance the potential activity in ribcage muscles, suggesting that a resting posture is ideal to recruit these muscles (de Mayo et al 2005, Druz & Sharp 1981, Reid et al 1976). In the current study, we investigated animals with their neck extended while resting on the sternum, as all three muscles of interest could potentially be utilized for posture when in a curled position or standing. Although we chose this position with the intent to alleviate postural roles of these muscles, the natural resting state for normal rats is in a curled position, and, as such, our findings may not represent muscle activity under normal breathing conditions. Future studies investigating different postures should be designed in order to determine the consequences of posture on muscle activity. Additionally, the study presented here looked at

the activity of non-diaphragm muscles in normal animals. Given that many conditions may alter either the central respiratory drive or the potential output of respiratory muscles, it would be pertinent to perform similar investigations in disease models before any hypotheses can be drawn as to the utility of intermittent hypoxia in human clinical disease states.

Here, we present data demonstrating the recruitment and coordination of non-diaphragm muscles to inspiratory events. Although the sternomastoid showed minimal inspiratory-related muscle activity under any condition studied, the scalene medius and the serratus dorsalis were utilized for inspiration under eupnea and were capable of augmenting their activity in response to increased chemosensory drive and following daily AIH. These findings provide the basis for future investigations into neuroplasticity in non-diaphragm muscles of breathing. Additional studies should be conducted to determine the signaling cascade leading to these phenomena as well as to understand how these muscles can be recruited for ventilation in the setting of diaphragm weakness or paralysis.

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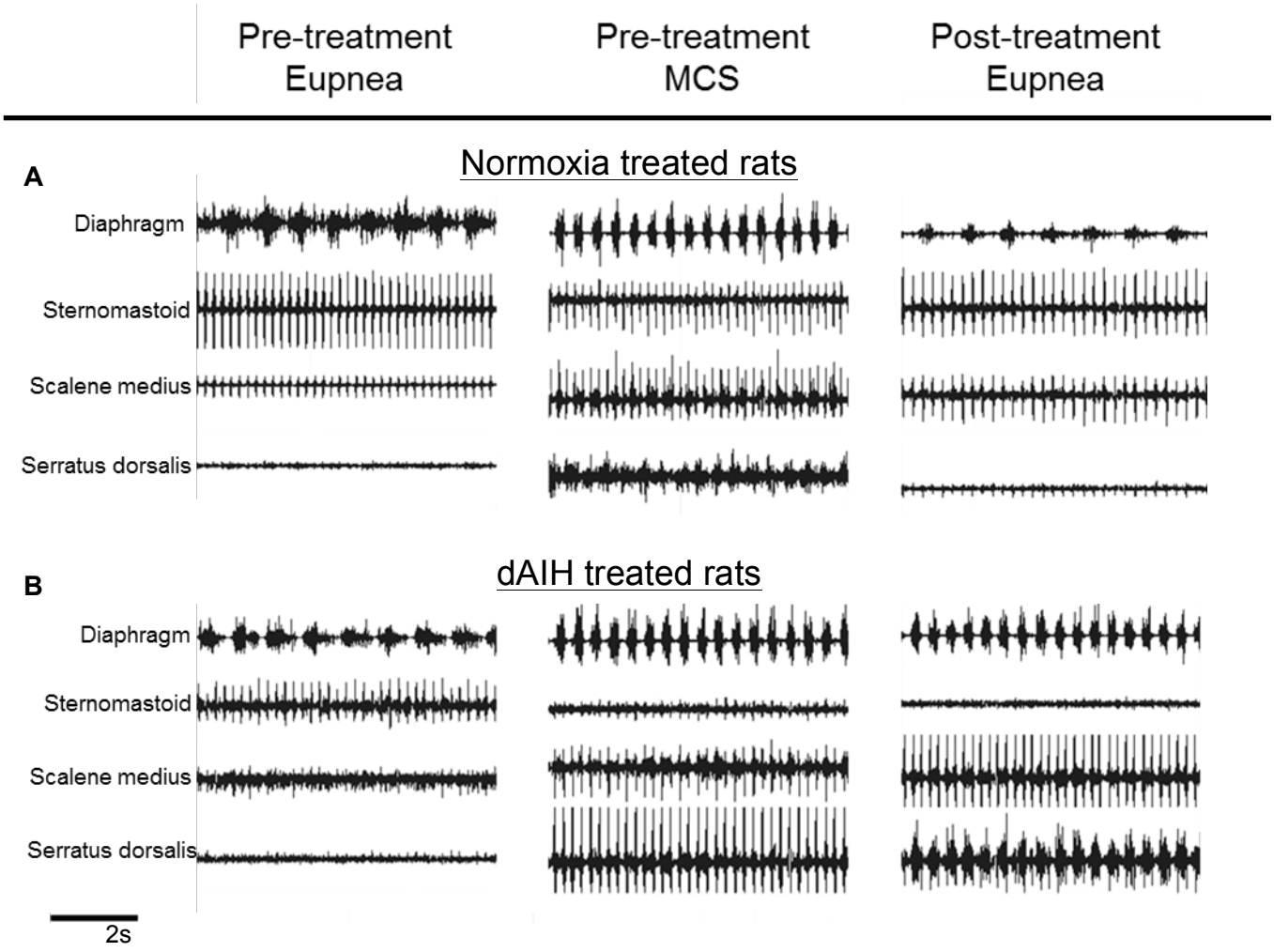

Figure 1. Representative electromyographic traces. **A/B**. Representative raw traces collected from one subject representing normoxia treatment (a) and AIH treatment (b) under normoxia (eupnea), increased chemoreceptor input (Pre-treatment MCS) and following treatment (Post-treatment eupnea).

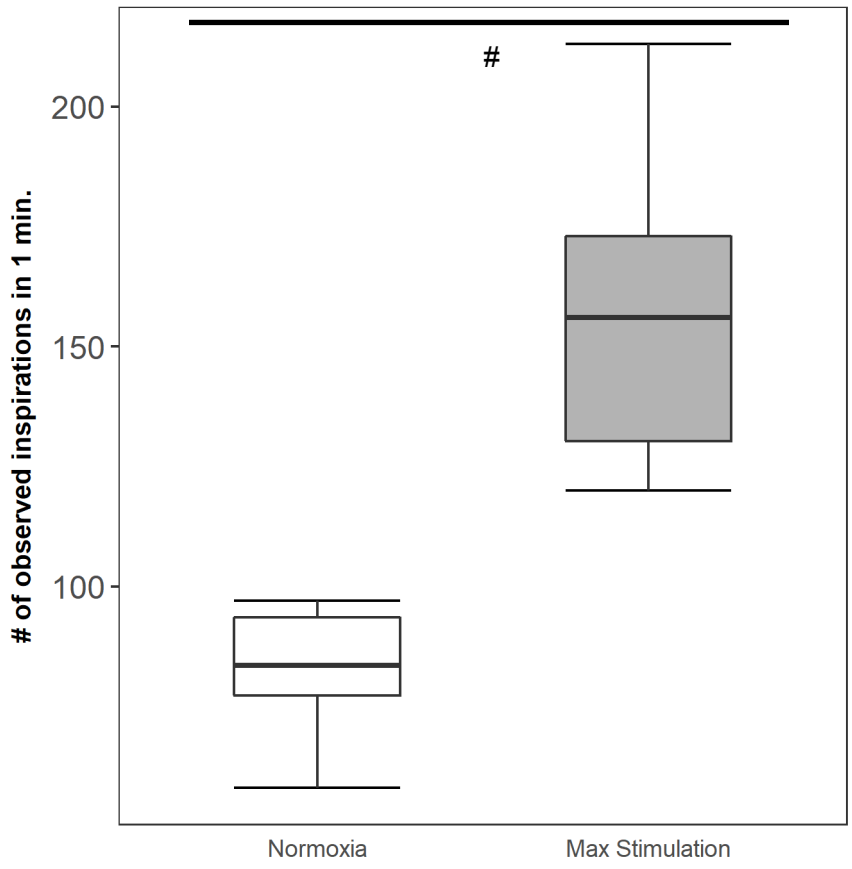

Figure 2. Maximum chemoreceptor stimulation increases diaphragm discharge frequency. The total number of diaphragm phasic discharges were counted over a period of one minute, representative of the respiratory frequency. Application of high chemosensory drive leads to a significant increase in the minute discharge frequency of the diaphragm as compared to normoxic breathing.

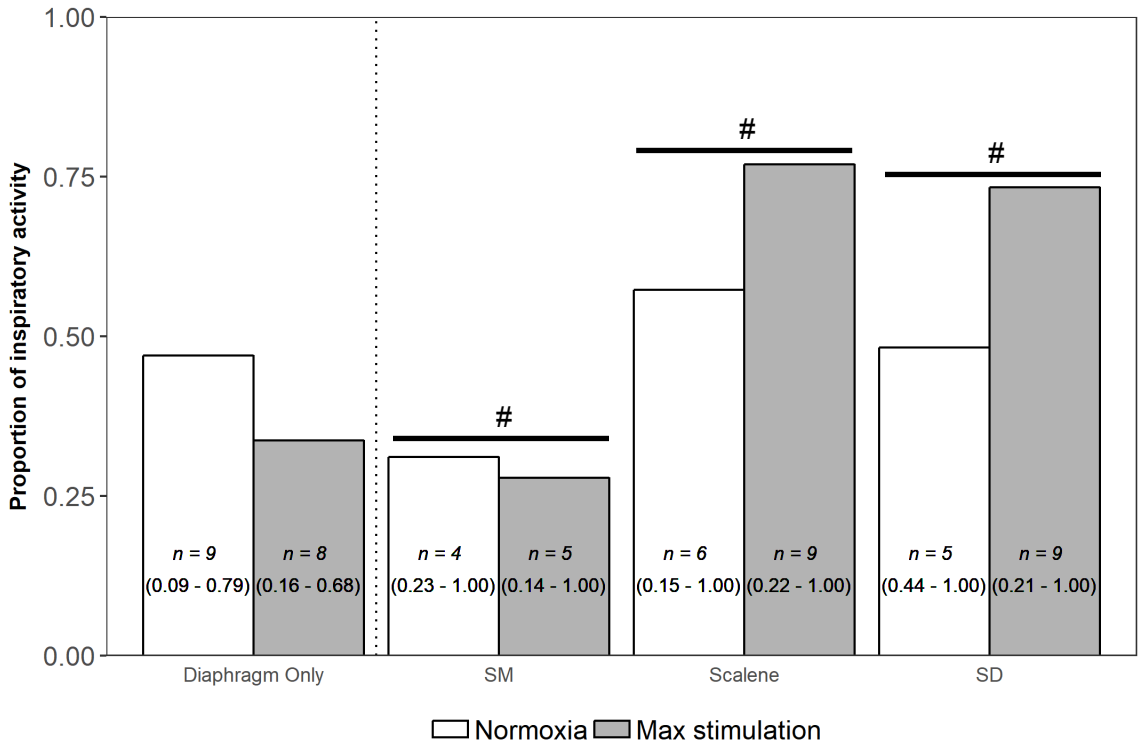

Figure 3. Maximum chemoreceptor stimulation enhances inspiratory related discharge events in the scalene medius and serratus dorsalis. To investigate the potential for inspiratory related activity, phasic electrical activity coinciding with diaphragm activity was compared prior to and during high chemosensory drive. While the sternomastoid demonstrated a small, but significant, reduction in activity, the scalene medius and serratus dorsalis demonstrate an increase in inspiratory related activity during maximum chemostimulation

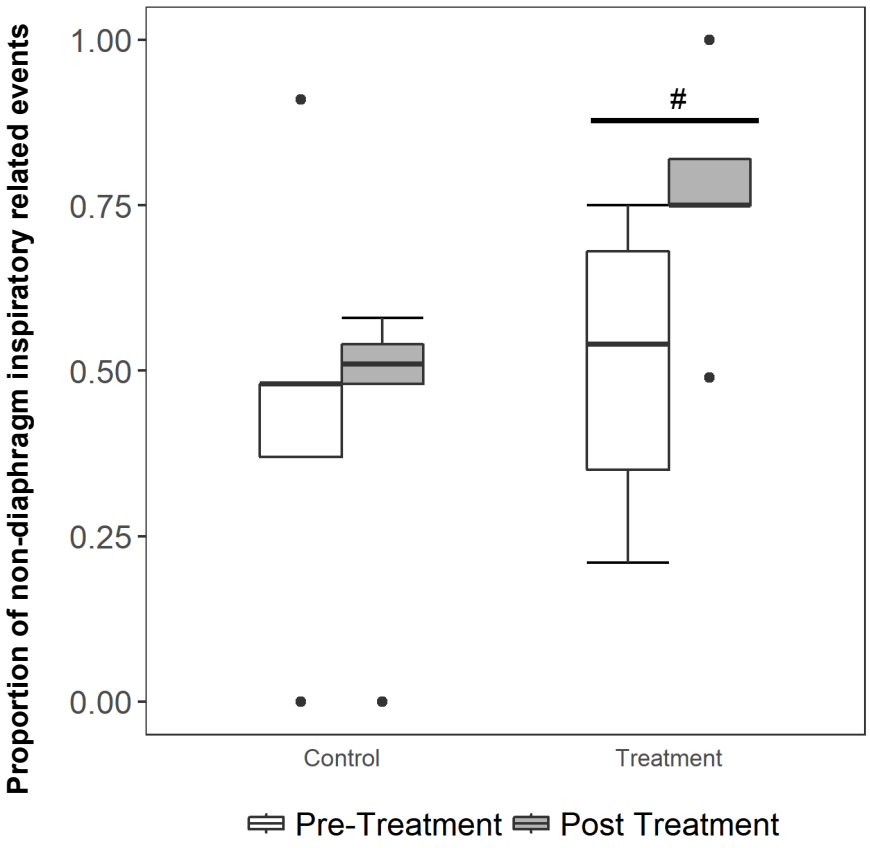

Figure 4. Daily intermittent hypoxia enhances overall coordination of accessory inspiratory muscles. The proportion of inspiratory episodes demonstrating any non-diaphragm muscle activity was compared prior to and following intermittent hypoxia. While no difference is observed prior to treatment, treatment with daily AIH leads to a significant increase in the frequency of coordinated non-diaphragm muscle activity. No difference was seen when comparing pre-normoxia treatment to post-normoxia treatment.

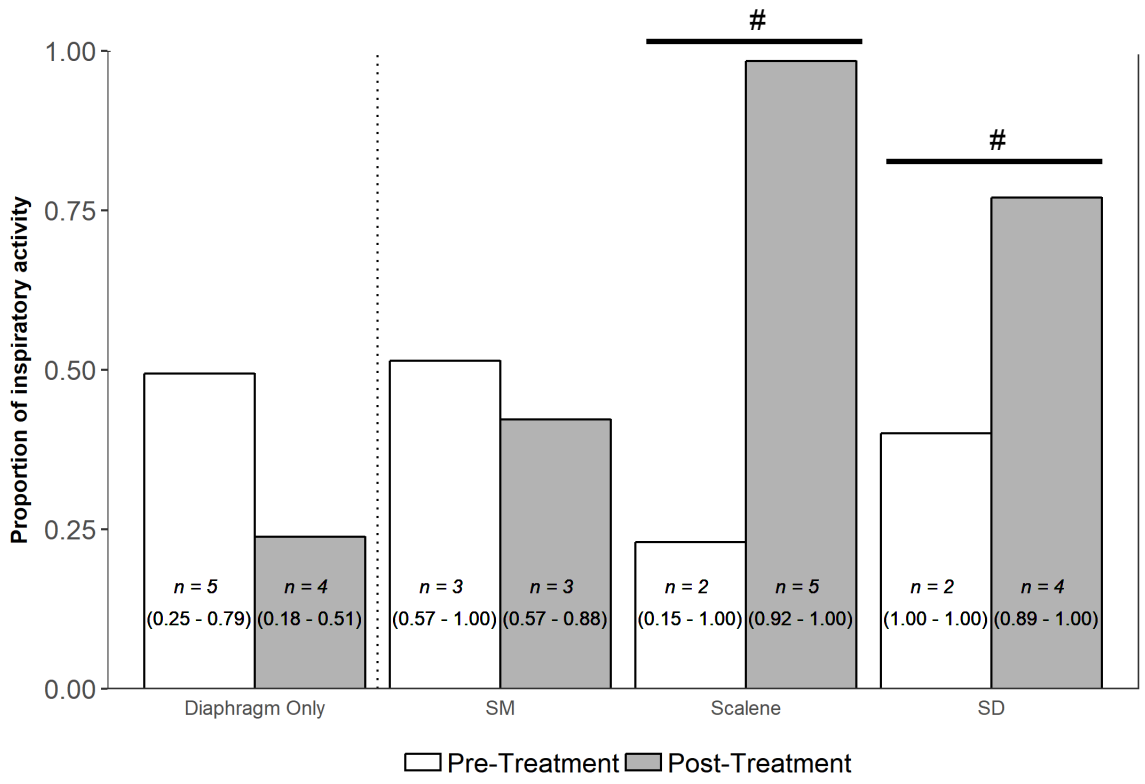

Figure 5. Daily acute intermittent hypoxia enhances eupneic non-diaphragm muscle inspiratory activity. The electrical activity of three muscles under investigation was gathered under normoxic breathing along with diaphragm activity prior to and following daily intermittent hypoxia treatment. Phasic activity coinciding with diaphragm activity was deemed an inspiration and data here are presented as fraction of total inspiratory bursts for each muscle. While a trend for reduced inspiratory activity was demonstrated in the sternomastoid, this failed to achieve significance. The scalene medius and serratus dorsalis, however, both demonstrate significant increases in coordinated inspiratory activity following intermittent hypoxia.

CHAPTER VI

Discussion

SUMMARY

The data presented within this dissertation provide the basis for continued studies into mechanisms that perturb breathing stability, particularly following episodic hypoxia, as seen with sleep apnea and many other neurodegenerative diseases. The fundamental goal of the projects presented here was to initiate investigations into neural processes influencing either propagation or limitation of respiratory disturbances during sleep and wakefulness. The main conclusions to be taken from these studies are 1) chronic intermittent hypoxia (CIH) disturbs expression of a homeostatic form of spinal neural plasticity called inactivity-induced phrenic motor facilitation (iPMF), 2) with the addition of mild doses of high fat diet, CIH disturbs the regularity of breathing frequency, 3) in the presence of astrocytic morphologic abnormalities, breathing demonstrates unstable characteristics but does not change the overall stability of breathing and 4) daily, low dose intermittent hypoxia improves the coordination of respiratory motor pools for inspiration. As a means of integrating these findings and to apply them in the context of disease, the following paragraphs explore these findings in the context of homeostatic spinal neuroplasticity and respiratory neural control and are succeeded by a discussion of diseases in which ventilation stability contributes to morbidity.

HOMEOSTATIC SPINAL NEUROPLASTICITY IN LIMITING RESPIRATORY DISTURBANCES

Chapter II of this manuscript tests the hypothesis that CIH, such as that experienced by individuals with sleep apnea, disrupts homeostatic plasticity in the control of breathing. We focused on homeostatic plasticity in phrenic motor neurons, wherein cells within the region of the phrenic motor pool locally sense reductions in

respiratory neural activity and shift their properties to maintain appropriate levels of inspiratory motor output, termed inactivity induced phrenic motor facilation (iPMF Baertsch & Baker-Herman 2015, Mahamed et al 2011, Streeter & Baker-Herman 2014). Homeostatic neuroplastic phenomena have been demonstrated elsewhere within the CNS (Lee et al 2014, Turrigiano 2008); however, to the authors' knowledge, iPMF represents the only known form of homeostatic plasticity in inspiratory motor neurons in adult animals. Previous reports have provided evidence that homeostatic adjustments in inspiratory motor output occur locally (Streeter & Baker-Herman 2014) and in response to varied causes and severity of reduced respiratory neural activity (Mahamed et al 2011, Streeter & Baker-Herman 2014). In fact, the magnitude of response has been demonstrated to be scaled to the magnitude of activity loss (Braegelmann et al 2017, Streeter & Baker-Herman 2014), suggesting mechanisms are at play to restrict excessive excitatory activity, which would be destabilizing. A working model has been developed over the course of the past several years wherein we believe that reduced activity is sensed by a reduction in local calcium concentrations, leading to a retinal dehydrogenase mediated conversion to retinoic acid, ultimately leading to a PKC-zeta dependent enhancement in phrenic motor output (figure 1A, Streeter & Baker-Herman 2014, Braegelmann et al 2017, Baetsch & Baker-Herman 2015).

As discussed in Chapter II, we have previously demonstrated a remarkable capacity of iPMF to improve inspiratory motor output stability by reducing the carbon dioxide level at which apnea develops (the apneic threshold; AT, (Baertsch & Baker 2017). Assuming CO2 of eupnea is unaltered, this would provide one means of stabilizing breathing. We propose iPMF mechanisms are constituently active, surveying

respiratory neural activity and shaping inspiratory motor output to maintain a set-point level of ventilation. Indeed, it has been previously demonstrated that, during sleep, people continually have alterations in their level of ventilation; however, this is manifested as only small changes in blood gas concentrations (Van den Aardweg & Karemaker 2002). It is possible that iPMF functions in limiting excursions from ideal blood gas concentrations; however, given the complexity of the respiratory neural network, it likely plays only a part in stabilizing respiratory patterns. In the case of a diseased neural system, however, loss of this function may be catastrophic.

In chapter II we found that previous exposure to high dose chronic intermittent hypoxia constrains the expression of iPMF in a self-limiting manner. Further, this inhibition is mediated by a unique PKC isoform, theta, which has been previously demonstrated to be required for the expression of a unique neuroplastic event, long-term facilitation (LTF; Devinney et al 2013). Given that LTF has been shown to require the activity of calcium permeable, NMDA type receptors (McGuire et al 2008), we have developed a working hypothesis wherein intermittent hypoxia initates an LTF-like mechanism via NMDA receptor activation, leading to increased intracellular calcium and inhitibition of iPMF (figure 1B). While we were unable to demonstrate a worsened phenotype of respiratory disturbances, previous reports have demonstrated the potential for augmented apnea and hypopnea frequency following similar doses of hypoxia. The differences in the studies may be attributable to our use of intrapleural siRNA, daily anesthesia, or inflammation from either. Regardless, with the addition of high fat diet, an additional destabilizing factor, we demonstrate increased irregularity of breathing frequency. Comparing results from studies in chapters II and III is challenging;

however, given differences in the species under investigation. It is not clear at this time what the effect of high fat diet would be in a rat model nor if CIH constrains iPMF in the mouse. Ongoing studies investigating high fat diet and CIH in rats are underway, and future work on the expression and constraint of neuroplasticity following CIH and high fat diet in mice is warranted to better interpret the data presented here.

Local mechanisms guiding iPMF expression have undergone fairly through investigation over the past several years. As mentioned above, we have demonstrated that reduced respiratory neural activity is sensed locally by blocking neurotransmission through the bulbospinal tract (Streeter & Baker-Herman 2014) and also have demonstrated the requirement of PKCζ activity during initiation (Baertsch & Baker-Herman 2015, Strey et al 2012). To date, no investigation has definitively demonstrated which cells are responsible for sensing respiratory neural activity. As discussed in chapter IV, glial cells have recently been demonstrated to have the capacity to sense and respond to altered oxygen and carbon dioxide levels; however, their role in sensing respiratory neural activity deprivation is unknown. If we consider the roles of astrocytes in removal and metabolism of excitatory neurotransmitters, as discussed in chapter IV, it is also feasible that these cells have the capacity to monitor activity levels. Astrocytes are ideally located to provide monitoring of neuronal activity in their proximity to synapses as well as their high distribution throughout the respiratory neural control network (Allen 2014). Further, astrocytes have defined roles in augmenting neuroplastic responses within other critical brain regions (Bonansco et al 2011, Panatier et al 2011, Pascual et al 2005, Theodosis et al 2008), including in response to neuronal activity deprivation (Beattie et al., 2002). Additional work in the initiation of iPMF mechanisms

should incorporate astroyctic manipulation, similar to that in chapter IV, to establish if these cells play a role in homeostatic neuroplastic phenomena.

When discussing homeostatic modulation of ventilation, a few further points warrant discussion. First is the concept of duration of effect. In the ideal situation, a stimulus which is self limiting will lead to a self limiting neuroplastic response, and in this sense would provide benefit to the overall stability of the system. The consequences of prolonged homeostatic enhancement of phrenic activity have not yet been investigated; however, these processes may become maladaptive and predispose an individual to muscle spasticity, fatigue, supraspinal accommodation or could enhance local counter-regulatory mechanisms. For these reasons, it is possible that iPMF may not improve long-term outcomes in neural dysfunction, but instead only play a role in maintaining ventilation during health. Future studies in irreversible conditions, such as spinal injury, may shed light on the consequences iPMF over long time courses. An additional consideration in regards to the homeostatic control in respiratory neural systems is the response seen following hyperactivity. It is simple to expect that a reduced activity would beget an increase in synaptic strength; however, if we consider the converse, i.e. a long-lasting reduction in phrenic activity in response to hyperactivity, this response could risk catastrophic hypoventilation, particularly during periods of high ventilatory need. Further studies into the phrenic neural response to hyperactivity are warranted to better understand how local systems adapt and respond in these situations.

Factors influencing the stability of respiratory neural circuits

Neuroplastic processes have the capacity to alter the stability of the respiratory network by influencing the 'gain' of the system, or the change in output for a given current input. The loop gain, an engineering term applied to the feedback mechanisms controlling breathing (Cherniack & Longobardo 2006, Khoo et al 1982), may be considered in three individual components: controller gain, plant gain and mixing gain (Javaheri & Dempsey 2013). While the three of these components work in conjunction to determine overall loop gain, we will consider each individually first within the context of the control of breathing. Controller gain refers to the magnitude of response of the ventilatory network to alterations in blood gas levels as detected by peripheral and central chemoreceptors (Cherniack & Longobardo 2006, Javaheri & Dempsey 2013). A high controller gain would mean there is a large change in ventilation following very small changes in carbon dioxide, which would be destabilizing. Plant gain is the change in blood gas concentration within the pulmonary capillary bed as a response to a given change in ventilation. Often this is considered in the context of a fixed production of carbon dioxide, and so at a high plant gain small changes in ventilation will lead to large changes in systemic carbon dioxide, predisposing to apnea development. Mixing gain refers to the time it takes for changes within the system to be detected, i.e. the time it takes for pulmonary capillary blood to reach the peripheral and central chemoreceptors, which is predominantly determined by circulation time and not likely to be influenced by neuroplastic events (Javaheri & Dempsey 2013). One final concept to discuss relevant to stability of breathing is that of the CO2 reserve, which is the difference between eupneic CO2 and the AT (Javaheri & Dempsey 2013). A small CO2 reserve is destabilizing and could be seen with high controller gain, high plant gain or both in

conjunction. As discussed above, the reduction in AT observed during induction and maintenance of iPMF would provide a stabilizing effect by widening the CO_2 reserve.

In the ideal situation, the system would be adequately responsive to resolve any excursions from a preferred set point but not so responsive as to overshoot and predispose to oscillatory patterns. While the calculation of loop gain is beyond the scope of this discussion, suffice to say that the calculated gain would be a representation of the response to a disturbance, i.e. a gain equal to one would mean the response is equivalent to the disturbance. Therefore, a low loop gain would be stabilizing to breathing in that the response to any fluctuation is smaller than the disturbance itself and so over several cycles, normal ventilation will be reestablished. A loop gain greater than one would indicate each response is larger than the initial disturbance and while this has the potential to reestablish normal blood gas concentrations rapidly, it also sets the system up for periodic swings in ventilation, which would manifest as alternations between hypopnea and hyperpnea (Cherniack & Longobardo 2006, Javaheri & Dempsey 2013).

The potential consequences of CIH on loop gain are much more complex. Previous studies have demonstrated influences of CIH on multiple levels of the chemoreflex system controlling breathing. The carotid bodies, which are responsible for peripheral oxygen sensing, demonstrate an augmentation in firing rate that is correlated to an enhancement in ventilation following CIH (Peng et al 2003, Rey et al 2004). This enhanced activity of the carotid bodies could serve to increase the controller gain of the overall system and would then be destabilizing. In the nucleus tractus solitarius,

however, pretreatment with CIH reduces excitatory neurotransmitter induced currents (Almado et al 2012, de Paula et al 2007, Kline et al 2007) which is seen alongside an increase in activity initiated by spontaneous neurotransmitter release (Kline et al 2007). These concurrent alterations in firing probability within the NTS have been proposed as a homeostatic regulation of activity and the authors suggest this results in minimal net change in excitability as compared to pre-CIH (Kline et al 2007). Further, CIH has been shown to sensitize the ventilatory response to acute hypoxia, resulting in a larger than expected response to chemosensory inputs (Del Rio et al 2010, Peng et al 2003, Peng & Prabhakar 2003, Rey et al 2004). Again, this enhanced response would be reflective of an increase in controller gain and would serve to destabilize breathing events. These findings make it challenging to speculate what effect CIH has on the loop gain of the entire network. In the studies to reported here, we utilized methods to selectively influence the activity of neuroplastic processes in the spinal cord in attempt to better understand how this segment of the respiratory control network may influence loop gain.

Studies specifically investigating spinal neuroplastic influences on loop gain are scant; however, some hypotheses may be proposed based on published data. As stated above, we observe a progressive and long-lasting reduction in AT over the course of iPMF induction and maintenance. Given that all studies investigating iPMF have been performed under anesthesia, we cannot determine the eupneic CO2; however, if we were to assume that no change is seen in these conditions, the effect of reducing AT would be to reduce controller gain and widen the CO2 reserve. This would provide a stabilizing effect on breathing. In contrast, the augmentation of phrenic burst

amplitude seen following either intermittent hypoxia or intermittent apnea, presuming there are no changes in carbon dioxide production, could serve to destabilize breathing by increasing plant gain. Studies contained within this document were designed to investigate the influence of iPMF on stability of breathing, and, as you will see, we were unable to provide further insight into these complex processes at this time.

STABILITY OF BREATHING IN NEURAL DISEASE

Multiple systems atrophy is a disorder of predominanty dysmyelination due to a accumulation of α-synuclein within oligodendrocytes and neurons (Overk et al 2017). While this disease is predominantly characterized by tremor, dysautonomia and ataxia, centrally mediated respiratory disturbances are common and contribute to altered levels of alertness on waking (Overk et al 2017). Individuals demonstrate both inadequate responses to hypoxia along with abnormal rhythm during hypoxia exposure (Lipp et al 2010, Shimohata et al 2007, Tsuda et al 2002). During wakefulness they demonstrate gasping, periodic breathing and during sleep central apnea, apneustic breathing, and Cheyne-Stokes ventilation patterns (Alfonsi et al 2016, Gaig & Iranzo 2012, Garcia-Sanchez et al 2016, Ohshima et al 2017). In addition, obstructive events are common and, based upon the findings presented here, may instigate a self-perpetuating cycle of intermittent hypoxia and worsened central apnea. Previous investigators have hypothesized that breathing abnormalities are a function of neuronal degeneration at the level of the pre-bötzinger complex and ventral arcurate nucleus.

Amyotrophic lateral sclerosis results from suite of potential mutatations culminating in the degeneration of upper and lower motor neurons alongside

considerable muscle loss, leading to both neural and muscular functional abnormalities (Hardiman et al 2017, Nichols et al 2015, Nichols et al 2013). Respiratory compromise has been well documented and is a consequence of paresis of muscles controlling the pharynx, larynx, as well as the muscles generating inspiration (Nichols et al 2013). Ventilatory failure is the most common cause of death in ALS. A relatively high proportion of individuals experience sleep disordered breathing, with reports of prevalence as high as 76% (Gaig & Iranzo 2012, Howell & Newman 2017). The most commonly reported abnormalities are nocturnal hypoventilation, central sleep apnea and obstructive sleep apnea. Intriguingly, these individuals show a high dependence on the non-diaphragm muscles of breathing, suggesting that strategies such as daily, acute, intermittent hypoxia may be of benefit, as discussed in chapter V. Apneic phenotype is predominantly central in origin and is found to be independent of the level of diaphragm weakness, suggesting abnormalities in the systems controlling loop gain are responsible for this particular aspect of ALS. While no investigation to date has demonstrated any effect of CIH nor iPMF on stability of breathing in ALS, LTF has shown some promise as a potential therapeutic for ventilatory insufficiency (Nichols et al 2015). Future studies focusing on manipulation of neuroplastic or metaplastic processes in this population are warranted.

The impact on ventilation in acute spinal cord injury, particularly in the cervical region, is straightforward. Disruption of descending bulbospinal projections to the phrenic motor pool leads to catastrophic and life-threatening reductions in ventilation. Intriguingly, recent work has also demonstrated a delayed, life-long disruption in the stability of breathing following both cervical and thoracic injury (Bascom et al 2015,

Chiodo et al 2016, Sankari & Badr 2016, Sankari et al 2014, Sankari et al 2015). In fact, upwards of 63 and 14% of individuals with chronic cervical and thoracic spinal injury (Sankari et al 2014), respectively, have central sleep apnea evidenced by high apnea-hypopnea indices compared to age matched controls (Sankari & Badr 2016, Sankari et al 2014). While reports of eupneic end tidal CO_2 in chronic CSI conflict (Sankari et al 2014, Zimmer et al 2007), development of central sleep apnea in CSI has been correlated to a narrowed difference between eupneic carbon dioxide level and the apneic threshold predisposing the development of apnea (Javaheri & Dempsey 2013). It is feasible that reduced expression of homeostatic plasticity, such as iPMF, in this chronic setting leads to an increase in apneic CO_2 levels predisposing to hypopnea and apnea. These questions should be investigated in future studies.

Rett syndrome is a neurodevelopmental condition wherein a loss of the gene encoding methyl CpG binding protein 2 (MeCP2) leads to dysfunction within both neuronal and glial cell populations during development (Kaufmann et al 2016). Stability of breathing is influenced during sleep and wakefulness and is typified by breath-holds, apnea and alternations between hyperventilation and hypoventilation (Bissonnette & Knopp 2006, Weese-Mayer et al 2006). In mice, disruption of expression of MeCP2, mutations of which cause Rett syndrome, leads to irregular breathing patterns and reduced ventilation at normoxic levels, but normal breathing patterns are demonstrated with increased chemosensory drive (Zhang et al 2011). With conditional astrocyte knockout of MeCP2, mice demonstrate altered chemosensory responses with a reduced response to elevated carbon dioxide, which resolves upon reinstitution of astrocyte MeCP2 expression (Garg et al 2015), suggesting abnormal

breathing is in part due to abnormal astrocytic activity. Further, Levitt and colleagues demonstrated a high frequency of respiratory disturbances and elevated irregularity score of breathing frequency in a model of Rett syndrome, which was alleviated by administration of serotonin receptor 1a receptor agonist (Levitt et al 2013). Indeed, data presented here would support the presence of elevated loop gain in a different model of astrocyte dysfunction (chapter IV). While these data support the role of astrocytes in stabilizing breathing, it is likely that neural dysfunction also plays a role in this condition. While investigations into neural plasticity in breathing have yet to be done in Rett syndrome, synaptic plasticity in the hippocampus is impaired in mice with MeCP2 knockout (Asaka et al 2006, Moretti et al 2006), suggesting that neuroplastic processes protecting breathing may also be impaired. Additional studies would be necessary to investigate neuroplastic expression in models of Rett syndrome.

FUTURE DIRECTIONS

The data contained within this dissertation provides impetus for future investigations on the stability of breathing in neural networks. Despite previous reports supporting the augmentation of apneic phenotype following chronic intermittent hypoxia exposure, in our hands we failed to worsen apnea frequency or duration. As discussed, our study design contained several divergent aspects from previous investigations. Most importantly, we required the use of daily anesthesia along with injection of non-sterile siRNA substrate into the pleural space. While iPMF was constrained in the reduced preparation utilized for phrenic nerve recording, it did not appear to result in a worsening of breathing stability as measured by whole-body plethysmography in the absence of anesthesia or positive pressure ventilation. It is possible that we captured the system on

the cusp of instability, and a longer duration of CIH would have been necessary to “push” the system toward overt effects. Alternatively, given the known influences of anesthesia on the control of breathing (Becker 1989, Knill 1988, Santiago & Edelman 1985) it is feasible that additional mechanisms are at play in conscious animals to maintain a normal level of ventilation and/or daily anesthesia induced alternative protective mechanisms to preserve breathing stability (Hengen and Behan 2015 Plos one paper). As variations gas dynamics and intermittent hypoxia delivery has been previously demonstrated to influence oxidative status of rodents (Lim et al 2015), a preliminary investigation into the efficacy of our gas exposure system in inducing apnea was performed, demonstrating an augmentation in the frequency of apneas and hypopneas observed following a similar CIH paradigm utilized in chapters II and III (n=2 per group, figure 2). To address potential confounding influences of inflammation or anesthesia, future studies should seek to utilize a long-lasting reduction in the phrenic expression of PKCζ, such as genetic or virally delivered knock-down, or pharmacologic intervention towards key mediators in iPMF expression, such as retinoic acid synthesis or signaling.

In the presence of additional means of destabilizing breathing, i.e. high fat diet, we demonstrated a worsened irregularity of breathing following CIH. These data suggest that dietary manipulation may be of benefit in the setting of sleep apnea or with sleep disordered breathing in neurodegenerative and neurodevelopmental conditions. The diet modifications utilized in these studies focused on saturated fats, which fails to fully recapitulate the diets leading to metabolic derangements in rodents or humans (Della Vedova et al 2016, Pranprawit et al 2013). Given that this relatively mild "dose" of

obesity induction was capable of inducing alterations in breathing, future studies should focus on longer duration and severity of obesity phenotype. Current studies are investigating the addition of high fructose corn syrup to high saturated fat diet, which was previously demonstrated to worsen metabolic effects of high fat diet (Della Vedova et al 2016). Further, given that we now have data to support the obstruction of iPMF expression after CIH (chapter II), future studies may investigate the application of substances known to initiate neurohomeostatic plasticity in the setting of metabolic syndrome.

Here we have only begun to scratch the surface regarding the role that astrocytes play in regulating breathing patterns. Chapter IV provides intriguing data supporting the role of these glial cells in restraining responses to chemosensory input. As discussed elsewhere in this manuscript, large responses to chemosensory input may contribute to the development of sleep disordered breathing, such as hypopnea or Cheyne-Stokes type ventilation. Given the relative simplicity of disease phenotype in the GFAP knock-in model used here, as compared to models of other neurodegenerative conditions, future investigations could be useful in looking at factors which appear to worsen respiratory disturbances, such as CIH or high fat diet. In addition, a reduced preparation investigating electrophysiologic activity at key regions of the brainstem and spinal cord would be beneficial to determine the level at which astroglial disease influences respiratory patterning.

Finally, in chapter V, we demonstrate a neuroplastic recruitment of non-diaphragm muscles of inspiration despite normal levels of diaphragmatic function. This provides evidence that 1) these muscles are capable of coordinating their activity to

inspiratory events and 2) pre-treatment with daily acute, intermittent hypoxia enhances the fidelity of these muscles in inspiratory activity. While ventilation was not measured in this initial investigation, it is not likely to have been largely influenced given that no change in requirements of ventilation was experienced. In future studies, this method could be applied alongside models in which loss of diaphragm activity would be expected, such as ALS or traumatic injury to the phrenic nerve.

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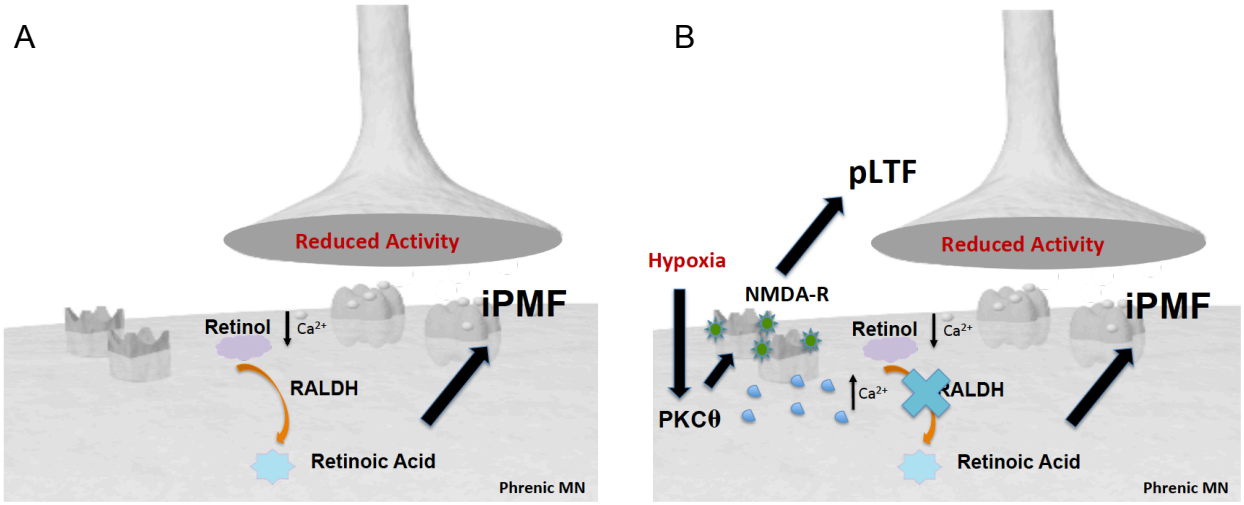

Figure 1: Working model of hypoxia induced constraint on iPMF mechanisms. **A**. A reduction in neural activity within the phrenic motor neuron (Phrenic MN) leads to a recution in intracellular calcium ion levels. This allows for the conversion of retinol to retinoic acid via the activity retinal dehydrogenase (RALDH), signaling iPMF. **B**. Intermittent hypoxia leads to a PKC-theta dependent increase in NMDA receptor activity leading to phrenic long term facitliation (pLTF). NMDA receptor activation additionally leads to an increase in intracellular calcium ion concentrations, which would limit the activity of RALDH and prevent normal expression of iPMF due to a loss of retinoic acid signaling, regardless of the level of activity present.

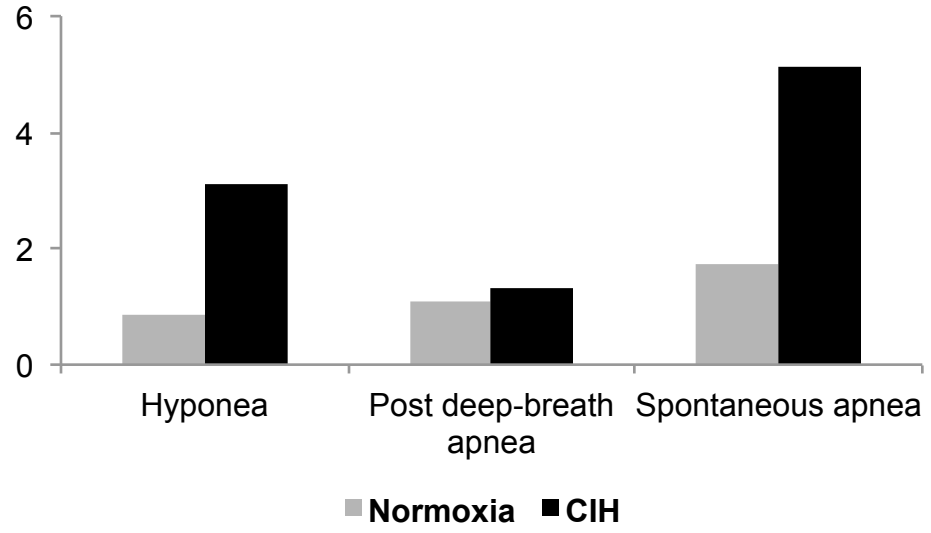

Figure 2: Chronic intermittent hypoxia augments the frequency of hypopnea and spontaneous apnea in rats. Immediately prior to and following exposure to chronic intermittent hypoxia (FiO_2=0.105, 2 min interspersed with FiO_2=0.21, 2 min, 8 hours/day, 7 days total) plethysmography was gathered. Periods of quiet breathing were manually identified and rates of apnea and hypopnea determined. Here we demonstrate that intermittent hypoxia administered in our hands leads to augmentation of spontaneous apnea and hypopnea when experienced in the absence of daily intrapleural injection and general anesthesia.

APPENDIX A

Does Inactivity-Induced Phrenic Motor Facilitation Play a Role in Spontaneous Recovery of Ventilation following Cervical Spinal Injury?

INTRODUCTION

Injury of the cervical spinal cord may lead to disruptions in the respiratory neural circuitry supplying signal to the phrenic motor nucleus. Intriguingly, spontaneous recovery of phrenic nerve signal has been demonstrated in relatively short time periods following injury (Goshgarian 2003, O'Hara & Goshgarian 1991). Previous work has shown that the phrenic motor nucleus is capable of responding to reductions in respiratory neural input by augmenting neural output to the diaphragm, thus maintaining adequate levels of ventilation despite catastrophic reduction in signal to breathe. This phenomenon, inactivity-induced phrenic motor facilitation (iPMF), is discussed thoroughly elsewhere in this publication (Baertsch & Baker-Herman 2013, Broytman et al 2013, Mahamed et al 2011, Strey et al 2012). We hypothesized that iPMF underlies the spontaneous recovery of ventilation following C2, mid-line spinal contusion injury, and preliminary data shown below support this hypothesis however due to high degree of variability in outcome measures this study was aborted and we are considering modifications to the study design to address these limitations.

METHODS

Animals

All experiments were performed on 12-16 week-old, male Sprague-Dawley rats (300-400g, colony 217, Harlan, Indianapolis, IN). Animals were co-housed in temperature and humidity controlled environment with 12:12 hour light/dark cycle. The

Animal Care and Use Committee of the School of Veterinary Medicine, University of Wisconsin, approved all experimental procedures used in this study.

Surgical preparation

Prior to surgery, the surgical suite and surgical tools were disinfected or sterilized. All surgeries were performed under isoflurane anesthesia in 100% inspired oxygen. Rats were treated pre-operatively with buprenorphine (0.03 mg/kg), carprofen (5 mg/kg) and enrofloxicin (10 mg/kg) subcutaneously to treat pain and minimize post-operative infection. Body temperature was maintained at 36-37.5°C with external heat support. An orotracheal tube was utilized for positive pressure ventilation (Tidal volume 6 mL/kg; Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA) with 1.5-3% isoflurane in 100% oxygen. Adequate anesthetic depth was determined by pedal withdrawal and corneal reflex. Oxygen saturation was monitored by pulse oximetry (model 8600; Nonin Medical Plymouth, MN) during surgical procedures.

Telemetry implantation procedure

Prior to surgery, approximately 2 mm of silicone insulation was removed from the distal end of each biopotential lead of the radiotelemetry units (4ET-S1/2; Data Sciences International, St. Paul, MN). Telemeter transmitter body, biopotential leads and battery unit were sterilized. Following midline celiotomy, two paired biopotential leads were tunneled subcutaneously to the dorsal skull base, wrapped in sterile drape and remained there until later in the surgical procedure. One paired biopotential lead was placed into the right, mid-costal diaphragm guided by 23g needle and adhered with

tissue adhesive (Vetbond 1469SB; 3M Animal Care Product, St. Paul, MN) and suture (6-0 elthilon; Ethicon US, LLC, Somerville, NJ). The same procedure was used to place paired biopotential leads in the left mid-costal diaphragm and the left nuchal musculature. Finally bolts serving as electroencephalographic leads were placed 2 mm anterior to bregma and 3 mm right lateral to midline and a reference lead was placed 1 mm right lateral to midline and 11 mm posterior to bregma. After connecting the radiotelemetry leads to cranial bolts, dental cement (Henry Schein, Melville, NY) was used to affix electrodes to the skull. The telemetry unit and battery were placed into the peritoneal cavity, the linea alba was opposed with simple interrupted pattern (3-0 Polysorb; Covidien Ltd., Dublin, Ireland) and the skin was opposed by wound clips (3M Precise disposable skin stapler; 3M Animal Care Products, St. Paul, MN). Post-operative pain was managed with once daily carprofen and twice daily buprenorphine at the same dose as preoperatively. Rats were visually monitored and weighed daily and given one week recovery prior to spinal cord injury.

Spinal cord injury procedure

The skin and superficial nuchal musculature were sharply dissected to reveal the epaxial musculature. The muscles were divided along their aponeuroses to expose the lamina of the second and third vertebrae. The muscles overlying the articular facets between the first to second and third to fourth cervical vertebrae were exposed by blunt dissection. The rat was suspended utilizing the brown adsen forceps attached to the contusion apparatus (Infinite Horizons Impator, Precision Systems and Instrumentation, Fairfax Station, VA). The muscle and bone superficial to the C2-C3 intervertebral space

were sharply removed to expose approximately 2 mm diameter segment of the spinal cord. 135 kD of force was applied to this space. The muscles were brought into opposition in a simple interrupted pattern (3-0 Polysorb; Covidien Ltd., Dublin, Ireland) and the skin was opposed by wound clips (3M Precise disposable skin stapler; 3M Animal Care Products, St. Paul, MN). Rats were positive pressure ventilated until spontaneous ventilation maintained an end-tidal carbon dioxide level of 35-45 mmHg. Post-operative pain was managed with once daily carprofen and twice daily buprenorphine at the same dose as preoperatively. Rats were visually monitored, weighed daily, and manual bladder expression and feeding were performed as necessary.

RNA interference in Respiratory Motor Neurons

To impair PKC isoforms in phrenic motor neurons, small interfering RNAs (siRNAs) targeting and PKC-zeta (siPKCζ) were delivered to the intrapleural space for 3 days prior to spinal contusion injury to prevent normal expression of iPMF. Intrapleural siRNAs are retrogradely transported to phrenic motor neurons and knockdown mRNA and protein levels within phrenic motor neurons. Under isoflurane anesthesia, bilateral intrapleural injections of siRNA (100 picomoles, Accell SMARTpool, Dharmacon; 30ul RNAse free water; 30ul/side) in the 5th intercostal space was performed daily 3 days prior to and daily during the recovery period following SCI until terminal measurements were taken. Rats serving as control received non-targeting siRNA (NTsi) under the same dosing interval and duration.

Whole body plethysmography

Rats were individually placed in whole body plethysmography chambers (4 liters, Data Sciences International model 600-1211-001, St. Paul, MN). Pressurized air continuously flowed through the chamber at 4 liters per minute, allowing precise control of inspired gas concentration and limiting expiratory gas accumulation. The chamber was positioned alongside a radiotelemetry receiver (see below) to measure EMG and ventilation simultaneously. Six hours of data were gathered under normoxic conditions and fifteen minutes under maximum chemoreceptor stimulation. Normoxia and MCS conditions were established in plethysmography chambers by mixing O_2, N_2, and CO_2 via a custom-made, computer controlled system of mass flow controllers to obtain desired gas concentrations as assessed by gas analyzer (CWE, model Gemini).

Telemetry signal acquisition

Rats were placed in whole-body plethysmography chambers (see above) and placed on radiotelemetry receiver unit (model RPC-2; Data Sciences International, St. Paul, MN). Signal was sent to a data exchange matrix (model ACQ-7700; Data Sciences International, St. Paul, MN) and collected through purposely designed software (PONEMAH Physiology Platform, Data Sciences International, St. Paul, MN).

Data analysis

Plethysmography and diaphragm EMG data were gathered during following three days of siRNA administration and at three days following spinal cord injury. Plethysmography data were averaged over 50 ms and periods of quiet breathing

suitable for analysis were manually identified. A purpose written MatLab script was utilized to assess for tidal volume (TV) respiratory frequency (RR), minute ventilation (MV) and hourly rates and duration of apnea and hypopnea utilizing correction for pressure and temperature (Drorbaugh & Fenn 1955, Flatau et al 1992). EEG signal was low pass filtered to 30 Hz and manually assessed in 10-second epochs to categorize into three different vigilance states: 1) quiet wakefulness—high frequency, low amplitude EEG activity with evidence of active nuchal EMG activity, 2) non-REM—low frequency, high amplitude EEG with minimal to no nuchal EMG activity, and 3) REM—low frequency, high amplitude EEG with minimal to no nuchal EMG activity. Diaphragm EMG was averaged over 50 ms and full-wave rectified prior to analysis. Peak amplitude was identified utilizing a purpose written MatLab script. Both plethysmography and EMG data were analyzed in 5-minute bins occurring during NREM sleep in normoxia and during quiet breathing periods of MCS.

Conclusions, limitations and future directions

Preliminary data provided here support the hypothesis that iPMF plays a role in the recovery of ventilation following spinal cord injury; however, the variability in all measures gathered limits the capability of assessing for differences in the populations studied. In reflecting on the study design, several factors contribute to the challenges in analyzing data from these studies. First the injury was performed on dorsal midline with the intention of injuring the bulbospinal tract bilaterally. The bulbospinal tract lies within the dorsolateral funniculus of the spinal cord, relatively distant from midline, which may explain the relatively mild deficits demonstrated in this study. Further, contusion injury is

performed under the guidance of a dissecting microscope and while every attempt is made to equally injure each side of the spinal cord, true midline contusion is challenging in the absence of visualizing the spinal cord at the cellular level. As such, data gathered by midline injury should be assessed in light of the degree of injury on each side of spinal cord. Being as though this was our first investigation into spinal contusion, we were unaware of this and post-mortem samples were not processed in a manner suitable to stratifying degree of injury per side. With the knowledge that a lateralized spinal injury leads to enhancement of neural signal on the contralateral neural circuit, we likely are minimizing the deficits in diaphragm EMG by averaging multiple animals without taking into account lateralization of injury.

Despite these limitations, data presented here provide a strong indication for future investigation into the role of iPMF in recovery following spinal injury. Future studies will utilize a lateralized laceration type injury called hemisection to reduce the amount of variability between test subjects, prevent the influence of non-iPMF compensatory mechanisms enhancing the ipsilateral neural circuitry and allow for comparisons in neural signals to contralateral phrenic nerve or diaphragm. While hemisection injury poorly models human spinal injury, it is superior to contusion in assessing functional responses to injury. Additional investigations with contusion injury may follow studies utilizing hemisection if indicated.

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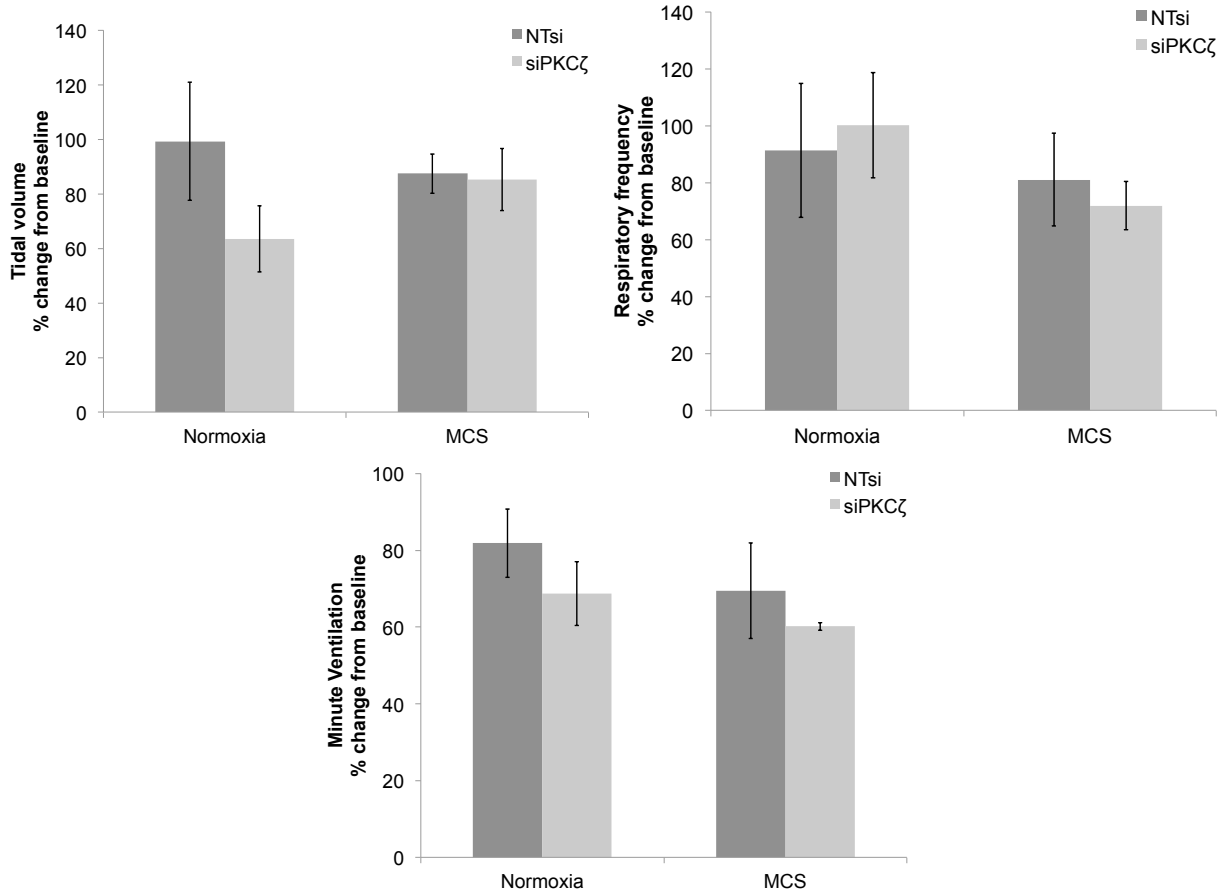

Figure 1. iPMF mechanisms may be responsible for the recovery of tidal volume following cervical spinal contusion under normoxic conditions. A) Rats administered intrapleural siRNA targeting PKCz, thus lacking normal iPMF expression, have lower tidal volume under normoxic conditions as compared to control rats (99.2 +/- 21.7 vs 63.5 +/- 12.1 %, n=5), but no difference is seen under enchanted chemosensory input (87.5 +/- 7.2 vs 85.3 +/- 11.4%, n=5). B-C) No appreciable differences in respiratory frequency or minute ventilation at either normoxia (91.4 +/- 23.6 vs 100.2 +/- 18.6%, n=5; 81.9 +/- 8.9 vs 68.7 +/- 8.3%, n=5) or MCS (81.0 +/- 16.3 vs 71.9 +/- 8.6%, n=5; 69.5 +/- 12.5 vs 60.17 +/- 1%, n=5) are noted between groups. Statistical analyses not performed due to high variability and low sample size.

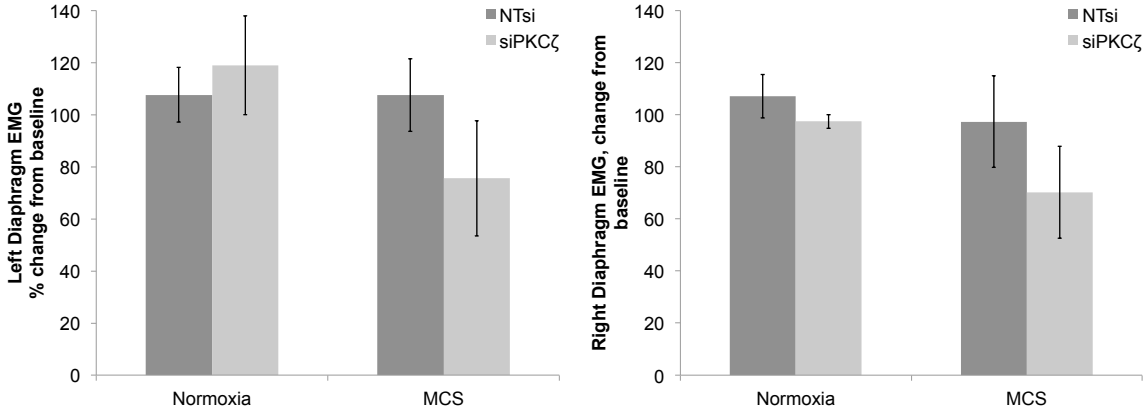

Figure 2. iPMF mechanisms may be responsible for maintaining normal diaphragm EMG amplitude following spinal injury. Reduced diaphragm EMG amplitude is seen with siPKCz following spinal injury as compared to NTsi controls under MCS (97.3 +/- 2.8 vs 70.3 +/- 5.1% right, 107.5 +/- 4.4 vs 75.6 +/- 3.6%, left, n=3) but not under normoxic conditions (100.7 +/- 2.6 vs 91.4 +/- 5.7% right, 107.6 +/- 3.8 vs 119 +/- 4%, left, n=3). Statistical analyses not performed due to high variability and low sample size.

APPENDIX B

Hypoxia-induced NMDA receptor activation impairs compensatory plasticity induced by reduced respiratory neural activity

D. P. Fields, J. Weltman, K. M. Braegelman, and T. L. Baker

In preparation

Abstract

Sleep apnea is characterized by a persistent pattern of unstable breathing, with recurrent periods of reduced or absent breathing, during sleep. A poor understanding of sleep apnea etiology has hindered development of drug therapies. Despite the prevalence of sleep apnea, the neural system controlling breathing exhibits an intrinsic capacity to sense and correct perturbations in breathing control (i.e. compensatory respiratory plasticity), suggesting breathing instability in sleep apnea may reflect a failure to initiate plasticity mechanisms. One form of compensatory respiratory plasticity is inactivity-induced phrenic motor facilitation (iPMF), a prolonged enhancement of inspiratory motor output triggered by episodes of reduced neural activity (i.e. neural apnea) within phrenic motor neurons controlling the diaphragm. Since clinically relevant apneas rarely occur without concomitant hypoxia, we sought to determine the impact of concurrent hypoxia and neural apnea on iPMF expression. In anesthetized and mechanically ventilated adult rats we demonstrate that while isolated neural apneas give rise to iPMF, concurrent exposure to moderate (but not mild) hypoxia abolishes iPMF expression. We show that hypoxia enhances spinal NR2B-containing NMDA receptors (NMDARs) to constrain signaling processes necessary for iPMF. Further, we demonstrate that spinal application of trans-retinoic acid bypasses NMDAR constraints, thereby rescuing respiratory plasticity following neural apnea with hypoxia. This study provides important mechanistic insight into understanding how clinically relevant neural apneas may undermine compensatory forms of respiratory plasticity, and indicates that

retinoic acid may be a novel pharmacological approach to improve breathing in individuals with sleep apnea.

Introduction

Sleep apnea is an insidious disease estimated to affect 1 in 15 Americans, with prevalence peaking in middle-aged individuals to 1 in 12 women and 1 in 4 men (Young et al., 1993; Heinzer et al., 2015). While the implications of sleep apnea are often underappreciated, recurrent breathing disruptions lead to sleep fragmentation, reduced blood oxygenation, and promotes hyperactivity of the sympathetic nervous system (Leung, 2009). Thus, sleep apnea is a dangerous precipitator of acute complications in patients with neurovascular and cardiovascular disease (Mehra and Redline, 2014; Abbott and Videnovic, 2016; Chowdhuri et al., 2016).

Sleep apnea presents as one of two diagnostically distinct subclasses: obstructive sleep apnea (OSA) or central (neural) sleep apnea (CSA). Whereas OSA is characterized by an anatomical narrowing of the upper airway impeding airflow during attempted breaths (Wheatley et al., 1993), CSA is characterized by insufficient neural motor output to the diaphragm, thus undermining breathing efforts despite significant hypoxemia (reviewed in Hernandez and Patil, 2016). Recent evidence suggests that these two conditions may not be as distinct as previously believed. In a large cohort of patients treated for OSA with continuous positive air pressure (CPAP), once OSA was resolved, previously unidentified CSA became apparent (Hoffman and Schulman, 2012; Westhoff et al., 2012), suggesting central apneas may be a precipitating factor for development of OSA. Alternatively, CSA and OSA may derive from a common origin related to impaired motor output within the neural respiratory network (Babcock and Badr, 1998; Aboubakr et al., 2001; Malhotra and White, 2002).

Despite the prevalence of sleep apnea, neural networks underlying breathing exhibit a profound capacity for compensatory plasticity, which adapts system properties following perceived respiratory failures (i.e. neural apneas/inactivity, hypoxia) and may prevent future failures (Mahamed et al., 2007; Bach and Mitchell, 1996; Mahamed and Mitchell, 2008). Compensatory respiratory plasticity presents in several ways: 1) enhanced phrenic inspiratory motor output (i.e. phrenic motor facilitation; **Figure 1A**), strengthening inspiratory efforts (Bach and Mitchell, 1996), 2) enhanced hypoglossal (XII) motor output (i.e. XII motor facilitation; **Figure 1B**), improving upper airway patency (Malhotra and White, 2002; Wilkinson et al., 2008), and 3) a reduction in apneic threshold (**Figure 1C**), reducing susceptibility to future apneas (Baertsch and Baker, 2017; Khoo et al., 1991; Carley et al., 1988).

Two well-studied models of compensatory respiratory plasticity are inactivity-induced phrenic motor facilitation (iPMF; Mahamed et al., 2011) and hypoxia-induced phrenic long-term facilitation (pLTF; Bach and Mitchell, 1996). Whereas iPMF is elicited by intermittent reductions in respiratory neural activity (i.e. neural apnea/inactivity; Streeter and Baker-Herman, 2014), pLTF develops following several episodes of moderate hypoxia (35-45 mmHg PaO_2; Fuller et al., 2000); both manifest as a persistent enhancement in phrenic inspiratory output. Although clinically significant reductions in respiratory neural activity rarely occur without concurrent hypoxia, hypoxia's capacity to modulate inactivity-induced plasticity has never been studied.

Recent developments in our understanding of iPMF and pLTF signaling processes have determined that these two forms of plasticity operate through independent signaling pathways (Strey et al., 2013; Fields and Mitchell, 2015).

Specifically, signaling mechanisms giving rise to iPMF and pLTF exhibit converging, yet contrasting, roles for NMDA receptors (NMDARs). Whereas spinal NMDAR activation is necessary for both induction and maintenance of pLTF (McGuire et al., 2005; 2008; Golder, 2008), spinal NMDAR activation constrains iPMF (Streeter et al., 2014). Thus, we propose that a contrasting drive to enhance and reduce NMDAR activity mechanistically undermines the capacity for either stimuli to elicit plasticity during concurrent respiratory neural hypoactivity with hypoxia, potentially explaining the lack of compensatory plasticity in individuals with breathing disorders characterized by insufficient respiratory drive (i.e. sleep apnea).

Here, we demonstrate that while neural apnea and hypoxia are independently sufficient for plasticity (iPMF and pLTF, respectively), concurrent reductions in respiratory neural activity with moderate (but not mild) hypoxia undermines expression of respiratory plasticity. Further, inhibition of hypoxia enhanced spinal NMDARs during concurrent neural apnea and hypoxia removes signaling constraints to enable plasticity. Alternatively, we show that hypoxia enhanced NMDARs can be bypassed with exogenous retinoic acid, a molecule necessary and sufficient for iPMF expression (Baetsch and Baker, 2017), thus rescuing several plasticity evoked breathing behaviors. This is the first study to provide a possible mechanistic explanation for why patients with sleep apnea lack compensatory respiratory behaviors, rendering them susceptible to recurrent apneas/hypoxia and secondary long-term complications associated with unstable breathing control. Recognition of hypoxia enhanced NMDARs as a key constraint to retinoic acid dependent inactivity-induced plasticity is an important first-

step in identifying novel targets for pharmacologically treating CSA, OSA, and other breathing disorders.

Methods

Animals.

Experiments were performed on 2.5-3.5 month old male Sprague-Dawley rats from Harlan Laboratories (HSD; colony 217). Rats were housed 2 per cage with 12hr light/dark cycles and food/water *ad libitum*. The Animal Care and Use Committee at the University of Wisconsin, Madison approved all experimental protocols.

Surgical Preparation.

Isoflurane anesthesia was induced in a closed container and continued on a nose cone of 2.5% isoflurane with 30/70 O_2/N_2 balance. A tail vein catheter was placed for urethane delivery and fluid infusion (10/5/1 lactated rings solution/hetastarch/8% sodium bicarbonate solution) in order to maintain anesthesia and blood pressure/pH homeostasis, respectively, throughout experimental protocol. The trachea was cannulated, and mechanical ventilation was begun (Model 683, Harvard Apparatus, Holliston Massachusetts; ~70 breaths/min, 2.5-3ml; 30/70 O_2/N_2 balance). Rats were slightly hyperventilated, so CO_2 was added in the inspired line to maintain an end-tidal CO_2 ($ETCO_2$) near ~45mmHg (assessed through an expired airflow capnograph; Respironics) and avoid unintended neural apneas during surgery or anesthesia conversion. The vagus nerve was isolated and cut bilaterally at the cervical level to prevent entrainment of spontaneous respiratory frequency to the ventilator. The right femoral artery was isolated and catheterized to monitor arterial blood pressure and draw

blood samples (0.3ml per sample) for pH and blood-gas analysis (ABL800; Radiometer, Copenhagen, Denmark). The C2 spinous process was exposed and a C2 laminectomy was performed over the spinal midline. A small hole was cut in the dura of the exposed spinal segment and a silicone catheter (2 French; Access Technologies) connected to a 50-µl Hamilton syringe was inserted into the intrathecal space and carefully advanced caudally (5 mm) so that the tip of the catheter laid on the dorsal surface of the C4 spinal segment. The left hypoglossal (XII) and phrenic nerves were dissected by dorsal approach, cut distally, de-sheathed, and placed on bipolar silver electrodes to record inspiratory motor output. Rats were then converted to urethane anesthesia (0.180g/100g rat i.v.) as isoflurane was gradually withdrawn over a period of ~15min. Following confirmation of adequate anesthetic depth, pancuronium bromide was infused through the tail vein (3mg/kg, i.v.) to induce neuromuscular paralysis. Periodically throughout the protocol, pressor and phrenic/XII nerve amplitude responses to toe pinch were assessed to ensure adequate maintenance of anesthesia depth. Body temperature was monitored by rectal thermometer and maintained near 37.5°C (± 1°C) with a custom heated surgery table.

Experimental Protocols.

A minimum of one hour following termination of isoflurane anesthesia, the apneic threshold (measured by $ETCO_2$) was identified by slowly reducing inspired CO_2 until phrenic/XII bursting ceased for at least 20sec. Inspired CO_2 was then slowly increased until nerve activity returned (i.e. recruitment threshold; RT; measured by $ETCO_2$) and raised an additional 2-3mmHg above RT for “baseline” nerve recordings. Baseline nerve activity was recorded for 15min with 2 arterial blood samples drawn 5min apart to obtain

baseline $PaCO_2$, PaO_2, and pH measurements (temperature corrected). For the remainder of the protocol, baseline parameters were maintained within ±1.5mmHg for $PaCO_2$ (of eupneic baseline), ±10mmHg for PaO_2 (baseline approx. 100mmHg PaO_2), ±1.5 for pH, and ±1.0°C for temperature. Blood gases were taken 15, 30, and 60min after the final neural/ventilator apnea challenges, and adjustments to inspired gases and fluid infusion were made after each blood test if necessary. At the end of each protocol, rats were exposed to a maximum respiratory challenge consisting of hypercapnia (90mmHg < $ETCO_2$ < 100mmHg) with hypoxia to ensure observed results were not due to deterioration of the preparation or a lack of dynamic range in respiratory motor output. Rats who did not experience at least a 100% increase in nerve activity during the maximum respiratory challenge were removed from data analysis.

Following baseline recordings, rats were exposed to one of five protocols: **1)** 5 neural apnea episodes (~1.25 min each, separated by 5 min of baseline blood gases; n = 8), **2)** 5, 25 sec ventilator apnea episodes (i.e. hypoxia challenges), in which mechanical ventilation was temporarily stopped while neural activity remained intact (each episode separated by 5min of baseline blood gases; n = 7), **3)** 5, 6 sec ventilator apnea episodes (each episode separated by 5 min of baseline blood gases; n = 8) with intact neural activity, **4)** 5, 25 sec ventilator apnea episodes during concurrent neural apnea (each episode separated by 5 min of baseline blood gases; n = 7), and **5)** 5, 6 sec ventilator apnea episodes during concurrent neural apnea (each episode separated by 5 min of baseline blood gases; n = 8). In order to assess potential drifts in nerve activity due to the surgical preparation alone, a “time control” group received the same surgical procedure without neural apnea or ventilator apnea experiences (n = 7).

Isolated neural apneas (i.e. neural inactivity)

To induce a neural apnea without hypoxia, we took advantage of the high sensitivity of the respiratory control system to hypocapnia. We induced a "neural apnea" by lowering inspired CO_2 until rhythmic phrenic and XII activity ceased, while maintaining ventilator settings (volume and frequency) at the same levels used during baseline recordings. Neural apnea was confirmed for 10 sec before inspired CO_2 was returned to baseline levels, though nerve activity did not return until ~ 1 min after $ETCO_2$ reached baseline levels. This delay in spontaneous respiratory activity is consistent with previously published work from our lab and others (Baertsch and Baker-Herman 2013; 2015). 5 episodes of neural apnea were given with each episode separated by 5 min of baseline inspired CO_2.

Isolated ventilator apneas (i.e. hypoxia)

To induce hypoxia without a reduction in respiratory neural activity, a "ventilator apnea" (i.e. hypoxic episode) was induced by turning off the ventilator for either 6 sec or 25 sec, depending on the experimental group. This was repeated 5 times with 5 min separating each ventilator apneic episode. Ventilatory apneas resulted in a consistent decrease in arterial O_2 (measured by pulse oximeter; 8600V, Nonin Medical Inc., Plymouth, MN, USA; **Supplementary Figure 1**). Following cessation of the hypoxic event, the ventilator was turned back on at baseline settings. Each animal naturally corrected arterial blood gases between hypoxic episodes without any adjustments being made to the ventilator or inspired gases.

Neural apneas with concurrent ventilator apneas

To induce neural apneas, inspired CO_2 was decreased until neural apnea was achieved. Following a 10 sec period of absent phrenic firing activity, the ventilator was turned off for either 6 sec or 25 sec. The acute hypoxic episode resulted in a natural rescue from neural apnea (approx. 4sec after turning the ventilator off), though the ventilator is kept off for the full 6sec or 25sec duration to maintain a consistent drop in arterial oxygen (confirmed with pulse oximetry). This experience was repeated 5 times, with 5min separating each episode.

Pharmacological Treatments. Separate groups of rats were exposed to intermittent neural apneas, intermittent ventilator apneas or both with intrathecal injection of the following pharmacological compounds: 4-Diethylaminobenzaldehyde **(**DEAB; Sigma-Aldrich St. Louis, MO), all-trans retinoic acid (RA; Sigma-Aldrich St. Louis, MO), amino-5-phosphonovaleric acid (APV; Sigma-Aldrich St. Louis, MO), Co 101244 (Tocris Bristol, UK), or eliprodil (Tocris Bristol, UK).

Solutions were dissolved in DMSO and stored at -20°C for up to 1 week. Prior to injecting, stock solutions were diluted with artificial CSF (aCSF; 120 NaCl, 3 KCl, 2 CaCl, 2 MgCl, 23 $NaHCO_3$, 10 glucose bubbled with 95% O_2/5% CO_2) to a concentration that was less than 1-10 of stock. Solutions were delivered intrathecally over spinal segments containing the phrenic motor nucleus over a 2 minute period. Vehicle treated control rats received 10-12 µl of a 20% DMSO in aCSF solution (vehicle). Separate groups of time control rats were also treated with each solution or

vehicle. There was no relative or absolute drift in nerve activity within time controls for drug injected or vehicle injected rats.

Immunohistochemistry (IHC). Following neural apnea, ventilator apnea, neural + ventilatory apnea or time control studies, rats were euthanized and perfused transcardially with 1mL/g chilled 0.01M PBS followed by 1mL/g chilled 4% paraformaldehyde (PFA) at a pH of 7.4. The spinal cords were harvested and placed into 4% PFA for 8 hours at 4^{O}C. The tissues were then transferred to 20% sucrose followed by 30% sucrose until sinking. 40µm transverse slices were cut using a microtome (SM2000R Leica, Buffalo Grove, IL) from C3-C5 and placed into antifreeze solution (30% glycerol; 30% ethylene glycol; 40% 0.1M PBS). For each group, 6 slices from 3 rats (18 total slices; 36 total images, per group) were selected for staining (slices were at least 200µm apart). Slices were washed with 0.05M tris-buffered saline with 0.1% Triton-X (TBS-Tx) and blocked with 5.0% bovine serum albumin (BSA) for 1hour. Tissues were stained with rabbit anti-phospho-NR2b-NMDAR (Santa Cruz Biotech), goat anti-CtB (Millipore), mouse anti-ALDH (Santa Cruz Biotech), and mouse anti-retinoic acid receptor alpha (Santa Cruz Biotech) at 4°C for 16h. Slices were washed with TBS-Tx and subsequently stained with conjugated donkey anti-goat Alexa Fluor 633 (Invitrogen), conjugated donkey anti-rabbit Alexa Fluor 488 (Invitrogen), conjugated donkey anti-mouse Alexa Fluor 595 (Invitrogen) for 2hr at room temperature. Tissue slices were washed a final time with TBS and mounted with coverslip and an anti-fade solution (Invitrogen). Slices were imaged on an epifluorescence confocal microscope at 40x using Nikon EZ-C1 software. The images were localized onto the phrenic nuclei

cluster bilaterally via focusing on the ventral region of cervical spinal cords. CtB was used as a retrograde tracer of phrenic motor neurons to confirm phrenic motor neuron location within respective slices as outlined in previous studies (Mantilla et al., 2009).

Data Analysis Neurophysiology.

Phrenic and XII burst activity was amplified (x10k), band-pass filtered (0.3-10kHz; AM Systems), integrated (time constant 50ms), and rectified. The resulting signal was digitized and analyzed with PowerLab (AD Instruments; Lab Chart 7.0 software). 60-breath bins were taken immediately prior to blood samples at baseline, 15, 30, and 60min post final neural apnea or hypoxic experience. Nerve burst amplitude was expressed as a percent change from baseline. Statistical differences between groups and individual time points were determined using a two-way repeated measures ANOVA design and a Bonferroni post-hoc test. Groups were considered significantly different if p-values were < 0.05. Data are shown as means ± SE. All p values are given relative to baseline amplitude and time matched control group nerve amplitude.

Data Analysis IHC.

NR2b-NMDA receptor quantification was done with NIH Image J Software. The control rat group was used as a reference; thus, changes in NR2b immunofluorescence in other groups were expressed relative to control rats. Quantification of NR2B was restricted to NeuN positive regions using overlay techniques available within Image J Software. There was no difference in the NeuN staining between the individual groups. Staining was completed in a single batch with one-way ANOVA and Fisher's LSD post-hoc tests to compare individual groups.

Results

Concurrent moderate hypoxia undermines inactivity-induced respiratory plasticity

To determine the impact of concurrent hypoxia on inactivity-induced plasticity, rats were exposed to neural apneas with and without hypoxia (**Figure 2B**). To induce a rapid change in blood oxygen levels that better mimics cessation of airflow expected with a neural apnea, we employed a 25 sec "ventilator apnea" (i.e., turned off the mechanical ventilator). This resulted in a consistant oxygen desaturation of ~70% (estimated 38 ± 0.7 mmHg PaO2). As expected, 5 intermittent neural apneas triggered a significant increase in phrenic inspiratory burst amplitude (i.e. iPMF; 54.3 ± 7.8 %; $p < 0.001$; **Figure 2Ai**). Similarly, 5 episodes of moderate hypoxia (via 25 sec ventilator apnea) triggered a significant increase in phrenic inspiratory burst amplitude (i.e. pLTF; 59.9 ± 4.1 %; $p < 0.001$; **Figure 2Aii**). However, 5 episodes of neural apnea with concurrent hypoxia did not elicit a significant change in phrenic motor output (7.7 ± 6.1 %; $p = 1.000$; **Figure 2Aiii**).

The onset of the ventilator apnea superimposed with neural apnea was met with a rapid return of inspiratory neural activity; as such, the duration of neural apnea was different in these rats when compared to neural apnea alone (**Supplemental Table 2**). To confirm that hypoxia's constraint of iPMF was not an artifact of the reduced apnea duration, we investigated a "mild" hypoxic experience with 6 sec ventilator apneas (vs above mentioned 25 sec ventilator apnea). First, we demonstrate that 5 episodes of 6 sec ventilator apnea resulted in a consistent oxygen desaturation of ~ 88% (estimated 56 ± 0.5 mmHg PaO_2), that was not sufficient to elicit pLTF (-0.9 ± 2.8 %; $p = 1.000$; **Figure 2Ci**). This is consistent with previous reports demonstrating moderate hypoxia

(35-45mmHg PaO_2) is necessary to initiate the signaling processes for hypoxia-induced respiratory plasticity (Fuller et a., 2000; **Supplemental Table 2**). Further, while neural apnea with concurrent mild hypoxia (6sec; 56mmHg PaO_2) and moderate hypoxia (25sec; 38mmHg PaO_2) similarly reduced the duration of the neural apneas (13-14 ± 0.6 sec; **Supplemental Table 2**), mild hypoxia did not disrupt expression of iPMF (66.6 ± 9.4 %; $p < 0.001$; **Figure 2Cii**). Collectively, these data suggest that signaling mechanisms giving rise to hypoxia-induced plasticity (i.e., pLTF) impair iPMF during concurrent inactivity with hypoxia.

Spinal retinoic acid synthesis is necessary for inactivity-induced, but not hypoxia-induced, plasticity.

Retinoic acid synthesis is necessary for several well-studied models of inactivity-induced plasticity (Aoto et al., 2008; Wang et al., 2011), including iPMF (Baetsch and Baker, 2017). Within the hippocampus, retinoic acid receptor alpha (RARα) mediates the necessary signaling cascades for plasticity expression (Aoto et al., 2008). We confirmed that retinaldehyde dehydrogenase (RALDH; synthesizes active retinoic acid from retinol) and RARα are present within CtB labeled phrenic (respiratory) motor neurons (**Figure 3A**). Thus, spinal respiratory neurons have the capacity to both synthesize and respond to local retinoic acid.

To date, there has been no evidence supporting a role for retinoic acid within hypoxia-induced pLTF. To confirm a mechanistic distinction between respiratory inactivity-induced plasticity (iPMF) and hypoxia-induced plasticity (pLTF) DEAB (10 µL x

1.0 mM), a RALDH inhibitor, was injected intrathecally at C3-C6. Consistent with previous results, DEAB pretreatment abolished expression of iPMF (5.0 ± 3.7 %; p = 1.000 relative to baseline; p = 0.001 relative to inactivity alone; **Figure 3Bii**). Conversely, DEAB pretreatment did not attenuate pLTF (60.1 ± 7.9%; p < 0.001 relative to baseline; p = 1.000 relative to hypoxia control; **Figure 3Biv**) demonstrating spinal retinoic acid synthesis is necessary for inactivity-induced, but not intermittent hypoxia-induced respiratory plasticity (**Figure 3C**).

Hypoxia enhanced NMDARs constrain iPMF

Spinal NMDAR activation is necessary for pLTF (McGuire et al., 2005; 2008; Golder 2010). By contrast, spinal NMDAR activation constrains retinoic acid dependent plasticity induced by neural activity deprivation within the hippocampus (Aoto et al., 2008) and spinal cord (Streeter and Baker-Herman 2014). To test the hypothesis that hypoxia-induced NMDAR activation occludes iPMF, APV (10 µL x 100 µM), a selective NMDAR antagonist, was injected intrathecally over C3-C6. We first confirmed respective roles for spinal NMDAR activation in pLTF and iPMF. Consistent with previous work, pretreatment with APV abolished pLTF (2.1 ± 3.1 %; p = 1.000; **Figure 4Aiv**). Conversely, intrathecal APV prior to neural apnea did not alter iPMF expression (73 ± 7.6%; p < 0.001 relative to baseline; p = 0.440 relative to inactivity control; **Figure 4Aii**), although there was a slight trend for an enhancement. Thus, inhibition of spinal NMDAR blocks hypoxia-induced pLTF, but not inactivity-induced iPMF (**Figure 4B**).

While basal activity of spinal NMDARs do not appear to constrain iPMF, hypoxia is known to enhance NMDAR signaling within the hippocampus (Takagi et al., 2003) and spinal cord (McGuire et al., 2005; 2008). Therefore, we sought to determine if hypoxia-enhanced NMDARs conditionally constrain plasticity during episodes of neural apnea with hypoxia. Pretreatment with intrathecal APV prior to neural apnea with hypoxia revealed a persistent enhancement in phrenic motor output (60.9 ± 8.0%; $p < 0.001$; **Figure 4Cii and 4D**), demonstrating hypoxia-enhanced NMDAR activation constrain iPMF.

Exact mechanisms by which hypoxia enhances NMDAR signaling for pLTF expression has not been determined but previous reports have demonstrated that NR2B-NMDAR subunits are phosphorylated in response to hypoxia (Takagi et al., 2003). In addition, phosphorylation of NR2B-NMDAR subunits increases calcium influx through NMDARs (Takasu et al., 2002; Paul and Connor; 2010) to enable some forms of plasticity (Xu et al., 2006); suggesting a potential mechanism for hypoxia-induced, NMDAR dependent, pLTF. To assess if these same post-translational events occur within the respiratory control system we investigated phosphorylation changes in cervical spinal NR2B-NMDARs. NeuN staining was used to selectively quantify NR2B changes within ventral C4-C5 spinal neurons. We demonstrate that NR2B phosphorylation was significantly increased within NeuN positive cells 60min following intermittent hypoxia ($p < 0.001$; **Figure 5A**). Conversely, neural apnea (with or without hypoxia) significantly decreased NR2B phosphorylation within NeuN positive cells ($p < 0.001$; **Figure 5A**) 60min after neural apnea. There was no significant difference in the

NR2B phosphorylation expression between neural apnea with or without hypoxia ($p = 0.213$). In addition, there was no difference in NeuN expression amongst the individual groups ($p > 0.05$). Of note, there is a significant increase in NR2B phosphorylation within astrocytes (**Figure 5A**). This was not explored further within this study.

Finally, to confirm that NR2B phosphorylation contributed to the obstruction of iPMF, CO101244 (10µL x 1.0mM) or eliprodil (10µL x 100µM), two NR2B selective NMDAR inhibitors (Bath et al., 1996; Zhou et al., 1999; Gill et al., 2002), were delivered in subsets of rats prior to co-presentation of hypoxia and neural apnea. Pretreatment of either CO101244 or eliprodil prior to neural apnea with concurrent hypoxia rescued respiratory plasticity expression (61.6 ± 5.01 %; $p < 0.001$ and 63.6 ± 11.2 %; $p < 0.001$; **Figure 5B**).

Exogenous retinoic acid rescues respiratory plasticity

Calcium inhibits RALDH to constrain inactivity-induced plasticity in the hippocampus (Wang et al., 2011). Since NR2B phosphorylation increases calcium influx through NMDA receptors (Viviani et al., 2003; Strack and Colbran, 1998), and hypoxia-enhanced NR2B-NMDA receptors constrain iPMF (**Figure 5**), we hypothesized that hypoxia may undermine iPMF by impairing calcium sensitive RALDH. Therefore, we hypothesized that exogenous retinoic acid would circumvent RALDH inhibition and rescue plasticity during concurrent neural apneas with hypoxia.

Cervical spinal injections of trans-retinoic acid (10µL x 50µM) in control rats (without neural apnea or hypoxia) gave rise to enhanced phrenic inspiratory burst

amplitude (41.6 ± 5.9%; $p < 0.001$; **Figure 6Aii and Figure 6B**), without a significant change in XII motor output (20.9 ± 9.0%; $p = 1.000$; **Figure 6C**) apneic threshold (-0.2 ± 0.4 mmHg $PaCO_2$ relative to baseline; $p = 1.000$; **Figure 6D**), or respiratory burst frequency (**Supplemental Table 1**). XII motor output, apneic threshold and burst frequency serve as internal controls ensuring intrathecal retinoic acid injections remained local to the spinal cord since brainstem retinoic acid application modulates activity of medullary respiratory centers (Guimarães et al., 2007) to increase firing frequency. Further, if retinoic acid induced phrenic motor plasticity was secondary to stimulation of medullary respiratory centers, a concomitant increase in XII motor output would have been observed as these motor pools share common innervation from the brainstem.

Conversely, spinal retinoic acid prior to neural apnea with hypoxia triggered an enhanced phrenic motor output (81.3 ± 14.4%; $p < 0.001$; **Figure 5Aiii, B**), enhanced XII motor output (99.0 ± 22.0%; $p < 0.001$; **Figure 5C**), and lead to a significant reduction in the apneic threshold (-7.8 ± 1.9mmHg $PaCO_2$ relative to baseline; $p = 0.007$; **Figure 5D**), without changing respiratory frequency (**Supplemental Table 1**). Thus, exogenous retinoic acid rescues protective respiratory behaviors during concurrent neural apneas with hypoxia.

Discussion

Rhythmic firing of the neural network controlling breathing is supported by intrinsic mechanisms (i.e. respiratory plasticity) designed to sense and correct

perturbations in breathing control (Strey et al., 2013). Despite this safeguard, breathing disorders characterized by periodic cessations in respiratory motor output exist. Previous investigations into respiratory plasticity have significantly advanced our understanding of the cellular events underlying respiratory plasticity, but unfortunately, most of these models have not mimicked the hallmark pattern of respiratory neural apnea *with* hypoxia that characterizes sleep apnea and many other breathing disorders. This gap in understanding of the signaling mechanisms underlying clinically relevant breathing dysfunction has hindered development of targeted pharmacological therapies for breathing disorders.

Here, we report that while neural apneas and hypoxia are independently sufficient to elicit compensatory respiratory plasticity (iPMF and pLTF, respectively), plasticity is abolished during concurrent neural apnea with hypoxia (**Figure 2**). With carefully designed control studies, we have shown that hypoxia undermines retinoic acid dependent iPMF by enhancing NR2B containing NMDARs (**Figure 5**). Further, plasticity can be rescued by either inhibiting NR2B-NMDARs (**Figure 5**) or by bypassing NMDARs with exogenous retinoic acid (**Figure 6**) thereby enabling 1) enhanced phrenic motor output, 2) enhanced XII motor output, and 3) a reduction in apneic threshold (**Figure 6)**. Together these behaviors are believed to stabilize neural respiratory activity for improved breathing control (**Figure 1**), an important clinical goal for patients with sleep apnea and related breathing disorders.

Hypoxia-enhanced spinal NMDARs constrain inactivity-induced iPMF

While enhanced NMDAR signaling is necessary for hypoxia-induced pLTF, NMDARs constrain some forms of hippocampal (Aoto et al., 2008) and spinal (Streeter

and Baker-Herman, 2014) inactivity-induced plasticity. Thus, we hypothesized that contending roles for NMDARs undermine respiratory plasticity expression during concurrent respiratory neural inactivity with hypoxia. The present study demonstrates that hypoxia-enhanced, but not basally active, spinal NMDARs constrain iPMF (**Figure 4**). While this is in contrast to other forms of inactivity-induced plasticity in which basal NMDAR activity constrains plasticity expression (Aoto et al., 2008; Steeter and Baker-Herman, 2014), slight differences in induction paradigms may account for the differences in NMDAR sensitivity. For example, we induced iPMF through several intermittent bouts of neural apneas while Streeter and Baker-Herman utilized a single prolonged (60min) neural apnea, similar to the single prolonged (24hr) episode of neural inactivity used by Aoto et al., 2008. Despite similar phenotypical expression, differing deprivation patterns can initiate unique signaling pathways within respiratory (Baertsch and Baker-Herman, 2013; Devinney et al., 2016), as well as non-respiratory (Sutton and Carew, 2000; Muller and Carew, 1998) neural networks.

Mechanisms rendering iPMF resistant to basal NMDAR activity are not well understood but may involve desensitization of post-synaptic membranes to NMDA activation. Although intermittent neural apnea is not intermittent stimulation per se, the periods separating individual inactivity episodes mimic intermittent stimulation and could lead to similar cellular adaptions. Through negative feedback, intermittent stimulation of NMDARs promotes their removal from the membrane (Joshi et al. 2007; Thiagarajan et al. 2002), decreasing NMDAR mediated calcium influx, effectively mimicking pharmacological inhibition with APV. This hypothesis may explain why neural apnea leads to a relative decrease in NR2B phosphorylation expression (Figure 5) and

reciprocates inhibition of hypoxia-induced plasticity (i.e. pLTF); enhanced NMDAR activity is required for induction and maintenance of pLTF (McGuire et al., 2005). Further work is still needed to test this hypothesis as well as mechanisms for bi-directional cross-talk inhibition of inactivity and hypoxia.

Exogenous application of trans-retinoic acid rescues compensatory respiratory behaviors

Here we demonstrate that iPMF and pLTF can be mechanistically distinguished by their requirement for retinoic acid synthesis; iPMF requires new retinoic acid synthesis and pLTF is retinoic acid synthesis independent (**Figure 3**). Further, we demonstrate that hypoxia-enhanced NMDARs constrain iPMF, likely by inhibiting calcium sensitive RALDH (Wang et al., 2011). While NMDAR inhibition rescues respiratory plasticity expression (**Figure 4**), previous failed attempts to treat OSA and CSA with NMDAR antagonists were limited by the psychotropic side effects that are characteristic of NMDAR modulation (Hedner et al., 1996; Torvaldsson et al., 2005). Investigators hypothesized that excess glutamate release in response to hypoxia was leading to neuronal excitotoxicity and secondary breathing instability. While NMDAR inhibition improved central apneas in a subset of patients (Hedner et al., 1996), glutamate levels did not change in response to hypoxia or following breathing stabilization (Torvaldsson et al., 2005). Conversely, our data suggest that hypoxia-enhanced NMDARs (vs glutamate release) interrupt intrinsic stabilizing mechanisms (vs excitotoxicity) for breathing control. We thus utilized selective NR2B-NMDAR antagonists and exogenous retinoic acid to bypass NMDAR constraints (without

associated psychotropic side effects) and enable compensatory respiratory behaviors during reduced respiratory neural activity with hypoxia.

To our surprise, spinal retinoic acid enabled phrenic motor plasticity, XII motor plasticity, and reduced the apneic threshold (**Figure 6**), without modulating respiratory frequency (**Supplemental Figure**). The absence of respiratory frequency changes suggest intrathecal retinoic acid injections remained local to the spinal cord and did not diffuse to the brainstem where retinoic acid stimulation of the PBC promotes hyperpneic breathing activity (Guimarães et al., 2007). Further, our IHC results demonstrate that spinal respiratory motor pools have the capacity to synthesize and respond to local retinoic acid synthesis (**Figure 2**) independent of PBC input, providing further evidence that these respiratory behaviors occur within the spine.

While aberrant firing of the respiratory rhythm generator, the PreBötzinger Complex (PBC), is thought to be the primary cause of many breathing disorders (McKay and Feldman, 2008), poor neural transmission of inspiratory effort from the PBC is emerging as a potentially important contributor to unstable breathing patterns (Kam et al., 2013; Garcia et al., 2016). For example, prolonged exposure to intermittent hypoxia disrupts PBC inspiratory transmission to respiratory motor nuclei without directly affecting the firing pattern of the PBC (Garcia et al., 2016). This results in non-synchronous firing patterns with failed transmission of inspiratory efforts from the PBC to the XII motor nuclei, a primary controller of upper airway patency. In patients, inspiratory transmission failure from the PBC to the XII and spinal phrenic motor pools could lead to OSA (Wheatley et al., 1993) and CSA (Dale et al., 2014), respectively. Thus, therapies improving transmission fidelity of the respiratory control network (i.e.

respiratory plasticity) may be one method for treating OSA, CSA, and related breathing disorders.

Still, the question remains, how can spinal application of retinoic acid affect XII motor output when the XII motor nuclei is located within the brainstem? While an anatomical connection between spinal motor nuclei and brainstem nuclei has not been definitively identified, there is evidence suggesting the existence of a spinal pattern integrator enabling communication between individual respiratory motor pools independent of the PBC (Butler et al., 2014; Rice et al., 2011). Further work is still needed to understand how spinal networks regulate non-spinal respiratory motor systems, but these current observations provide some evidence of its existence.

Clinical implications for CSA, OSA and other periodic breathing disorders

Previous studies investigating periodic breathing disorders have focused on aberrant PBC activity within the brainstem. We take an alternative approach and seek to understand the absence of compensatory respiratory behaviors during breathing events. Specifically, we demonstrate that compensatory plasticity induced by a reduction in respiratory neural activity, which is hypothesized to stabilize breathing activity, is conditionally constrained during concurrent induction of hypoxia-induced plasticity. These experimental observations are of central significance as they begin to explain several important clinical observations.

First, patients with mixed/complex sleep apnea receiving CPAP or tracheostomy will continue to have apneas initially upon treatment. However, in many of these patients, central events will spontaneously resolve through mechanisms that are not understood (Arzt et al., 2009, Deacon and Catcheside, 2015; Salloum et al., 2010).

Secondly, many patients with primary CSA experience a reduction in central events following treatment with inspired oxygen (Chowdhuri et al., 2012). Finally, periodic breathing is common in both full-term and premature infants whom experience transient episodes of mild apnea associated hypoxic events. While many infants receive supplemental oxygen, most infants with mild hypoxic events naturally correct their breathing pattern within 6 months of life, without intervention (Kelly et al., 1985). Collectively, these data are consistent with the hypothesis that moderate hypoxia (35-45 mmHg PaO_2) constrains compensatory mechanisms that correct breathing instability and when hypoxia is resolved (or mild in intensity) endogenous mechanisms emerge to restore respiratory stability.

During sleep apnea, and other breathing disorders, neural apneas often lead to acute hypoxic events. Recent clinical observations suggest, and our evidence supports, hypoxia may undermine compensatory mechanisms initiated by neural apneas, thus propagating subclinical apneic events into pathological breathing disorders. By addressing hypoxemia with CPAP or oxygen therapy, intrinsic compensatory mechanisms will be revealed to stabilize breathing control. Alternatively, retinoic acid treatment may rescue compensatory signaling mechanisms to stabilize breathing control when hypoxemia cannot be corrected; i.e patients with chronic cardiopulmonary disease. Further work is still needed to confirm the efficacy of retinoic acid treatment in patients with unstable breathing patterns.

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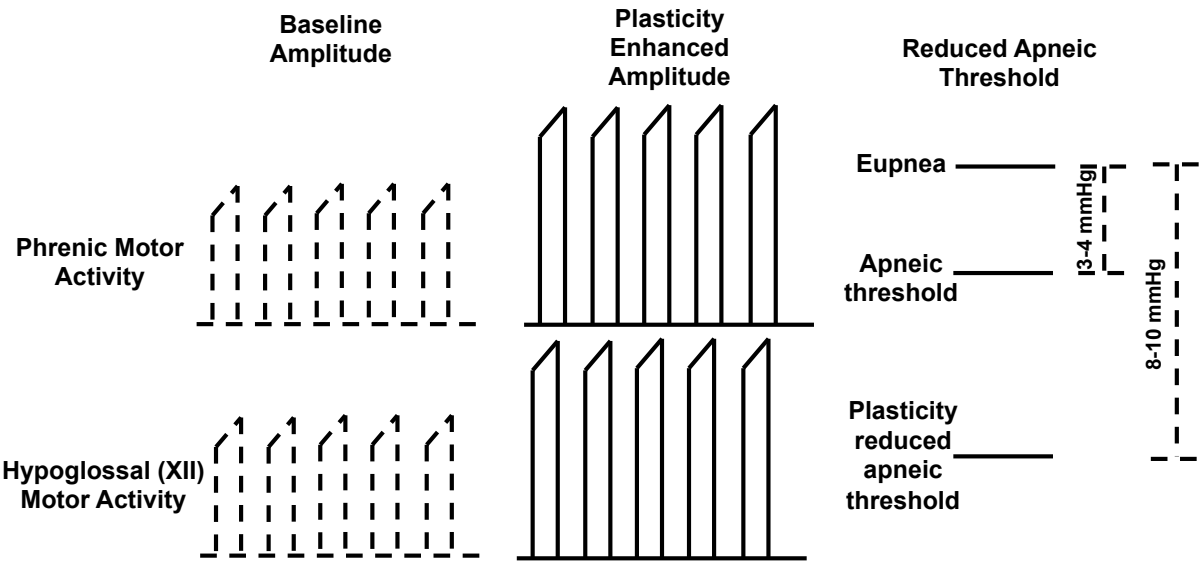

Figure 1. Compensatory respiratory behaviors. Expression of long lasting respiratory plasticity presents in several ways. Three well studied examples are: phrenic motor facilitation for enhanced diaphragm muscle control, XII motor facilitation for improved upper airway patency, and apneic threshold reduction to prevent subsequent apneas. These behaviors serve to enhance respiratory activity for improved breathing stability.

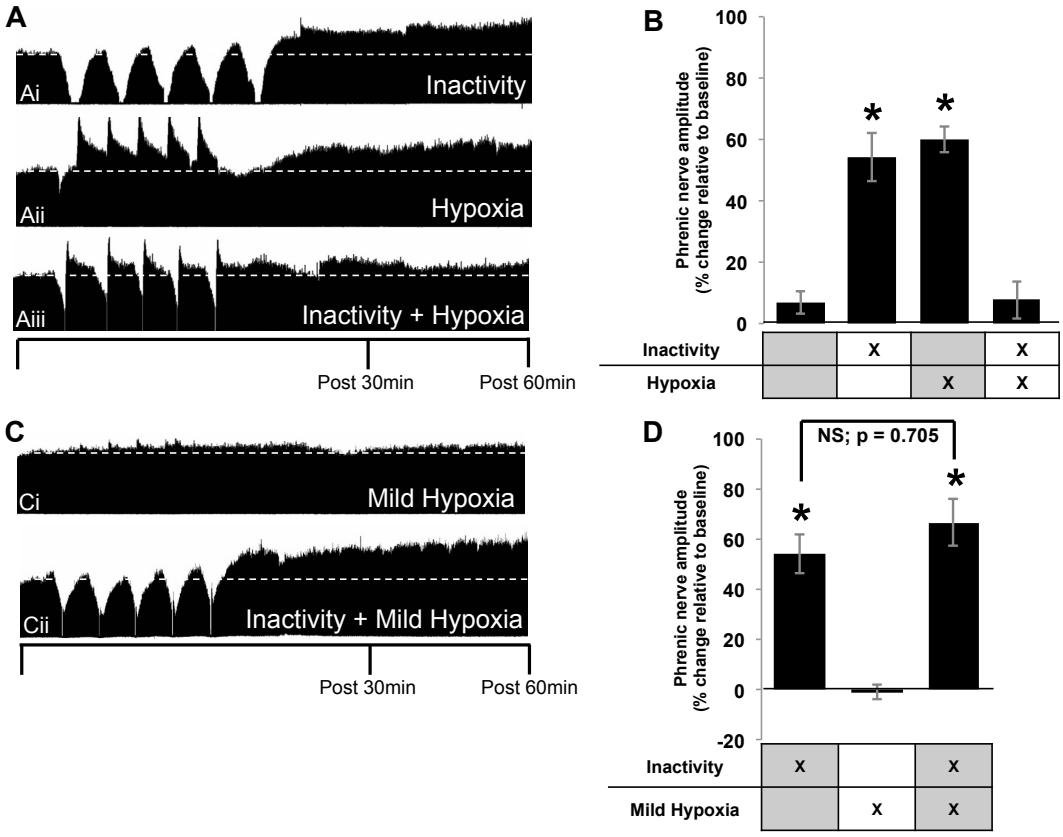

Figure 2. Concurrent hypoxia undermines inactivity-induced respiratory plasticity. **A)** Representative phrenic neurogram traces: **i)** neural apnea elicited iPMF, **ii)** hypoxia episodes elicited long-lasting pLTF, **iii)** neural apnea with concurrent hypoxia does not elicit long-lasting plasticity. **B)** Average data. Neural apnea gave rise to a long-lasting enhancement in phrenic motor output (60min: 54.3 ± 7.8%; n = 8; $p < 0.001$). Hypoxia gave rise to a long-lasting enhancement in phrenic motor output (60min: 59.9 ± 4.1%; n = 7; $p < 0.001$). Neural apnea with concurrent hypoxia did not significantly affect phrenic motor output (60min: 7.7 ± 6.1%; n = 7; $p = 1.000$). Combined neural apnea with hypoxia was significantly different from average burst amplitude following neural apnea

or hypoxia alone ($p < 0.001$). **C)** Representative phrenic neurogram traces: **i)** mild hypoxia does not elicit pLTF, **ii)** neural apnea with concurrent mild hypoxia elicits iPMF. **D)** Average data. Mild hypoxia neither elicits pLTF (60min: -0.9 ± 2.8%; $n = 8$; $p = 1.000$) nor obstructs expression of iPMF (60min: 66.6 ± 9.4%; $n = 8$; $p < 0.001$). There was no difference in phrenic motor amplitude between the neural apnea and neural apnea with mild hypoxia groups ($p = 0.705$). Data represents mean values ± SEM at 60min post challenge experience. Significant differences from baseline and control are denoted with (*). NS denotes non-significant difference $p > 0.05$.

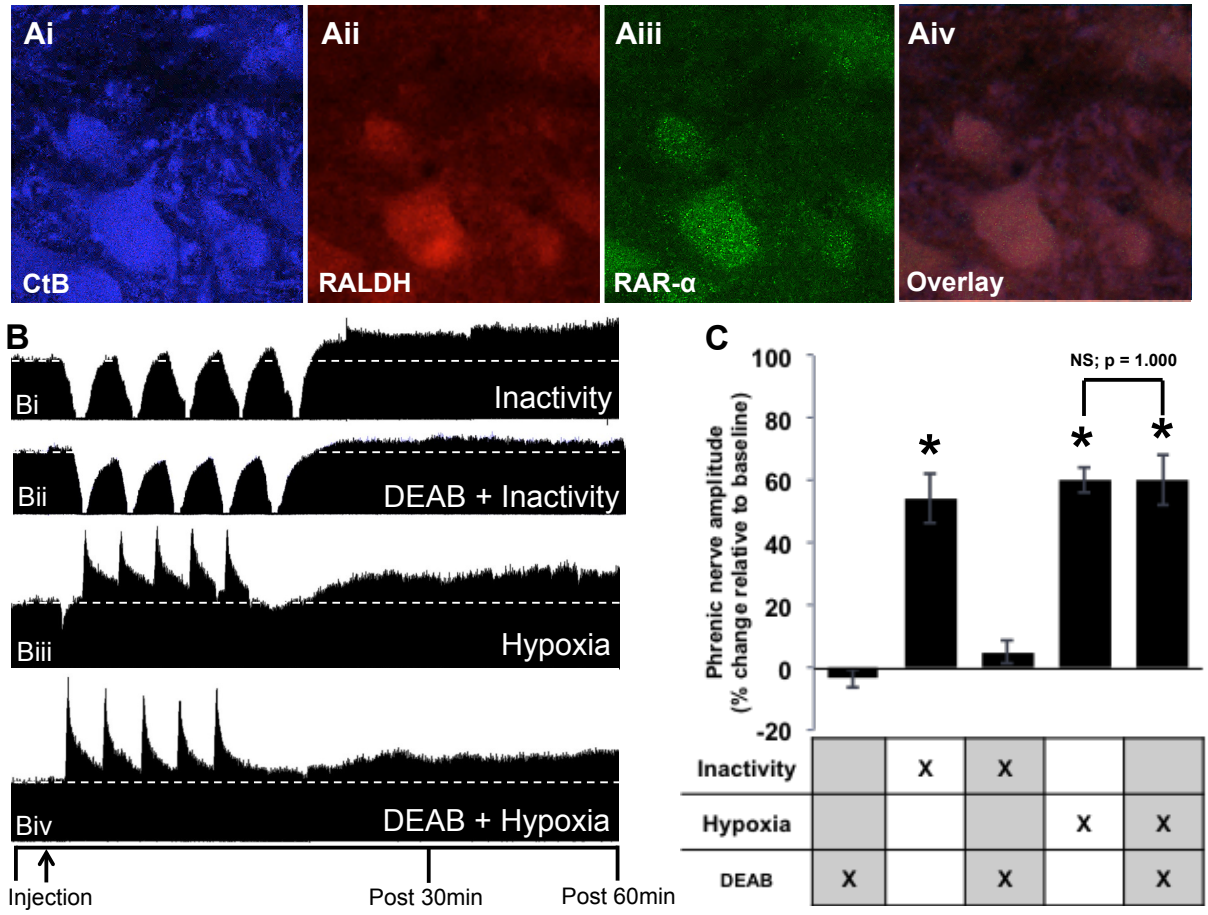

Figure 3. New retinoic acid synthesis is necessary for inactivity-induced, but not hypoxia-induced plasticity. **A)** Representative immunofluorescence images (100x magnification) identifying RALDH and RARα within CtB labeled cervical spinal phrenic motor neurons. Ai) CtB labeled (blue) phrenic respiratory neurons within the cervical spinal cord. Aii) RALDH (red) within cervical spinal cord tissue. Aiii) RARα (green) within cervical spinal cord tissue. Aiv) Overlay of CtB, RALDH, and RARα within cervical spinal cord tissue. **B)** Representative phrenic neurogram traces: **i)** neural apnea elicits iPMF, **ii)** pretreatment with DEAB, a RALDH inhibitor, prevents iPMF, **iii)** hypoxia elicits pLTF, and **iv)** DEAB pretreatment does not effect hypoxia-induced pLTF. **C)** Summary of

DEAB treated groups. DEAB pretreatment abolished iPMF (5.0 ± 3.7%; n = 6; p = 1.000). DEAB pretreated neural apnea group was statistically different from vehicle treated neural apnea control group ($p < 0.001$). Conversely, DEAB pretreatment did not effect hypoxia induced pLTF (60.1 ± 7.9%; n = 7; $p < 0.001$). Comparing DEAB pretreated hypoxia with the hypoxia control group there was no statistical difference (p = 1.000). Data represent mean values ± SEM. Significant differences from baseline and control are denoted with (*). NS denotes non-significant differences $p > 0.05$.

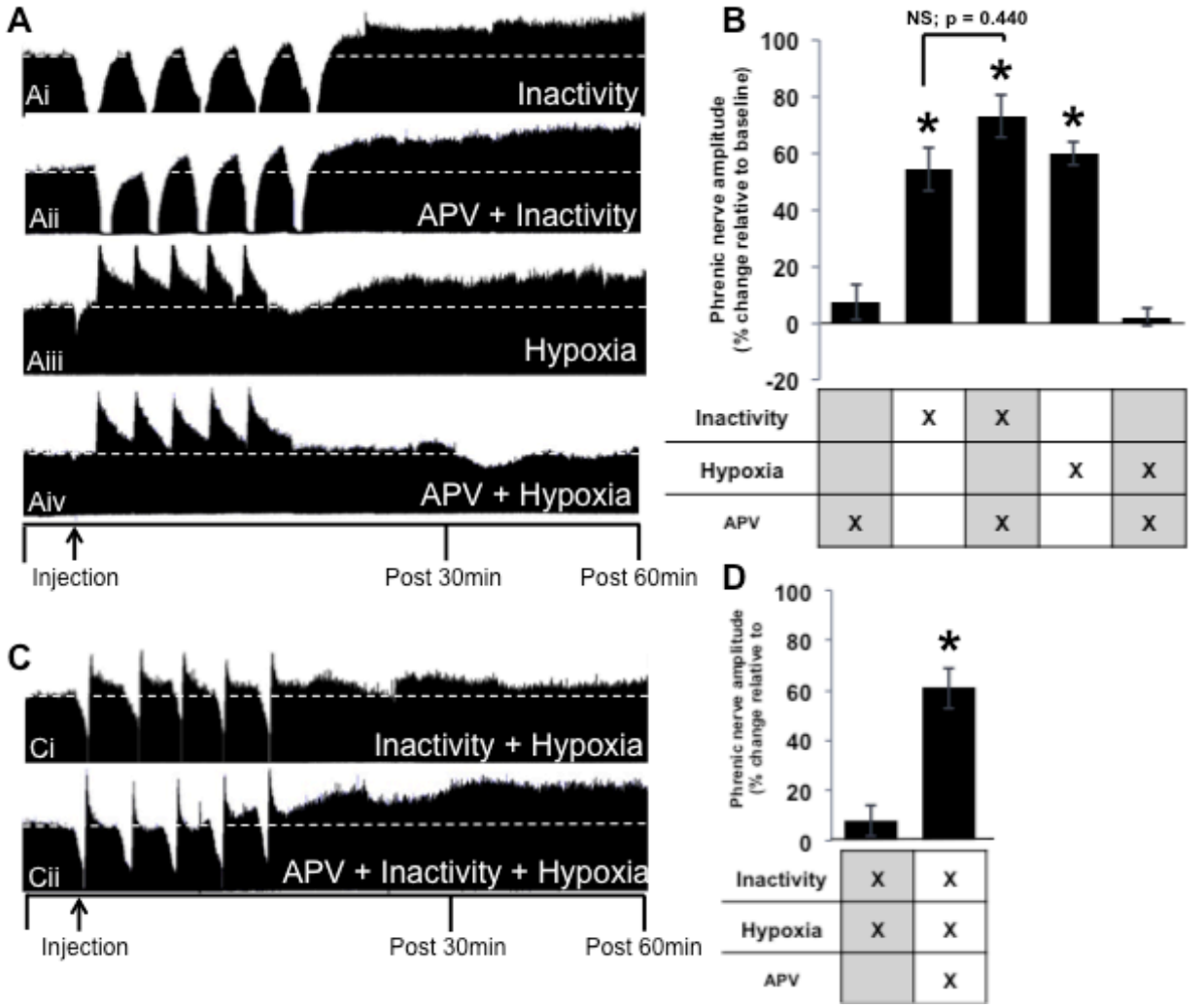

Figure 4. Hypoxia-enhanced NMDA receptors constrain inactivity-induced iPMF. **A)** Representative phrenic neurogram traces: **i)** neural apnea elicits iPMF expression, **ii)** Pretreatment with APV, NMDA receptor inhibitor, does not effect iPMF, **iii)** hypoxia elicits pLTF, and **iv)** pretreatment with APV abolishes pLTF. **B)** Summary of APV treated groups. Pretreatment with APV does not effect iPMF (73 ± 7.6%; n = 8; $p < 0.001$). Comparing APV pretreated neural apnea group with vehicle treated neural apnea control group, there was no significant difference ($p = 0.440$). APV pretreated

hypoxia animals did not express an enhancement in phrenic motor output (2.1 ± 3.1%; n = 8; p = 1.000) Comparing APV pretreated hypoxia group with vehicle treated hypoxia control group, APV pretreatment group was significantly different from hypoxia control (p = 0.001). **C)** Representative neurogram traces: **i)** neural apnea with hypoxia abolishes plasticity expression, and **ii)** APV pretreatment rescues plasticity during neural apnea with hypoxia. **D)** Summary of APV treated groups. Pretreatment with APV rescued plasticity expression in animals experiencing neural apnea with hypoxia (60.9 ± 8.0%; p < 0.001) Data represent mean values ± 1 SEM. Significant differences from baseline and control are denoted with (*). NS denotes non-significant differences p > 0.05.

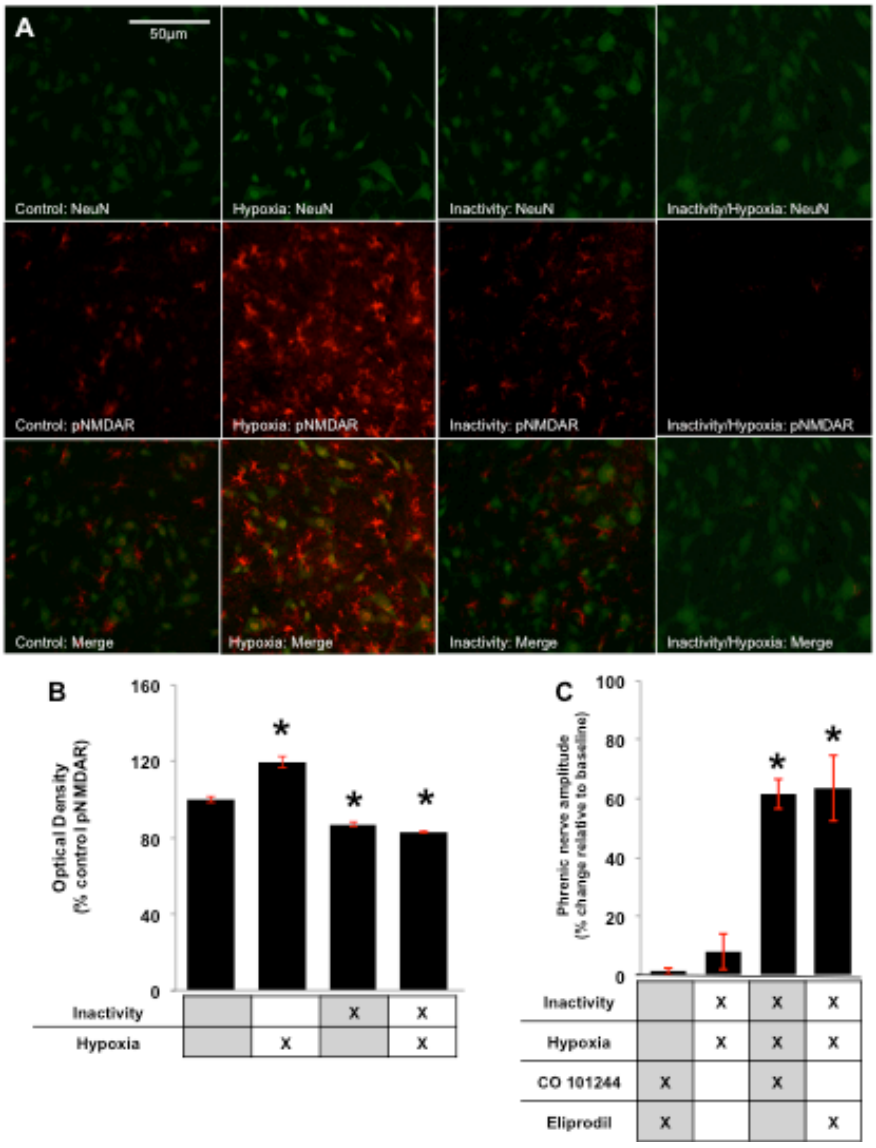

Figure 5. NR2B modulated NMDA receptors constrain inactivity-induced iPMF. **A)** Hypoxia enhanced NR2B phosphorylation of cervical spinal NMDA receptors; control NeuN, control pNMDA, control NeuN – pNMDA overlay, hypoxia NeuN, hypoxia pNMDA, hypoxia NeuN – pNMDA overaly, inactivity NeuN, inactivity pNMDA, inactivity NeuN – pNMDA overaly, inactivity/hypoxia NeuN, inactivity/hypoxia pNMDA, inactivity/hypoxia NeuN – pNMDA overlay. **B)** Summary of pNMDAR optical density.

Density is expressed as precent control values. Intermittent hypoxia leads to an increase in the pNMDAR density in NeuN positive neurons, whereas inactivity leads to an overall reduction in the pNMDAR density. The experience of simltanous inactivity and hypoxia also reduces pNMDAR density. **C)** Summary of NR2B data. Pretreatment with selective NR2B inhibitors Co 101244 (61.6 ± 5.01%; $n = 5$; $p < 0.001$) and eliprodil (63.6 ± 11.2%; $n = 5$; $p < 0.001$) revealed enhanced phrenic motor output following inactivity with hypoxia. When compared to inactivity with hypoxia control groups, both Co 101244 and eliprodil pretreated groups were significantly different ($p < 0.001$). There was no significant difference between CO 101244 and eliprodil pretreated inactivity with hypoxia groups ($p = 1.000$). Data represents mean values ± SEM. Significant differences from baseline and control are denoted with (*****).

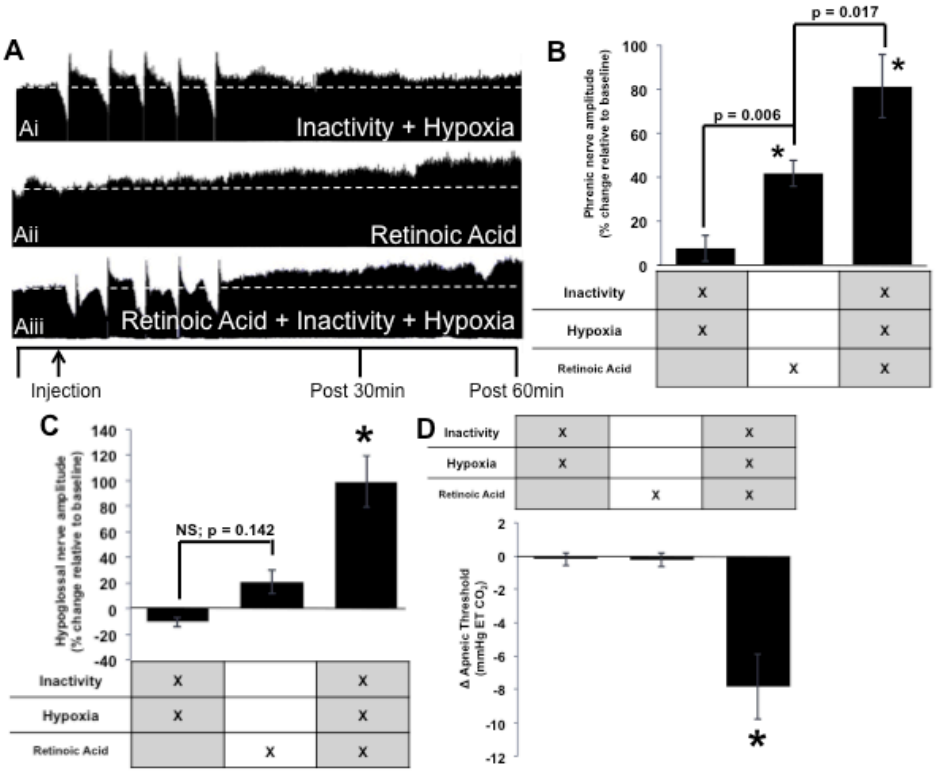

Figure 6. Exogenous retinoic acid reveals phrenic plasticity following reduced respiratory neural activity with concurrent hypoxia. **A)** Representative phrenic neurogram traces: **i)** neural apnea with hypoxia does not elicit a long-lasting change in phrenic motor output, **ii)** exogenous retinoic acid elicits a progressive enhancement in phrenic motor output, and **iii)** exogenous retinoic acid pretreatment prior to neural apnea with hypoxia revealed enhanced phrenic motor output. **B)** Summary of phrenic retinoic acid data. Retinoic acid enhances phrenic motor output in controls (41.6 ± 5.9%; n = 7; $p < 0.001$) and following neural apnea with hypoxia (81.3 ± 14.4%; n = 6; $p < 0.001$). Phrenic motor output was significantly higher in retinoic acid with neural apnea and hypoxia group relative to retinoic acid alone ($p < 0.001$). **C)** Summary of XII retinoic

acid data. Retinoic acid alone does not modulate XII motor output (20.9 ± 9.9%; n = 6; p = 0.142), but does enhance XII motor output following neural apnea with hypoxia (99.0 ± 22.0%; n = 6; p < 0.001). Summary of apneic threshold retinoic acid data. Retinoic acid alone does not modulate apneic threshold (-0.2 ± 0.4; n = 7; p = 1.000), but does reduce apneic threshold following neural apnea with hypoxia (-7.8 ± 1.9mmHg $PaCO_2$; n = 6; p = 0.007). Data represent mean values ± SEM. Significant differences from baseline and control are denoted with (*). NS denotes non-significant differences p > 0.05.

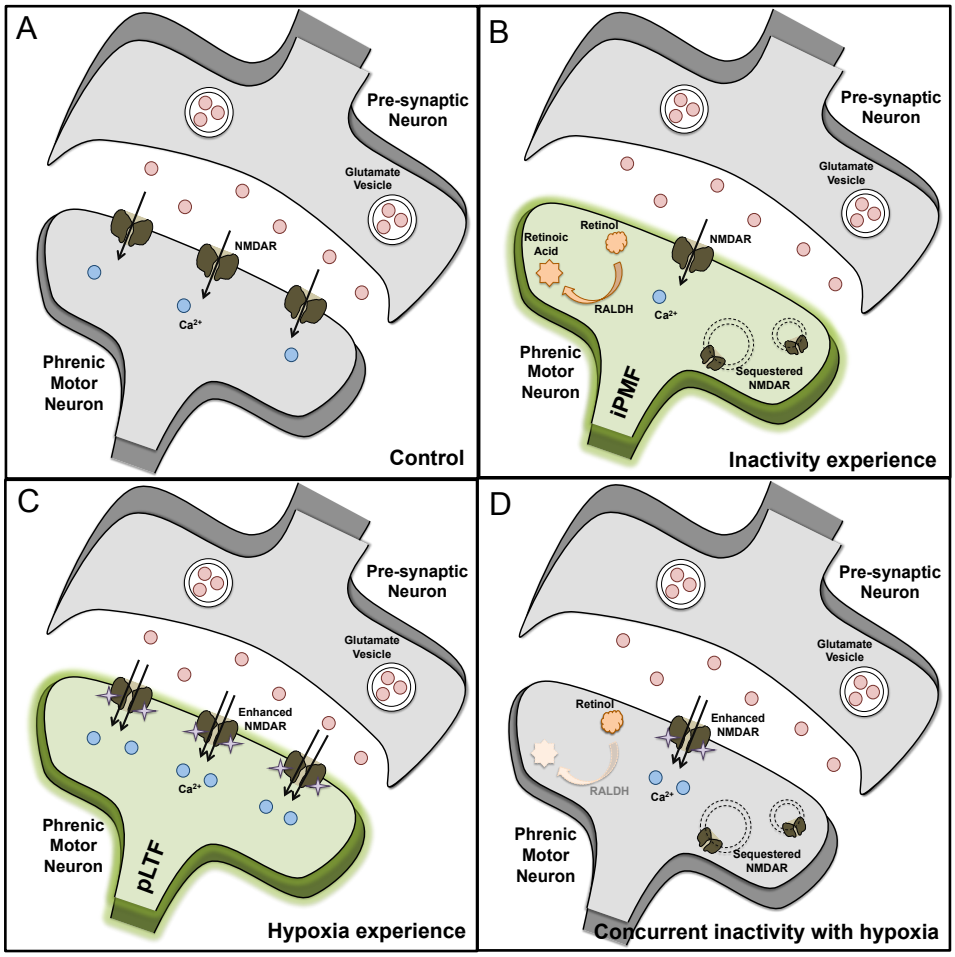

Figure 7. Proposed concurrent hypoxia mediated signaling mechanisms constrain iPMF. **A)** Basal phrenic motor activity is dependent on presynaptic glutamate release, driving cation influx through glutamate sensitive channels (NMDA receptors; NMDAR) on phrenic motor neurons. **B)** Following reduced respiratory neural activity, transient suppression of calcium ion influx decreases NMDAR dependent currents, enabling retinaldehyde dehydrogenase (RALDH) activity to synthesize new retinoic acid from retinol. Through activation of phrenic RARα, RA initiates plasticity. **C)** Hypoxia-induced phosphorylation of NR2B subunits potentiates NMDA currents to enable pLTF. **D)** Concurrent reductions in respiratory neural activity with hypoxia promotes contending

drives to enhance (hypoxia-induced) and blunt (inactivity-induced) NMDAR activity, resulting in a net obstruction of respiratory plasticity.

		Vehicle Control (n = 7)	Inactivity (n = 8)	Hypoxia (n = 7)	Inactivity + Hypoxia (n = 7)	Mild Hypoxia (n = 8)	Inactivity + Mild Hypoxia (n = 8)	DEAB Control (n = 5)	DEAB + Inactivity (n = 6)	DEAB + Hypoxia (n = 7)
Temp (°C)	Baseline	37.4 ± 0.2	37.4 ± 0.1	37.3 ± 0.2	37.3 ± 0.1	37.3 ± 0.1	37.5 ± 0.1	37.4 ± 0.1	37.4 ± 0.1	37.4 ± 0.1
	Post 15min	37.6 ± 0.1	37.6 ± 0.1	37.2 ± 0.2	37.2 ± 0.2	37.4 ± 0.1	37.6 ± 0.1	37.6 ± 0.0	37.4 ± 0.1	37.4 ± 0.1
	Post 30min	37.5 ± 0.1	37.4 ± 0.2	37.3 ± 0.1	37.3 ± 0.1	37.3 ± 0.1	37.7 ± 0.1	37.4 ± 0.2	37.4 ± 0.1	37.4 ± 0.1
	Post 60min	37.4 ± 0.2	37.4 ± 0.2	37.4 ± 0.1	37.4 ± 0.1	37.3 ± 0.1	37.5 ± 0.1	37.3 ± 0.1	37.6 ± 0.1	37.3 ± 0.1
pO2 (mmHg)	Baseline	108.4 ± 2.6	107.7 ± 1.2	100.9 ± 1.7	104.3 ± 2.1	103.0 ± 2.2	105.2 ± 1.2	101.3 ± 2.9	105.2 ± 1.2	106.3 ± 1.5
	Post 15min	108.2 ± 3.7	107.5 ± 2.8	102.0 ± 3.0	102.4 ± 3.5	102.7 ± 1.3	107.2 ± 2.7	98.4 ± 4.3	105.7 ± 3.9	103.5 ± 3.3
	Post 30min	106.9 ± 2.6	109.1 ± 3.6	102.3 ± 1.2	101.1 ± 2.0	102.4 ± 1.6	103.8 ± 1.4	99.2 ± 2.8	102.5 ± 1.9	101.4 ± 2.6
	Post 60min	106.2 ± 2.7	105.4 ± 1.6	96.9 ± 1.9	103.9 ± 1.8	104.3 ± 2.0	104.6 ± 2.5	101.6 ± 3.8	104.1 ± 1.6	103.1 ± 1.5
pCO2 (mmHg)	Baseline	44.7 ± 1.6	45.9 ± 1.7	45.7 ± 1.4	47.7 ± 1.2	44.9 ± 1.2	46.1 ± 1.9	44.2 ± 0.6	45.3 ± 1.7	44.5 ± 2.0
	Post 15min	44.0 ± 1.7	46.6 ± 1.4	45.6 ± 1.3	48.0 ± 1.5	45.3 ± 1.2	46.0 ± 2.3	44.8 ± 0.8	45.0 ± 1.7	44.4 ± 1.7
	Post 30min	45.1 ± 1.3	47.4 ± 1.4	46.6 ± 1.3	47.8 ± 1.1	45.0 ± 1.0	46.2 ± 2.0	45.2 ± 0.6	45.6 ± 1.6	45.6 ± 2.0
	Post 60min	45.0 ± 1.6	47. 7 ± 1.6	46.6 ± 1.5	47.7 ± 1.2	45.0 ± 1.1	46.5 ± 1.8	44.7 ± 0.8	44.6 ± 1.7	44.7 ± 2.0
Frequency (beats / min)	Baseline	50 ± 1	48 ± 2	49 ± 1	50 ± 2	52 ± 2	48 ± 3	•49 ± 1	51 ± 3	52 ± 3
	Post 15min	49 ± 1	50 ± 2	48 ± 1	48 ± 3	53 ± 2	53 ± 2	45 ± 1	51 ± 3	50 ± 3
	Post 30min	50 ± 1	49 ± 2	49 ± 1	49 ± 2	49 ± 1	52 ± 2	*43 ± 1	51 ± 3	52 ± 3
	Post 60min	49 ± 1	48 ± 2	50 ± 2	48 ± 2	48 ± 2	52 ± 3	*42 ± 2	51 ± 3	52 ± 3

		APV Control (n = 5)	APV + Inactivity (n = 8)	APV + Hypoxia (n = 8)	APV + Inactivity + Hypoxia (n = 7)	CO 101244 Control (n = 3)	CO 101244 + Inactivity + Hypoxia (n = 5)	Eliprodil Control (n = 3)	Eliprodil + Inactivity + Hypoxia (n = 5)	Retinoic Acid (n = 7)	Retinoic Acid + Inactivity + Hypoxia (n = 6)
Temp (°C)	Baseline	37.6 ± 0.1	37.5 ± 0.1	37.4 ± 0.1	37.4 ± 0.1	37.1 ± 0.1	37.4 ± 0.2	36.9 ± 0.1	*37.0 ± 0.1	37.5 ± 0.1	37.3 ± 0.2
	Post 15min	37.5 ± 0.1	37.5 ± 0.1	37.5 ± 0.2	37.5 ± 0.2	37.0 ± 0.1	37.6 ± 0.2	37.2 ± 0.0	37.3 ± 0.1	37.4 ± 0.1	37.5 ± 0.1
	Post 30min	37.5 ± 0.1	37.5 ± 0.1	37.6 ± 0.1	37.6 ± 0.1	37.0 ± 0.1	37.5 ± 0.2	37.2 ± 0.0	37.2 ± 0.0	37.4 ± 0.1	37.5 ± 0.0
	Post 60min	37.6 ± 0.1	37.5 ± 0.1	37.6 ± 0.1	37.5 ± 0.2	37.8 ± 0.1	37.4 ± 0.2	37.0 ± 0.0	37.1 ± 0.0	37.5 ± 0.1	37.4 ± 0.1
pO2 (mmHg)	Baseline	103.3 ± 2.4	100.6 ± 1.9	99.1 ± 2.6	100.0 ± 3.1	102.7 ± 1.2	104.7 ± 2.1	107.0 ± 1.7	106.9 ± 2.7	101.6 ± 2.4	108.4 ± 2.7
	Post 15min	*95.7 ± 2.3	99.7 ± 3.0	98.5 ± 2.0	98.0 ± 1.2	98.3 ± 0.6	102.7 ± 1.2	100.6 ± 3.8	102.0 ± 1.8	103.1 ± 2.1	105.4 ± 5.3
	Post 30min	99.2 ± 2.5	102.8 ± 2.3	98.9 ± 1.6	98.5 ± 1.6	105.7 ± 1.8	103.4 ± 2.1	107.0 ± 4.7	102.6 ± 1.9	103.5 ± 2.2	107.6 ± 2.6
	Post 60min	100.9 ± 1.6	102.2 ± 2.2	101.5 ± 2.7	101.9 ± 0.9	103.7 ± 1.7	103.4 ± 2.2	106.7 ± 4.4	108.9 ± 2.9	106.0 ± 2.7	108.0 ± 2.4
pCO2 (mmHg)	Baseline	45.1 ± 1.9	46.2 ± 1.0	43.2 ± 1.0	48.0 ± 1.2	47.7 ± 0.4	47.8 ± 1.4	47.4 ± 1.3	46.8 ± 0.7	44.3 ± 1.0	47.4 ± 1.3
	Post 15min	45.9 ± 1.6	46.6 ± 1.0	43.8 ± 1.2	*50.7 ± 2.1	47.6 ± 1.6	47.8 ± 1.6	48.5 ± 2.1	46.8 ± 0.6	44.2 ± 1.2	47.4 ± 1.2
	Post 30min	45.4 ± 1.9	46.2 ± 0.9	43.8 ± 1.0	48.7 ± 1.3	47.9 ± 0.2	47.8 ± 1.4	47.6 ± 1.1	46.7 ± 0.7	44.3 ± 1.2	47.2 ± 1.3
	Post 60min	45.1 ± 2.0	46.7 ± 1.0	43.2 ± 1.2	48.1 ± 1.2	47.6 ± 0.3	47.5 ± 1.2	47.2 ± 1.0	47.2 ± 0.9	44.2 ± 1.2	46.8 ± 1.4
Frequency (beats / min)	Baseline	•54 ± 2	50 ± 2	51 ± 2	49 ± 1	46 ± 4	48 ± 1	52 ± 3	52 ± 2	51 ± 2	52 ± 2
	Post 15min	48 ± 2	52 ± 2	48 ± 2	53 ± 1	46 ± 3	51 ± 2	51 ± 2	53 ± 1	51 ± 2	54 ± 2
	Post 30min	48 ± 2	50 ± 2	48 ± 2	51 ± 2	45 ± 3	52 ± 2	49 ± 3	54 ± 0	52 ± 2	54 ± 2
	Post 60min	47 ± 2	52 ± 2	46 ± 2	51 ± 2	*42 ± 4	52 ± 3	51 ± 2	55 ± 1	53 ± 2	54 ± 1

Supplemental Table 1. Physiology variables. There were no consistent differences of temperature (Temp), PaCO2, PaO2, or neural firing frequency within any individual group or among different groups. Differences between groups at a given time point (*) and differences from baseline within individual groups (#) are denoted within the table; $p < 0.05$. Values expressed as means ± SEM.

	Apnea Duration (seconds)	% O_2 Saturation	Estimated PaO_2
Inactivity	82	-	-
Inactivity/Hypoxia (25 sec)	14	71	38mmHg
Inactivity/Hypoxia (6 sec)	13	87.5	56mmHg

Supplemental Table 2. Comparable apnea duration and oxygen saturation levels. Apnea episodes averaged 82sec. Coupling neural apnea with hypoxia or mild hypoxia reduced the apnea duration to 14 and 13sec respectively. Oxygen desaturation for hypoxia group were 71% (~38mmHg). Conversely desaturation for mild hypoxia group was 87.5% (56mmHg). Desaturation for hypoxia and mild hypoxia group was significantly lower than the 98% baseline oxygen saturation ($p = 0.018$)