Mechanisms of respiratory neuroplasticity elicited by

reduced respiratory neural activity

By

Kristi Ann Strey

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Comparative Biomedical Science)

at the

UNIVERSITY OF WISCONSIN-MADISON

2013

Date of final oral examination: 9/30/2013

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

Tracy L. Baker-Herman, Assistant Professor, Comparative Biosciences
Gordon S. Mitchell, Professor and Department Chair, Comparative Biosciences
Jyoti J. Watters, Associate Professor, Comparative Biosciences
Becky A. Johnson, Clinical Associate Professor, Surgical Sciences
Jerry C. Yin, Professor, Genetics

Table of Contents

Acknowledgements……...……..……...ii

Abstract……...…….……..iii

I. Introduction…….....1

II. Spinal atypical protein kinase C activity is necessary to stabilize inactivity-induced phrenic motor facilitation……....43

Figures……...75

III. Decreased spinal synaptic inputs to phrenic motor neurons elicit localized inactivity-induced phrenic motor facilitation……..…….….82

Figures……...……..109

IV. Inactivity-induced phrenic motor facilitation is differentially regulated among Sprague-Dawley rat sub-strains……...……..……..…..…....116

Figures……...……..147

V. Discussion…….……..……156

Appendix A: Inactivity-induced respiratory plasticity: Protecting the drive to breathe in disorders that reduce respiratory neural activity…...……...….……..…...170

Figures……...……..210

ACKNOWLEDGEMENTS

Throughout my graduate studies, many people have helped me complete this thesis that I want to thank. First of all, I want to thank my mentor Dr. Tracy Baker-Herman. Four years ago we started a journey at UW-Madison together and now that journey (at least for me) comes to an end. I want to thank you for your motivation, guidance, support and most of all, for believing in me. You're truly an amazing person, mentor and scientist and without you I couldn't have made it this far. I'd also like to thank Dr. Gordon Mitchell, for introducing me to Dr. Tracy Baker-Herman many years ago, I'm so thankful for our serendipitous encounter. To my committee members Dr. Gordon Mitchell, Dr. Jyoti Watters, Dr. Becky Johnson and Dr. Jerry Yin, thank you for your time and ideas and for challenging me, you have made me a stronger person and a better scientist. To my colleagues in the Baker-Herman Lab, Mitchell Lab, Watters Lab and Johnson Lab that I've had the pleasure working with, thank you for making my time as a graduate student filled with fun times, great scientific debates and for the memories and friendships we have made. Also, I'd like to thank Bradley Wathen, an amazing technician, who always has gone above and beyond to help with everything and Kalen Nichols for her devotion and time in keeping the ABL in working order. I'd also like to thank Dr. Peter Lipton for allowing me to be the TA for his cellular mechanisms of memory course. To my wonderful fiancé Todd, who has been by my side from the beginning, thanks for being my greatest supporter and encouraging me when I needed it the most, especially in these last few months; I'm so excited to start our next chapter together. One final thank you to my parents, Mike and Cindy who have supported and guided me along my journey as a graduate student, your love and support has undoubtedly helped me get this far.

ABSTRACT

One mechanism that confers flexibility to the respiratory control system to enable system wide adaptations is respiratory neuroplasticity. In ventilated Harlan Sprague-Dawley (HSD) rats, central neural apnea elicits a TNFα-dependent form of plasticity called inactivity-induced phrenic motor facilitation (iPMF); a long-lasting (60 min) rebound increase in phrenic burst amplitude. Mechanisms downstream of TNFα may involve atypical PKC (aPKC) isoforms since exogenous application of spinal TNFα induces an aPKC-dependent phrenic facilitation. The first study in this thesis tested the hypothesis that aPKC is necessary for iPMF by delivering an aPKC inhibitor to spinal regions containing phrenic motor neurons before, 10 or 45 min after central neural apnea. We show that iPMF has two mechanistically distinct phases: 1) an early, labile phase that requires spinal aPKC activity to transition to, 2) a long-lasting aPKC-independent phase. Further, we demonstrate that the relevant isoforms necessary for iPMF are PKCζ and/or PKCι/λ. The second study tested two key questions: does reduced neural activity *per se* elicit iPMF and where in the CNS is reduced neural activity sensed? We reversibly blocked axon conduction in descending axons driving ipsilateral phrenic motor neurons with intraspinal C2 injections of procaine. Following axon conduction recovery, long-lasting iPMF is expressed ipsilateral to reduced phrenic synaptic inputs and a novel form of plasticity was revealed in the contralateral phrenic nerve that did not receive reduced synaptic inputs, which we termed crossed spinal phrenic motor facilitation (csPMF). Ipsilateral iPMF, but not contralateral csPMF requires spinal TNFα and aPKC signaling. The final study presented in this thesis shows that Charles River Sprague-Dawley (CRSD) rats express a transient (*versus* long-lasting) iPMF. We show that spinal NMDA receptor activation inhibits long-lasting iPMF by constraining spinal TNFα and aPKC-dependent signaling, indicating that iPMF duration in CRSD rats is regulated by spinal regulatory mechanisms. Collectively, this

dissertation describes a fundamental property of respiratory control: reduced respiratory neural activity elicits a local form of plasticity near phrenic motor nucleus (iPMF) and identifies cellular mechanisms giving rise to iPMF. We hypothesize that iPMF may be one mechanism to assure neural activity is maintained in respiratory motor neurons throughout life.

CHAPTER I

INTRODUCTION

Breathing is a fundamental and essential motor behavior. From birth until death, respiratory motor neurons must rhythmically activate respiratory musculature to produce an adequate breath; thus, neural activity within respiratory motor neurons is critical for life. In addition, respiratory neural output must remain flexible in order to adapt to physiological changes including growth, development, exercise and changes in arousal state or posture. This neural output must also be coordinated with other non-ventilatory behaviors that utilize the upper airway such as swallowing, coughing and vocalization. To assure ventilation does not cease, the respiratory control system utilizes multiple strategies including feedback, feedforward and adaptive control (i.e. respiratory plasticity) mechanisms.

Respiratory plasticity refers to a persistent change in system performance based on a prior experience (Mitchell and Johnson, 2003). In recent years, respiratory plasticity has been the focus of much research and has challenged the previous perception that the respiratory control system is rigid and unmodifiable. It is now generally accepted that the respiratory control system expresses a great deal of plasticity in response to many diverse stimuli (Mitchell and Johnson, 2003). While most forms of respiratory plasticity require neuromodulators for their induction and/or maintenance (for review Feldman et al., 2003; Mitchell and Johnson, 2003; Dale-Nagle et al., 2010), activity-dependent forms of respiratory plasticity following increases (McCrimmon et al., 1997; Zhou et al., 1997; Johnson and Mitchell, 2002) and decreases in activity have been reported (Castro-Moure and Goshgarian, 1996, 1997; Zhang et al., 2003). Recently, we described a novel TNFα-dependent form of respiratory plasticity in ventilated Harlan Sprague-Dawley (HSD) rats elicited by reduced respiratory neural activity called inactivity-induced motor facilitation (iPMF; Mahamed et al., 2011; Broytman et al., 2013), a long-lasting (60 min) rebound increase in phrenic burst amplitude. Although activity-dependent mechanisms may

allow respiratory neurons to adapt to persistent increases and decreases in neural activity, we are particularly interested in those activated following decreased neural activity, since a lack of respiratory neural activity imposes a critical risk to life.

Since plasticity is elicited based on prior experience, a logical question is: when would organisms experience reductions in respiratory neural drive? Although breathing is a remarkably dependable behavior, various conditions during health and disease are accompanied by reduced respiratory neural activity. For example, healthy individuals experience reductions in respiratory neural output that may lead to central apnea during behaviors such as sleep (Uliel et al., 2004; Javaheri and Dempsey, 2013), voluntary diving (Gooden, 1994; Dutschmann and Paton, 2002) or at high altitudes (Berssenbrugge et al., 1984). Periods of reduced or absent respiratory motor output, may accompany a variety of other conditions, such as prematurity (Gaultier and Gallego, 2005) and aging (Ancoli-Israel et al., 1987), or may be a secondary consequence of various pathologies, including genetic disorders (Goridis et al., 2010), neurodegenerative diseases (Gaig and Iranzo, 2012) or heart failure (Yumino and Bradley, 2008). Finally, reduced respiratory neural activity is often experienced following spinal cord injury (Strakowski et al., 2007) and by patients requiring ventilatory support (Tobin, 2001; Epstein, 2011). A discussion of the situations accompanied by reduced respiratory neural activity and the role iPMF may play in these conditions has recently been reviewed (Strey et al., 2013) and is included in Appendix A to provide structural context to the importance of maintaining respiratory neural activity.

The overarching question guiding this dissertation is: ***How do respiratory motor neurons respond to reduced respiratory neural activity*****?** We addressed this fundamental question by investigating the response of phrenic motor neurons to reduced respiratory neural activity. Since phrenic motor neurons drive the main inspiratory muscle in mammals (i.e. the diaphragm),

activity in these motor neurons is critical to live. I will begin by describing the respiratory circuit responsible for generating the basic neural drive to breathe, with a focus on inspiratory neural drive and outline the pathways that transmit excitatory neural drive to the phrenic motor circuit. I will then discuss two strategies that maintain respiratory homeostasis, chemoreceptor mediated feedback and respiratory plasticity. I will introduce the form of respiratory plasticity studied in this dissertation called inactivity-induced phrenic motor facilitation (iPMF) and review what is currently known about this novel form of plasticity. I will transition away from the respiratory control system and introduce an activity-dependent form of plasticity observed within the brain and spinal cord thought to be necessary to maintain stable neural output in response to changes in neural activity called homeostatic plasticity. This will be followed by a discussion of three components relevant to our signaling cascade and their role in neuroplasticity: NMDA receptors (NMDARs), tumor necrosis factor alpha (TNFα) and atypical protein kinase C (aPKC) isoforms. Finally, I will summarize my dissertation aims, highlighting the key findings of each study.

Respiratory control system

In mammals, eupnic breathing is divided into three phases: inspiration, post-inspiration and expiration (Richter and Spyer, 2001; Hilaire and Pasaro, 2003). These phases are generated by brainstem respiratory neurons that are active during discrete temporal windows relative to the breathing cycle. Here I discuss the generation of respiratory pattern and rhythm with an emphasis on the production of inspiratory neural drive. I will also describe the pathways supplying respiratory neural drive to the phrenic motor neurons and key features of the phrenic motor nucleus relevant to this dissertation.

Central pattern and rhythm generation

The mammalian respiratory central pattern generator (CPG) is located within the caudal portion of the brainstem, spanning the pons and the medulla. The respiratory CPG contains a neural network that intrinsically generates rhythmic respiratory neural drive (Marder and Calabrese, 1996). After decades of research, it is generally agreed upon that the key site responsible for inspiratory breathing rhythmicity is the pre-Bötzinger Complex located within the medulla (pre-BötC) (Smith et al., 1991; Rekling and Feldman, 1998; Hilaire and Duron, 1999; Gray et al., 2001; Vandam et al., 2008; Okada et al., 2012). Indeed, rhythmic respiratory activity in phase with hypoglossal motor output is detected in slices of the ventral medulla containing the pre-BötC (Smith et al., 1991). Further, conditional inactivation of select pre-BötC neurons (expressing somatostatin) in adult rats eliminates rhythmic inspiratory neural activity (Tan et al., 2008), strongly suggesting that the pre-BötC is required for normal respiratory rhythm generation *in vivo*. Located slightly rostral to the pre-BötC, the Bötzinger Complex (BötC) predominately contains expiratory neurons (Jiang and Lipski, 1990; Ezure et al., 2003a; Ezure et al., 2003b), thought to inhibit inspiratory motor neurons during expiration to facilitate phase alternation.

Two main hypotheses have been put forth regarding the mechanism of inspiratory rhythm generation: 1) the pace-maker hypothesis (Feldman and Smith, 1989), and 2) dual-oscillator or reciprocal inhibition hypothesis (Burns, 1963; Richter et al., 1992). The first hypothesis suggests that a population of excitatory interneurons within the pre-BötC have “pace-maker” like properties which gives rise to a basic inspiratory rhythm (Feldman et al., 2013). This “kernel” of intrinsic cells is embedded in a network of inhibitory and/or excitatory follower cells which are necessary for the completion of the rhythm (Smith et al., 2000). The second hypothesis proposes

that mutually inhibitory neurons within the pre-BötC and the BötC active during early inspiration and post-inspiration/expiration respectively, form an inhibitory "ring" to generate rhythmic activity (Janczewski et al., 2013). New evidence suggests that reciprocal inhibition between preBötC and BötC is not required for respiratory rhythm generation in adult (anesthetized) mammals (Janczewski et al., 2013). Instead, inhibition may play a key role in shaping respiratory pattern and frequency (Janczewski et al., 2013). However, future studies are necessary to fully understand respiratory rhythm generation in adult animals during wakefulness.

Axons from the pre-BötC and BötC, project caudally to respiratory pre-motor neurons distributed in two bilateral columns in the medulla: the dorsal and ventral respiratory groups (DRG and VRG respectively). The DRG contains bulbospinal inspiratory neurons that innervate phrenic and intercostal motor neurons. The relative contribution of excitatory input during inspiration from DRG pre-motor neurons appears to be species specific since inspiratory neural drive to phrenic motor neurons is observed in cats (Dick et al., 1988), but not in rats (Tian and Duffin, 1998). The VRG is divided into two anatomically distinct segments based on neuronal function. The rostral portion of the VRG (rVRG) contains inspiratory pre-motor neurons that receive excitatory synaptic inputs from the pre-BötC during inspiration and inhibition during expiration (Ellenberger and Feldman, 1990; Ellenberger et al., 1990; Smith et al., 2007). The caudal section of the VRG (cVRG) is mainly composed of expiratory pre-motor neurons innervated by BötC neurons. Respiratory motor drive from VRG pre-motor neurons is transmitted to respiratory motor neurons found within the brainstem and spinal cord; projections to the phrenic motor nucleus are considered below.

Phrenic motor circuit

In mammals, the diaphragm is the main inspiratory muscle. The diaphragm receives excitatory input from phrenic motor neurons distributed along two longitudinal columns within the ventral cervical spinal cord extending from ~C3-C7, depending on the species. For example, the phrenic motor nucleus is distributed from: C3-C6 in the mouse (Qiu et al., 2010), rat (Goshgarian and Rafols, 1984) and rabbit (Ullah, 1978), C4-C7 in the guinea pig (Johnson and Getting, 1988) and C4-C6 in the cat (Berger et al., 1984). In the rat, the majority of phrenic motor neurons are found at C4 and C5 (Goshgarian and Rafols, 1981). Dendritic arbors of phrenic motor neurons run in a rostrocaudal direction within the phrenic column in a "bundled" fashion (Berger et al., 1984). Although, in neonates dendrites also extend out of the phrenic column and cross the midline to the contralateral phrenic motor pool; the number of contralateral dendritic projections generally decrease with age (Prakash et al., 2000).

Phrenic motor neurons receive three main types of synaptic inputs: medullary (i.e. bulbospinal; Ellenberger et al., 1990), cortical (i.e. corticospinal; Butler, 2007) and cervical spinal inputs from neighboring interneurons (Lee and Fuller, 2011). Bulbospinal inputs from the rVRG are the main source of inspiratory neural drive and are mainly monosynaptic projections (Ellenberger et al., 1990). rVRG pre-motor neurons (in rats) mainly innervate ipsilateral phrenic motor neurons, although a small number of fibers decussate in the brainstem and/or spinal cord and innervate contralateral phrenic motor neurons (Ellenberger et al., 1990; Tian and Duffin, 1998; Duffin and Li, 2006). Anterograde tracing studies suggest that VRG axons travel to the phrenic motor nucleus *via* ventrolateral and ventromedial funiculi (Feldman et al., 1985; Lipski et al., 1994). The ventrolateral funiculus primarily contains axons traveling to the ipsilateral phrenic motor pool while projections crossing to the contralateral phrenic motor pool are located

in ventromedial tracts (Lipski et al., 1994). Bulbospinal axons to phrenic motor neurons crossing the midline are referred to as "crossed spinal phrenic pathways" and are generally thought to be latent pathways under "normal" circumstances (Goshgarian and Rafols, 1984; Goshgarian et al., 1989; Sperry and Goshgarian, 1993), but can become functionally active following certain respiratory stressors (Goshgarian, 2003). More recent studies suggest that polysynaptic pathways to the phrenic motor nucleus are also present (Lane et al., 2008). Polysynaptic pathways from bulbospinal neurons to the phrenic motor pool involve propriospinal interneurons of two neuronal populations: 1) renshaw cells and 2) pre-phrenic interneurons (Lee and Fuller, 2011). Although the functional contribution of interneurons to eupnic breathing is still unclear, it is postulated that they modulate supraspinal input to lower motor neurons, integrate multiple neural circuits (e.g., phrenic and intercostal) and contribute to respiratory neural plasticity (Lane, 2011). In contrast to inputs that supply "automatic" respiratory neural drive, corticospinal neurons mediate voluntary breathing and coordinate the myriad of tasks that must be integrated with breathing like vocalization, breath holding and whistling (Butler, 2007).

Strategies that maintain ventilation

The respiratory control system must respond to acute and chronic physiological situations like exercise, sleep, aging, pregnancy, gain or loss of weight and pathophysiological conditions like central sleep apnea and neurodegenerative disorders. In order to maintain adequate ventilation, yet respond to physiological or pathophysiological changes, the respiratory control system uses multiple strategies including feedback, feedforward and adaptive control (plasticity) mechanisms (Feldman et al., 2003). Chemoreceptor mediated feedback and respiratory plasticity

are particularly important to studies presented in this dissertation and are therefore discussed below.

Feedback: Chemoreception

Arterial CO_2 and O_2 must be maintained within a narrow physiological range. Unlike ventilatory responses to changes in O_2, responses to CO_2 are induced by comparatively smaller changes in $PaCO_2$ and are made at the expense of PaO_2 levels (Haldane and Priestley, 1905). During wakefulness, breathing is generally a stable motor behavior and arterial blood gases are maintained within this narrow physiological range. However, during sleep the "wakefulness" drive to breathe is lost exposing a CO_2 dependent "apneic threshold" (Dempsey, 2005). This apneic threshold is defined as the point at which respiratory neural activity ceases. During sleep, even small reductions in $PaCO_2$ (2-5mmHg) can lead to temporary pauses in inspiratory efforts resulting in central neural apneas (Skatrud and Dempsey, 1983). Rhythmic inspiration resumes once $PaCO_2$ rises sufficiently above the level observed during eupnic breathing. The resumption of respiratory neural drive following neural apnea is referred to as the recruitment threshold.

Feedback from central and peripheral chemoreceptors in response to changes in cerebral spinal fluid and/or blood O_2, CO_2 and pH alter ventilation to maintain blood gas homeostasis. Peripheral chemoreceptors found in the carotid bodies function as the main O_2 sensor, but also respond to changes in CO_2 and pH (Lahiri and Forster, 2003; Prabhakar and Peng, 2004; Forster and Smith, 2010). Central CO_2 chemoreceptors detect changes in CO_2/H^+/pH and are found in nuclei throughout the brainstem including the retrotrapezoid nucleus (RTN; Guyenet et al., 2008), the medullary raphé (Hodges and Richerson, 2010), the locus coeruleus (LC; Gargaglioni

et al., 2010), the nucleus tractus solitarius (NTS; Nichols et al., 2008), the lateral hypothalamus and the caudal ventrolateral medulla (Nattie, 2011).

An elegant study conducted by Smith and colleagues determined the relative contribution of central *versus* peripheral chemoreceptors to CO_2 induced ventilatory responses in unanesthetized and intact dogs (Smith et al., 2006). Specifically, the carotid sinus was perfused to maintain eupnic $PaCO_2$/pH values constant while inspired CO_2 was increased in a step wise fashion; thereby, the contribution of central chemoreceptor input to the CO_2-induced ventilatory response could be determined. Results from this study demonstrated that although central chemoreceptors provide a greater percentage (~68%) of the CO_2-induced ventilatory response, carotid body chemoreceptors allow for a more rapid ventilatory response (Smith et al., 2006). The relative contribution of each central chemoreceptive nuclei to the CO_2 ventilatory response are largely state dependent (Nattie, 2000). For example, focal acidification of the medullary raphé has no effect during wakefulness, but results in a 15-20% increase in ventilation and frequency during sleep (Nattie and Li, 2001). This is in contrast to other central chemoreceptor regions like the RTN which results in increased ventilation during wakefulness, but not sleep following a similar focal acidification (Li et al., 1999).

Adaptive control: respiratory plasticity

Respiratory plasticity is defined as a persistent change in system performance based on a prior experience (Mitchell and Johnson, 2003). A variety of forms of respiratory plasticity have been described in response to a myriad of stimuli including: hypoxia (Fuller et al., 2000; Devinney et al., 2013), hypercapnia (Baker et al., 2001; Bavis et al., 2006), exercise (Bach et al., 1993; Babb et al., 2010), increased respiratory activity (Johnson and Mitchell, 2002; Kline et al.,

2007), carotid body denervation (Forster, 2003; Prabhakar, 2011) and decreased respiratory activity (Castro-Moure and Goshgarian, 1996; Prakash et al., 1999; Mahamed et al., 2011). It is postulated that plasticity may allow respiratory motor output to adapt to acute or chronic changes while maintaining adequate ventilation (Feldman et al., 2003; Mitchell and Johnson, 2003).

The most studied form of respiratory plasticity was originally described by Millhorn and colleagues and is called phrenic long-term facilitation (pLTF; Millhorn et al., 1980b, a). pLTF is induced *via* direct carotid nerve stimulation (Millhorn et al., 1980b, a; Fregosi and Mitchell, 1994) and acute intermittent hypoxia (Baker and Mitchell, 2000; Mitchell et al., 2001; Bavis and Mitchell, 2003; Wilkerson et al., 2007; MacFarlane and Mitchell, 2008; Hoffman et al., 2012; Nichols et al., 2012; Pavlinac Dodig et al., 2012; Devinney et al., 2013) and is a progressive and long-lasting (60min) increase in phrenic burst amplitude. Long-term facilitation is elicited in multiple respiratory-related signals including hypoglossal and intercostal motor output (hLTF and iLTF respectively; Baker-Herman and Strey, 2011). Although LTF is most commonly observed in anesthetized animals, ventilatory LTF is elicited in during sleep (Terada et al., 2008; Terada and Mitchell, 2011) and wakefulness (Turner and Mitchell, 1997; Olson et al., 2001).

Many forms of respiratory plasticity, including pLTF, are induced by neuromodulators (Mitchell and Johnson, 2003) and may be independent of increased activity (Bocchiaro and Feldman, 2004; MacFarlane and Mitchell, 2009). However, increases in neural activity during chronic intermittent hypoxia can induce activity dependent decreases in excitatory neurotransmission to NTS neurons (Kline et al., 2007). Similarly, decreases in respiratory neural activity lead to compensatory increase in respiratory neural output. Although many early reports provided anecdotal evidence (Webber and Pleschka, 1984; Budzińska et al., 1985; Martin et al., 1994), Castro-Moure and Goshgarian published one of the first studies demonstrating that a

reversible reduction in phrenic synaptic inputs results in an increase in respiratory motor output (Castro-Moure and Goshgarian, 1996). A subsequent study suggested that this compensatory increase was due (at least in part) to central neural mechanisms, since reduced phrenic synaptic inputs elicited morphological plasticity within the phrenic motor nucleus (Castro-Moure and Goshgarian, 1997). However, chemoreflex induced modulation of respiratory neural activity could not be ruled out since rats were not ventilated during reduced phrenic synaptic inputs. Further, the underlying cellular mechanisms giving rise this compensation were unknown.

Inactivity-induced phrenic motor facilitation (iPMF)

To better understand the long-lasting impact of reduced respiratory neural activity on phrenic motor output, we exposed anesthetized and ventilated rats to a 30 min central neural apnea using three methods: 1) hypocapnia, 2) isoflurane and, 3) high frequency vagal stimulation (Mahamed et al., 2011). Although these stimuli induce central neural apnea, each stimulus manipulates respiratory neural activity indirectly by altering other physiological parameters, which could conceivably have led to the observed increase phrenic burst amplitude. Hypocapnia induced respiratory depression causes moderate brain hypocapnia/alkalosis, reduced cerebral perfusion and decreased oxygen unloading within tissues including the brain (Vogel et al., 1996; Brian, 1998; Nwaigwe et al., 2000); isoflurane increases excitability of brainstem respiratory neurons (Doi et al., 1989; Stuth et al., 1992) and motor neurons (Rampil and King, 1996; Antognini et al., 1999; Brandes et al., 2007); and high frequency vagal stimulation results in enhanced serotonin release (Manta et al., 2009) and increased activation of the high affinity BDNF receptor TrkB (Furmaga et al., 2012). Similar to early anecdotal literature reports, once respiratory neural activity was restored, phrenic motor output was enhanced. Although the mode

of action for each stimulus was different, all treatments reversibly reduced central respiratory neural activity; therefore, we hypothesized that reduced respiratory neural activity rather than unintended effects associated with each stimulus gave rise to increased respiratory motor output. We termed this form of plasticity inactivity-induced phrenic motor facilitation (iPMF; Mahamed et al., 2011). iPMF is a long-lasting (60 min) rebound increase in respiratory burst amplitude following the resumption of respiratory neural activity.

Preliminary data suggest that following iPMF expression, the cytokine tumor necrosis factor alpha (TNFα) is increased in ventral spinal homogenates containing the phrenic motor nucleus (Broytman and Baker-Herman, unpublished data). A subsequent report demonstrated that spinal TNFα is necessary for late, but not early iPMF (Broytman et al., 2013). Consistent with this report, exogenous spinal TNFα (in absence of neural apnea) gives rise to phrenic motor facilitation (i.e. PMF), suggesting TNFα is sufficient to increase phrenic burst amplitude (Broytman et al., 2013). Interestingly, TNFα induced PMF requires spinal atypical protein kinase C (aPKC) activity, indicating aPKC isoforms may be a key downstream target necessary for increased phrenic burst amplitude. Critical questions regarding the response to reduced respiratory neural activity were left unanswered from these studies: 1) Are atypical PKC isoforms necessary for iPMF (*Chapter 2*)? 2) is iPMF induced by reduced neural activity *per se (Chapter 3)*? 3) Where in the CNS is activity sensed and responded to (*Chapter 3*)? 4) Is long-lasting iPMF expressed by all rat sub-strains? (*Chapter 4*)? 5) What is the physiological or pathophysiological relevance of iPMF (*Appendix A*)?

Homeostatic plasticity

Neural networks must produce a stable neural output throughout life. In a seminal study, Liu and Tsien showed that hippocampal neuron innervation density is inversely related to the amplitude of the excitatory post-synaptic currents (EPSCs), such that neurons receiving many synaptic inputs generate smaller EPSCs and those receiving fewer synaptic inputs have substantially larger EPSCs (Liu and Tsien, 1995). This compensatory response to altered activity has been coined "homeostatic plasticity" necessary to counterbalance prolonged increases or decreases in neuronal activity by altering neuronal synaptic strength to maintain stable network output (Turrigiano et al., 1998) and is thought to be a fundamental property of central circuits (Turrigiano and Nelson, 2004; Turrigiano, 2008). Indeed, homeostatic plasticity has been documented in a variety of neural networks including hippocampal (O'Brien et al., 1998; van Welie et al., 2004; Hou et al., 2008; Sutton, 2010), cortical (Turrigiano, 1999; Desai et al., 2002; Nelson et al., 2002; Benucci et al., 2013) and spinal circuits (Galante et al., 2001; Gonzalez-Islas et al., 2010; Garcia-Bereguiain et al., 2013).

In order to be an effective mechanism, homeostatic plasticity must have fine spatial and temporal resolution to adjust and maintain neural activity within functional constraints of a given system. Indeed, following increases and/or decreases in activity many neuronal properties can be altered including: voltage-dependent conductance or "intrinsic excitability" (Desai et al., 1999b; Zhang and Linden, 2003; Gonzalez-Islas et al., 2010), inhibitory and/or excitatory synaptic strength (Turrigiano and Nelson, 2004; Shepherd et al., 2006; Gainey et al., 2009; Garcia-Bereguiain et al., 2013) or neuromodulatory inputs (Queenan et al., 2012). Intrinsic excitability refers to the ability of a neuron to generate an action potential in response to a given input and is a function of the properties, kinetics, distribution and abundance of membrane bound ion

channels (e.g., sodium channels, L-type calcium channels, potassium channels, etc.). The cellular mechanisms of enhanced intrinsic excitability following decreased activity include, up-regulation of voltage gated sodium channels (Aptowicz et al., 2004) and down regulation of persistent potassium currents (Desai et al., 1999a); these homeostatic adaptations may be mediated by changes in intracellular [Ca^{2+}] (Turrigiano et al., 1994).

The most well studied form of homeostatic plasticity observed at excitatory synapses following *global* changes in activity is synaptic scaling. Synaptic scaling is a process in which negative feedback mechanisms are activated in response to altered activity to "scale" all synapses back to a target firing range, while maintaining the relative strength of individual synapses (Turrigiano et al., 1998; Turrigiano, 2008). This mechanism is postulated to allow neural circuits to remain at an appropriate level of activity in order to respond to destabilizing influences exerted on the system on a daily basis. For instance, hippocampal neurons face plastic changes associated with learning and memory including long-term potentiation and long-term depression (i.e. LTP and LTD respectively) and changes in synapse number/strength during growth and development, etc. (Turrigiano, 2012). A key feature of synaptic scaling is to prevent activity from reaching "zero" or becoming saturated while maintaining so called "information storage" or memory. Generally, synaptic scaling is thought to bidirectionally alter synaptic strength through the insertion and removal of GluA2-containing AMPA receptors (AMPARs) (Ibata et al., 2008; Gainey et al., 2009; Tatavarty et al., 2013) although other cellular mechanisms may also be required (Perez-Otano and Ehlers, 2005; Gonzalez-Islas et al., 2010). Regardless of the specific cellular mechanism required, synaptic scaling is typically thought to be the integrated response to changes in activity occurring over extended time periods (24-48hrs; Desai, 2003). However, some forms of homeostatic plasticity are more adept for responding to

brief changes in neural activity (Alkon, 1984; Aizenman and Linden, 2000; Sutton et al., 2006); although these may require diverse signaling cascades.

NMDA receptors

NMDA receptors (NMDARs) are ionotropic glutamate receptors found throughout the CNS and mediate excitatory neurotransmission. NMDARs are tetramers composed of two obligatory GluN1 subunits and either two GluN2 (A-D) subunits or two GluN3 (A-B) subunits. NMDAR subunit expression patterns vary greatly across CNS regions, developmental stage and sub-cellular location. For example, GluN2A-containing NMDARs are predominantly located at the synapse and GluN2B-containing NMDARs are localized to the extrasynaptic sites (Groc et al., 2009). The sub-cellular location of NMDAR subunits determines the functional properties of the receptor. Activation of synaptic NMDARs typically increases synaptic strength, while activation of extrasynaptic receptors tends to promote the weakening of synaptic connections (Fong et al., 2002; Massey et al., 2004). Adding to the diversity of NMDAR signaling, these receptors are subject to several post-translational modifications including phosphorylation, palmitoylation and nitrosylation (Sanz-Clemente et al., 2013).

NMDARs and plasticity

Typically defined as coincidence detectors (i.e. coincidence of glutamate release and post-synaptic depolarization), NMDARs are involved in many types of plasticity. Activation of NMDARs induces Hebbian forms of plasticity including LTP and LTD, as well as homeostatic forms of plasticity including synaptic scaling (Soares et al., 2013). NMDARs in homeostatic plasticity will be discussed. In addition to bidirectional modification of membrane bound

AMPARs (Beattie et al., 2000; Lu et al., 2009), mounting evidence indicates that NMDARs themselves are subject to activity-dependent regulation (Perez-Otano and Ehlers, 2005; Harris and Pettit, 2008; Li et al., 2009; Hunt and Castillo, 2012). Indeed, synaptic scaling induced by a global reduction in neural activity blockade increases NMDAR mediated current (Watt et al., 2000) *via* upregulation of postsynaptic NMDARs (Rao and Craig, 1997).

In addition to synaptic scaling following global reductions in neural activity described thus far, global blockade of neuronal firing coupled with local NMDAR blockade (with APV) induces a local form of synaptic scaling (Sutton et al., 2006). This form of scaling operates over an order of magnitude faster (<60 min) than global synaptic scaling and suggests that spontaneous activation of NMDARs by miniature EPSCs or "minis" plays a key role in stabilizing synaptic strength. The authors remark that "*during plasticity, the presence of absence of minis may serve as a critical signal for whether a synapse will be strengthened, maintained, or perhaps eventually lost*" (Sutton et al., 2006). This local form of plasticity requires protein synthesis and new insertion of GluA2-lacking AMPARs (Sutton et al., 2006), suggesting cellular mechanisms of this response are distinct from global forms of homeostatic synaptic scaling.

Tumor necrosis factor alpha, receptors and signaling

Tumor necrosis factor alpha (TNFα) is a multifunctional cytokine typically thought of as a mediator of inflammatory and immune responses. However, TNFα plays a critical role in neural signaling in the healthy CNS (Stellwagen et al., 2005; Stellwagen and Malenka, 2006; Turrigiano, 2006; Kaneko et al., 2008; Knogler et al., 2010; Steinmetz and Turrigiano, 2010; Broytman et al., 2013). TNFα is synthesized by neurons, microglia and astrocytes as a transmembrane protein (tmTNF; Lieberman et al., 1989; Morganti-Kossman et al., 1997; Chung

et al., 2005) and is subject to cleavage by the enzyme matrix metalloprotease TNFα converting enzyme (TACE) producing soluble TNFα (MacEwan, 2002b). Both forms of TNFα activate a number of signaling pathways including activation of caspases, lipases, kinases and phosphatases (MacEwan, 2002a). Interestingly, signaling through TNFα can generate ceramide and arachodonic acid (Kolesnick and Kronke, 1998), key molecules responsible for activation of atypical PKC isoforms (discussed below).

Signaling heterogeneity of TNFα stems (in part) from its interaction with two transmembrane receptors (TNFR1 and TNFR2) with distinct structural domains, intracellular sequences and functions (Grell et al., 1994). These intracellular binding regions located on TNFRs recruit diverse adaptor/scaffold molecules following receptor activation such as TRAFs (TRAF2/5), TRADD, RIP, etc (Schrofelbauer and Hoffmann, 2011). Recruitment of these adaptor/scaffolding molecules to TNFRs form stimulus specific protein complexes and bridge two or more components of a cellular pathway, thus encoding signaling specificity and giving rise to activation of numerous cascades (Good et al., 2011).

Tumor necrosis factor alpha and plasticity

TNFα has been implicated in activity-dependent forms of plasticity such as synaptic scaling (Beattie et al., 2002; Stellwagen and Malenka, 2006). For example, in hippocampal neurons prolonged periods (48hrs) of activity blockade (with TTX) triggers release of soluble TNFα and increases miniature excitatory post-synaptic currents (mEPSCs; Stellwagen and Malenka, 2006). This increase in mEPSC is mediated by enhanced cell-surface expression of AMPARs (Beattie et al., 2002; Stellwagen et al., 2005; Stellwagen and Malenka, 2006). Further, TNFα appears to bidirectionally regulate AMPARs since blockade of TNFα signaling decreases

surface AMPARs, while exogenous TNFα increases AMPARs (Beattie et al., 2002). In addition to regulating synaptic strength, TNFα can influence neuronal intrinsic excitability by modulating Na^+, Ca^{2+} and K^+ channels (Czeschik et al., 2008). The relevant cell type responsible for secreting TNFα in response to reduced neural activity may be glia, since *TNF* $^{-/-}$ neurons plated onto wild-type glia, but not *TNF* $^{-/-}$ glia expressed synaptic scaling (Stellwagen and Malenka, 2006). Glial-derived TNFα plays a permissive rather than instructive role in synaptic scaling by allowing neurons to undergo synaptic scaling (Steinmetz and Turrigiano, 2010).

Consistent with an activity-dependent role of TNFα, following spinal cord injury TNFα is increased in spinal segments caudal to injury within minutes (Wang et al., 1996; Pineau and Lacroix, 2007), although this increase in TNFα may be due to tissue trauma in addition to (or rather than) decreased synaptic input. Nevertheless, increased TNFα following spinal injury rapidly increases synaptic AMPAR expression in neurons caudal to the lesion (Ferguson et al., 2008). Additional reports suggest that TNFα induces neuroanatomical plasticity leading to collateral sprouting within the cervical spinal cord and promotes functional motor recovery following CNS injury (Oshima et al., 2009). The role of TNFα induced plasticity following injury appears to be complex and may be a function of the source of TNFα, concentration and receptor subtype activated (Santello and Volterra, 2012), since increased TNFα following injury may also impair plasticity (Huie et al., 2012).

Protein kinase C: structure, activation and regulation

The mammalian protein kinase C (PKC) family is composed of nine genes encoding ten *full-length* serine/threonine kinases making up three sub-families: conventional (α, βI, βII, γ), novel (δ, ε, θ, η) and atypical (ζ, ι/λ; human/murine isozymes; Nishizuka, 1995). These sub-

families are classified based on kinase structure, cofactor requirements and amino acid sequences (Nishizuka, 1995). All PKC isoforms are composed of two domains, 1) a catalytic domain containing three phosphorylation sites and 2) a regulatory domain comprised of a ligand binding domain and an autoinhibitory pseudosubstrate region. Although the catalytic domain of all three sub-families is remarkably conserved, the regulatory domain is unique, as reflected in the stimuli necessary for activation (Moscat and Diaz-Meco, 2000; Newton, 2001). Conventional isoforms bind DAG (diacylglycerol) and phorbol esters through the C1A and C1B domains and Ca^{2+} through the C2 domain. Novel isoforms also bind DAG and phorbol esters, but have a unique or "novel" C2 domain that renders it Ca^{2+} insensitive. Unlike conventional and novel PKCs, atypical PKCs (aPKC) do not require Ca^{2+}, DAG or phorbol esters for activation; instead they contain a single C1 domain that binds ceramide (Lozano et al., 1994; Wang et al., 2009), arachidonic acid (Nakanishi and Exton, 1992; Muller et al., 1995), or phosphatidylinositol 3,4,5-triphosphate (PIP_3; Nishizuka, 1995).

To be fully active, all PKC isoforms require phosphorylation at three sites: the activation motif, hydrophobic motif and turn motif. Following synthesis PKCs exist in an "open" conformation, allowing access to these phosphorylation sites. In this conformation, the first phosphorylation occurs within the activation loop *via* phosphoinositide-dependent protein kinase-1 (PDK-1; Newton, 2010). This is a constitutive step in conventional and novel PKCs, but is agonist dependent for atypical PKCs. The mammalian target of rapamycin complex 2 (mTORC2; Jacinto and Lorberg, 2008) phosphorylates the turn motif, triggering autophosphorylation of the hydrophobic motif (Newton, 2001). Under basal conditions, PKCs reside in the cytosol with the pseudosubstrate region bound to the catalytic domain, in a "closed" conformation, rendering the kinase inactive. Upon ligand binding, PKC isozymes become more

hydrophobic resulting in their translocation and interaction with hydrophilic membranes (Newton, 2001).

A property unique to full-length aPKCs is the presence of a PB1 binding domain allowing for protein-protein interactions (Newton, 2001). Interactions between aPKCs and scaffolding/adaptor proteins are thought to confer specificity to the ubiquitously activated kinases (Mochly-Rosen, 1995). Adaptor proteins containing PB1 domains known to interact with aPKC isoforms include p62/ZIP, MEKK2/MEKK3, MEK5, Par-3, Par-4 and Par-6 (Moscat et al., 2006). Interactions between aPKC and adaptor proteins direct kinase signaling by forming multimeric signaling complexes to recruit aPKCs to specific sites. For example, the aPKC-p62/ZIP complex interacts with TNFα, IL-1, AMPA and $GABA_c$ receptors (Moscat and Diaz-Meco, 2000; Sanz et al., 2000; Croci et al., 2003; Jiang et al., 2009).

Within the aPKC sub-family, a truncated version of PKCζ also exists, referred to as PKMζ. PKMζ lacks the regulatory domain (including the autoinhibitory pseudosubstrate region) and, as such, PKMζ is a constitutively active kinase. Invertebrates express one ζ mRNA and through a proteolysis step, calpain cleaves the regulatory domain to produce PKMζ protein (Bougie et al., 2009). Although vertebrates utilize calpain cleavage in some tissues, within the brain a separate mRNA for PKMζ is produced from a distinct promoter within the PKCζ mRNA (Marshall et al., 2000; Hernandez et al., 2003).

Atypical protein kinase C in plasticity

Atypical PKCs are involved in multiple forms of neural plasticity including spinal nociceptive plasticity (Laferriere et al., 2011), sensory gating (Wan et al., 2012) and the most widely studied form of plasticity, hippocampal LTP (Sacktor et al., 1993). The cellular

mechanisms giving rise to LTP can be separated into two phases: induction (e-LTP) and maintenance (l-LTP). E-LTP begins shortly after post-tetanus and lasts ~1-2/3 hrs and is generally thought to be protein synthesis independent (Bliss and Collingridge, 1993; Malenka and Bear, 2004); while l-LTP lasts for at least 5 hrs and requires *de novo* protein synthesis (Stanton and Sarvey, 1984; Frey et al., 1988; Cracco et al., 2005). Kinases including full-length aPKC isoforms are thought to mediate early-LTP. For example, following LTP-inducing tetanus, NMDAR activation and Ca^{2+} entry, PKCζ/ι is immediately translocated to the membrane (~15 sec post-tetanus); however, at later time points (30 min) PKCζ/ι is no longer elevated within membrane fractions (Sacktor et al., 1993). One possible explanation for this translocation is phosphorylation of AMPARs necessary for e-LTP (Dozmorov et al., 2006). Consistent with this result, a recent study showed that the scaffolding protein p62/sequestosome1 (SQSTM1) binds aPKC isoforms PKCζ/ι (Moscat et al., 2007) to tether PKCζ/ι to the GluA1 subunit to promote phosphorylation (Jiang et al., 2009; Ren et al., 2013). Indeed, PKCζ/ι phosphorylates the GluA1 subunit at S818 (Boehm et al., 2006; Ren et al., 2013). The interaction between p62/ZIP and PKCζ/ι may also promote the delivery of AMPARs to the membrane (Jiang et al., 2009).

The first molecule shown to play a critical role in the maintainence of l-LTP is the truncated and persistently active PKMζ (Sacktor et al., 1993; Ling et al., 2002). PKMζ mRNA is transported to distal dendrites where it remains translationally repressed (Muslimov et al., 2004). Following synaptic stimulation and activation of multiple kinases, translational supression is relieved and local PKMζ mRNA is translated (Osten et al., 1996; Muslimov et al., 2004; Kelly et al., 2007). The requirement for *de novo* protein synthesis of PKMζ appears to be a key aspect of l-LTP (Osten et al., 1996; Hernandez et al., 2003; Kelly et al., 2007). Immediately after PKMζ is translated, it is constitutively phosphorylated by PDK1 within the activation loop (pT410;

Kelly et al., 2007), allowing for maximal activity. Activated PKMζ interacts with multiple proteins including N-ethylmaleimide-sensitive factor (NSF) and PKC 1 (PICK1) to increase/maintain GLUA2--containing AMPARs within the post-synaptic density (Yao et al., 2008; Migues et al., 2010).

Recent reports have called into question if PKMζ is indeed "the" aPKC isoform required for long-lasting forms of plasticity including hippocampal LTP and spinal nociceptive plasticity (Matt and Hell, 2013; Price and Ghosh, 2013; Ren et al., 2013; Volk et al., 2013). Following genetic ablation of PKCζ/PKMζ, LTP remains intact and subsequent delivery of the pharmacological aPKC inhibitor, PKCζ-PS, reverses LTP (Volk et al., 2013). Since PKCζ is not found within the brain (Hernandez et al., 2003) the molecule suspected to maintain LTP in this case is PKCι/λ (Volk et al., 2013). However, these results may reflect conditional induced functional redundancies of aPKC isoforms rather than the "normal" physiological roles of aPKCs (Price and Ghosh, 2013). Future studies are necessary to reconcile these disparate results.

Summary of AIMS

This goal of this dissertation was to examine how phrenic motor neurons respond to reduced respiratory neural activity. To isolate the properties of this response and investigate the cellular mechanisms, I investigated a novel form of respiratory neural plasticity elicited by reduced respiratory neural activity called inactivity-induced phrenic motor facilitation (iPMF).

In chapter two, I present experiments testing the hypothesis that ***spinal atypical protein kinase C (aPKC) isoforms are necessary for iPMF*.** Our data suggest that long-lasting iPMF consists of at least two mechanistically distinct phases: 1) an early, labile phase that requires spinal aPKC activity to transition to, 2) a long-lasting aPKC-independent phase. We show that

the relevant isoforms necessary for iPMF are PKCζ and/or PKCι/λ. Consistent with this finding early (but not late) iPMF is associated with increased interaction between PKCζ/ι and the scaffolding protein p62/ZIP in spinal regions associated with the phrenic motor pool. In contrast to iPMF, phrenic long-term facilitation (pLTF) does not require aPKC isoforms, suggesting iPMF is a unique form of respiratory plasticity requiring distinct spinal mechanisms.

Since methods used to reduce respiratory neural activity and elicit iPMF indirectly manipulated activity and it was unknown where in the CNS reduced neural activity was sensed and responded to. Therefore, in chapter three I tested the hypothesis that ***local spinal mechanisms sense and respond to reduced phrenic synaptic inputs independent of chemoreflexes***. Unilateral axon conduction blockade gives rise to: 1) a TNFα and aPKC dependent iPMF within the ipsilateral phrenic motor pool receiving reduced phrenic synaptic inputs and 2) a crossed spinal phrenic motor facilitation (csPMF) independent of TNFα and aPKC within the contralateral motor pool that did not receive reduced phrenic synaptic inputs. Both ipsilateral iPMF and contralateral csPMF are associated with a proportional increase in the phrenic response to hypercapnia.

In the final chapter, chapter four, I tested the hypothesis that ***iPMF is differentially regulated in Sprague-Dawley rat sub-strains.*** Our data suggest iPMF is differentially regulated in Sprague-Dawley rat sub-strains since Charles River Sprague-Dawley (CRSD) rats express a transient (*versus* long-lasting) iPMF following central neural apnea. Further, we show that spinal NMDA receptor activation inhibits long-lasting iPMF by constraining spinal TNFα and aPKC-dependent signaling, indicating that iPMF duration in CRSD rats is regulated by spinal regulatory mechanisms.

Finally, appendix A is a review discussing physiological and pathophysiological conditions associated with reduced respiratory neural activity and evidence that respiratory plasticity is elicited in these conditions. The current theories regarding the relevance of iPMF to these situations and whether iPMF would play an adaptive or maladaptive role during these conditions is also considered.

REFERENCES

Aizenman CD, Linden DJ (2000) Rapid, synaptically driven increases in the intrinsic excitability of cerebellar deep nuclear neurons. Nat Neurosci 3:109-111.

Alkon DL (1984) Changes of membrane currents during learning. The Journal of experimental biology 112:95-112.

Ancoli-Israel S, Kripke DF, Mason W (1987) Characteristics of obstructive and central sleep apnea in the elderly: an interim report. Biol Psychiatry 22:741-750.

Antognini JF, Carstens E, Buzin V (1999) Isoflurane depresses motoneuron excitability by a direct spinal action: an F-wave study. Anesthesia and analgesia 88:681-685.

Aptowicz CO, Kunkler PE, Kraig RP (2004) Homeostatic plasticity in hippocampal slice cultures involves changes in voltage-gated Na+ channel expression. Brain Res 998:155-163.

Babb TG, Wood HE, Mitchell GS (2010) Short- and long-term modulation of the exercise ventilatory response. Med Sci Sports Exerc 42:1681-1687.

Bach KB, Lutcavage ME, Mitchell GS (1993) Serotonin is necessary for short-term modulation of the exercise ventilatory response. Respir Physiol 91:57-70.

Baker-Herman TL, Strey KA (2011) Similarities and differences in mechanisms of phrenic and hypoglossal motor facilitation. Respir Physiol Neurobiol 179:48-56.

Baker TL, Mitchell GS (2000) Episodic but not continuous hypoxia elicits long-term facilitation of phrenic motor output in rats. J Physiol 529 Pt 1:215-219.

Baker TL, Fuller DD, Zabka AG, Mitchell GS (2001) Respiratory plasticity: differential actions of continuous and episodic hypoxia and hypercapnia. Respir Physiol 129:25-35.

Bavis RW, Mitchell GS (2003) Intermittent hypoxia induces phrenic long-term facilitation in carotid-denervated rats. J Appl Physiol 94:399-409.

Bavis RW, Johnson RA, Ording KM, Otis JP, Mitchell GS (2006) Respiratory plasticity after perinatal hypercapnia in rats. Respir Physiol Neurobiol 153:78-91.

Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, Von Zastrow M, Beattie MS, Malenka RC (2002) Control of synaptic strength by glial TNFalpha. Science 295:2282-2285.

Benucci A, Saleem AB, Carandini M (2013) Adaptation maintains population homeostasis in primary visual cortex. Nat Neurosci 16:724-729.

Berger AJ, Cameron WE, Averill DB, Kramis RC, Binder MD (1984) Spatial distributions of phrenic and medial gastrocnemius motoneurons in the cat spinal cord. Exp Neurol 86:559-575.

Berssenbrugge AD, Dempsey JA, Skatrud JB (1984) Effects of sleep state on ventilatory acclimatization to hypoxia in humans. J Appl Physiol 57:1089-1096.

Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361:31-39.

Bocchiaro CM, Feldman JL (2004) Synaptic activity-independent persistent plasticity in endogenously active mammalian motoneurons. Proc Natl Acad Sci U S A 101:4292-4295.

Boehm J, Kang MG, Johnson RC, Esteban J, Huganir RL, Malinow R (2006) Synaptic incorporation of AMPA receptors during LTP is controlled by a PKC phosphorylation site on GluR1. Neuron 51:213-225.

Bougie JK, Lim T, Farah CA, Manjunath V, Nagakura I, Ferraro GB, Sossin WS (2009) The atypical protein kinase C in Aplysia can form a protein kinase M by cleavage. Journal of neurochemistry 109:1129-1143.

Brandes IF, Zuperku EJ, Stucke AG, Hopp FA, Jakovcevic D, Stuth EA (2007) Isoflurane depresses the response of inspiratory hypoglossal motoneurons to serotonin in vivo. Anesthesiology 106:736-745.

Brian JE (1998) Carbon dioxide and the cerebral circulation. Anesthesiology 88:1365-1386.

Broytman O, Baertsch NA, Baker-Herman TL (2013) Spinal TNFα is necessary for inactivity-induced phrenic motor facilitation. J Physiol.

Budzińska K, von Euler C, Kao FF, Pantaleo T, Yamamoto Y (1985) Effects of graded focal cold block in rostral areas of the medulla. Acta Physiol Scand 124:329-340.

Burns BD (1963) The central control of respiratory movements. British medical bulletin 19:7-9.

Butler JE (2007) Drive to the human respiratory muscles. Respir Physiol Neurobiol 159:115-126.

Castro-Moure F, Goshgarian HG (1996) Reversible cervical hemispinalization of the rat spinal cord by a cooling device. Exp Neurol 141:102-112.

Castro-Moure F, Goshgarian HG (1997) Morphological plasticity induced in the phrenic nucleus following cervical cold block of descending respiratory drive. Exp Neurol 147:299-310.

Chung CY, Seo H, Sonntag KC, Brooks A, Lin L, Isacson O (2005) Cell type-specific gene expression of midbrain dopaminergic neurons reveals molecules involved in their vulnerability and protection. Hum Mol Genet 14:1709-1725.

Cracco JB, Serrano P, Moskowitz SI, Bergold PJ, Sacktor TC (2005) Protein synthesis-dependent LTP in isolated dendrites of CA1 pyramidal cells. Hippocampus 15:551-556.

Croci C, Brandstatter JH, Enz R (2003) ZIP3, a new splice variant of the PKC-zeta-interacting protein family, binds to GABAC receptors, PKC-zeta, and Kv beta 2. The Journal of biological chemistry 278:6128-6135.

Czeschik JC, Hagenacker T, Schafers M, Busselberg D (2008) TNF-alpha differentially modulates ion channels of nociceptive neurons. Neurosci Lett 434:293-298.

Dale-Nagle EA, Hoffman MS, MacFarlane PM, Mitchell GS (2010) Multiple pathways to long-lasting phrenic motor facilitation. Advances in experimental medicine and biology 669:225-230.

Dempsey JA (2005) Crossing the apnoeic threshold: causes and consequences. Exp Physiol 90:13-24.

Desai NS (2003) Homeostatic plasticity in the CNS: synaptic and intrinsic forms. Journal of physiology, Paris 97:391-402.

Desai NS, Rutherford LC, Turrigiano GG (1999a) Plasticity in the intrinsic excitability of cortical pyramidal neurons. Nat Neurosci 2:515-520.

Desai NS, Rutherford LC, Turrigiano GG (1999b) BDNF regulates the intrinsic excitability of cortical neurons. Learn Mem 6:284-291.

Desai NS, Cudmore RH, Nelson SB, Turrigiano GG (2002) Critical periods for experience-dependent synaptic scaling in visual cortex. Nat Neurosci 5:783-789.

Devinney MJ, Huxtable AG, Nichols NL, Mitchell GS (2013) Hypoxia-induced phrenic long-term facilitation: emergent properties. Annals of the New York Academy of Sciences 1279:143-153.

Dick TE, Viana F, Berger AJ (1988) Electrophysiological determination of the axonal projections of single dorsal respiratory group neurons to the cervical spinal cord of cat. Brain Res 454:31-39.

Doi K, Kasaba T, Kosaka Y (1989) [A comparative study of the depressive effects of halothane and isoflurane on medullary respiratory neurons in cats]. Masui The Japanese journal of anesthesiology 38:1427-1437.

Dozmorov M, Li R, Abbas AK, Hellberg F, Farre C, Huang FS, Jilderos B, Wigstrom H (2006) Contribution of AMPA and NMDA receptors to early and late phases of LTP in hippocampal slices. Neuroscience research 55:182-188.

Duffin J, Li YM (2006) Transmission of respiratory rhythm: midline-crossing connections at the level of the phrenic motor nucleus? Respir Physiol Neurobiol 153:139-147.

Dutschmann M, Paton JF (2002) Influence of nasotrigeminal afferents on medullary respiratory neurones and upper airway patency in the rat. Pflugers Archiv : European journal of physiology 444:227-235.

Ellenberger HH, Feldman JL (1990) Brainstem connections of the rostral ventral respiratory group of the rat. Brain Res 513:35-42.

Ellenberger HH, Feldman JL, Goshgarian HG (1990) Ventral respiratory group projections to phrenic motoneurons: electron microscopic evidence for monosynaptic connections. J Comp Neurol 302:707-714.

Epstein SK (2011) How often does patient-ventilator asynchrony occur and what are the consequences? Respir Care 56:25-38.

Ezure K, Tanaka I, Kondo M (2003a) Glycine is used as a transmitter by decrementing expiratory neurons of the ventrolateral medulla in the rat. J Neurosci 23:8941-8948.

Ezure K, Tanaka I, Saito Y (2003b) Brainstem and spinal projections of augmenting expiratory neurons in the rat. Neuroscience research 45:41-51.

Feldman JL, Smith JC (1989) Cellular mechanisms underlying modulation of breathing pattern in mammals. Annals of the New York Academy of Sciences 563:114-130.

Feldman JL, Loewy AD, Speck DF (1985) Projections from the ventral respiratory group to phrenic and intercostal motoneurons in cat: an autoradiographic study. J Neurosci 5:1993-2000.

Feldman JL, Mitchell GS, Nattie EE (2003) Breathing: rhythmicity, plasticity, chemosensitivity. Annu Rev Neurosci 26:239-266.

Feldman JL, Del Negro CA, Gray PA (2013) Understanding the rhythm of breathing: so near, yet so far. Annu Rev Physiol 75:423-452.

Ferguson AR, Christensen RN, Gensel JC, Miller BA, Sun F, Beattie EC, Bresnahan JC, Beattie MS (2008) Cell death after spinal cord injury is exacerbated by rapid TNF alpha-induced trafficking of GluR2-lacking AMPARs to the plasma membrane. J Neurosci 28:11391-11400.

Fong DK, Rao A, Crump FT, Craig AM (2002) Rapid synaptic remodeling by protein kinase C: reciprocal translocation of NMDA receptors and calcium/calmodulin-dependent kinase II. J Neurosci 22:2153-2164.

Forster HV (2003) Plasticity in the control of breathing following sensory denervation. J Appl Physiol 94:784-794.

Forster HV, Smith CA (2010) Contributions of central and peripheral chemoreceptors to the ventilatory response to CO2/H+. J Appl Physiol 108:989-994.

Fregosi RF, Mitchell GS (1994) Long-term facilitation of inspiratory intercostal nerve activity following carotid sinus nerve stimulation in cats. J Physiol 477 (Pt 3):469-479.

Frey U, Krug M, Reymann KG, Matthies H (1988) Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro. Brain Res 452:57-65.

Fuller DD, Bach KB, Baker TL, Kinkead R, Mitchell GS (2000) Long term facilitation of phrenic motor output. Respir Physiol 121:135-146.

Furmaga H, Carreno FR, Frazer A (2012) Vagal nerve stimulation rapidly activates brain-derived neurotrophic factor receptor TrkB in rat brain. PloS one 7:e34844.

Gaig C, Iranzo A (2012) Sleep-disordered breathing in neurodegenerative diseases. Curr Neurol Neurosci Rep 12:205-217.

Gainey MA, Hurvitz-Wolff JR, Lambo ME, Turrigiano GG (2009) Synaptic scaling requires the GluR2 subunit of the AMPA receptor. J Neurosci 29:6479-6489.

Galante M, Avossa D, Rosato-Siri M, Ballerini L (2001) Homeostatic plasticity induced by chronic block of AMPA/kainate receptors modulates the generation of rhythmic bursting in rat spinal cord organotypic cultures. Eur J Neurosci 14:903-917.

Garcia-Bereguiain MA, Gonzalez-Islas C, Lindsly C, Butler E, Hill AW, Wenner P (2013) In vivo synaptic scaling is mediated by GluA2-lacking AMPA receptors in the embryonic spinal cord. J Neurosci 33:6791-6799.

Gargaglioni LH, Hartzler LK, Putnam RW (2010) The locus coeruleus and central chemosensitivity. Respir Physiol Neurobiol 173:264-273.

Gaultier C, Gallego J (2005) Development of respiratory control: evolving concepts and perspectives. Respir Physiol Neurobiol 149:3-15.

Gonzalez-Islas C, Chub N, Garcia-Bereguiain MA, Wenner P (2010) GABAergic synaptic scaling in embryonic motoneurons is mediated by a shift in the chloride reversal potential. J Neurosci 30:13016-13020.

Good MC, Zalatan JG, Lim WA (2011) Scaffold proteins: hubs for controlling the flow of cellular information. Science 332:680-686.

Gooden BA (1994) Mechanism of the human diving response. Integrative physiological and behavioral science : the official journal of the Pavlovian Society 29:6-16.

Goridis C, Dubreuil V, Thoby-Brisson M, Fortin G, Brunet JF (2010) Phox2b, congenital central hypoventilation syndrome and the control of respiration. Seminars in cell & developmental biology 21:814-822.

Goshgarian HG (2003) The crossed phrenic phenomenon: a model for plasticity in the respiratory pathways following spinal cord injury. J Appl Physiol 94:795-810.

Goshgarian HG, Rafols JA (1981) The phrenic nucleus of th albino rat: a correlative HRP and Golgi study. J Comp Neurol 201:441-456.

Goshgarian HG, Rafols JA (1984) The ultrastructure and synaptic architecture of phrenic motor neurons in the spinal cord of the adult rat. J Neurocytol 13:85-109.

Goshgarian HG, Yu XJ, Rafols JA (1989) Neuronal and glial changes in the rat phrenic nucleus occurring within hours after spinal cord injury. J Comp Neurol 284:519-533.

Gray PA, Janczewski WA, Mellen N, McCrimmon DR, Feldman JL (2001) Normal breathing requires preBotzinger complex neurokinin-1 receptor-expressing neurons. Nat Neurosci 4:927-930.

Grell M, Zimmermann G, Hulser D, Pfizenmaier K, Scheurich P (1994) TNF receptors TR60 and TR80 can mediate apoptosis via induction of distinct signal pathways. Journal of immunology 153:1963-1972.

Groc L, Bard L, Choquet D (2009) Surface trafficking of N-methyl-D-aspartate receptors: physiological and pathological perspectives. Neuroscience 158:4-18.

Guyenet PG, Bayliss DA, Mulkey DK, Stornetta RL, Moreira TS, Takakura AT (2008) The retrotrapezoid nucleus and central chemoreception. Advances in experimental medicine and biology 605:327-332.

Haldane JS, Priestley JG (1905) The regulation of the lung-ventilation. J Physiol 32:225-266.

Harris A, Pettit D (2008) Recruiting extrasynaptic NMDA receptors augments synaptic signaling. J Neurophysiol 99:524-533.

Hernandez AI, Blace N, Crary JF, Serrano PA, Leitges M, Libien JM, Weinstein G, Tcherapanov A, Sacktor TC (2003) Protein kinase M zeta synthesis from a brain mRNA encoding an independent protein kinase C zeta catalytic domain. Implications for the molecular mechanism of memory. The Journal of biological chemistry 278:40305-40316.

Hilaire G, Duron B (1999) Maturation of the mammalian respiratory system. Physiological reviews 79:325-360.

Hilaire G, Pasaro R (2003) Genesis and control of the respiratory rhythm in adult mammals. News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society 18:23-28.

Hodges MR, Richerson GB (2010) Medullary serotonin neurons and their roles in central respiratory chemoreception. Respir Physiol Neurobiol 173:256-263.

Hoffman MS, Nichols NL, Macfarlane PM, Mitchell GS (2012) Phrenic long-term facilitation after acute intermittent hypoxia requires spinal ERK activation but not TrkB synthesis. J Appl Physiol 113:1184-1193.

Hou Q, Zhang D, Jarzylo L, Huganir RL, Man HY (2008) Homeostatic regulation of AMPA receptor expression at single hippocampal synapses. Proc Natl Acad Sci U S A 105:775-780.

Huie JR, Baumbauer KM, Lee KH, Bresnahan JC, Beattie MS, Ferguson AR, Grau JW (2012) Glial tumor necrosis factor alpha (TNFalpha) generates metaplastic inhibition of spinal learning. PloS one 7:e39751.

Hunt DL, Castillo PE (2012) Synaptic plasticity of NMDA receptors: mechanisms and functional implications. Curr Opin Neurobiol 22:496-508.

Ibata K, Sun Q, Turrigiano GG (2008) Rapid synaptic scaling induced by changes in postsynaptic firing. Neuron 57:819-826.

Jacinto E, Lorberg A (2008) TOR regulation of AGC kinases in yeast and mammals. The Biochemical journal 410:19-37.

Janczewski WA, Tashima A, Hsu P, Cui Y, Feldman JL (2013) Role of inhibition in respiratory pattern generation. J Neurosci 33:5454-5465.

Javaheri S, Dempsey JA (2013) Central sleep apnea. Compr Physiol 3:141-163.

Jiang C, Lipski J (1990) Extensive monosynaptic inhibition of ventral respiratory group neurons by augmenting neurons in the Botzinger complex in the cat. Exp Brain Res 81:639-648.

Jiang J, Parameshwaran K, Seibenhener ML, Kang MG, Suppiramaniam V, Huganir RL, Diaz-Meco MT, Wooten MW (2009) AMPA receptor trafficking and synaptic plasticity require SQSTM1/p62. Hippocampus 19:392-406.

Johnson SM, Getting PA (1988) Phrenic motor nucleus of the guinea pig: dendrites are bundled without clustering of cell somas. Exp Neurol 101:208-220.

Johnson SM, Mitchell GS (2002) Activity-dependent plasticity in descending synaptic inputs to respiratory spinal motoneurons. Respir Physiol Neurobiol 131:79-90.

Kaneko M, Stellwagen D, Malenka RC, Stryker MP (2008) Tumor necrosis factor-alpha mediates one component of competitive, experience-dependent plasticity in developing visual cortex. Neuron 58:673-680.

Kelly MT, Crary JF, Sacktor TC (2007) Regulation of protein kinase Mzeta synthesis by multiple kinases in long-term potentiation. J Neurosci 27:3439-3444.

Kline DD, Ramirez-Navarro A, Kunze DL (2007) Adaptive depression in synaptic transmission in the nucleus of the solitary tract after in vivo chronic intermittent hypoxia: evidence for homeostatic plasticity. J Neurosci 27:4663-4673.

Knogler LD, Liao M, Drapeau P (2010) Synaptic scaling and the development of a motor network. J Neurosci 30:8871-8881.

Kolesnick RN, Kronke M (1998) Regulation of ceramide production and apoptosis. Annu Rev Physiol 60:643-665.

Laferriere A, Pitcher MH, Haldane A, Huang Y, Cornea V, Kumar N, Sacktor TC, Cervero F, Coderre TJ (2011) PKMzeta is essential for spinal plasticity underlying the maintenance of persistent pain. Molecular pain 7:99.

Lahiri S, Forster RE, 2nd (2003) CO2/H(+) sensing: peripheral and central chemoreception. The international journal of biochemistry & cell biology 35:1413-1435.

Lane MA (2011) Spinal respiratory motoneurons and interneurons. Respir Physiol Neurobiol 179:3-13.

Lane MA, White TE, Coutts MA, Jones AL, Sandhu MS, Bloom DC, Bolser DC, Yates BJ, Fuller DD, Reier PJ (2008) Cervical prephrenic interneurons in the normal and lesioned spinal cord of the adult rat. J Comp Neurol 511:692-709.

Lee KZ, Fuller DD (2011) Neural control of phrenic motoneuron discharge. Respir Physiol Neurobiol 179:71-79.

Li A, Randall M, Nattie EE (1999) CO(2) microdialysis in retrotrapezoid nucleus of the rat increases breathing in wakefulness but not in sleep. J Appl Physiol 87:910-919.

Li GH, Jackson MF, Orser BA, Macdonald JF (2009) Reciprocal and activity-dependent regulation of surface AMPA and NMDA receptors in cultured neurons. Int J Physiol Pathophysiol Pharmacol 2:47-56.

Lieberman AP, Pitha PM, Shin HS, Shin ML (1989) Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus. Proc Natl Acad Sci U S A 86:6348-6352.

Ling DS, Benardo LS, Serrano PA, Blace N, Kelly MT, Crary JF, Sacktor TC (2002) Protein kinase Mzeta is necessary and sufficient for LTP maintenance. Nat Neurosci 5:295-296.

Lipski J, Zhang X, Kruszewska B, Kanjhan R (1994) Morphological study of long axonal projections of ventral medullary inspiratory neurons in the rat. Brain Res 640:171-184.

Liu G, Tsien RW (1995) Properties of synaptic transmission at single hippocampal synaptic boutons. Nature 375:404-408.

Lozano J, Berra E, Municio MM, Diaz-Meco MT, Dominguez I, Sanz L, Moscat J (1994) Protein kinase C zeta isoform is critical for kappa B-dependent promoter activation by sphingomyelinase. The Journal of biological chemistry 269:19200-19202.

MacEwan DJ (2002a) TNF receptor subtype signalling: differences and cellular consequences. Cellular signalling 14:477-492.

MacEwan DJ (2002b) TNF ligands and receptors--a matter of life and death. British journal of pharmacology 135:855-875.

MacFarlane PM, Mitchell GS (2008) Respiratory long-term facilitation following intermittent hypoxia requires reactive oxygen species formation. Neuroscience 152:189-197.

MacFarlane PM, Mitchell GS (2009) Episodic spinal serotonin receptor activation elicits long-lasting phrenic motor facilitation by an NADPH oxidase-dependent mechanism. J Physiol 587:5469-5481.

Mahamed S, Strey KA, Mitchell GS, Baker-Herman TL (2011) Reduced respiratory neural activity elicits phrenic motor facilitation. Respir Physiol Neurobiol 175:303-309.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

Manta S, Dong J, Debonnel G, Blier P (2009) Enhancement of the function of rat serotonin and norepinephrine neurons by sustained vagus nerve stimulation. Journal of psychiatry & neuroscience : JPN 34:272-280.

Marder E, Calabrese RL (1996) Principles of rhythmic motor pattern generation. Physiological reviews 76:687-717.

Marshall BS, Price G, Powell CT (2000) Rat protein kinase c zeta gene contains alternative promoters for generation of dual transcripts with 5'-end heterogeneity. DNA and cell biology 19:707-719.

Martin RJ, Dreshaj IA, Miller MJ, Haxhiu MA (1994) Hypoglossal and phrenic responses to central respiratory inhibition in piglets. Respir Physiol 97:93-103.

Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, Bashir ZI (2004) Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. J Neurosci 24:7821-7828.

Matt L, Hell JW (2013) PKClambda: a new player in LTP coming to the rescue of PKCzeta's faltering role in LTP? The EMBO journal 32:1348-1349.

McCrimmon DR, Zuperku EJ, Hayashi F, Dogas Z, Hinrichsen CF, Stuth EA, Tonkovic-Capin M, Krolo M, Hopp FA (1997) Modulation of the synaptic drive to respiratory premotor and motor neurons. Respir Physiol 110:161-176.

Migues PV, Hardt O, Wu DC, Gamache K, Sacktor TC, Wang YT, Nader K (2010) PKMzeta maintains memories by regulating GluR2-dependent AMPA receptor trafficking. Nat Neurosci 13:630-634.

Millhorn DE, Eldridge FL, Waldrop TG (1980a) Prolonged stimulation of respiration by a new central neural mechanism. Respir Physiol 41:87-103.

Millhorn DE, Eldridge FL, Waldrop TG (1980b) Prolonged stimulation of respiration by endogenous central serotonin. Respir Physiol 42:171-188.

Mitchell GS, Johnson SM (2003) Neuroplasticity in respiratory motor control. J Appl Physiol 94:358-374.

Mitchell GS, Baker TL, Nanda SA, Fuller DD, Zabka AG, Hodgeman BA, Bavis RW, Mack KJ, Olson EB, Jr. (2001) Invited review: Intermittent hypoxia and respiratory plasticity. J Appl Physiol 90:2466-2475.

Mochly-Rosen D (1995) Localization of protein kinases by anchoring proteins: a theme in signal transduction. Science 268:247-251.

Morganti-Kossman MC, Lenzlinger PM, Hans V, Stahel P, Csuka E, Ammann E, Stocker R, Trentz O, Kossmann T (1997) Production of cytokines following brain injury: beneficial and deleterious for the damaged tissue. Molecular psychiatry 2:133-136.

Moscat J, Diaz-Meco MT (2000) The atypical protein kinase Cs. Functional specificity mediated by specific protein adapters. EMBO reports 1:399-403.

Moscat J, Diaz-Meco MT, Wooten MW (2007) Signal integration and diversification through the p62 scaffold protein. Trends in biochemical sciences 32:95-100.

Moscat J, Diaz-Meco MT, Albert A, Campuzano S (2006) Cell signaling and function organized by PB1 domain interactions. Molecular cell 23:631-640.

Muller G, Ayoub M, Storz P, Rennecke J, Fabbro D, Pfizenmaier K (1995) PKC zeta is a molecular switch in signal transduction of TNF-alpha, bifunctionally regulated by ceramide and arachidonic acid. The EMBO journal 14:1961-1969.

Muslimov IA, Nimmrich V, Hernandez AI, Tcherepanov A, Sacktor TC, Tiedge H (2004) Dendritic transport and localization of protein kinase Mzeta mRNA: implications for molecular memory consolidation. The Journal of biological chemistry 279:52613-52622.

Nakanishi H, Exton JH (1992) Purification and characterization of the zeta isoform of protein kinase C from bovine kidney. The Journal of biological chemistry 267:16347-16354.

Nattie E (2000) Multiple sites for central chemoreception: their roles in response sensitivity and in sleep and wakefulness. Respir Physiol 122:223-235.

Nattie E (2011) Julius H. Comroe, Jr., distinguished lecture: central chemoreception: then ... and now. J Appl Physiol 110:1-8.

Nattie EE, Li A (2001) CO2 dialysis in the medullary raphe of the rat increases ventilation in sleep. J Appl Physiol 90:1247-1257.

Nelson SB, Sjöström PJ, Turrigiano GG (2002) Rate and timing in cortical synaptic plasticity. Philos Trans R Soc Lond B Biol Sci 357:1851-1857.

Newton AC (2001) Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem Rev 101:2353-2364.

Newton AC (2010) Protein kinase C: poised to signal. American journal of physiology Endocrinology and metabolism 298:E395-402.

Nichols NL, Dale EA, Mitchell GS (2012) Severe acute intermittent hypoxia elicits phrenic long-term facilitation by a novel adenosine-dependent mechanism. J Appl Physiol 112:1678-1688.

Nichols NL, Hartzler LK, Conrad SC, Dean JB, Putnam RW (2008) Intrinsic chemosensitivity of individual nucleus tractus solitarius (NTS) and locus coeruleus (LC) neurons from neonatal rats. Advances in experimental medicine and biology 605:348-352.

Nishizuka Y (1995) Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 9:484-496.

Nwaigwe CI, Roche MA, Grinberg O, Dunn JF (2000) Effect of hyperventilation on brain tissue oxygenation and cerebrovenous PO2 in rats. Brain Res 868:150-156.

O'Brien RJ, Kamboj S, Ehlers MD, Rosen KR, Fischbach GD, Huganir RL (1998) Activity-dependent modulation of synaptic AMPA receptor accumulation. Neuron 21:1067-1078.

Okada Y, Sasaki T, Oku Y, Takahashi N, Seki M, Ujita S, Tanaka KF, Matsuki N, Ikegaya Y (2012) Preinspiratory calcium rise in putative pre-Botzinger complex astrocytes. J Physiol 590:4933-4944.

Olson EB, Bohne CJ, Dwinell MR, Podolsky A, Vidruk EH, Fuller DD, Powell FL, Mitchel GS (2001) Ventilatory long-term facilitation in unanesthetized rats. J Appl Physiol 91:709-716.

Oshima T, Lee S, Sato A, Oda S, Hirasawa H, Yamashita T (2009) TNF-alpha contributes to axonal sprouting and functional recovery following traumatic brain injury. Brain Res 1290:102-110.

Osten P, Valsamis L, Harris A, Sacktor TC (1996) Protein synthesis-dependent formation of protein kinase Mzeta in long-term potentiation. J Neurosci 16:2444-2451.

Pavlinac Dodig I, Pecotic R, Valic M, Dogas Z (2012) Acute intermittent hypoxia induces phrenic long-term facilitation which is modulated by 5-HT1A receptor in the caudal raphe region of the rat. J Sleep Res 21:195-203.

Perez-Otano I, Ehlers MD (2005) Homeostatic plasticity and NMDA receptor trafficking. Trends Neurosci 28:229-238.

Pineau I, Lacroix S (2007) Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved. J Comp Neurol 500:267-285.

Prabhakar NR (2011) Sensory plasticity of the carotid body: role of reactive oxygen species and physiological significance. Respir Physiol Neurobiol 178:375-380.

Prabhakar NR, Peng YJ (2004) Peripheral chemoreceptors in health and disease. J Appl Physiol 96:359-366.

Prakash YS, Miyata H, Zhan WZ, Sieck GC (1999) Inactivity-induced remodeling of neuromuscular junctions in rat diaphragmatic muscle. Muscle & nerve 22:307-319.

Prakash YS, Mantilla CB, Zhan WZ, Smithson KG, Sieck GC (2000) Phrenic motoneuron morphology during rapid diaphragm muscle growth. J Appl Physiol 89:563-572.

Price TJ, Ghosh S (2013) ZIPping to pain relief: the role (or not) of PKMzeta in chronic pain. Molecular pain 9:6.

Qiu K, Lane MA, Lee KZ, Reier PJ, Fuller DD (2010) The phrenic motor nucleus in the adult mouse. Exp Neurol 226:254-258.

Queenan BN, Lee KJ, Pak DT (2012) Wherefore art thou, homeo(stasis)? Functional diversity in homeostatic synaptic plasticity. Neural Plast 2012:718203.

Rampil IJ, King BS (1996) Volatile anesthetics depress spinal motor neurons. Anesthesiology 85:129-134.

Rao A, Craig AM (1997) Activity regulates the synaptic localization of the NMDA receptor in hippocampal neurons. Neuron 19:801-812.

Rekling JC, Feldman JL (1998) PreBotzinger complex and pacemaker neurons: hypothesized site and kernel for respiratory rhythm generation. Annu Rev Physiol 60:385-405.

Ren SQ, Yan JZ, Zhang XY, Bu YF, Pan WW, Yao W, Tian T, Lu W (2013) PKClambda is critical in AMPA receptor phosphorylation and synaptic incorporation during LTP. The EMBO journal 32:1365-1380.

Richter DW, Spyer KM (2001) Studying rhythmogenesis of breathing: comparison of in vivo and in vitro models. Trends Neurosci 24:464-472.

Richter DW, Ballanyi K, Schwarzacher S (1992) Mechanisms of respiratory rhythm generation. Curr Opin Neurobiol 2:788-793.

Sacktor TC, Osten P, Valsamis H, Jiang X, Naik MU, Sublette E (1993) Persistent activation of the zeta isoform of protein kinase C in the maintenance of long-term potentiation. Proc Natl Acad Sci U S A 90:8342-8346.

Santello M, Volterra A (2012) TNFalpha in synaptic function: switching gears. Trends Neurosci 35:638-647.

Sanz-Clemente A, Nicoll RA, Roche KW (2013) Diversity in NMDA receptor composition: many regulators, many consequences. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry 19:62-75.

Sanz L, Diaz-Meco MT, Nakano H, Moscat J (2000) The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. The EMBO journal 19:1576-1586.

Schrofelbauer B, Hoffmann A (2011) How do pleiotropic kinase hubs mediate specific signaling by TNFR superfamily members? Immunological reviews 244:29-43.

Shepherd JD, Rumbaugh G, Wu J, Chowdhury S, Plath N, Kuhl D, Huganir RL, Worley PF (2006) Arc/Arg3.1 mediates homeostatic synaptic scaling of AMPA receptors. Neuron 52:475-484.

Skatrud JB, Dempsey JA (1983) Interaction of sleep state and chemical stimuli in sustaining rhythmic ventilation. J Appl Physiol 55:813-822.

Smith CA, Rodman JR, Chenuel BJ, Henderson KS, Dempsey JA (2006) Response time and sensitivity of the ventilatory response to CO2 in unanesthetized intact dogs: central vs. peripheral chemoreceptors. J Appl Physiol 100:13-19.

Smith JC, Ellenberger HH, Ballanyi K, Richter DW, Feldman JL (1991) Pre-Botzinger complex: a brainstem region that may generate respiratory rhythm in mammals. Science 254:726-729.

Smith JC, Abdala AP, Koizumi H, Rybak IA, Paton JF (2007) Spatial and functional architecture of the mammalian brain stem respiratory network: a hierarchy of three oscillatory mechanisms. J Neurophysiol 98:3370-3387.

Smith JC, Butera RJ, Koshiya N, Del Negro C, Wilson CG, Johnson SM (2000) Respiratory rhythm generation in neonatal and adult mammals: the hybrid pacemaker-network model. Respir Physiol 122:131-147.

Soares C, Lee KF, Nassrallah W, Beique JC (2013) Differential Subcellular Targeting of Glutamate Receptor Subtypes during Homeostatic Synaptic Plasticity. J Neurosci 33:13547-13559.

Sperry MA, Goshgarian HG (1993) Ultrastructural changes in the rat phrenic nucleus developing within 2 h after cervical spinal cord hemisection. Exp Neurol 120:233-244.

Stanton PK, Sarvey JM (1984) Blockade of long-term potentiation in rat hippocampal CA1 region by inhibitors of protein synthesis. J Neurosci 4:3080-3088.

Steinmetz CC, Turrigiano GG (2010) Tumor necrosis factor-α signaling maintains the ability of cortical synapses to express synaptic scaling. J Neurosci 30:14685-14690.

Stellwagen D, Malenka RC (2006) Synaptic scaling mediated by glial TNF-alpha. Nature 440:1054-1059.

Stellwagen D, Beattie EC, Seo JY, Malenka RC (2005) Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-alpha. J Neurosci 25:3219-3228.

Strakowski JA, Pease WS, Johnson EW (2007) Phrenic nerve stimulation in the evaluation of ventilator-dependent individuals with C4- and C5-level spinal cord injury. American journal of physical medicine & rehabilitation / Association of Academic Physiatrists 86:153-157.

Strey KA, Baertsch NA, Baker-Herman TL (2013) Inactivity-induced respiratory plasticity: Protecting the drive to breathe in disorders that reduce respiratory neural activity. Respir Physiol Neurobiol.

Stuth EA, Tonkovic-Capin M, Kampine JP, Zuperku EJ (1992) Dose-dependent effects of isoflurane on the CO2 responses of expiratory medullary neurons and the phrenic nerve activities in dogs. Anesthesiology 76:763-774.

Sutton MA (2010) Homeostatic plasticity: single hippocampal neurons see the light. Neuron 68:326-328.

Sutton MA, Ito HT, Cressy P, Kempf C, Woo JC, Schuman EM (2006) Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. Cell 125:785-799.

Tan W, Janczewski WA, Yang P, Shao XM, Callaway EM, Feldman JL (2008) Silencing preBotzinger complex somatostatin-expressing neurons induces persistent apnea in awake rat. Nat Neurosci 11:538-540.

Tatavarty V, Sun Q, Turrigiano GG (2013) How to scale down postsynaptic strength. J Neurosci 33:13179-13189.

Terada J, Mitchell GS (2011) Diaphragm long-term facilitation following acute intermittent hypoxia during wakefulness and sleep. J Appl Physiol 110:1299-1310.

Terada J, Nakamura A, Zhang W, Yanagisawa M, Kuriyama T, Fukuda Y, Kuwaki T (2008) Ventilatory long-term facilitation in mice can be observed during both sleep and wake periods and depends on orexin. J Appl Physiol 104:499-507.

Tian GF, Duffin J (1998) The role of dorsal respiratory group neurons studied with cross-correlation in the decerebrate rat. Exp Brain Res 121:29-34.

Tobin MJ (2001) Advances in mechanical ventilation. N Engl J Med 344:1986-1996.

Turner DL, Mitchell GS (1997) Long-term facilitation of ventilation following repeated hypoxic episodes in awake goats. J Physiol 499 (Pt 2):543-550.

Turrigiano G (2012) Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function. Cold Spring Harb Perspect Biol 4:a005736.

Turrigiano G, Abbott LF, Marder E (1994) Activity-dependent changes in the intrinsic properties of cultured neurons. Science 264:974-977.

Turrigiano GG (1999) Homeostatic plasticity in neuronal networks: the more things change, the more they stay the same. Trends Neurosci 22:221-227.

Turrigiano GG (2006) More than a sidekick: glia and homeostatic synaptic plasticity. Trends Mol Med 12:458-460.

Turrigiano GG (2008) The self-tuning neuron: synaptic scaling of excitatory synapses. Cell 135:422-435.

Turrigiano GG, Nelson SB (2004) Homeostatic plasticity in the developing nervous system. Nat Rev Neurosci 5:97-107.

Turrigiano GG, Leslie KR, Desai NS, Rutherford LC, Nelson SB (1998) Activity-dependent scaling of quantal amplitude in neocortical neurons. Nature 391:892-896.

Uliel S, Tauman R, Greenfeld M, Sivan Y (2004) Normal polysomnographic respiratory values in children and adolescents. Chest 125:872-878.

Ullah M (1978) Localization of the phrenic nucleus in the spinal cord of the rabbit. J Anat 125:377-386.

van Welie I, van Hooft JA, Wadman WJ (2004) Homeostatic scaling of neuronal excitability by synaptic modulation of somatic hyperpolarization-activated Ih channels. Proc Natl Acad Sci U S A 101:5123-5128.

Vandam RJ, Shields EJ, Kelty JD (2008) Rhythm generation by the pre-Botzinger complex in medullary slice and island preparations: effects of adenosine A(1) receptor activation. BMC Neurosci 9:95.

Vogel J, Abounader R, Schröck H, Zeller K, Duelli R, Kuschinsky W (1996) Parallel changes of blood flow and heterogeneity of capillary plasma perfusion in rat brains during hypocapnia. Am J Physiol 270:H1441-1445.

Volk LJ, Bachman JL, Johnson R, Yu Y, Huganir RL (2013) PKM-ζ is not required for hippocampal synaptic plasticity, learning and memory. Nature 493:420-423.

Wan Q, Jiang XY, Negroiu AM, Lu SG, McKay KS, Abrams TW (2012) Protein kinase C acts as a molecular detector of firing patterns to mediate sensory gating in Aplysia. Nat Neurosci 15:1144-1152.

Wang CX, Nuttin B, Heremans H, Dom R, Gybels J (1996) Production of tumor necrosis factor in spinal cord following traumatic injury in rats. Journal of neuroimmunology 69:151-156.

Wang G, Krishnamurthy K, Umapathy NS, Verin AD, Bieberich E (2009) The carboxyl-terminal domain of atypical protein kinase Czeta binds to ceramide and regulates junction formation in epithelial cells. The Journal of biological chemistry 284:14469-14475.

Watt AJ, van Rossum MC, MacLeod KM, Nelson SB, Turrigiano GG (2000) Activity coregulates quantal AMPA and NMDA currents at neocortical synapses. Neuron 26:659-670.

Webber CL, Pleschka K (1984) Respiratory effects of high cervical cord cold blockade on efferent vagal and phrenic discharges in the rabbit. Pflugers Archiv : European journal of physiology 402:10-17.

Wilkerson JE, Macfarlane PM, Hoffman MS, Mitchell GS (2007) Respiratory plasticity following intermittent hypoxia: roles of protein phosphatases and reactive oxygen species. Biochem Soc Trans 35:1269-1272.

Yao Y, Kelly MT, Sajikumar S, Serrano P, Tian D, Bergold PJ, Frey JU, Sacktor TC (2008) PKM zeta maintains late long-term potentiation by N-ethylmaleimide-sensitive factor/GluR2-dependent trafficking of postsynaptic AMPA receptors. J Neurosci 28:7820-7827.

Yumino D, Bradley TD (2008) Central sleep apnea and Cheyne-Stokes respiration. Proc Am Thorac Soc 5:226-236.

Zhang W, Linden DJ (2003) The other side of the engram: experience-driven changes in neuronal intrinsic excitability. Nat Rev Neurosci 4:885-900.

Zhang Y, McGuire M, White DP, Ling L (2003) Episodic phrenic-inhibitory vagus nerve stimulation paradoxically induces phrenic long-term facilitation in rats. J Physiol 551:981-991.

Zhou Z, Champagnat J, Poon CS (1997) Phasic and long-term depression in brainstem nucleus tractus solitarius neurons: differing roles of AMPA receptor desensitization. J Neurosci 17:5349-5356.

CHAPTER II

Spinal atypical protein kinase C activity is necessary to stabilize inactivity-induced phrenic motor facilitation

K.A. Strey, N.L. Nichols, N.A. Baertsch, O. Broytman, T.L. Baker-Herman

J Neurosci. 32(46):16510-20, 2012.

ABSTRACT

The neural network controlling breathing must establish rhythmic motor output at a level adequate to sustain life. Reduced respiratory neural activity elicits a novel form of plasticity in circuits driving the diaphragm known as inactivity-induced phrenic motor facilitation (iPMF), a rebound increase in phrenic inspiratory output observed once respiratory neural drive is restored. The mechanisms underlying iPMF are unknown. Here, we demonstrate in anesthetized rats that spinal mechanisms give rise to iPMF, and that iPMF consists of at least two mechanistically distinct phases: 1) an early, labile phase that requires atypical PKC (PKCζ and/or PKCι/λ) activity to transition to a 2) late, stable phase. Early (but not late) iPMF is associated with increased interactions between PKCζ/ι and the scaffolding protein p62/ZIP in spinal regions associated with the phrenic motor pool. Although PKCζ/ι activity is necessary for iPMF, spinal aPKC activity is not necessary for phrenic long-term facilitation (pLTF) following acute intermittent hypoxia, an activity-independent form of spinal respiratory plasticity. Thus, while iPMF and pLTF both manifest as prolonged increases in phrenic burst amplitude, they arise from distinct spinal cellular pathways. Our data are consistent with the hypotheses that: 1) local mechanisms sense and respond to reduced respiratory-related activity in the phrenic motor pool, and 2) inactivity-induced increases in phrenic inspiratory output require local PKCζ/ι activity to stabilize into a long-lasting iPMF. Although the physiological role of iPMF is unknown, we suspect that iPMF represents a compensatory mechanism, assuring adequate motor output in a physiological system where prolonged inactivity ends life.

INTRODUCTION

To sustain life, neurons controlling breathing must transmit a continuous rhythmic motor output of appropriate magnitude to respiratory muscles, thereby assuring adequate breathing. Since even the briefest pause in breathing threatens life, the respiratory control system may exhibit unique characteristics, such as rapid responses to periods of reduced neural activity. We recently described a novel form of plasticity elicited by reduced central respiratory neural activity (in the absence of hypoxia) known as inactivity-induced phrenic motor facilitation (iPMF; Baker-Herman and Strey, 2011; Mahamed et al., 2011), a rebound increase in inspiratory motor output to the diaphragm revealed when central respiratory neural activity is restored. iPMF is induced by multiple forms of brainstem-initiated (i.e., central) apnea, including those caused by hyperventilation, increased inhibitory sensory feedback and anesthesia-induced respiratory depression (Baker-Herman and Strey, 2011; Mahamed et al., 2011). Because of the diverse nature of these initiating stimuli, we suggested that iPMF arises from a common factor: reduced respiratory neural activity (Baker-Herman and Strey, 2011).

Although a central neural apnea results in reduced activity in many respiratory-related neurons throughout the neuraxis, we hypothesized that mechanisms operating within or near the phrenic motor pool give rise to iPMF (Baker-Herman and Strey, 2011). Consistent with this hypothesis, a 4 hour disruption in descending synaptic inputs to phrenic motor neurons *via* unilateral axon conduction block enhances ipsilateral diaphragm EMG activity once axon conduction is restored (Castro-Moure and Goshgarian, 1996) suggesting that mechanisms downstream from brainstem respiratory networks are sufficient to induce plasticity in response to phrenic/diaphragm inactivity. However, it is currently unknown if inactivity-induced diaphragm EMG facilitation is due to mechanisms operating in the phrenic motor circuit or at the

neuromuscular junction. Indeed, both the diaphragm (Mantilla and Sieck, 2009) and phrenic motor pool (Castro-Moure and Goshgarian, 1997) exhibit profound morphological changes in response to prolonged inactivity.

Here, we provide the first evidence that reduced central respiratory neural activity elicits mechanisms of plasticity in or near the phrenic motor pool that manifest as a prolonged enhancement of phrenic burst amplitude once respiratory neural activity is restored (i.e., iPMF). Further, we demonstrate that iPMF requires activity of atypical PKC (aPKC) isoforms, PKCζ and/or PKCιλ. Specifically, spinal PKCζ/ι activity following a neural apnea is necessary to transition from an early, transient facilitation to a longer-lasting, stable iPMF; however, once formed, continued PKCζ/ι activity is no longer necessary to maintain late iPMF. Finally, early (but not late) iPMF is associated with increased interaction between PKCζ/ι and the scaffolding protein ZIP (PKCζ interacting protein; also known as p62) in spinal regions associated with the phrenic motor pool, suggesting activation of PKCζ/ι in the relevant time frame at relevant sites. By contrast, spinal aPKC is not necessary for an activity-independent form of phrenic plasticity, phrenic long-term facilitation (pLTF) following acute intermittent hypoxia (Dale-Nagle et al., 2010), suggesting that these forms of spinal plasticity occur *via* distinct cellular mechanisms. To our knowledge, this is the first report describing a key role for local PKCζ/ι activity in stabilizing plasticity in any spinal motor output.

METHODS

Animals: Experiments were performed on 2 to 5 month old male Sprague-Dawley rats (Harlan colony 217). Rats were housed two per cage in a controlled environment (12h light/dark cycle),

with food and water *ad libitum*. The Institutional Animal Care and Use Committee at the University of Wisconsin, Madison, approved all experiments.

Surgical Preparation: Anesthesia was induced with isoflurane in a closed chamber, followed by inhalation of isoflurane through a nose cone (2.5-3.5% in 50% O_2, balance N_2). Rats were tracheotomized and pump ventilated (tidal volume = 2-2.5 ml; Harvard Apparatus, Rodent Ventilator 683), and isoflurane was continued through the ventilator. End tidal CO_2 was monitored (Respironics Novametrix) and maintained at ~45 mmHg throughout surgery by adjusting ventilation rate and/or adding CO_2 to the inspired gas mix. A bilateral vagotomy was performed to prevent entrainment of respiratory frequency with the ventilator. Tracheal pressure was monitored throughout the surgery to assure rats continued respiratory efforts during surgery. The femoral artery was catheterized for blood pressure measurement and periodic blood gas sampling. Depending on surgeon preference, the tail or femoral vein was catheterized for fluid infusion. C2 laminectomy was performed, and a small hole was cut in the dura to place a soft silicone catheter (2 French, Access Technologies) connected to a Hamilton syringe in the intrathecal space over spinal segment C4. The left phrenic nerve was exposed dorsally, cut distally, desheathed and submersed in mineral oil. Rats were converted to urethane anesthesia (1.7-1.8 mg/kg, *i.v.*) and isoflurane was withdrawn. Rats were then paralyzed with pancuronium bromide (2.5 mg/kg, *i.v.*), and 1-3 ml/h fluid infusion of 1:4 hetastarch (Hespan, 6% hetastarch in 0.9% sodium chloride) and lactated ringers solution was started, and continued throughout the experiment. Body temperature was maintained at 37^{o}C using a rectal probe and a custom-designed heated table.

Electrophysiological measurements: The phrenic nerve was placed on a bipolar silver electrode. Phrenic activity was amplified (x10k), band-pass filtered (0.3-20kHz) and integrated (time constant: 50 ms). Raw and integrated traces were recorded using either the WinDAQ (DATAQ Instruments) or PowerLab (AD Instruments) data acquisition systems.

End tidal CO_2 was used as an index of arterial PCO_2. These values were confirmed by periodically sampling arterial blood at key time points for blood gas analysis (Radiometer Copenhagen, ABL 500). Blood pressure was monitored and used as an indicator of the stability of the preparation and depth of anesthesia by pressor responses to paw pad pinch.

Pharmacological treatments: Stock solutions of the following compounds were prepared in artificial CSF (aCSF; in mM: 120 NaCl, 3 KCl, 2 CaCl, 2 MgCl, 23 $NaHCO_3$, 10 glucose bubbled with 95%O_2/5%CO_2): myristoylated ζ-pseudosubstrate inhibitory peptide (PKCζ-PS; 2 µg/µl; Tocris Bioscience), myristoylated scrambled ζ-pseudosubstrate peptide (scrPKCζ-PS; 2 µg/µl; Tocris Bioscience), NPC-15437 (2.4 µg/µl; Enzo Life Sciences). Bisindolylmaleimide I (BIM; Tocris Bioscience) was prepared in 1.7% DMSO (in aCSF; 10ng/µl) or in 17% DMSO (100 ng/µl). It should be noted that PKCζ-PS is also frequently referred to as "ZIP" (ζ-pseudosubstrate inhibitory peptide) in the literature; however, to avoid confusion with the scaffolding protein p62/ZIP, we refer to this compound as "PKCζ-PS". All compounds were delivered intrathecally at doses consistent with those reported in the literature that block different forms of spinal plasticity: PKCζ-PS (10 nmoles; Asiedu et al., 2011; Laferriere et al., 2011), scrPKCζ-PS (10 nmoles), NPC-15437 (50 nmoles; Laferriere et al., 2011) and BIM (0.24 and 2.4 nmoles; Yashpal et al., 1995; Hua et al., 1999; Ferguson et al., 2008) (M. Devinney and G.

Mitchell, personal communication). Vehicle treated rats received aCSF. For all compounds, a total of 10 µl was delivered in the intrathecal space in 1-2 µl boluses over 2 min.

Electrophysiological protocols: Approximately 1h after conversion to urethane anesthesia, CO_2 apneic and recruitment thresholds for phrenic activity were determined in order to set baseline phrenic nerve activity. Apneic threshold was determined by monitoring end tidal CO_2 and slowly increasing ventilator rate and/or lowering inspired CO_2 until rhythmic phrenic burst activity ceased. Recruitment threshold was determined by slowly lowering ventilator rate and/or increasing inspired CO_2 until phrenic activity resumed. End tidal CO_2 was raised a further 2 mmHg to establish baseline phrenic discharge. After 20-30 min of stable phrenic burst amplitude and frequency, an arterial blood sample was drawn; arterial PCO_2 and phrenic burst activity at this time point was considered "baseline" for all subsequent measurements. Rats were then subjected to one of the following three protocols: 1) neural apnea, 2) acute intermittent hypoxia, or 3) time control (see below).

Central (brainstem) respiratory neural activity was eliminated by clamping $PaCO_2$ ~5 mmHg below the CO_2 threshold for breathing. This "neural apnea" was achieved by increasing ventilator rate and/or decreasing inspired CO_2 until end tidal CO_2 was just below the apneic threshold and phrenic bursting ceased. Neural apnea was maintained for 30 min, and then respiratory neural activity was resumed by returning arterial PCO_2 to baseline values. Since rats were mechanically ventilated, arterial oxygen levels were maintained throughout the neural apnea. Blood gas analysis confirmed that arterial oxygen levels were >150 mmHg throughout the protocol, and that $PaCO_2$ levels returned to within 1.5 mmHg of baseline following restoration of central respiratory neural activity. Rats exposed to a neural apnea received intrathecal injections

of: 1) aCSF (n=10), scrPKCζ-PS (n=8) or PKCζ-PS (n=8) ~20 min prior to neural apnea, 2) scrPKCζ-PS (n=7), PKCζ-PS (n=6), NPC-15437 (n=5) or BIM (n=3, each dose) 10 min following the resumption of respiratory neural activity or 3) PKCζ-PS (n=3) 45 min following the resumption of respiratory neural activity.

Acute intermittent hypoxia (IH) consisted of 3, 5 min episodes of hypoxia (11%; PaO_2 = 36 ± 1 mmHg), separated by a 5 min return to baseline oxygen conditions. Following IH, baseline oxygen conditions were restored. Blood gas analysis confirmed that arterial PO_2 was between 35-45 mmHg during hypoxia and that arterial CO_2 was maintained within 1.5 mmHg of baseline following IH exposure. Rats exposed to IH received intrathecal injections of either: 1) scrPKCζ-PS (n=3) or PKCζ-PS (n=8) ~20 min prior to IH or 2) scrPKCζ-PS (n=3) or PKCζ-PS (n=5) 10 min following IH exposures.

To control for any time dependent changes in phrenic motor output not related to our treatments, results were compared to "time controls" that received similar surgery and experimental duration, but no neural apnea or IH. Time control rats received either intrathecal aCSF (n = 6), PKCζ-PS (n = 7) or NPC-15437 (n = 3).

Phrenic burst amplitude was monitored continuously before, during and for 60-90 min following the above protocols. Arterial blood samples were analyzed before, during and at 10-15, 25-30, 60 and 90 min after each treatment protocol to ensure adequate maintenance of baseline arterial PCO_2, PO_2, SBE (base excess) and pH (note – 90 min blood gas sample was only collected in rats receiving post-stimulus drug injections). At the end of each protocol, a maximal CO_2 response ($90<P_{ET}CO_2<100$) was assessed to ensure that observed responses were not influenced by deterioration of the preparation. To be included in the analysis, rats had to meet the following criteria: arterial PO_2 above 150 mmHg (except during hypoxia in IH rats), arterial

PCO_2 maintained within 1.5 mmHg of baseline (except during neural apnea) and base excess within 3 mEq/L from baseline values.

Tissue preparation for protein analysis: In separate subgroups of rats, the association of PKCζ and PKCι with p62/ZIP was measured at different time points following a neural apnea, and were compared to a similar surgical duration in time controls. Rats were prepared as described above. At 10 (neural apnea, n=6; time control, n=6), 20 (neural apnea, n=3; time control, n=3) or 60 (neural apnea, n=8; time control, n=4) min following restoration of respiratory neural activity, the C4 spinal segment was harvested and stored *en bloc* at -80 for future analysis.

Segments were placed on a freezing microtome, and successive 40-µm sections of the dorsal horn were removed and discarded until the ventral aspect of the central canal was visible. Ventral white matter was removed in the same manner until ventral gray matter was visible. Lateral white matter was removed with a #11 scalpel blade under a dissecting microscope. The remaining ventral horn was placed in 200µl of ice-cold IP buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM EDTA, 1% (v/v) Nonidet® P 40, 1x Protease Inhibitor Cocktail (Sigma-Aldrich), 1x Halt Phosphatase Inhibitor Cocktail (Pierce Biotechnology) and homogenized in a glass-teflon Dounce homogenizer with 15 – 20 strokes of the pestle. Homogenates were centrifuged at 2500g for 10 min at 4°C. Protein concentration of the supernatant was measured using the BCA Protein Assay Kit (Pierce Biotechnology) according to the manufacturer's instructions. Supernatants were diluted to 2.21 µg/µl with IP buffer and 442µg total protein was used for immunoprecipitation.

Immunoprecipitation: Supernatants were incubated with 1µg of anti-PKCζ polyclonal antibody (C20; Santa Cruz Biotechnology) or 1µg pre-immune rabbit IgG (Sigma-Aldrich) at 4 °C overnight. The substrate-antibody complex was precipitated with protein G Sepharose (Sigma-Aldrich) at 4 °C for 1 hour, washed 4 times in 15x volumes of IP buffer and eluted by incubation in SDS-PAGE loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 5% (v/v) β-mercaptoethanol) at 96 °C for 5 min.

Immunoblotting: Samples were resolved by polyacrylamide gel electrophoresis on 4-15% Tris-HCl gels in Tris/Glycine/SDS buffer (Bio-Rad) and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Blots were blocked in 5% milk in 1× TBST (20 mM Tris, 500 mM NaCl, 0.1% Tween) for 1 h and probed with anti-p62 monoclonal antibody (1:8000; Abcam) overnight at 4 °C. Immunoblots were rinsed in 1× TBST and probed with HRP-conjugated horse anti-mouse IgG (1:10,000; Cell Signaling Technology) for 1 h at room temperature. The immunoblot label was expressed using Pierce Super Signal West Dura extended duration chemiluminescent kit (Pierce Biotechnology). Chemiluminescence was detected and digitized using an AutoChemi Imaging system (UVP Bioimaging Systems) with LabWorks 4.6. To ensure equal loading, blots were stripped using Pierce Restore Western blot Stripping Buffer (Pierce Biotechnology) and reprobed with anti-PKCζ polyclonal antibody (1:1000; Santa Cruz Biotechnology) and HRP-conjugated goat anti-rabbit IgG (1:10,000; Cell Signaling Technology).

Immunofluorescence: In separate subgroups of rats, immunofluorescence was used to assess changes in aPKC levels following a neural apnea and to determine if aPKC and p62/ZIP were

co-localized in identified phrenic motor neurons. Rats were anesthetized with isoflurane anesthesia through a nose cone (1 – 2% isoflurane in 50% O_2, balance N_2). A 25-µl Hamilton syringe was used to bilaterally inject 10 µl of 5mg/ml Cholera Toxin B subunit solution (CTB, List Biological Laboratories) in the intrapleural space (Mantilla et al., 2009). Rats were allowed to recover for 5 days prior to electrophysiological experiments described above (neural apnea n=3; time control n=4). Following electrophysiological measurements, animals were transcardially perfused with 4% paraformaldehyde (PFA) in 1× phosphate-buffered saline (PBS, pH 7.4).

Spinal segments C3-C6 were collected, post-fixed for 24 hours in 4% PFA, cryoprotected by sequential 24-hour incubations in 20% sucrose and 30% sucrose in 1× PBS, and 40 µm coronal sections were prepared using a Leica SM200R sliding microtome. Spinal cord sections were incubated with antibodies against CTB (1:10,000 anti-CTB, Calbiochem), aPKC (1:1,000 anti-PKCζ/ι C-20, Santa Cruz Biotechnology) and p62/ZIP (1:500 anti-SQSTM/p62, Abcam) to localize aPKC and p62/ZIP with CTB-back labeled phrenic motor neurons. All secondary antibodies were conjugated to Alexa Fluor® fluorescent dyes (Invitrogen). Sections were mounted on slides with ProLong Gold (Invitrogen). Immunofluorescence images were obtained as described in (Guenther et al., 2012). All images were collected with the same laser and EZ-C1 software settings.

Statistical Analysis: Integrated phrenic burst amplitude was analyzed in 30-60 sec bins before (baseline), and 5, 15, 30, 60 and 90 min following neural apnea, intermittent hypoxia or an equivalent duration in time controls (90 min time point was only analyzed in rats receiving intrathecal compounds post-neural apnea). Since there were no differences in phrenic burst

amplitude at any time point between time controls receiving intrathecal aCSF or PKCζ-PS, burst amplitude data from these groups were combined. Integrated phrenic burst amplitude was expressed as a percent change from baseline. A two-way repeated measures ANOVA was used to detect significant differences (Prism 5, GraphPad Software). Individual comparisons were made *via* Bonferroni *post-hoc* tests at a significance level of 0.05.

Arterial PCO_2, PO_2 and pH were analyzed using a two-way repeated measures ANOVA (Prism 5, GraphPad Software) before (baseline), and 10-15, 25-30, 60 and 90 min following neural apnea, intermittent hypoxia or an equivalent duration in time controls (90 min time point was only analyzed in rats receiving intrathecal compounds post-neural apnea).

p62/ZIP and PKCζ/ι immunoblot images were analyzed with ImageJ software. Images were converted to an 8-bit format, thresholds were set manually, and masked. p62/ZIP and PKCζ/ι bands were outlined; the resulting regions were superimposed on the original 8-bit version of the same image and mean gray value of each region was measured. p62/ZIP density was normalized to PKCζ/ι density, and ANOVA was used to detect significant differences (Prism 5, GraphPad Software) between groups. Individual differences were determined by Student Newman-Keuls *post-hoc* tests at a significance level of 0.05. All data are presented as means ± SEM.

RESULTS

To induce a reversible disruption in central respiratory neural activity, rats were hyperventilated just below the CO_2 apneic threshold for breathing. This "neural apnea" was maintained for 30 min while continuing arterial oxygen support with the ventilator. Central respiratory neural activity was resumed by restoring arterial PCO_2 to baseline levels. Table 1 lists

average arterial PCO_2, PO_2 and pH during baseline and at the end of the protocol; no time dependent changes in post-neural apnea blood gases were apparent in any rat group.

iPMF requires spinal aPKC activity

Figure 1A depicts representative compressed phrenic neurograms, illustrating phrenic burst amplitude during baseline, a 30 min neural apnea, and for 60 min following restoration of respiratory neural activity (representative time control traces not shown). Figure 1B represents average change in phrenic burst amplitude from baseline for 60 min following restoration of respiratory neural activity after neural apnea (or equivalent points in time controls) in all rats. In rats with vehicle (aCSF) injections, phrenic burst amplitude following neural apnea was significantly increased relative to baseline (all time points, $p<0.05$) and significantly increased relative to time control rats that experienced the same surgical procedures, but no neural apnea (all time points, $p<0.05$; Figures 1A and 1B), indicating iPMF. iPMF lasted for at least 60 min following restoration of respiratory neural activity. These data demonstrate that reduced central respiratory neural activity elicits iPMF, consistent with other reports (Baker-Herman and Strey, 2011; Mahamed et al., 2011).

To determine the role of aPKC activity in iPMF, rats received an intrathecal injection of PKCζ-PS, a cell-permeable pseudosubstrate inhibitory peptide that binds to and inhibits the catalytic domain of all aPKC isoforms. As expected, rats receiving intrathecal scrambled PKCζ-PS (scrPKCζ-PS) prior to a neural apnea exhibited significant increases in phrenic burst amplitude relative to baseline and time control rats at all time points following restoration of respiratory neural activity (all $p<0.05$; Figures 1A and 1B), indicating scrPKCζ-PS does not interfere with iPMF. By contrast, rats pretreated with intrathecal PKCζ-PS expressed significant

increases in phrenic burst amplitude relative to baseline shortly after restoration of respiratory neural activity (5 and 15 min; $p<0.05$), which was significantly lower than rats receiving scrPKCζ-PS ($p<0.05$) and was not significantly different from time controls ($p>0.05$; Figures 1A and 1B). These data suggest that spinal aPKC inhibition attenuates, but does not eliminate early phases of iPMF. This modest facilitation was only transient since phrenic burst amplitude was no longer significantly different from baseline or time controls by 30 and 60 min following restoration of respiratory neural activity ($p>0.05$). Thus, whereas expression of late iPMF requires spinal aPKC activity, early iPMF is only partially dependent on its activity.

Spinal aPKC activity is required to stabilize iPMF

To determine if continued spinal aPKC activity following resumption of respiratory neural activity is required for iPMF, rats were exposed to a 30 min neural apnea, and then respiratory neural activity was restored; 10 min following restoration of respiratory neural activity, rats received intrathecal scrPKCζ-PS or PKCζ-PS (i.e., after early iPMF had developed). Figure 2A depicts representative compressed phrenic neurograms, illustrating phrenic burst amplitude during baseline, 30 min neural apnea and for 90 min following the restoration of respiratory neural activity. Figure 2B illustrates average change in phrenic burst amplitude from baseline during the first 90 min after neural apnea. Rats receiving intrathecal scrPKCζ-PS or PKCζ-PS 10 min following restoration of respiratory neural activity (scrPKCζ-PS-10 and PKCζ-PS-10, respectively) expressed a similar significant increase in phrenic burst amplitude immediately prior to intrathecal drug application (both $p<0.05$), indicating that both rat groups began with a similar iPMF magnitude. As expected, intrathecal scrPKCζ-PS-10 did not impair subsequent iPMF expression since phrenic burst amplitude remained significantly increased

relative to baseline at all time points following scrPKCζ-PS-10 injections ($p<0.05$); these values were not significantly different from phrenic activity prior to scrPKCζ-PS-10 treatment ($p>0.05$). By contrast, phrenic burst amplitude steadily decreased in rats receiving intrathecal PKCζ-PS-10, such that by 30 min post-neural apnea, phrenic burst amplitude was significantly reduced relative to that observed prior to intrathecal PKCζ-PS-10 injections, and remained below pre-injection levels until the end of the recording (all $p<0.05$). In addition, phrenic burst amplitude following intrathecal PKCζ-PS-10 was significantly reduced relative to scrPKCζ-PS-10 at 60 and 90 min post-neural apnea ($p<0.05$), suggesting that aPKC inhibition during early phases of iPMF attenuates late iPMF.

To determine if aPKC activity is required to maintain late iPMF, we delivered intrathecal PKCζ-PS 45 min (PKCζ-PS-45) following restoration of respiratory neural activity. As expected, rats expressed a significant iPMF immediately prior to PKCζ-PS-45 application ($p<0.05$; Figures 2A and 2B). However, contrary to intrathecal PKCζ-PS-10, intrathecal PKCζ-PS-45 did not interfere with subsequent iPMF expression. Indeed, rats receiving intrathecal PKCζ-PS-45 continued to express significant increases in phrenic burst amplitude from baseline at all measured time points after intrathecal PKCζ-PS-45 ($p<0.05$), which was not significantly different from phrenic amplitude prior to PKCζ-PS-45 injections or scrPKCζ-PS values at any time point (all $p>0.05$). Collectively, these data suggest that spinal aPKC activity is necessary to transition from early to late iPMF, but continued aPKC activity is not necessary to maintain late iPMF.

pLTF does not require aPKC activity

To determine if spinal aPKC activity is a general requirement for spinal phrenic plasticity, we investigated the role of aPKC in a contrasting model, phrenic long-term facilitation (pLTF) following moderate acute intermittent hypoxia (IH; Dale-Nagle et al., 2010; Baker-Herman and Strey, 2011). In contrast to iPMF, pLTF is activity-independent (MacFarlane and Mitchell, 2009) and is expressed as a progressive ramping of phrenic burst amplitude that is often not significantly increased from baseline until 30 min following IH in anesthetized rats. Figure 3A depicts representative compressed phrenic neurograms, illustrating phrenic burst amplitude before, during and for 60 min following 3 hypoxic episodes. Figure 3B illustrates average change in phrenic burst amplitude from baseline for 60 min post-IH. In rats receiving control injections of intrathecal scrPKCζ-PS prior to IH, phrenic burst amplitude was significantly greater than baseline at all measured time points post-IH ($p<0.05$) and significantly different than time controls at 60 min post-IH ($p<0.05$). Similarly, in rats receiving intrathecal PKCζ-PS prior to IH, phrenic burst amplitude was significantly increased relative to baseline and time controls at 30 and 60 min ($p<0.05$). Importantly, there were no significant differences in phrenic burst amplitude between PKCζ-PS and scrPKCζ-PS treated rats at any time point following IH ($p>0.05$). These data suggest that spinal pre-treatment with an aPKC inhibitor did not impair pLTF following IH.

A separate group of rats received intrathecal scrPKCζ-PS or PKCζ-PS 10 min after IH exposure (scrPKCζ-PS-10 and PKCζ-PS-10, respectively; Figures 3A and 3C; representative trace for scrPKCζ-PS-10 not shown). As expected, scrPKCζ-PS-10 did not impair pLTF; rats receiving intrathecal scrPKCζ-PS-10 expressed significantly increased phrenic burst amplitude relative to baseline at 30, 60 and 90 min post-IH (all $p<0.05$). Similarly, rats receiving intrathecal

PKCζ-PS-10 expressed significant increases in phrenic burst amplitude relative to baseline at 30, 60 and 90 min post-IH (all $p<0.05$). Importantly, increased phrenic burst amplitude following IH was not significantly different between rats receiving intrathecal PKCζ-PS-10 and those receiving intrathecal scrPKCζ-PS-10. Together, these data suggest that unlike iPMF, pLTF does not require spinal aPKC activity.

iPMF requires PKCζ/ι activity

aPKC isoforms include PKCζ and PKCι/λ (PKCλ is the mouse homolog of PKCι, but for simplicity, we refer to PKCι/λ as PKCι) and a constitutively active form of PKCζ that lacks a regulatory subunit, known as PKMζ (Reyland, 2009).To determine if iPMF stabilization requires activation of PKCs containing both a regulatory and catalytic subunit, a subgroup of rats received intrathecal NPC-15437 10 min following restoration of respiratory neural activity (Figure 4). NPC-15437 binds to the regulatory domain of all PKC isoforms and, as such, does not inhibit PKMζ activity (Sullivan et al., 1991). Intrathecal NPC-15437 had no effect on phrenic burst amplitude in time control rats ($p>0.05$; data not shown), suggesting no time dependent change in phrenic burst amplitude due to NPC-15437 alone. As expected, rats exposed to a central neural apnea expressed a significant increase in phrenic burst amplitude relative to baseline and time controls 5 min following return of respiratory neural activity ($p<0.05$), indicating iPMF. However, following intrathecal NPC-15437 in this group, phrenic burst amplitude steadily declined, such that by 15 min post-neural apnea, phrenic burst amplitude was significantly lower relative to the 5 min time point and remained at or below these levels until the end of the recording (all $p<0.05$). In addition, all measured time points following intrathecal NPC-15437 were not significantly different than baseline or time controls ($p>0.05$). These data

suggest that an isoform of PKC containing a regulatory subunit is necessary for iPMF expression.

Since NPC-15437 inhibits full-length PKCs in all sub-families (i.e., classical, novel and atypical), we sought to rule out an additional requirement for other, non-atypical PKC isoforms in iPMF. Thus, iPMF was induced by a neural apnea, and then intrathecal BIM (0.24 or 2.5 nmoles) was delivered 10 min following restoration of respiratory neural activity (Figure 4). BIM inhibits classical and novel PKCs with 30 – 300x higher affinity than aPKCs (Toullec et al., 1991; Martiny-Baron et al., 1993). The 0.24 nmole dose of BIM is consistent with doses previously used to block other forms of spinal plasticity (Yashpal et al., 1995; Hua et al., 1999; Ferguson et al., 2008); further, the 2.5 nmole spinal dose of BIM is known to block phrenic motor facilitation induced by intrathecal injections of a classical and novel PKC isoform activator, phorbol-12-myristate-13-acetate (PMA; M. Devinney and G. Mitchell, personal communication). In contrast to NPC-15437 or PKCζ-PS-10, intrathecal BIM (0.24 or 2.5 nmoles) 10 min post-neural apnea had no effect on iPMF since phrenic burst amplitude remained elevated from baseline at all time points post-drug injection (all $p<0.05$). Indeed, phrenic amplitude (and iPMF magnitude) at all time points following intrathecal BIM were not significantly different than pre-BIM levels (0.24 or 2.5 nmoles, $p>0.05$). Thus, classical and novel PKCs are not necessary to develop late-iPMF.

Collectively, our data strongly demonstrate that: 1) spinal PKCζ and/or PKCι activity is/are necessary in the transition to late iPMF; 2) once late iPMF is established, neither PKCζ and/or PKCι activity are necessary to maintain iPMF; and 3) neither novel nor conventional PKC isoforms are necessary in the transition to late iPMF.

Increased association of PKCζ/ι with p62 during early, but not late iPMF.

As previously reported (Guenther et al., 2010), aPKCs were expressed in retrogradely labeled phrenic motor neurons (Figure 5). aPKC levels in identified phrenic motor neurons did not change 60 min following restoration of respiratory neural activity after a neural apnea (immunofluorescence intensity for time control: 1 $\pm$ 0.2 A.U.; 60 min post-neural apnea: 1 $\pm$ 0.1 A.U.; $p>0.05$; data not shown). Confirming these findings, immunoblot analysis indicated that aPKC levels in ventral spinal regions associated with the phrenic motor pool did not change 5 or 60 min following restoration of respiratory neural activity (data not shown).

Following activation, PKCζ and PKCι bind to specific binding proteins to assemble a multimeric signaling complex, which confers specificity in aPKC signal transduction (Moscat et al., 2006; Moscat et al., 2007). One such binding protein is the scaffolding protein p62/ZIP (ZIP, zeta interacting peptide, is the rat homolog of human p62, also known as sequestosome [SQSTM1]). p62/ZIP binds to the PB1 domain in the regulatory subunit of PKCζ and PKCι, and as such, does not interact with PKMζ. Immunofluorescence confirmed that p62/ZIP and aPKC are co-localized in CTB-labeled phrenic motor neurons (Figure 6A). p62/ZIP also labeled large, putative motor neurons in the ventral horn that were not labeled with CTB, suggesting that p62/ZIP is highly expressed in alpha motor neurons.

To determine if iPMF is associated with an increase in the interaction between PKCζ/ι and p62/ZIP, spinal segments containing the bulk of the phrenic motor pool (C4) were collected at 10, 20 or 60 min following the restoration of respiratory neural activity or equivalent time points in time controls. aPKC in ventral C4 gray matter homogenates was then immunoprecipitated, and immunoblot analysis for p62/ZIP was performed on the immunoprecipitates to assess p62/ZIP binding to endogenous aPKC (Figures 6B and 6C).

p62/ZIP density in rats exposed to neural apnea was expressed relative to time controls. Consistent with our neuropharmacology results, rats exposed to a neural apnea exhibited increased p62/ZIP binding with PKCζ/ι in spinal segments associated with the phrenic motor nucleus at 10 ($p<0.05$), but not 60 min ($p>0.05$) following restoration of respiratory neural activity. We observed a trend toward increased p62/ZIP binding with PKCζ/ι at 20 min following restoration of respiratory neural activity ($p=0.056$). Collectively, these data suggest that early, but not late phases of iPMF are associated with an increased interaction between p62/ZIP and PKCζ/ι in regions of the spinal cord containing phrenic motor neurons. Collectively, our data indicate that spinal PKCζ and/or PKCι (but not PKMζ) in or near the phrenic motor pool is/are required to transition from early, labile increases in phrenic burst amplitude into longer-lasting, stable iPMF, likely *via* the formation of a p62/ZIP-PKCζ/ι signaling cassette.

DISCUSSION

We report a key role for spinal PKCζ/ι in iPMF, a novel form of plasticity in phrenic motor output induced by respiratory neural inactivity. iPMF consists of at least two mechanisms: 1) an early, transient phase that is only partially dependent on spinal aPKC activity, and 2) a longer-lasting phase that requires spinal aPKC activity, specifically isoforms PKCζ or PKCι/λ to develop but not to maintain. We also report that neural apnea induces an early, transient increase in PKCζ/ι binding to p62/ZIP in spinal regions associated with the phrenic motor pool. We hypothesize that neural apnea leads to formation of a PKCζ/ι-p62/ZIP signaling cassette within the phrenic motor pool, which stabilizes inactivity-induced increases in phrenic burst amplitude and creates long-lasting iPMF. The role of spinal PKCζ/ι activity is unique to iPMF since aPKC inhibition had no effect on pLTF following IH. Overall, our data suggest that spinal mechanisms

sense and respond to reduced respiratory-related inputs to phrenic motor neurons. To our knowledge, this is the first report demonstrating a key role for PKCζ/ι in any form of spinal motor plasticity.

PKCζ/ι is required for iPMF but not pLTF

In recent years, multiple, distinct cellular cascades leading to phrenic motor facilitation (pMF) have been described (Zhang et al., 2003; Golder et al., 2008; Dale-Nagle et al., 2010; Hoffman and Mitchell, 2011; MacFarlane et al., 2011; Mahamed et al., 2011; Nichols et al., 2012). The most frequently studied form of pMF is pLTF following acute IH (Dale-Nagle et al., 2010; Terada and Mitchell, 2011). The working model of pLTF suggests that IH leads to serotonin release near phrenic motor neurons, activating 5-HT_2 receptors (Baker-Herman and Mitchell, 2002; Dale-Nagle et al., 2010) and increasing brain derived neurotrophic factor (BDNF) synthesis; BDNF subsequently activates its high affinity receptor, TrkB (Baker-Herman et al., 2004), and leads to ERK MAP kinase activation (Wilkerson and Mitchell, 2009). Unlike iPMF, pLTF is activity-independent, since intermittent spinal serotonin receptor activation elicits pLTF without changes in phrenic activity and in the absence of hypoxia (MacFarlane and Mitchell, 2009).

Mechanisms giving rise to iPMF were previously unknown. We originally hypothesized that both pLTF and iPMF required spinal aPKC activity since the constitutively active aPKC isoform, PKMζ, plays a prominent role in several models of synaptic plasticity (Sacktor et al., 1993; Drier et al., 2002; Ling et al., 2002; Sutton et al., 2004; Pastalkova et al., 2006; Sacktor, 2008; Villareal et al., 2009) including spinal nociceptive plasticity (Laferriere et al., 2012; Zhang et al., 2012). In many studies (including ours), aPKC activity was inhibited with ζ-

pseudosubstrate inhibitory peptide (PKCζ-PS), which mimics the auto-inhibitory pseudosubstrate sequence in the regulatory domain of PKCζ. Although frequently claimed to be a selective inhibitor for PKMζ, PKCζ-PS binds to and inhibits the catalytic domain of all aPKC isoforms (Selbie et al., 1993; Suzuki et al., 2003; Moscat et al., 2006). Thus, it is not possible to identify the specific aPKC isoform using this compound. Contrary to our original hypothesis, we found that rats pre-treated with intrathecal PKCζ-PS exhibited an attenuated, transient iPMF after neural apnea, with no effect on pLTF following IH.

Since late, but not early iPMF was abolished by spinal aPKC inhibition, we sought to determine critical times during iPMF that require spinal aPKC activity. When delivered shortly after early iPMF induction (10 min), PKCζ-PS "erased" the ongoing iPMF; when delivered 45 min after the establishment of iPMF, PKCζ-PS had no effect. Thus, spinal aPKC activity is necessary to transition from a transient to a longer-lasting form of iPMF, suggesting a role for spinal aPKC in stabilization of inactivity-induced facilitation. It is unlikely that the relevant isoform for iPMF stabilization is PKMζ since NPC-15437, which inhibits all PKC isoforms *via* the regulatory domain (Sullivan et al., 1991) also "erased" the ongoing iPMF. Further, since BIM had no effect on iPMF (a PKC inhibitor with 30–300x greater affinity for novel/classical *versus* aPKC isoforms;Toullec et al., 1991; Martiny-Baron et al., 1993), we suggest that the aPKC isoform(s) necessary for iPMF stabilization is/are PKCζ and/or PKCι.

In contrast to iPMF, spinal aPKC activity is not necessary for pLTF following acute intermittent hypoxia since intrathecal PKCζ-PS had no effect on the magnitude of pLTF when delivered either before or after pLTF induction. Thus, the requirement for spinal aPKC activity in phrenic plasticity appears to characterize inactivity-induced phrenic plasticity.

Interactions between PKCζ/ι and p62/ZIP

Scaffolding/adaptor proteins coordinate signaling networks and confer specificity in cell signaling processes by targeting multifunctional kinases to particular sites, and restraining access to unwanted substrates (Mochly-Rosen, 1995; Moscat et al., 2007; Good et al., 2011). One scaffolding/adaptor protein targeting PKCζ/ι is p62/ZIP (Puls et al., 1997; Sanchez et al., 1998). PKCζ and PKCι interact with p62/ZIP *via* the PB1 protein-protein interaction domain unique to the regulatory subunit of aPKCs; thus, PKMζ or classical or novel PKC isoforms are not expected to interact with ZIP/62 (Puls et al., 1997; Sanchez et al., 1998). In response to specific stimuli, PKCζ/ι binds to p62/ZIP, which relocates and anchors the activated kinase to a context-specific signaling complex (Mochly-Rosen, 1995; Samuels et al., 2001). p62/ZIP is not a substrate, nor does it affect kinase activity of PKCζ or PKCι; rather, p62/ZIP contains protein binding motifs suggesting a role in shuttling/binding PKCζ/ι to stimulus-specific multimeric signaling complexes (Puls et al., 1997; Sanchez et al., 1998; Suzuki et al., 2003), including complexes associated with AMPA (Jiang et al., 2009), TrkA (Wooten et al., 2001), $GABA_C$ (Croci et al., 2003), TNFα (Sanz et al., 1999; Sanz et al., 2000) or IL-1 (Sanz et al., 2000) receptors. Thus, p62/ZIP imparts specificity in substrate selectivity to PKCζ/ι in response to a given stimulus (Moscat et al., 2006; Moscat et al., 2007).

Neural apnea induced an early, transient interaction between PKCζ/ι and p62/ZIP in spinal regions associated with the phrenic motor nucleus, consistent with our neuropharmacology data showing that spinal PKCζ/ι inhibition shortly following restoration of respiratory neural activity impaired subsequent iPMF. We hypothesize that spinal PKCζ/ι stabilizes early, labile increases in phrenic burst amplitude *via* interactions with p62/ZIP, leading to long-lasting iPMF. In our working model, reduced synaptic inputs to the phrenic motor pool activates PKCζ/ι and

leads to formation of a PKCζ/ι-p62/ZIP signaling cassette, which anchors activated PKCζ/ι at critical sub-cellular sites to give rise to iPMF. Since phrenic motor neurons richly express both aPKC and p62/ZIP, we speculate that PKCζ/ι -p62/ZIP complex formation occurs within phrenic motor neurons, although we cannot rule out a similar effect in pre-synaptic neurons.

Mechanisms activating PKCζ/ι and inducing p62/ZIP-PKCζ/ι complex formation are not yet known. TNF-receptor 1 (TNF-R1) activation induces p62/ZIP-PKCζ/ι complex formation, a key step in TNFα signaling (Sanz et al., 1999). Our preliminary data suggest that iPMF requires spinal TNFα (unpublished observations); thus p62/ZIP may recruit PKCζ/ι to the TNF-R1 complex during or following phrenic inactivity. p62/ZIP-PKCζ/ι may traffic relevant proteins (Geetha and Wooten, 2003; Jiang et al., 2009) to or from the membrane, thereby increasing synaptic strength between bulbo-spinal respiratory pre-motor neurons and phrenic motor neurons. Consistent with this hypothesis, the p62/ZIP-PKCζ/ι cassette recruits AMPA receptors to synapses during early hippocampal LTP (Jiang et al., 2009).

iPMF significance

Although motor neuron pools driving respiratory muscles must be rhythmically active from birth until death, mechanisms enabling respiratory neurons to adapt to prolonged changes in neuronal excitability and/or synaptic inputs throughout life are unknown. iPMF may represent one component in a continuum of "homeostatic plasticity" mechanisms (Turrigiano, 2008) that enable adjustments in neuronal activity during challenges to breathing. In particular, iPMF may preserve inspiratory motor output at times when an animal is at risk from inadequate respiratory stimulation or excessive inhibition (Hengen et al., 2012).

An extreme example of inadequate respiratory stimulation is cervical spinal injury. Traumatic injury induces many changes in the spinal cord, including tissue damage, inflammation, ischemia and withdrawal of synaptic inputs caudal to injury (Hausmann, 2003).Immediately following an incomplete high cervical injury, an initially silent phrenic nerve slowly and spontaneously regains (partial) function over days to weeks post-injury (Nantwi et al., 1999; Golder et al., 2001; Golder et al., 2003; Goshgarian, 2003; Golder and Mitchell, 2005; Fuller et al., 2006; Vinit et al., 2007; Lane et al., 2008). Remodeling spinal circuits post-injury may restore phrenic motor ouptut ipsilateral to injury by recruiting latent contralateral pathways crossing the midline at the level of the phrenic pool (ie. the "crossed phrenic phenomenon"; Goshgarian, 2009; Lane et al., 2009; Darlot et al., 2012) or strengthening spared ipsilateral pathways (Vinit et al., 2008; Vinit and Kastner, 2009). Inactivity may play a prominent role in restoring function by strengthening spared pathways *via* the same mechanisms responsible for iPMF. Consistent with this hypothesis, increased aPKC enzymatic activity and phosphorylated aPKC expression are observed in spinal segments associated with the phrenic motor pool following cervical spinal injury at times consistent with a role in spontaneous functional recovery (Guenther et al., 2012). While we do not yet know the role for iPMF in compensating for impaired respiratory neural output, understanding mechanisms of iPMF may guide novel therapeutic strategies to treat ventilatory control disorders in patients where endogenous plasticity is impaired or insufficient.

Acknowledgements: We would like to thank Gordon S. Mitchell for his comments on this manuscript. This work was supported by a grant from the National Institutes of Health (HL105511).

The authors declare no conflicts of interest.

REFERENCES

Asiedu MN, Tillu DV, Melemedjian OK, Shy A, Sanoja R, Bodell B, Ghosh S, Porreca F, Price TJ (2011) Spinal protein kinase M zeta underlies the maintenance mechanism of persistent nociceptive sensitization. J Neurosci 31:6646-6653.

Baker-Herman TL, Mitchell GS (2002) Phrenic long-term facilitation requires spinal serotonin receptor activation and protein synthesis. J Neurosci 22:6239-6246.

Baker-Herman TL, Strey KA (2011) Similarities and differences in mechanisms of phrenic and hypoglossal motor facilitation. Respir Physiol Neurobiol 179:48-56.

Baker-Herman TL, Fuller DD, Bavis RW, Zabka AG, Golder FJ, Doperalski NJ, Johnson RA, Watters JJ, Mitchell GS (2004) BDNF is necessary and sufficient for spinal respiratory plasticity following intermittent hypoxia. Nat Neurosci 7:48-55.

Castro-Moure F, Goshgarian HG (1996) Reversible cervical hemispinalization of the rat spinal cord by a cooling device. Exp Neurol 141:102-112.

Castro-Moure F, Goshgarian HG (1997) Morphological plasticity induced in the phrenic nucleus following cervical cold block of descending respiratory drive. Exp Neurol 147:299-310.

Croci C, Brandstatter JH, Enz R (2003) ZIP3, a new splice variant of the PKC-zeta-interacting protein family, binds to GABAC receptors, PKC-zeta, and Kv beta 2. The Journal of biological chemistry 278:6128-6135.

Dale-Nagle EA, Hoffman MS, MacFarlane PM, Mitchell GS (2010) Multiple pathways to long-lasting phrenic motor facilitation. Advances in experimental medicine and biology 669:225-230.

Darlot F, Cayetanot F, Gauthier P, Matarazzo V, Kastner A (2012) Extensive respiratory plasticity after cervical spinal cord injury in rats: axonal sprouting and rerouting of ventrolateral bulbospinal pathways. Exp Neurol 236:88-102.

Drier EA, Tello MK, Cowan M, Wu P, Blace N, Sacktor TC, Yin JC (2002) Memory enhancement and formation by atypical PKM activity in Drosophila melanogaster. Nat Neurosci 5:316-324.

Ferguson AR, Bolding KA, Huie JR, Hook MA, Santillano DR, Miranda RC, Grau JW (2008) Group I metabotropic glutamate receptors control metaplasticity of spinal cord learning through a protein kinase C-dependent mechanism. J Neurosci 28:11939-11949.

Fuller DD, Golder FJ, Olson EB, Mitchell GS (2006) Recovery of phrenic activity and ventilation after cervical spinal hemisection in rats. J Appl Physiol 100:800-806.

Geetha T, Wooten MW (2003) Association of the atypical protein kinase C-interacting protein p62/ZIP with nerve growth factor receptor TrkA regulates receptor trafficking and Erk5 signaling. The Journal of biological chemistry 278:4730-4739.

Golder FJ, Mitchell GS (2005) Spinal synaptic enhancement with acute intermittent hypoxia improves respiratory function after chronic cervical spinal cord injury. J Neurosci 25:2925-2932.

Golder FJ, Reier PJ, Davenport PW, Bolser DC (2001) Cervical spinal cord injury alters the pattern of breathing in anesthetized rats. J Appl Physiol 91:2451-2458.

Golder FJ, Fuller DD, Davenport PW, Johnson RD, Reier PJ, Bolser DC (2003) Respiratory motor recovery after unilateral spinal cord injury: eliminating crossed phrenic activity decreases tidal volume and increases contralateral respiratory motor output. J Neurosci 23:2494-2501.

Golder FJ, Ranganathan L, Satriotomo I, Hoffman M, Lovett-Barr MR, Watters JJ, Baker-Herman TL, Mitchell GS (2008) Spinal adenosine A2a receptor activation elicits long-lasting phrenic motor facilitation. J Neurosci 28:2033-2042.

Good MC, Zalatan JG, Lim WA (2011) Scaffold proteins: hubs for controlling the flow of cellular information. Science 332:680-686.

Goshgarian HG (2003) The crossed phrenic phenomenon: a model for plasticity in the respiratory pathways following spinal cord injury. J Appl Physiol 94:795-810.

Goshgarian HG (2009) The crossed phrenic phenomenon and recovery of function following spinal cord injury. Respir Physiol Neurobiol 169:85-93.

Guenther CH, Vinit S, Windelborn JA, Behan M, Mitchell GS (2010) Atypical protein kinase C expression in phrenic motor neurons of the rat. Neuroscience 169:787-793.

Guenther CH, Windelborn JA, Tubon TC, Jr., Yin JC, Mitchell GS (2012) Increased atypical PKC expression and activity in the phrenic motor nucleus following cervical spinal injury. Exp Neurol 234:513-520.

Hausmann ON (2003) Post-traumatic inflammation following spinal cord injury. Spinal cord 41:369-378.

Hengen KB, Nelson NR, Stang KM, Johnson SM, Crader SM, Watters JJ, Mitchell GS, Behan M (2012) Increased GABA(A) receptor epsilon-subunit expression on ventral respiratory column neurons protects breathing during pregnancy. PloS one 7:e30608.

Hoffman MS, Mitchell GS (2011) Spinal 5-HT7 receptor activation induces long-lasting phrenic motor facilitation. J Physiol 589:1397-1407.

Hua XY, Chen P, Yaksh TL (1999) Inhibition of spinal protein kinase C reduces nerve injury-induced tactile allodynia in neuropathic rats. Neurosci Lett 276:99-102.

Jiang J, Parameshwaran K, Seibenhener ML, Kang MG, Suppiramaniam V, Huganir RL, Diaz-Meco MT, Wooten MW (2009) AMPA receptor trafficking and synaptic plasticity require SQSTM1/p62. Hippocampus 19:392-406.

Laferriere A, Pitcher MH, Haldane A, Huang Y, Cornea V, Kumar N, Sacktor TC, Cervero F, Coderre TJ (2011) PKMzeta is essential for spinal plasticity underlying the maintenance of persistent pain. Molecular pain 7:99.

Lane MA, Lee KZ, Fuller DD, Reier PJ (2009) Spinal circuitry and respiratory recovery following spinal cord injury. Respir Physiol Neurobiol 169:123-132.

Lane MA, White TE, Coutts MA, Jones AL, Sandhu MS, Bloom DC, Bolser DC, Yates BJ, Fuller DD, Reier PJ (2008) Cervical prephrenic interneurons in the normal and lesioned spinal cord of the adult rat. J Comp Neurol 511:692-709.

Ling DS, Benardo LS, Serrano PA, Blace N, Kelly MT, Crary JF, Sacktor TC (2002) Protein kinase Mzeta is necessary and sufficient for LTP maintenance. Nat Neurosci 5:295-296.

MacFarlane PM, Mitchell GS (2009) Episodic spinal serotonin receptor activation elicits long-lasting phrenic motor facilitation by an NADPH oxidase-dependent mechanism. J Physiol 587:5469-5481.

MacFarlane PM, Vinit S, Mitchell GS (2011) Serotonin 2A and 2B receptor-induced phrenic motor facilitation: differential requirement for spinal NADPH oxidase activity. Neuroscience 178:45-55.

Mahamed S, Strey KA, Mitchell GS, Baker-Herman TL (2011) Reduced respiratory neural activity elicits phrenic motor facilitation. Respir Physiol Neurobiol 175:303-309.

Mantilla CB, Sieck GC (2009) Neuromuscular adaptations to respiratory muscle inactivity. Respir Physiol Neurobiol 169:133-140.

Mantilla CB, Zhan WZ, Sieck GC (2009) Retrograde labeling of phrenic motoneurons by intrapleural injection. J Neurosci Methods 182:244-249.

Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D, Schachtele C (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. The Journal of biological chemistry 268:9194-9197.

Mochly-Rosen D (1995) Localization of protein kinases by anchoring proteins: a theme in signal transduction. Science 268:247-251.

Moscat J, Diaz-Meco MT, Wooten MW (2007) Signal integration and diversification through the p62 scaffold protein. Trends in biochemical sciences 32:95-100.

Moscat J, Diaz-Meco MT, Albert A, Campuzano S (2006) Cell signaling and function organized by PB1 domain interactions. Molecular cell 23:631-640.

Nantwi KD, El-Bohy AA, Schrimsher GW, Reier PJ, Goshgarian HG (1999) Spontaneous Functional Recovery in a Paralyzed Hemidiaphragm Following Upper Cervical Spinal Cord Injury in Adult Rats. . Neurorehabil Neural Repair 13: :225-234. .

Nichols NL, Dale EA, Mitchell GS (2012) Severe acute intermittent hypoxia elicits phrenic long-term facilitation by a novel adenosine-dependent mechanism. J Appl Physiol 112:1678-1688.

Pastalkova E, Serrano P, Pinkhasova D, Wallace E, Fenton AA, Sacktor TC (2006) Storage of spatial information by the maintenance mechanism of LTP. Science 313:1141-1144.

Puls A, Schmidt S, Grawe F, Stabel S (1997) Interaction of protein kinase C zeta with ZIP, a novel protein kinase C-binding protein. Proc Natl Acad Sci U S A 94:6191-6196.

Reyland ME (2009) Protein kinase C isoforms: Multi-functional regulators of cell life and death. Front Biosci 14:2386-2399.

Sacktor TC (2008) PKMzeta, LTP maintenance, and the dynamic molecular biology of memory storage. Prog Brain Res 169:27-40.

Sacktor TC, Osten P, Valsamis H, Jiang X, Naik MU, Sublette E (1993) Persistent activation of the zeta isoform of protein kinase C in the maintenance of long-term potentiation. Proc Natl Acad Sci U S A 90:8342-8346.

Samuels IS, Seibenhener ML, Neidigh KB, Wooten MW (2001) Nerve growth factor stimulates the interaction of ZIP/p62 with atypical protein kinase C and targets endosomal localization: evidence for regulation of nerve growth factor-induced differentiation. Journal of cellular biochemistry 82:452-466.

Sanchez P, De Carcer G, Sandoval IV, Moscat J, Diaz-Meco MT (1998) Localization of atypical protein kinase C isoforms into lysosome-targeted endosomes through interaction with p62. Molecular and cellular biology 18:3069-3080.

Sanz L, Diaz-Meco MT, Nakano H, Moscat J (2000) The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. The EMBO journal 19:1576-1586.

Sanz L, Sanchez P, Lallena MJ, Diaz-Meco MT, Moscat J (1999) The interaction of p62 with RIP links the atypical PKCs to NF-kappaB activation. The EMBO journal 18:3044-3053.

Selbie LA, Schmitz-Peiffer C, Sheng Y, Biden TJ (1993) Molecular cloning and characterization of PKC iota, an atypical isoform of protein kinase C derived from insulin-secreting cells. The Journal of biological chemistry 268:24296-24302.

Sullivan JP, Connor JR, Tiffany C, Shearer BG, Burch RM (1991) NPC 15437 interacts with the C1 domain of protein kinase C. An analysis using mutant PKC constructs. FEBS letters 285:120-123.

Sutton MA, Bagnall MW, Sharma SK, Shobe J, Carew TJ (2004) Intermediate-term memory for site-specific sensitization in aplysia is maintained by persistent activation of protein kinase C. J Neurosci 24:3600-3609.

Suzuki A, Akimoto K, Ohno S (2003) Protein kinase C lambda/iota (PKClambda/iota): a PKC isotype essential for the development of multicellular organisms. Journal of biochemistry 133:9-16.

Terada J, Mitchell GS (2011) Diaphragm long-term facilitation following acute intermittent hypoxia during wakefulness and sleep. J Appl Physiol 110:1299-1310.

Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, et al. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. The Journal of biological chemistry 266:15771-15781.

Turrigiano GG (2008) The self-tuning neuron: synaptic scaling of excitatory synapses. Cell 135:422-435.

Villareal G, Li Q, Cai D, Fink AE, Lim T, Bougie JK, Sossin WS, Glanzman DL (2009) Role of protein kinase C in the induction and maintenance of serotonin-dependent enhancement of the glutamate response in isolated siphon motor neurons of Aplysia californica. J Neurosci 29:5100-5107.

Vinit S, Kastner A (2009) Descending bulbospinal pathways and recovery of respiratory motor function following spinal cord injury. Respir Physiol Neurobiol 169:115-122.

Vinit S, Stamegna JC, Boulenguez P, Gauthier P, Kastner A (2007) Restorative respiratory pathways after partial cervical spinal cord injury: role of ipsilateral phrenic afferents. Eur J Neurosci 25:3551-3560.

Vinit S, Darlot F, Stamegna JC, Sanchez P, Gauthier P, Kastner A (2008) Long-term reorganization of respiratory pathways after partial cervical spinal cord injury. Eur J Neurosci 27:897-908.

Wilkerson JE, Mitchell GS (2009) Daily intermittent hypoxia augments spinal BDNF levels, ERK phosphorylation and respiratory long-term facilitation. Exp Neurol 217:116-123.

Wooten MW, Seibenhener ML, Mamidipudi V, Diaz-Meco MT, Barker PA, Moscat J (2001) The atypical protein kinase C-interacting protein p62 is a scaffold for NF-kappaB activation by nerve growth factor. The Journal of biological chemistry 276:7709-7712.

Yashpal K, Pitcher GM, Parent A, Quirion R, Coderre TJ (1995) Noxious thermal and chemical stimulation induce increases in 3H-phorbol 12,13-dibutyrate binding in spinal cord dorsal horn as well as persistent pain and hyperalgesia, which is reduced by inhibition of protein kinase C. J Neurosci 15:3263-3272.

Zhang Y, McGuire M, White DP, Ling L (2003) Episodic phrenic-inhibitory vagus nerve stimulation paradoxically induces phrenic long-term facilitation in rats. J Physiol 551:981-991.

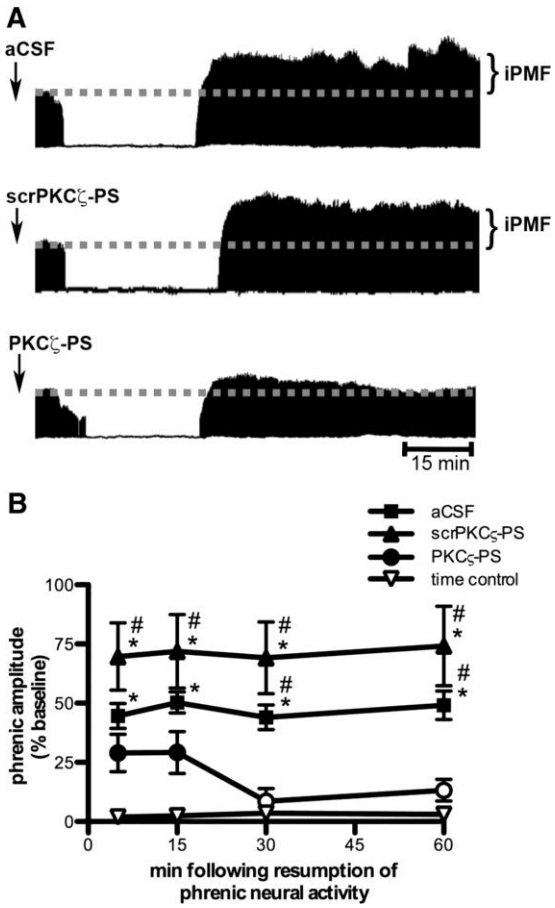

Figure 1: Spinal aPKC activity is necessary for iPMF. ***A.*** Representative compressed and integrated phrenic neurograms before, during and for 60 min following a 30 min neural apnea, illustrating that rats receiving control injections (arrows) of intrathecal aCSF (top) or scrPKCζ-PS (middle) prior to neural apnea expressed a prolonged increase in phrenic burst amplitude following resumption of respiratory neural activity, indicating iPMF. Only modest, transient increases in phrenic burst amplitude post-neural apnea were observed rats receiving intrathecal PKCζ-PS (bottom) prior to a neural apnea, indicating that spinal aPKC inhibition attenuated and shortened iPMF. ***B.*** Average change in phrenic burst amplitude from baseline for 60 min following resumption of respiratory neural activity in rats receiving intrathecal aCSF (squares), scrPKCζ-PS (triangles) or PKCζ-PS (circles) 20 min prior to neural apnea. Prolonged iPMF is expressed in rats receiving a control injection of aCSF or scrPKCζ-PS, when compared to baseline or time controls (inverted triangles). Rats receiving intrathecal PKCζ-PS (circles) expressed only transient, modest iPMF since phrenic burst amplitude was significantly increased from baseline only at 5 and 15 min following resumption of respiratory neural activity, which was not significantly different from time controls. No changes in phrenic burst amplitude were observed in time controls receiving similar surgery, but no neural apnea. Mean values $\pm$ SEM. Filled symbols indicate significantly different than baseline; *significantly different from time controls; # significantly different from PKCζ-PS rats; $p<0.05$.

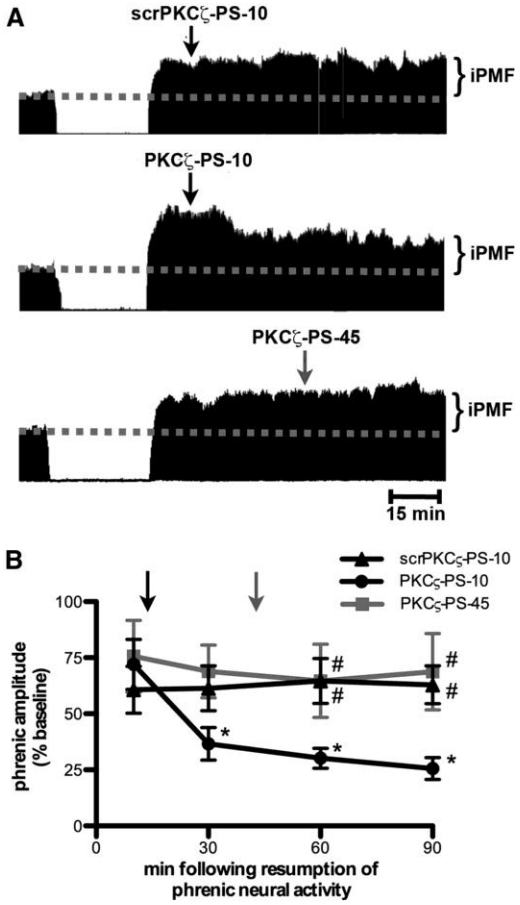

Figure 2: Spinal aPKC activity following a neural apnea stabilizes, but does not maintain iPMF. ***A.*** Representative compressed and integrated phrenic neurograms before, during and for 90 min following a 30 min neural apnea, illustrating that a control injection of intrathecal scrPKCζ-PS 10 min (scrPKCζ-PS-10; top) following resumption of respiratory neural activity did not impair neural apnea-induced iPMF. By contrast, intrathecal PKCζ-PS delivered 10 min (PKCζ-PS-10; middle), but not 45 min (PKCζ-PS-45; bottom), following resumption of respiratory neural activity returned phrenic burst amplitude toward baseline. Arrows indicate time points of drug delivery. ***B.*** Average change in phrenic burst amplitude from baseline for 90 min following neural apnea in rats receiving intrathecal scrPKCζ-PS-10 (triangles), PKCζ-PS-10 (circles) or PKCζ-PS-45 (gray square) at time points after resuming respiratory neural activity. Arrows indicate time points of drug delivery (black arrow for scrPKCζ-PS-10 and PKCζ-PS-10; gray arrow for PKCζ-PS-45). All rats expressed significant increases in phrenic burst amplitude relative to baseline or time controls (not shown) immediately prior to drug injections, indicating iPMF. Significant iPMF was observed in rats receiving intrathecal scrPKCζ-PS-10 for up to 90 min (vs. baseline or time controls), whereas iPMF progressively declined following PKCζ-PS-10 injections, such that by 30 min following resumption of respiratory neural activity, iPMF was significantly decreased from the pre-PKCζ-PS-10 injection value. No impairment in iPMF was observed in PKCζ-PS-45 rats. Intrathecal PKCζ-PS-10 was significantly lower than scrPKCζ-PS-10 and PKCζ-PS-45 at 60 and 90 min following neural apnea. Mean values $\pm$ SEM. Filled symbols indicate significantly different than baseline. *significantly different from pre-injection time point; # significantly different from PKCζ-PS-10 rats; $p<0.05$.

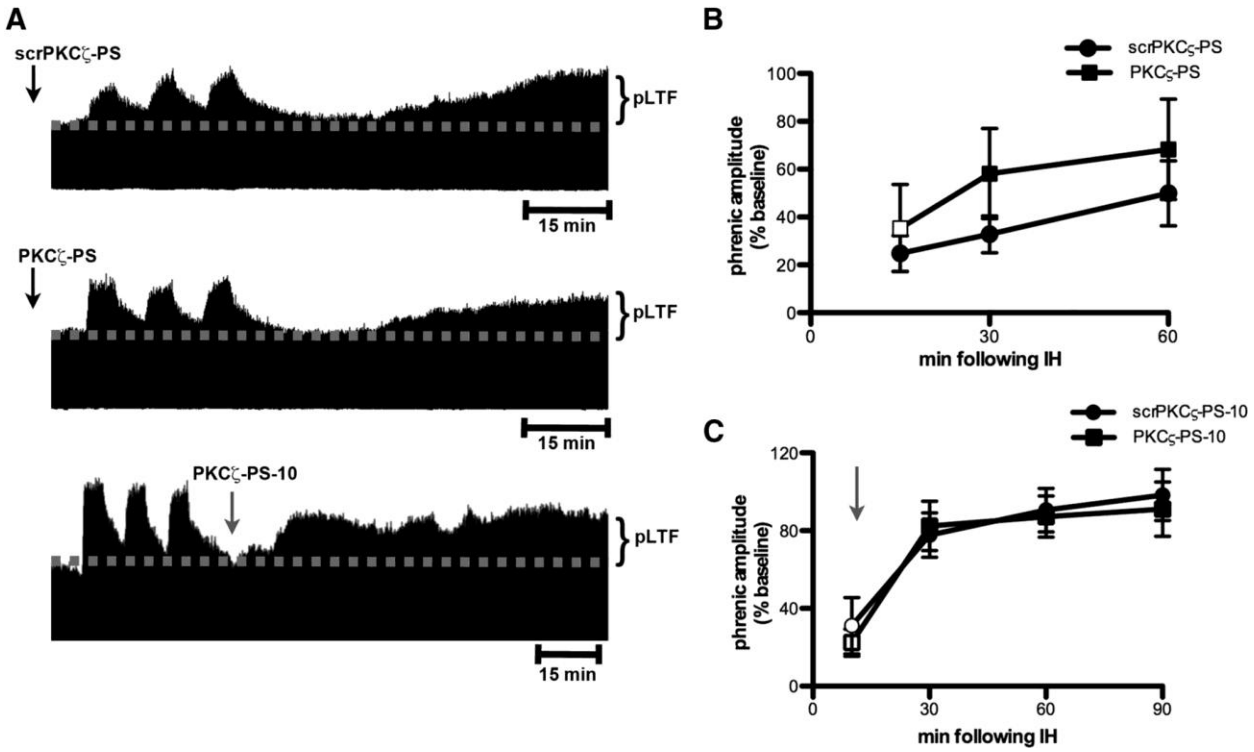

Figure 3: Intermittent hypoxia induced pLTF does not require spinal aPKC activity. ***A.*** Representative compressed and integrated phrenic neurograms before, during and for 60-90 min following 3, 5 min episodes of hypoxia (11% O_2), illustrating the development of pLTF in rats receiving intrathecal scrPKCζ-PS (top) or PKCζ-PS (middle) 20 min prior to IH, or PKCζ-PS delivered 10 min (PKCζ-PS-10; bottom) following IH. Arrows indicate time points of drug delivery. ***B.*** Average change in phrenic burst amplitude from baseline for 60 min following IH in rats receiving intrathecal scrPKCζ-PS (circles) or PKCζ-PS (squares) 20 min prior to IH. Both rat groups exhibited significantly increased phrenic burst amplitude 60 min following IH, relative to baseline and time controls (time controls not shown). No differences were observed between rats receiving intrathecal scrPKCζ-PS or PKCζ-PS at any point. *C.* Average change in phrenic burst amplitude from baseline following IH in rats receiving intrathecal scrPKCζ-PS (circles) or PKCζ-PS (squares) 10 min following IH. Black arrow indicates time point of drug delivery. Both groups exhibited significant increases in phrenic burst amplitude at 30, 60 and 90 min following IH, relative to baseline and time controls (time controls not shown). No differences were observed between rats receiving intrathecal scrPKCζ-PS-10 or PKCζ-PS-10. Mean values $\pm$ SEM. Filled symbols indicate significantly different than baseline. * significantly different from time controls; $p<0.05$.

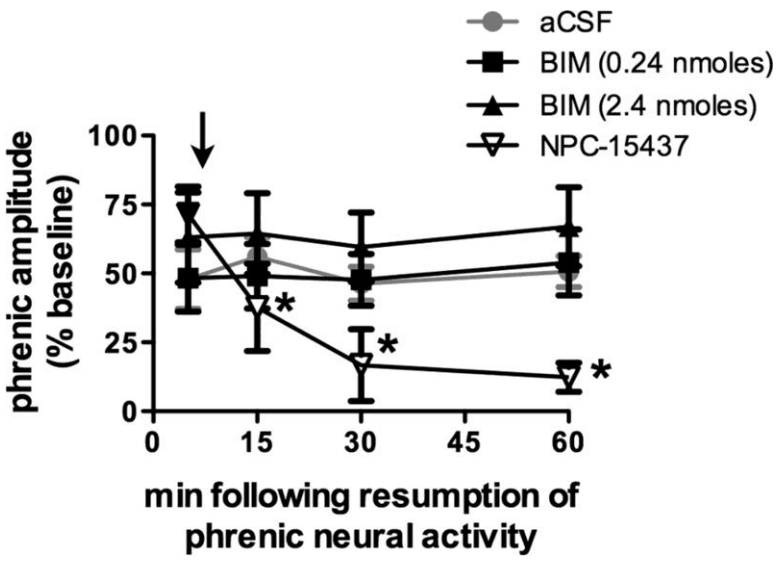

Figure 4: Stabilization of iPMF requires activity of spinal PKCζ/ι, but not classical or novel PKC isoforms. Average change in phrenic burst amplitude from baseline for 60 min following neural apnea in rats receiving intrathecal NPC-15437 (inverted triangles; inhibits PKC isoforms with a regulatory subunit), 0.24 nmoles BIM (squares; novel and classical PKC inhibitor) or 2.9 nmoles BIM (triangle) 10 min after resumption of respiratory neural activity. Black arrow indicates time point of drug delivery. All rats expressed significant increases in phrenic burst amplitude relative to baseline or time controls (not shown) immediately prior to drug injections, indicating iPMF. iPMF progressively declined following NPC-15437 injections; by 15 min following resumption of respiratory neural activity, iPMF was significantly decreased from pre-injection value and no longer significantly different from baseline or time controls. No change in iPMF magnitude was observed following intrathecal BIM (0.24 or 2.9 nmoles). Rats receiving intrathecal aCSF prior to neural apnea (from figure 1) are shown in gray circles for comparison. Mean values $\pm$ SEM. Filled symbols indicate significantly different from baseline. *significantly different from pre-injection time point; # significantly different from time controls; $p<0.05$.

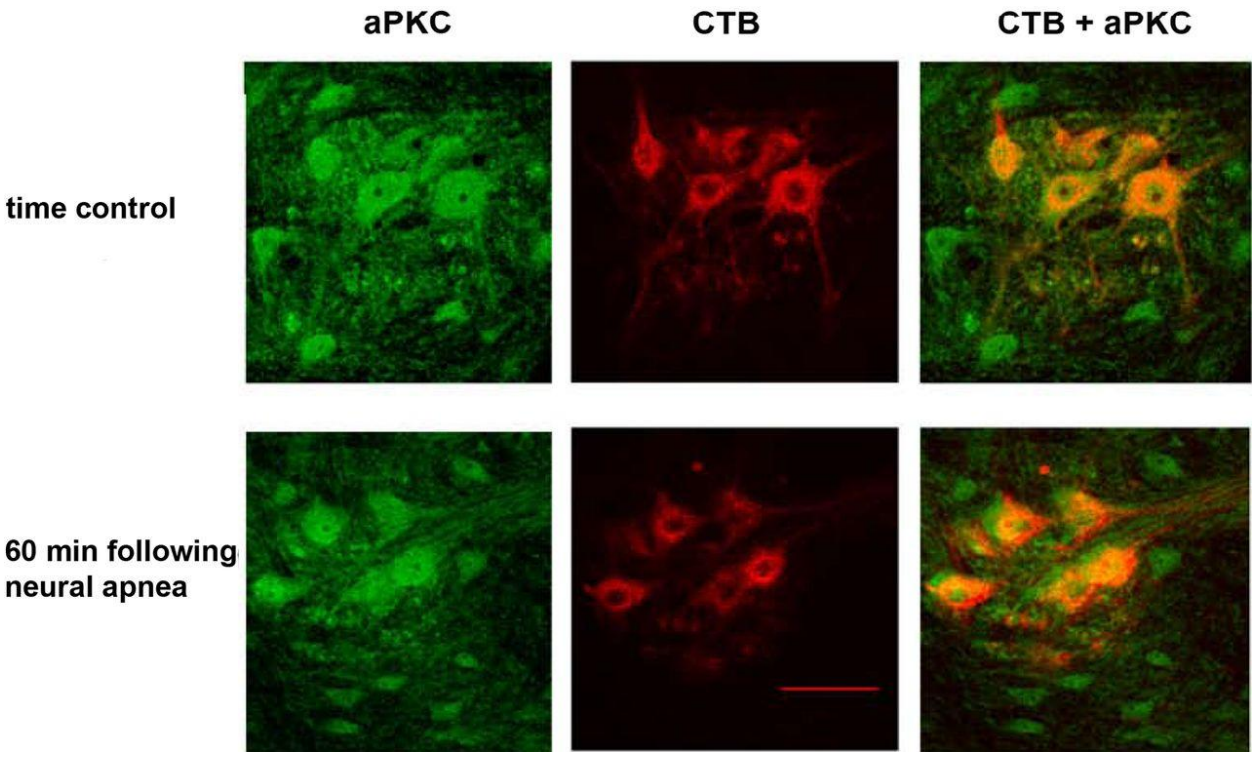

Figure 5: aPKC levels in CTB-labeled phrenic motor neurons do not change 60 min following neural apnea. Representative photomicrographs depicting aPKC (green) and CTB (red) in the C4 ventral horn of time controls (top panel) or 60 min following neural apnea (iPMF; bottom panel). Merged image on right demonstrates co-localization of CTB (+) phrenic motor neurons and aPKC. Scale bar = 100 μm (at 20X).

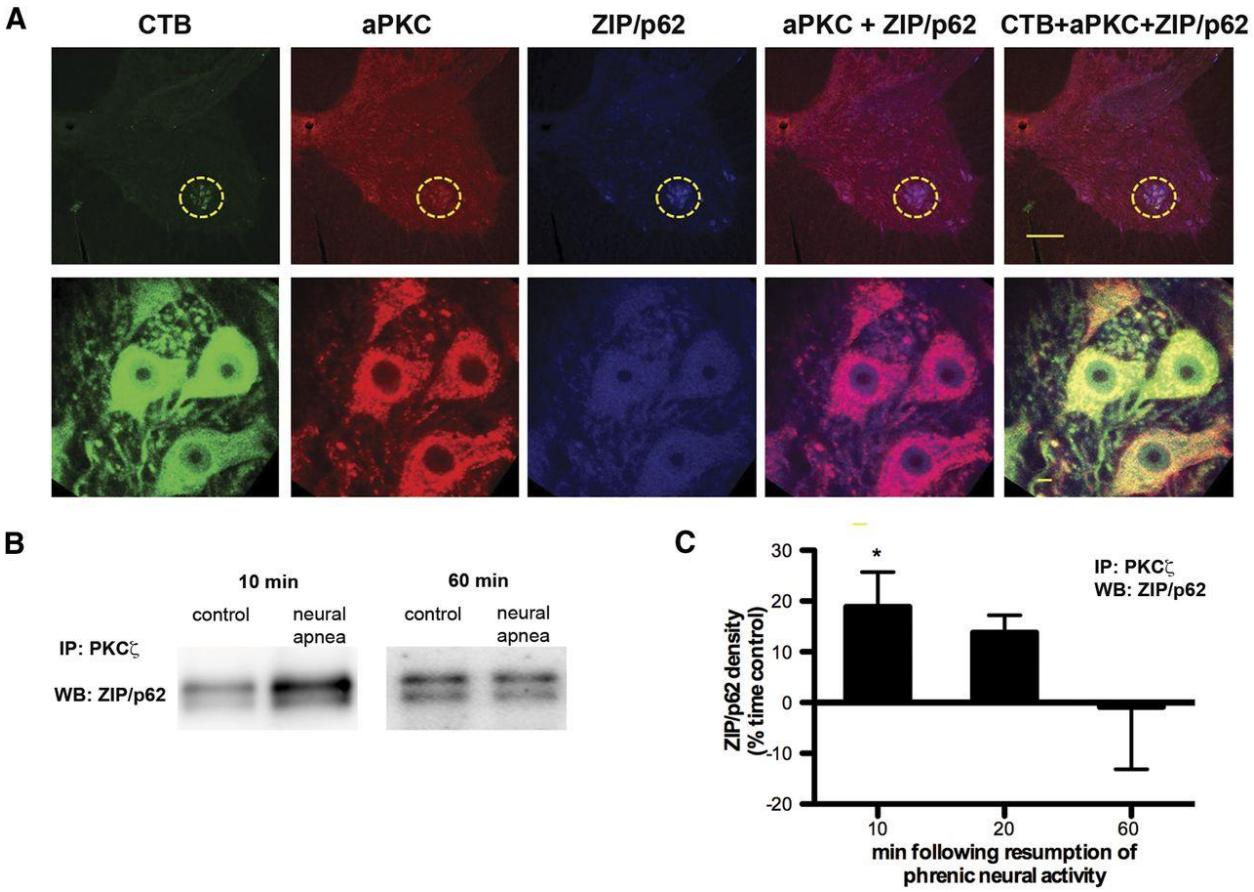

Figure 6: Increased association between PKCζ/ι and p62/ZIP in ventral spinal segments containing phrenic motor neurons. ***A.*** Representative images depicting CTB (green), aPKC (red) and p62/ZIP (blue) in the C4 ventral horn. Merged images on right demonstrate co-localization of p62/ZIP and aPKC in CTB+ phrenic motor neurons. The phrenic motor nucleus is encircled in top panels. Scale bar on top panel = 200 µm, bottom panel = 20 µm. ***B, C.*** PKCζ/ι was immunoprecipitated from ventral C4 gray matter homogenates; immunoprecipitates were then immunoblotted for p62/ZIP. ***B.*** Representative immunoblots of p62/ZIP 10 or 60 following resumption of respiratory neural activity after a neural apnea, or equivalent time points in time controls. ***C.*** Average change in p62/ZIP density from time controls illustrating significant increases in p62/ZIP binding to PKCζ/ι 10, but not 20 or 60 min after resumption of respiratory neural activity. *significantly different from zero; $p<0.05$.

		PO_2	PCO_2	pH
Time Control	baseline	250 ± 7	47.4 ± 1.5	7.36 ± 0.01
	60 min	253 ± 3	47.4 ± 1.5	7.35 ± 0.01
Neural apnea + pre-aCSF	baseline	253 ± 10	47.3 ± 1.2	7.35 ± 0.01
	60 min	249 ± 13	47.6 ± 1.3	7.34 ± 0.01
Neural apnea + pre-PKCζ-PS	baseline	239 ± 5	47 ± 1.9	7.34 ± 0.01
	60 min	246 ± 6	46.4 ± 1.9	7.33 ± 0.02
Neural apnea + pre-scrPKCζ-PS	baseline	240 ± 12	49.4 ± 1.5	7.34 ± 0.02
	60 min	241 ± 10	49.6 ± 1.6	7.33 ± 0.01
IH + pre-PKCζ-PS	baseline	266 ± 12	50.3 ± 1.2	7.34 ± 0.01
	60 min	270 ± 11	50.6 ± 1	7.30 ± 0.01
IH + pre-scrPKCζ-PS	baseline	277 ± 21	48.6 ± 2	7.37 ± 0.04
	60 min	270 ± 11	48.9 ± 2	7.33 ± 0.01
Neural apnea + PKCζ-PS-10	baseline	276 ± 24	51.8 ± 0.9	7.34 ± 0.01
	90 min	297 ± 7	51.4 ± 1	7.34 ± 0.01
Neural apnea + scrPKCζ-PS-10	baseline	276 ± 17	49.2 ± 1	7.33 ± 0.01
	90 min	281 ± 19	49.8 ± 1	7.34 ± 0.02
IH + PKCζ-PS-10	baseline	254 ± 28	47.3 ± 2.6	7.35 ± 0.02
	90 min	288 ± 13	47.1 ± 2.1	7.36 ± 0.01
IH + scrPKCζ-PS-10	baseline	265 ± 50	46.7 ± 1.9	7.36 ± 0.01
	90 min	286 ± 25	46.6 ± 1.7	7.38 ± 0.01
Neural apnea + PKCζ-PS-45	baseline	258 ± 4	42.3 ± 2	7.36 ± 0.02
	60 min	226 ± 26	43 ± 2.3	7.37 ± 0.01
Time control +NPC15437	baseline	299 ± 20	45.4 ± 1.6	7.37 ± 0.02
	90 min	290 ± 6	45 ± 1.3	7.38 ± 0.01
Neural apnea + NPC15437	baseline	282 ± 18	42.3 ± 1.3	7.38 ± 0.01
	90 min	303 ± 19	42.4 ± 1.4	7.40 ± 0.02
Neural apnea + BIM	baseline	275 ± 6	43 ± 0.4	7.36 ±0.01
	90 min	244 ± 15	43.1 ± 0.6	7.38 ± 0.03

Table 1: Arterial levels of PO_2, PCO_2 and pH in rat groups before and at the end of the protocol. No significant differences were observed over time in any group. Mean values ± SEM.

CHAPTER III

Decreased spinal synaptic inputs to phrenic motor neurons elicit localized inactivity-induced phrenic motor facilitation

K.A. Strey and T.L. Baker-Herman

Submitted to Exp. Neurol., 2013

ABSTRACT

In ventilated rats, central neural apnea reduces respiratory neural activity throughout the neuraxis and elicits a form of neuroplasticity called inactivity-induced phrenic motor facilitation (iPMF). We hypothesized that activity-dependent mechanisms localized to the phrenic motor pool (i.e. spinal) sense and respond to reduced phrenic synaptic inputs to give rise to iPMF. To test this hypothesis directly, ventilated rats received nano-injections of procaine in the C2 ventrolateral funiculus (VLF) to temporarily block conduction in bulbospinal respiratory axons innervating the one phrenic motor pool, while activity in the phrenic motor pool contralateral to conduction block was maintained. Upon resumption of respiratory neural activity, phrenic amplitude ipsilateral to C2 axon conduction block was significantly increased indicating iPMF. Interestingly, contralateral phrenic burst amplitude was also enhanced suggesting a crossed spinal phrenic motor facilitation (csPMF). In addition, the maximum phrenic response to a severe respiratory stimulus (hypercapnia) was significantly enhanced in the ipsilateral and contralateral motor pool, suggesting a proportional increase in the phrenic dynamic range. Finally, we demonstrate that iPMF, but not csPMF requires spinal tumor necrosis factor alpha (TNFα) signaling and atypical PKC (aPKC) activity. Collectively, our results suggest that local, spinal mechanisms sense and respond to reduced respiratory neural activity--in and of itself. In addition, we describe a novel form of plasticity elicited in the contralateral motor pool (csPMF) following reduced ipsilateral phrenic activity. We hypothesize that iPMF and csPMF may assure neural activity is maintained within phrenic motor neurons throughout life.

INTRODUCTION

Breathing is a fundamental and essential behavior. From birth until death, the respiratory control system must transmit a regular, rhythmic motor output to the respiratory musculature of appropriate magnitude to enable optimal gas exchange. If activity in respiratory motor neurons ceases for longer than minutes, death quickly follows. An emerging principle of neuroscience is that perturbations in neural activity are sensed and locally adjusted to assure neurons continue to operate within an optimal range (Turrigiano, 2008). It is unknown if similar activity-dependent mechanisms maintain neural activity within respiratory neural networks.

In ventilated rats, a prolonged reduction in central respiratory neural activity elicits a rebound increase in respiratory motor output in the phrenic nerve (innervating the diaphragm), a form of neuroplasticity known as inactivity-induced phrenic motor facilitation (iPMF; Mahamed et al., 2011; Strey et al., 2012; Baertsch and Baker-Herman, 2013). Since multiple forms of central neural apnea with different mechanisms of action elicit iPMF (Mahamed et al., 2011), we proposed that iPMF arises from a feature common to all forms: reduced respiratory neural activity (Baker-Herman and Strey, 2011) However, since all forms elicit reduced respiratory neural activity indirectly, it remains possible other factors contribute to iPMF expression. For example, the most common method to induce iPMF is *via* hyperventilation, leading to neural apnea by lowering arterial CO_2 levels below the so-called apneic threshold for breathing. However, in addition to stopping the neural drive to breathe, hypocapnia and/or associated alkalosis results in decreased cerebral blood flow and reduced oxygen unloading at the tissues (Vogel et al., 1996; Brian, 1998), both of which could lead to brain hypoxia (Schneider et al., 1998; Nwaigwe et al., 2000); a stimulus known to elicit prolonged increases in respiratory motor output (Blitz and Ramirez, 2002; Bavis and Mitchell, 2003). Thus, we cannot rule out a role for

local ischemia in iPMF following hyperventilation-induced central neural apnea. Further, since central neural apnea reduces respiratory neural activity throughout the neuraxis, it is unknown if global (brainstem) or local (spinal) mechanisms give rise to iPMF. The central question driving this study is: are changes in respiratory neural activity --in and of itself--regulated independent of chemoreflexes?

Here we used nano-injections of procaine to reversibly reduce descending synaptic inputs to the ipsilateral phrenic motor pool in ventilated (and therefore blood gas controlled) rats. We show for the first time that: 1) a temporary reduction in ipsilateral phrenic synaptic inputs elicits a rebound increase in phrenic burst amplitude (i.e. iPMF) and a novel form of compensatory plasticity in the contralateral phrenic motor pool (i.e. csPMF), 2) iPMF, but not csPMF, requires spinal TNFα and aPKC activity and 3) the dynamic range of phrenic motor output is proportionately enhanced following iPMF and csPMF. Collectively, these data suggest that local, spinal mechanisms sense and respond to reduced phrenic neural activity independent of chemoreflexes.

METHODS

Animals. Experiments were performed on adult (3-5 month), male Harlan Sprague-Dawley rats (colony 211a and 217; Harlan Laboratories). Rats were housed two per cage in a controlled environment (12h light/dark cycle), with food and water *ad libitum.* All experiments were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison.

Electrophysiology preparation. Rats were anesthetized with isoflurane in a closed chamber and transferred to a heated table where anesthesia (3% isoflurane, in 50% O2:N2 balance) was continued through a nose cone. A rectal probe (Physitemp) was used to maintain core body temperature at 37.0 ± 1.0°C by adjusting water temperature circulated to the table. The trachea was exposed, cannulated and immediately connected to a pump ventilator (Harvard Apparatus, Rodent Ventilator 683). A bilateral vagotomy was performed to prevent ventilator entrainment and tracheal pressure was monitored to verify that rats continued to generate respiratory efforts throughout the surgery (i.e. did not inadvertently experience neural apnea due to anesthetic-induced respiratory depression; Mahamed et al., 2011). A femoral arterial catheter was placed to monitor blood pressure and sample arterial blood gases throughout the protocol (ABL-500; Radiometer, Copenhagen, Denmark). The left tail vein was catheterized (Surflo *i.v.* catheter and injection plug) and rats were converted to urethane anesthesia (1.7-1.8 g/kg *i.v.*) while inspired isoflurane was slowly withdrawn. Using a dorsal approach, both phrenic nerves were isolated and de-sheathed. A partial cervical C2 laminectomy and durotomy were performed to expose the left dorsal rootlets. An intrathecal catheter (2 French, Access Technologies) connected to a Hamilton syringe was placed underneath the dura and advanced caudally to spinal segment C4. Following surgery, rats were paralyzed with pancuronium bromide (2.5 mg/kg, *i.v.*), followed by a slow infusion (1-3 mL/h) of a bicarbonate/lactated ringers (1:4) solution to maintain fluid and acid base homeostasis.

Intrathecal compounds. The following compounds were dissolved in artificial CSF (aCSF; in mM: 120 NaCl, 3 KCl, 2 CaCl, 2 MgCl, 23 NaHCO3, 10 glucose bubbled with 95%O_2/5%CO_2 pH 7.4): myristoylated ζ-pseudosubstrate inhibitory peptide (PKCζ-PS; 2 mg/ml; Tocris

Bioscience), myristoylated scrambled ζ-pseudosubstrate peptide (scrPKCζ-PS; 2 mg/ml; Tocris Bioscience), and the soluble TNFα receptor 1 (sTNFR1; .1µg/µl; R&D Systems). For all intrathecal compounds, the total injection volume was 10µl, delivered in 1-2 µl boluses over 2 min. Vehicle treated rats received equivalent volumes of intrathecal aCSF.

Electrophysiology protocols. Following surgery, the left and right phrenic nerves were placed on silver bipolar recording electrodes and each cavity was filled with mineral oil. Compound action potentials were amplified, band-pass filtered (300-10,000Hz), and integrated (time constant 50 msec). Raw and integrated signals were digitized and recorded with PowerLab 7 data acquisition system (AD Instruments). One hour after isoflurane was discontinued, baseline phrenic nerve activity was established by manipulating inspired CO_2 until phrenic burst frequency was ~45 bursts/min.

To reversibly block axon conduction in respiratory neurons innervating the left phrenic motor pool, targeted nano-injections of procaine were delivered to the C2 ventrolateral funiculus (VLF). We targeted VLF axons since they supply the majority of synaptic input to phrenic motor neurons during eupnic breathing (Vinit and Kastner, 2009). Using published coordinates (Fuller et al., 2003), a micropipette (tip diameter ~18µm) filled with procaine (20% in aCSF; Sigma Aldrich) or aCSF was positioned over the left hemi-cord rostral to the C2 dorsal rootlets ~1-1.25mm lateral to the midline and advanced ~1.5-1.75 mm into the spinal cord. Using a pneumatic pico-injector (~1-2 psi; Harvard apparatus, PLI-100), ~200nl of procaine or aCSF was injected into the C2 VLF while monitoring bilateral phrenic motor output. Synaptic inputs to one phrenic motor pool (ipsilateral to injections) were reduced, while contralateral synaptic inputs were not affected. Since procaine rapidly impairs voltage-gated sodium channels and has a short

half-life ($t_{1/2}$=1-2 min; (Ting et al., 1949; Reidenberg et al., 1972), phrenic burst amplitude in some procaine-injected rats began to recover after only 15 min of reduced burst amplitude; thus, additional injection/s of procaine was necessary to maintain reduced phrenic burst amplitude for 30 min.

The following experimental groups were included: 1) intraspinal procaine (n=7); 2) intrathecal aCSF 20 min prior to intraspinal procaine (n=6); 3) intrathecal sTNFR1 20 min prior to intraspinal procaine (n=7), 4) intrathecal PKCζ-PS 20 min prior to intraspinal procaine (n=7) or 5) intrathecal scrPKCζ-PS 20 min prior to intraspinal procaine (n=7). To control for time-dependent variations associated with the surgery, pressure nano-injections or pharmacological compounds, a subgroup of rats received similar volumes of intraspinal injections of aCSF into the C2 VLF (time controls). Four time control groups were included: 1) intraspinal aCSF (n=3); 2) intrathecal vehicle 20 min prior to intraspinal aCSF (n=3); 3) intrathecal sTNFR1 20 min prior to intraspinal aCSF (n=3), and 4) intrathecal PKCζ-PS 20 min prior to intraspinal aCSF (n=3).

For all experiments, bilateral phrenic motor output was monitored for 60 min following recovery of axon conduction (or equivalent duration in time controls). Blood gases in all experiments were sampled immediately before (baseline), during and 5, 15, 30 and 60 min following C2 axon conduction block (or equivalent duration in controls) to ensure observed effects were not due to changes in arterial blood gases. At the end of each protocol, rats received a brief (~5 min) high CO_2 challenge ($ETCO_2$ ~98) to assess maximum phrenic burst amplitude.

Fluorescent staining. To verify the location and spread of procaine, two procaine treated rats also received the red fluorescent lipophilic membrane dye DiI (1,1' - Dioctadecyl - 3,3,3',3' - tetramethylindocarbocyanine iodide; Life Technologies) (5%) in the micropipette. Following the

electrophysiology protocol, rats were transcardially perfused with heparinized phosphate-buffered saline (1X PBS; 150ml) followed by 4% paraformaldehyde (pH 7.4) in 1X PBS (100ml/100g of rat). The C2 spinal cord segment was harvested, cryoprotected in sequential 24-hour incubations of 20% and 30% sucrose/1X PBS solutions. Coronal sections (40μm) were prepared using a Leica SM200R sliding microtome, mounted, dried and coverslipped in ProLong Gold. Immunofluorescent images were captured at 4X using a Nikon C1 confocal microscope. To visualize the location of the injectate within the spinal tissue, the gain was increased until the autofluorescence of the gray/white matter was detected.

Statistical Analysis. Criteria to include data in statistical analyses were: a mean arterial blood pressure above 60mmHg, PaO_2 >140 mmHg and $PaCO_2$ within 1.5mmHg of baseline throughout the protocol. Integrated phrenic burst amplitude and frequency were averaged over 30 second periods at baseline, 5, 15, 30 and 60 min post-C2 axon conduction recovery or an equivalent duration in time controls. Integrated phrenic amplitude was expressed as a percent increase over baseline (normalized to zero), while phrenic burst frequency was expressed as an absolute change from baseline. No significant differences in phrenic burst amplitude or frequency were observed between time control groups ($p>0.05$); therefore all time controls were combined and used for statistical analysis. For comparison purposes, this combined "time control" group was expressed in Figures 2-4. A two-way analysis of variance with repeated measures was used for statistical comparisons of ipsilateral phrenic burst amplitude, contralateral phrenic burst amplitude, frequency and blood gas parameters; individual comparisons were made using Fisher LSD post-hoc tests (Prism 6, GraphPad Software). A one-way analysis of variance was used to detect significant differences in the maximum phrenic response to a high CO_2 challenge, and

individual comparisons were made using a Fisher LSD post-hoc test (Prism 6, GraphPad Software). Linear regression analysis was performed to detect significant correlations between phrenic burst amplitude at 60 min and during hypercapnic challenge. For the latter two analyses, rats receiving axon conduction block with or without intrathecal vehicle were combined for clarity. For all analyses, the significance level was set at 0.05 and the results were expressed as means ± SEM.

RESULTS

Table 1 lists average $PaCO_2$, PaO_2, pH and mean arterial pressure (MAP) before, during and 60 min after unilateral C2 axon conduction recovery or an equivalent duration in rats receiving unilateral C2 control injections ("time controls"). No time dependent changes in $PaCO_2$ or MAP were observed in any group ($p>0.05$) Rats receiving vehicle prior to intraspinal procaine expressed a small, but significant decrease in pH at 60 min compared to baseline ($p<0.05$). In addition, at 60 min pH was significantly lower in procaine treated rats compared to time controls ($p<0.05$). Rats receiving PKCζ-PS prior to intraspinal procaine expressed a significant reduction in PaO_2 at 60 min compared to baseline ($p<0.05$). Although small, but significant decreases in PaO_2 and pH were detected we do not expect this influenced our results since: 1) changes in PaO_2 were small and while reduced from baseline, PaO_2 was still maintained above 290 mmHg and 2) pH changes were not associated with iPMF expression or lack thereof.

Unilateral intraspinal procaine reduces ipsilateral, but not contralateral phrenic burst activity

To temporarily disrupt spinal synaptic inputs to one phrenic motor pool, procaine was injected into the left C2 VLF to block action potential transmission in a portion of the descending axons that provide excitatory neural drive to the ipsilateral phrenic motor pool (Fig. 1A, B). Unilateral C2 procaine resulted in a significant reduction in ipsilateral phrenic burst amplitude relative to baseline ($p<0.05$), with no significant change apparent in contralateral phrenic burst amplitude ($p>0.05$; Table 2). As expected, no significant changes in ipsilateral ($p>0.05$) or contralateral ($p>0.05$) phrenic burst amplitude were observed in time control rats receiving unilateral control injections of aCSF in the C2 VLF. In addition, ipsilateral phrenic burst amplitude during axon conduction block was significantly lower than time controls ($p<0.05$), indicating that reduced phrenic burst amplitude was not due to physical trauma caused by pressure nano-injections into the C2 VLF.

In a subgroup of rats, the fluorescent dye (DiI) was simultaneously injected with procaine to verify the location of injectate. Figure 1C depicts a representative photomicrograph of DiI (red) staining and indicates that procaine injections were in or near the left VLF, suggesting that procaine remained restricted to the ipsilateral spinal cord.

Unilateral reduction in synaptic inputs to the phrenic motor pool elicits ipsilateral iPMF

To determine if a prolonged reduction in phrenic synaptic inputs elicits iPMF, axon conduction was allowed to recover while continuously monitoring bilateral phrenic motor output. Representative compressed ipsilateral and contralateral phrenic neurograms, depicting baseline, ~30 min of unilateral C2 conduction block and 60 min following axon conduction recovery, or

an equivalent duration in rats receiving unilateral C2 control injections (time controls) are shown in Figure 2A. Average percent change in ipsilateral and contralateral phrenic burst amplitude relative to baseline for 60 min following axon conduction recovery or an equivalent duration in controls not receiving reduced neural activity is shown in Figure 2B and 2C. Ipsilateral and contralateral phrenic burst amplitude in time controls was not significantly increased relative to baseline at any time point during the recording period (all $p>0.05$; Fig. 2B,C), suggesting pressure nano-injections into the C2 VLF do not alter phrenic burst amplitude. By contrast, rats receiving unilateral intraspinal procaine expressed a significant increase in ipsilateral phrenic burst amplitude relative to baseline and time controls at all time points following axon conduction recovery (all time points $p<0.05$; Fig. 2B), suggesting ipsilateral iPMF. A small but significant increase in contralateral phrenic burst amplitude relative to baseline and time controls was observed 60 min after ipsilateral axon conduction recovery ($p<0.05$; Fig. 2C), suggesting crossed spinal phrenic motor facilitation (csPMF). Collectively, these data suggest that unilateral disruption of phrenic synaptic inputs to one phrenic motor pool elicits a long-lasting rebound increase in ipsilateral phrenic burst amplitude (i.e. iPMF) and a smaller, progressive increase in contralateral burst amplitude (i.e. csPMF).

Average change in phrenic burst frequency from baseline for 60 min following C2 axon conduction recovery, or an equivalent duration in rats receiving C2 control injections (time controls) is shown in Figure 2D. Phrenic burst frequency in time control rats was significantly increased relative to baseline at 60 min ($p>0.05$; Fig. 2D), indicating a small progressive increase in phrenic burst frequency is inherent to our preparation. In rats receiving intraspinal procaine, phrenic burst frequency was not significantly increased compared to time controls at any time point following axon conduction recovery (all $p>0.05$), although a small, but significant increase

relative to baseline was observed at 30 and 60 min (both $p<0.05$; Fig. 2D); together these results indicate that phrenic burst frequency facilitation was not elicited.

Ipsilateral iPMF, but not csiPMF, requires spinal TNFα activity

We recently described a critical role for spinal TNFα in iPMF following a central (i.e. brainstem-initiated) reduction in respiratory neural activity (Broytman et al., 2013). To test the hypothesis that spinal TNFα is necessary for ipsilateral iPMF following C2 axon conduction block, the soluble TNFα receptor (sTNFR1), which binds and prevents endogenous TNFα signaling, was delivered intrathecally prior to C2 intraspinal procaine. Representative compressed ipsilateral and contralateral phrenic neurograms depicting baseline, ~30 min of unilateral C2 conduction block and 60 min following axon conduction recovery in rats receiving intrathecal vehicle or sTNFR1 are shown in Figure 3A. Intraspinal procaine significantly decreased ipsilateral, but not contralateral phrenic burst amplitude in rats receiving intrathecal sTNFR1 ($p<0.05$; Fig. 3B, C), which was not significantly different in magnitude or duration of reduced activity than rats receiving intrathecal vehicle prior to intraspinal procaine ($p>0.05$; Table 2).

Average change in ipsilateral and contralateral phrenic burst amplitude in rats receiving either intrathecal vehicle or intrathecal sTNFR1 prior to C2 axon conduction block (or an equivalent duration in time controls) is shown in Figure 3B and 3C. In rats receiving vehicle prior to intraspinal procaine ipsilateral phrenic burst amplitude was significantly increased relative to baseline and time controls at all time points (all $p<0.05$; Fig. 3B), indicating iPMF. By contrast, ipsilateral phrenic burst amplitude in rats receiving intrathecal sTNFR1 was not significantly increased from time controls at any time point following axon conduction recovery

(all $p>0.05$; Fig. 3B), despite a significant increase compared to baseline at 15 and 30 min (both $p<0.05$). Further, ipsilateral phrenic burst amplitude at 60 min post-axon conduction recovery was significantly lower in rats receiving intrathecal sTNFR1 than vehicle treated rats ($p<0.05$; Fig. 3B), suggesting long-lasting iPMF is blocked in rats receiving sTNFR1. In vehicle treated rats, contralateral phrenic burst amplitude was significantly increased compared to baseline and time controls at 60 min ($p<0.05$; Fig 3C), suggesting csPMF. Interestingly, rats receiving intrathecal sTNFR1 also expressed a significant increase relative to baseline at 15, 30 and 60 min (all $p<0.05$) and time controls at 60 min ($p<0.05$; Fig. 3C) suggesting spinal inhibition of TNFα did not impair csPMF. Collectively, these data suggest that ipsilateral iPMF, but not contralateral csPMF requires spinal TNFα.

Average change in phrenic burst frequency in rats receiving either intrathecal vehicle or sTNFR1 prior to C2 axon conduction block (or an equivalent duration in time controls) is shown in Figure 3D. Phrenic burst frequency in rats receiving vehicle prior to intraspinal procaine was not significantly increased from time controls at any point (all $p>0.05$); however, a small but significant increase from baseline was observed at all time points (all $p<0.05$). Similarly, in rats receiving intrathecal sTNFR1 prior to intraspinal procaine phrenic burst frequency was not significantly different than baseline or time controls at any time point (all $p>0.05$). Together these results indicate phrenic burst frequency facilitation was not elicited in either group.

Ipsilateral iPMF requires spinal aPKC activity

Since iPMF following central neural apnea requires spinal atypical protein kinase C (aPKC) activity (Strey et al., 2012), we hypothesized that spinal aPKC activity is necessary for ipsilateral iPMF following C2 axon conduction block. To test this hypothesis, a cell-permeable

pseudosubstrate inhibitory peptide (PKCζ-PS) which binds and inhibits all aPKC isoforms (i.e., PKCζ, PKCι/λ and PKMζ; Reyland, 2009) or the inactive scrambled version of the peptide (scrPKCζ-PS) was delivered intrathecally prior to intraspinal procaine. Representative compressed ipsilateral and contralateral phrenic neurograms depicting baseline, ~30 min of unilateral C2 conduction block and 60 min following axon conduction recovery in rats pretreated with intrathecal scrPKCζ-PS or PKCζ-PS are shown in Figure 4A. Intraspinal procaine significantly decreased ipsilateral ($p<0.05$), but not contralateral ($p>0.05$), phrenic burst amplitude relative to baseline and time controls; however, the magnitude and duration of reduced activity was not significantly different between rats receiving scrPKCζ-PS or PKCζ-PS ($p>0.05$; Table 2).

Average percent change in ipsilateral and contralateral phrenic burst amplitude from baseline for 60 min following C2 axon conduction recovery (or an equivalent duration in time controls) is shown in Figure 4B and 4C. Rats pretreated with intrathecal scrPKCζ-PS prior to intraspinal procaine expressed a significant increase in ipsilateral phrenic burst amplitude relative to baseline and time controls at all time points (all $p<0.05$) suggesting scrPKCζ-PS did not impair ipsilateral iPMF (Fig. 4B). By contrast, phrenic burst amplitude in rats pretreated with intrathecal PKCζ-PS was not significantly increased from time controls at 60 min ($p>0.05$) although moderate increases from baseline (15, 30 and 60 min; all $p<0.05$) and time controls (15 and 30 min; both $p<0.05$; Fig. 4B) were apparent. Indeed, ipsilateral phrenic burst amplitude in rats receiving intrathecal PKCζ-PS was significantly lower than rats receiving intrathecal scrPKCζ-PS at 60 min; suggesting spinal aPKC inhibition impaired late, but not early iPMF($p<0.05$; Fig. 4B). Contralateral burst amplitude in rats pretreated with scrPKCζ-PS was significantly increase from baseline and time controls at 15 and 30 min (all $p<0.05$) and from

baseline at 60 min ($p<0.05$; Fig. 4C) confirming csPMF. Surprisingly, rats receiving intrathecal PKCζ-PS expressed a significant increase in contralateral burst amplitude relative to baseline and time controls at all time points (all $p<0.05$), suggesting that csPMF was not attenuated by spinal aPKC inhibition. Collectively our results suggest that spinal aPKC activity is necessary for late ipsilateral iPMF, but not contralateral csPMF.

Average change in phrenic burst frequency in rats receiving either intrathecal scrPKCζ-PS or PKCζ-PS prior to C2 axon conduction block (or an equivalent duration in time controls) is shown in Figure 4D. Phrenic burst frequency in rats pretreated with scrPKCζ-PS prior to intraspinal procaine was not significantly increased from time controls at any time point (all $p<0.05$), although a small and significant increase from baseline was observed at all time points (all $p<0.05$; Fig. 4D). Similarly, in rats pretreated with PKCζ-PS phrenic burst frequency was not significantly increased compared to time controls at any time point (all $p<0.05$) despite significant increases relative to baseline at 5, 30 and 60 min (all $p>0.05$; Fig. 4D). Together these results indicate that burst frequency facilitation was not elicited in either group.

Reduced phrenic synaptic inputs increases the dynamic range of phrenic motor output

To assess maximum phrenic burst amplitude and assure our preparation did not deteriorate, all rats were presented with ~10% CO_2 at the end of the protocol. Average ipsilateral and contralateral phrenic burst amplitude (relative to baseline) during hypercapnia in rats receiving unilateral C2 axon conduction block or time controls is shown in Figure 5A and B. As expected, phrenic burst amplitude was significantly increased during hypercapnia relative to baseline in all groups ($p<0.05$). In rats receiving unilateral C2 axon conduction block, ipsilateral phrenic burst amplitude during hypercapnia was significantly increased relative to ipsilateral

time controls not receiving reduced activity ($p<0.05$; Fig. 5A). Confirming this observation, rats pretreated with scrPKCζ-PS prior to C2 axon conduction block expressed a significant increase in the ipsilateral phrenic hypercapnic response relative to the response of ipsilateral time controls ($p<0.05$; Fig. 5A). Spinal treatments that block ipsilateral iPMF, impair this enhanced hypercapnic response since rats receiving intrathecal sTNFR1 or PKCζ-PS no longer expressed a significant increase in phrenic burst amplitude during hypercapnia compared to the ipsilateral time control response (both $p>0.05$). Although only rats pretreated with sTNFR1 prior to axon conduction block expressed a significant increase in contralateral phrenic burst amplitude during hypercapnia relative to time controls ($p<0.05$), contralateral phrenic burst amplitude among other groups expressed a trend similar to the ipsilateral response, however the contralateral increase during hypercapnia was of smaller magnitude ($p<0.05$; Fig. 5B).

Linear regression analysis indicated a strong positive relationship between the magnitude of ipsilateral iPMF at 60 min post-axon conduction recovery and ipsilateral phrenic burst amplitude during hypercapnia ($p<0.05$; Fig. 5C). A smaller, but positive relationship was also observed between the magnitude of contralateral csPMF at 60 min and the contralateral phrenic hypercapnic response ($p<0.05$; Fig. 5D). In contrast, regression analysis of time controls not receiving axon conduction block did not express a significant relationship between the magnitude of ipsilateral or contralateral phrenic burst amplitude at 60 min and the corresponding phrenic response during hypercapnia (both $p>0.05$; Fig. 5E, 5F). Collectively, our results suggest iPMF and csPMF following unilateral axon conduction block are associated with a proportional increase in the phrenic dynamic range during hypercapnia.

DISCUSSION

Multiple mechanisms assure ventilation and maintain arterial blood gases (Feldman et al., 2003). However, it is unknown if respiratory neural activity--in and of itself--is sensed and responded to independent of arterial blood gas changes (i.e. chemoreflexes). Here, we demonstrate that unilateral disruption of spinal synaptic inputs to one phrenic motor pool in ventilated rats elicits two distinct forms of plasticity: 1) a robust, rebound increase in phrenic burst amplitude, ipsilateral to reduced synaptic inputs (i.e. iPMF) and 2) a smaller and progressive increase in phrenic burst amplitude in the contralateral motor pool where synaptic inputs were maintained (csPMF). Ipsilateral iPMF, but not contralateral csPMF, requires spinal TNFα signaling and aPKC activity in or near the phrenic motor nucleus. By contrast, both ipsilateral iPMF and contralateral csPMF are associated with a proportional increase in the phrenic response to a severe respiratory challenge (hypercapnia). Collectively, our results indicate that mechanisms local to the phrenic motor nucleus sense and respond to reduced phrenic synaptic inputs independent of chemoreflexes.

Local spinal mechanisms sense and respond to reduced phrenic synaptic inputs

iPMF was first described following central neural apnea (Mahamed et al., 2011). In this study, several diverse methods were used to induce central neural apnea including hyperventilation, anesthetic induced respiratory depression and increased inhibitory sensory feedback. Although we hypothesized that iPMF was due to a feature common to all methods—reduced respiratory neural activity—a direct demonstration of reduced activity *per se* was lacking. Further, since central neural apnea reduces respiratory activity throughout the neuraxis and elicits facilitation in multiple respiratory-related neural signals, including phrenic (iPMF;

Mahamed et al., 2011), hypoglossal (iHMF;Baker-Herman and Strey, 2011) and intercostal motor output (iIMF; Strey et al., 2013) it remained possible that an input common to all signals (i.e. brainstem respiratory neurons) gives rise to this plasticity. Indeed, central neural apnea elicits respiratory burst frequency plasticity (Mahamed et al., 2011) which likely occurs in brainstem respiratory rhythm generating neurons (Powell et al., 1998; Blitz and Ramirez, 2002; Baker-Herman and Mitchell, 2008). However, spinal mechanisms appear to be at least partially involved since iPMF following central apnea requires spinal TNFα (Broytman et al., 2013) and aPKC (Strey et al., 2012).

To directly test the hypotheses that: 1) reduced respiratory neural activity itself elicits iPMF and 2) spinal mechanisms sense and respond to reduced phrenic synaptic inputs, procaine was used to reversibly block axon conduction in descending axons in the C2 VLF innervating one phrenic motor pool. Procaine and a similar compound, lidocaine, decrease sodium currents and temporarily block neural transmission within the brain (Larkin, 1975; Dean et al., 1982; Yim and Mogenson, 1983; Holahan and Routtenberg, 2011) and spinal cord (Gonzalez-Islas and Wenner, 2006). Unlike central neural apnea, which reduces activity throughout the respiratory network, pressure micro-injections of local anesthetics into the central nervous system have an estimated diffusion of ~200-400μm (Malpeli and Schiller, 1979; Tehovnik and Sommer, 1997). Indeed, a major benefit of using this experimental approach is the ability to reduce activity in one phrenic motor pool while maintaining activity in the contralateral phrenic motor pool.

Data presented here clearly demonstrate that spinal mechanisms in/near the phrenic motor pool sense and respond to reduced phrenic synaptic inputs and elicit ipsilateral iPMF. Since phrenic burst frequency facilitation was not elicited following axon conduction block, contribution of brainstem rhythmogenic neurons to iPMF are expected to be negligible. This is

consistent with a previous finding suggesting that supraspinal centers are not required for increased phrenic burst output following local changes in phrenic synaptic inputs (Hayashi et al., 2003). In addition to ipsilateral iPMF, we show a novel crossed spinal phrenic motor facilitation (csPMF) induced in the contralateral motor pool that received continuous synaptic inputs.

Mechanisms of iPMF and csPMF

Previous results suggest that iPMF following a global reduction in respiratory neural activity requires spinal TNFα (Broytman et al., 2013) and aPKC activity (Strey et al., 2012). Here we show that ipsilateral iPMF elicited by spinal axon conduction block requires both TNFα and aPKC activity, indicating that regardless of whether reduced respiratory neural activity is of central or local origin, similar spinal mechanisms give rise to iPMF. The requirement for TNFα following reduced synaptic inputs may be a conserved response of all central circuits since TNFα is sufficient for compensatory increases in neural activity within cortical (Kaneko et al., 2008; Steinmetz and Turrigiano, 2010), hippocampal (Beattie et al., 2000; Stellwagen et al., 2005) and spinal neurons (Ferguson et al., 2008; Stück et al., 2012; Broytman et al., 2013). The mechanisms whereby TNFα leads to iPMF may involve aPKC isoforms since exogenous TNFα applied to spinal segments containing phrenic motor neurons elicits an aPKC dependent phrenic motor facilitation (i.e. PMF; Broytman et al., 2013).

The aPKC family includes two full-length isoforms PKCζ, PKCι/λ and one truncated and persistently active isoform PKMζ (Newton, 2001) and are implicated in multiple forms of neural plasticity (Ling et al., 2002; Laferriere et al., 2011; Ren et al., 2013). Our data indicate that full-length PKCζ and/or PKCι/λ (referred to as PKCζ/ι) are necessary during early phases of iPMF, to transition to long-lasting iPMF (Strey et al., 2012). Early, but not late iPMF, is associated with

an increased interaction between the aPKC isoform(s) PKCζ/ι and the scaffolding molecule p62/ZIP (Strey et al., 2012). This PKCζ/ι–p62/ZIP signaling complex may promote signaling specificity to this ubiquitously activated kinase (Moscat et al., 2007). Mechanisms downstream of the PKCζ/ι-p62/ZIP signaling cassette giving rise to iPMF are completely unknown.

In contrast to ipsilateral iPMF, contralateral csPMF induced by unilateral C2 axon conduction block does not require spinal TNFα or aPKC activity. Although the cellular mechanisms giving rise to csPMF are unknown, future studies testing if cAMP and PKA, molecules necessary for the activation of crossed spinal pathways are warranted (Kajana and Goshgarian, 2008).

Does iPMF represent a form of homeostatic plasticity?

Homeostatic plasticity is a fundamental property of neural networks allowing neurons to bidirectionally respond to changes in synaptic inputs while maintaining activity within an optimal firing range (Turrigiano, 2012). At excitatory synapses, homeostatic responses involve compensatory alterations in excitatory/inhibitory synaptic strength and/or intrinsic excitability (Desai et al., 1999; Turrigiano, 2008). Although typically thought to require prolonged periods of altered activity, (hours-days;Desai et al., 1999; Stellwagen and Malenka, 2006; Garcia-Bereguiain et al., 2013), homeostatic mechanisms operate over various temporal domains including in response to brief changes in activity (minutes; van Welie et al., 2004; Ibata et al., 2008).

Although iPMF shares key features similar to homeostatic plasticity observed in other central circuits, whether iPMF represents “true” homeostatic plasticity remains unknown. Consistent with the idea that iPMF represents a homeostatic mechanism, reduced synaptic inputs

per se elicit iPMF and phrenic burst amplitude is enhanced during future challenges following iPMF expression, suggesting phrenic dynamic range is maintained. However, an inconsistency of this suggestion is that phrenic burst amplitude does not spontaneously return to baseline as one would expect from a "true" homeostatic form of plasticity. Since, inactivity-induced facilitation within the hypoglossal and intercostal motor pools (iHMF and iIMF respectively) are of similar magnitude to iPMF, but spontaneously return to baseline after ~30 min (Baker-Herman and Strey, 2011; Strey et al., 2013), we suspect the propensity for a long-lasting increase may be unique to the phrenic neural circuit which must necessarily remain active to sustain life.

Significance of iPMF and csPMF

Although the physiological significance of either form of plasticity has not been shown, we suspect that iPMF csPMF may maintain activity in respiratory neurons during physiological situations (e.g., sleep) and pathophysiological conditions (e.g., central sleep apnea and spinal cord injury) associated with reduced respiratory neural drive (Strey et al., 2013). An extreme example of reduced phrenic synaptic inputs is cervical spinal cord injury. In rodent models, cervical spinal injury results in complete silencing of ipsilateral phrenic nerve and hemidiaphragm activity (Vinit and Kastner, 2009). Within days-weeks, respiratory activity spontaneously resumes (albeit much reduced), suggesting endogenous compensatory mechanisms restore phrenic motor output (El-Bohy et al., 1998; Golder and Mitchell, 2005; Baussart et al., 2006; Fuller et al., 2006; Vinit et al., 2007; Lane et al., 2009; Golder et al., 2011). Although bulbospinal projections crossing the midline are latent synaptic inputs (Goshgarian and Rafols, 1984), respiratory stress can give rise to functionally active pathways (Goshgarian, 2009). Although it is unclear whether iPMF and/or csPMF enable the spontaneous recovery of

respiratory output following spinal cord injury, an understanding of how ongoing activity or a lack thereof influences respiratory motor output is essential in order to intervene in situations where ventilation is compromised.

Acknowledgements: We would like to thank Nathan A. Baertsch for his comments on this manuscript. This work was supported by a grant from the National Institutes of Health (HL105511).

The authors declare no conflicts of interest.

REFERENCES

Baertsch NA, Baker-Herman TL (2013) Inactivity-induced phrenic and hypoglossal motor facilitation are differentially expressed following intermittent vs. sustained neural apnea. J Appl Physiol 114:1388-1395.

Baker-Herman TL, Mitchell GS (2008) Determinants of frequency long-term facilitation following acute intermittent hypoxia in vagotomized rats. Respir Physiol Neurobiol 162:8-17.

Baker-Herman TL, Strey KA (2011) Similarities and differences in mechanisms of phrenic and hypoglossal motor facilitation. Respir Physiol Neurobiol 179:48-56.

Baussart B, Stamegna JC, Polentes J, Tadié M, Gauthier P (2006) A new model of upper cervical spinal contusion inducing a persistent unilateral diaphragmatic deficit in the adult rat. Neurobiol Dis 22:562-574.

Bavis RW, Mitchell GS (2003) Intermittent hypoxia induces phrenic long-term facilitation in carotid-denervated rats. J Appl Physiol 94:399-409.

Beattie EC, Carroll RC, Yu X, Morishita W, Yasuda H, von Zastrow M, Malenka RC (2000) Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. Nat Neurosci 3:1291-1300.

Blitz DM, Ramirez JM (2002) Long-term modulation of respiratory network activity following anoxia in vitro. J Neurophysiol 87:2964-2971.

Brian JE (1998) Carbon dioxide and the cerebral circulation. Anesthesiology 88:1365-1386.

Broytman O, Baertsch NA, Baker-Herman TL (2013) Spinal TNFα is necessary for inactivity-induced phrenic motor facilitation. J Physiol.

Dean P, Redgrave P, Lewis G (1982) Locomotor activity of rats in open field after microinjection of procaine into superior colliculus or underlying reticular formation. Behav Brain Res 5:175-187.

Desai NS, Rutherford LC, Turrigiano GG (1999) BDNF regulates the intrinsic excitability of cortical neurons. Learn Mem 6:284-291.

El-Bohy AA, Schrimsher GW, Reier PJ, Goshgarian HG (1998) Quantitative assessment of respiratory function following contusion injury of the cervical spinal cord. Exp Neurol 150:143-152.

Feldman JL, Mitchell GS, Nattie EE (2003) Breathing: rhythmicity, plasticity, chemosensitivity. Annu Rev Neurosci 26:239-266.

Ferguson AR, Christensen RN, Gensel JC, Miller BA, Sun F, Beattie EC, Bresnahan JC, Beattie MS (2008) Cell death after spinal cord injury is exacerbated by rapid TNF alpha-induced trafficking of GluR2-lacking AMPARs to the plasma membrane. J Neurosci 28:11391-11400.

Fuller DD, Johnson SM, Olson EB, Mitchell GS (2003) Synaptic pathways to phrenic motoneurons are enhanced by chronic intermittent hypoxia after cervical spinal cord injury. J Neurosci 23:2993-3000.

Fuller DD, Golder FJ, Olson EB, Mitchell GS (2006) Recovery of phrenic activity and ventilation after cervical spinal hemisection in rats. J Appl Physiol 100:800-806.

Garcia-Bereguiain MA, Gonzalez-Islas C, Lindsly C, Butler E, Hill AW, Wenner P (2013) In vivo synaptic scaling is mediated by GluA2-lacking AMPA receptors in the embryonic spinal cord. J Neurosci 33:6791-6799.

Golder FJ, Mitchell GS (2005) Spinal synaptic enhancement with acute intermittent hypoxia improves respiratory function after chronic cervical spinal cord injury. J Neurosci 25:2925-2932.

Golder FJ, Fuller DD, Lovett-Barr MR, Vinit S, Resnick DK, Mitchell GS (2011) Breathing patterns after mid-cervical spinal contusion in rats. Exp Neurol 231:97-103.

Gonzalez-Islas C, Wenner P (2006) Spontaneous network activity in the embryonic spinal cord regulates AMPAergic and GABAergic synaptic strength. Neuron 49:563-575.

Goshgarian HG (2009) The crossed phrenic phenomenon and recovery of function following spinal cord injury. Respir Physiol Neurobiol 169:85-93.

Goshgarian HG, Rafols JA (1984) The ultrastructure and synaptic architecture of phrenic motor neurons in the spinal cord of the adult rat. J Neurocytol 13:85-109.

Hayashi F, Hinrichsen CF, McCrimmon DR (2003) Short-term plasticity of descending synaptic input to phrenic motoneurons in rats. J Appl Physiol 94:1421-1430.

Holahan MR, Routtenberg A (2011) Lidocaine injections targeting CA3 hippocampus impair long-term spatial memory and prevent learning-induced mossy fiber remodeling. Hippocampus 21:532-540.

Ibata K, Sun Q, Turrigiano GG (2008) Rapid synaptic scaling induced by changes in postsynaptic firing. Neuron 57:819-826.

Kajana S, Goshgarian HG (2008) Spinal activation of the cAMP-PKA pathway induces respiratory motor recovery following high cervical spinal cord injury. Brain Res 1232:206-213.

Kaneko M, Stellwagen D, Malenka RC, Stryker MP (2008) Tumor necrosis factor-alpha mediates one component of competitive, experience-dependent plasticity in developing visual cortex. Neuron 58:673-680.

Laferriere A, Pitcher MH, Haldane A, Huang Y, Cornea V, Kumar N, Sacktor TC, Cervero F, Coderre TJ (2011) PKMzeta is essential for spinal plasticity underlying the maintenance of persistent pain. Molecular pain 7:99.

Lane MA, Lee KZ, Fuller DD, Reier PJ (2009) Spinal circuitry and respiratory recovery following spinal cord injury. Respir Physiol Neurobiol 169:123-132.

Larkin RP (1975) Effect of ventromedial hypothalamic procaine injections on feeding, lever pressing, and other behavior in rats. J Comp Physiol Psychol 89:1100-1108.

Ling DS, Benardo LS, Serrano PA, Blace N, Kelly MT, Crary JF, Sacktor TC (2002) Protein kinase Mzeta is necessary and sufficient for LTP maintenance. Nat Neurosci 5:295-296.

Mahamed S, Strey KA, Mitchell GS, Baker-Herman TL (2011) Reduced respiratory neural activity elicits phrenic motor facilitation. Respir Physiol Neurobiol 175:303-309.

Malpeli JG, Schiller PH (1979) A method of reversible inactivation of small regions of brain tissue. J Neurosci Methods 1:143-151.

Moscat J, Diaz-Meco MT, Wooten MW (2007) Signal integration and diversification through the p62 scaffold protein. Trends in biochemical sciences 32:95-100.

Newton AC (2001) Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem Rev 101:2353-2364.

Nwaigwe CI, Roche MA, Grinberg O, Dunn JF (2000) Effect of hyperventilation on brain tissue oxygenation and cerebrovenous PO2 in rats. Brain Res 868:150-156.

Powell FL, Milsom WK, Mitchell GS (1998) Time domains of the hypoxic ventilatory response. Respir Physiol 112:123-134.

Reidenberg MM, James M, Dring LG (1972) The rate of procaine hydrolysis in serum of normal subjects and diseased patients. Clin Pharmacol Ther 13:279-284.

Ren SQ, Yan JZ, Zhang XY, Bu YF, Pan WW, Yao W, Tian T, Lu W (2013) PKClambda is critical in AMPA receptor phosphorylation and synaptic incorporation during LTP. The EMBO journal 32:1365-1380.

Reyland ME (2009) Protein kinase C isoforms: Multi-functional regulators of cell life and death. Front Biosci 14:2386-2399.

Schneider GH, Sarrafzadeh AS, Kiening KL, Bardt TF, Unterberg AW, Lanksch WR (1998) Influence of hyperventilation on brain tissue-PO2, PCO2, and pH in patients with intracranial hypertension. Acta Neurochir Suppl 71:62-65.

Steinmetz CC, Turrigiano GG (2010) Tumor necrosis factor-α signaling maintains the ability of cortical synapses to express synaptic scaling. J Neurosci 30:14685-14690.

Stellwagen D, Malenka RC (2006) Synaptic scaling mediated by glial TNF-alpha. Nature 440:1054-1059.

Stellwagen D, Beattie EC, Seo JY, Malenka RC (2005) Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-alpha. J Neurosci 25:3219-3228.

Strey KA, Baertsch NA, Baker-Herman TL (2013) Inactivity-induced respiratory plasticity: Protecting the drive to breathe in disorders that reduce respiratory neural activity. Respir Physiol Neurobiol.

Strey KA, Nichols NL, Baertsch NA, Broytman O, Baker-Herman TL (2012) Spinal atypical protein kinase C activity is necessary to stabilize inactivity-induced phrenic motor facilitation. J Neurosci 32:16510-16520.

Stück ED, Christensen RN, Huie JR, Tovar CA, Miller BA, Nout YS, Bresnahan JC, Beattie MS, Ferguson AR (2012) Tumor necrosis factor alpha mediates GABA(A) receptor trafficking to the plasma membrane of spinal cord neurons in vivo. Neural Plast 2012:261345.

Tehovnik EJ, Sommer MA (1997) Effective spread and timecourse of neural inactivation caused by lidocaine injection in monkey cerebral cortex. J Neurosci Methods 74:17-26.

Ting KS, COON JM, CONWAY AC (1949) A spectrophotometric method for determination of procaine and p-aminobenzoic acid. J Lab Clin Med 34:822-829.

Turrigiano G (2012) Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function. Cold Spring Harb Perspect Biol 4:a005736.

Turrigiano GG (2008) The self-tuning neuron: synaptic scaling of excitatory synapses. Cell 135:422-435.

van Welie I, van Hooft JA, Wadman WJ (2004) Homeostatic scaling of neuronal excitability by synaptic modulation of somatic hyperpolarization-activated Ih channels. Proc Natl Acad Sci U S A 101:5123-5128.

Vinit S, Kastner A (2009) Descending bulbospinal pathways and recovery of respiratory motor function following spinal cord injury. Respir Physiol Neurobiol 169:115-122.

Vinit S, Stamegna JC, Boulenguez P, Gauthier P, Kastner A (2007) Restorative respiratory pathways after partial cervical spinal cord injury: role of ipsilateral phrenic afferents. Eur J Neurosci 25:3551-3560.

Vogel J, Abounader R, Schröck H, Zeller K, Duelli R, Kuschinsky W (1996) Parallel changes of blood flow and heterogeneity of capillary plasma perfusion in rat brains during hypocapnia. Am J Physiol 270:H1441-1445.

Yim CY, Mogenson GJ (1983) Response of ventral pallidal neurons to amygdala stimulation and its modulation by dopamine projections to nucleus accumbens. J Neurophysiol 50:148-161.

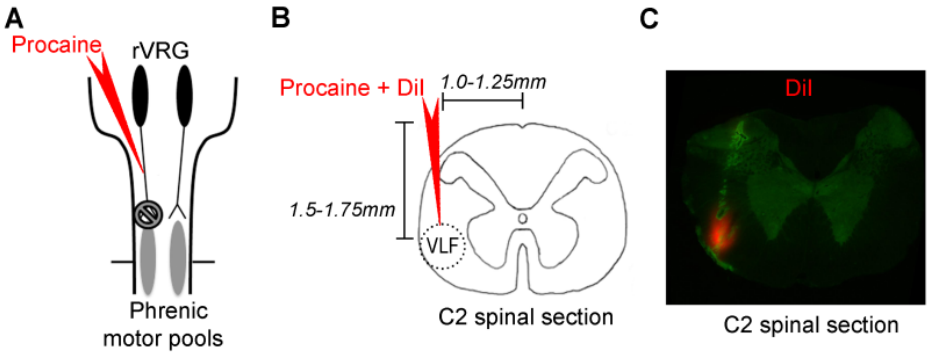

Figure 1: Fluorescent labeling of intraspinal injectate. ***A.*** Schematic of the brainstem and spinal cord depicting bulbospinal respiratory axons originating in the rostral ventral respiratory group (rVRG; *black ovals*) projecting to the phrenic motor pools (*gray ovals*). ***B.*** Illustration of a transverse C2 spinal section and coordinates used to target descending respiratory axons in the ventrolateral funiculus (VLF; outlined by the dotted circle). ***C.*** Representative photomicrograph (4X) of the injectate labeled with DiI (*red*) within the C2 spinal tissue (*green*), demonstrating that pressure nano-injections into the spinal cord remain localized to the ipsilateral VLF.

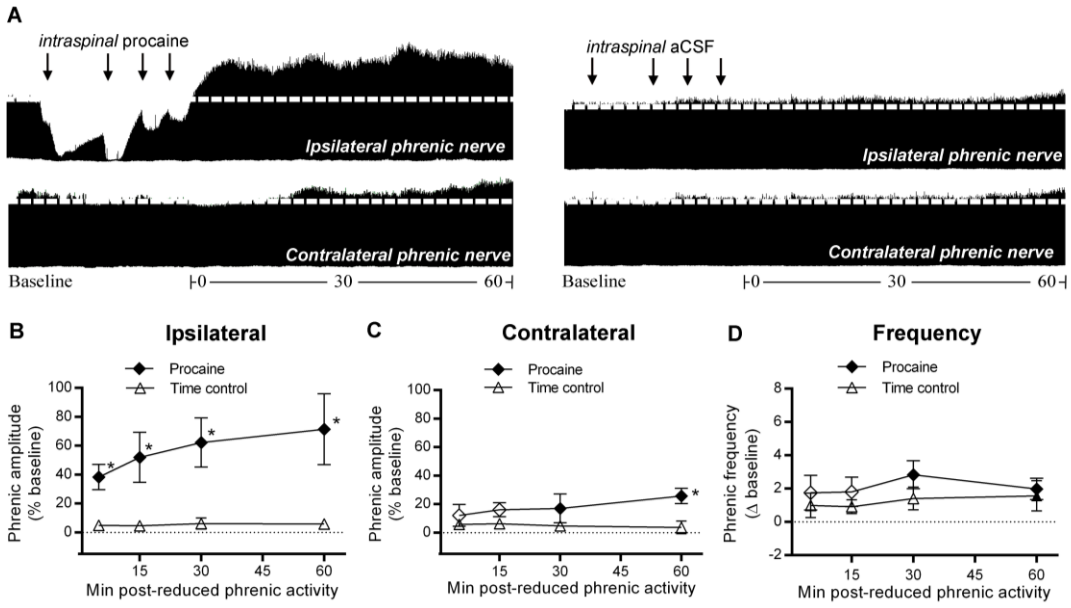

Figure 2: Unilateral C2 reduction in phrenic synaptic inputs elicits ipsilateral iPMF and contralateral csPMF. ***A.*** Representative compressed ipsilateral (*top*) and contralateral (*bottom*) phrenic neurograms during baseline, ~30 min of unilateral C2 axon conduction block (*left*), or control injection (*right*) and 60 min following axon conduction recovery or equivalent duration after control injection "time controls". Black arrows denote intraspinal injections. Average change in ***B.*** ipsilateral or ***C.*** contralateral phrenic burst amplitude from baseline and for 60 min after axon conduction recovery in rats receiving intraspinal procaine (*diamonds*) or an equivalent duration in time controls (*triangles*). In time controls, ipsilateral and contralateral phrenic burst amplitude was not significantly different than baseline at any point. Following unilateral axon conduction block, ipsilateral phrenic amplitude was significantly increased relative to baseline and time controls at all time points, indicating iPMF. A small but significant increase in contralateral phrenic amplitude relative to baseline and time controls at 60 min was detected, suggesting contralateral csPMF. ***D.*** Average change in phrenic burst frequency from baseline for 60 min after axon conduction recovery in rats receiving intraspinal procaine (*diamonds*) or an equivalent duration in time controls (*triangles*). Phrenic burst frequency was not significantly different than time controls at any time point post-conduction block, suggesting no phrenic burst frequency facilitation. Values presented are mean ± SEM. Filled symbols indicate significantly different from baseline. *significantly different from time controls; $p<0.05$.

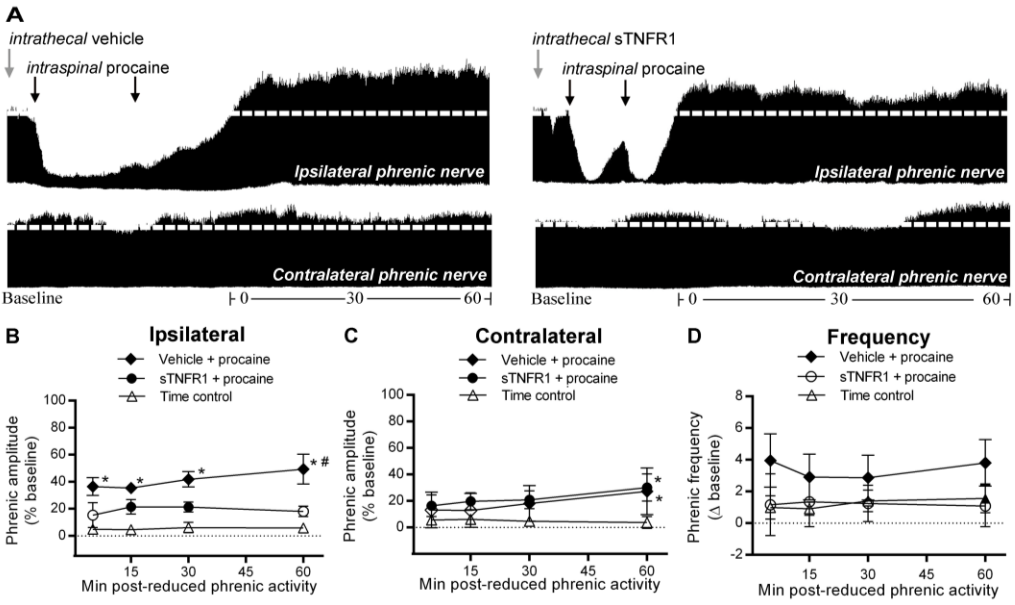

Figure 3: Spinal TNFα is required for ipsilateral iPMF, but not csPMF following unilateral C2 axon conduction block. ***A.*** Representative compressed ipsilateral (*top*) and contralateral (*bottom*) phrenic neurograms depicting phrenic burst amplitude during baseline, ~30 min of unilateral C2 axon conduction block and 60 min following axon conduction recovery in rats receiving intrathecal vehicle (*left*) or sTNFR1 (*right*). Gray arrows indicate intrathecal delivery; black arrows denote intraspinal procaine injections. Average change in ***B.*** ipsilateral or ***C.*** contralateral phrenic burst amplitude from baseline for 60 min after axon conduction recovery in rats receiving intrathecal vehicle (*diamonds*) or intrathecal sTNFR1 (*circles*) or an equivalent duration in time controls (*triangles*). In vehicle treated rats, a significant increase in ipsilateral phrenic burst amplitude compared to baseline and time controls was observed at all time points, indicating iPMF. By contrast, when pretreated with sTNFR1, ipsilateral phrenic amplitude was not significantly different than time controls, although small increases from baseline were observed at 15 and 30 min, suggesting iPMF was not elicited. Phrenic burst amplitude in sTNFR1 treated rats was significantly lower than vehicle treated rats at 60 min. In vehicle treated rats, contralateral phrenic burst amplitude was significantly increased relative to baseline and time controls at 60 min, suggesting csPMF was induced. Likewise, rats receiving sTNFR1 expressed a significant increase in contralateral phrenic burst amplitude compared to baseline and time controls at 60 min, indicating csPMF was not blocked following TNFα scavenging. ***D.*** Average change in phrenic burst frequency from baseline for 60 min after axon conduction recovery in rats receiving vehicle (*diamonds*) or sTNFR1 (*circles*) prior to intraspinal procaine or an equivalent duration in time controls (*triangles*). In vehicle or sTNFR1 treated rats, phrenic burst frequency post-axon conduction block was not significantly different than time controls indicating no phrenic burst frequency facilitation in either group. The filled symbols indicate significantly different from baseline. Values presented are mean ± SEM. *significantly different from time controls; # significantly different from sTNFR1 rats; $p<0.05$.

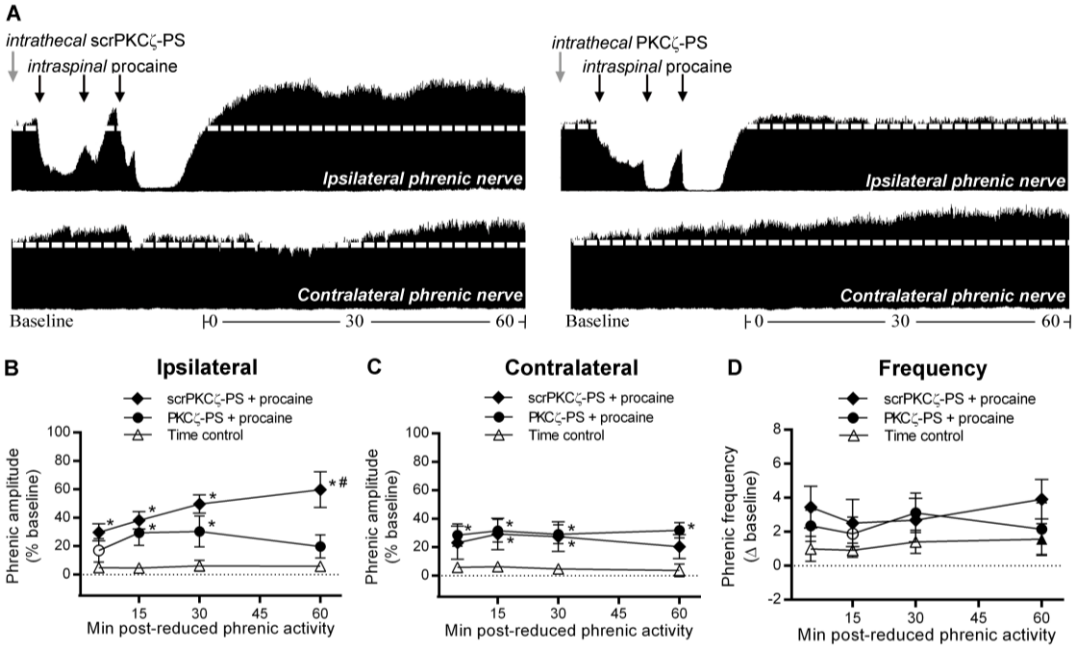

Figure 4: Spinal aPKC activity is required for ipsilateral iPMF, but not csPMF following unilateral C2 axon conduction block. ***A.*** Representative compressed ipsilateral (*top*) and contralateral (*bottom*) phrenic neurograms in rats pretreated with intrathecal scrPKCζ-PS (*left*) or PKCζ-PS (*right*) depicting baseline, ~30 min of unilateral C2 axon conduction block and 60 min following axon conduction recovery. Gray arrows indicate intrathecal delivery; black arrows denote intraspinal injections. Average change in ***B.*** ipsilateral and ***C.*** contralateral phrenic burst amplitude in rats receiving intrathecal scrPKCζ-PS (*diamonds*) or PKCζ-PS (*circles*) prior to C2 axon conduction block or an equivalent duration in time controls (*triangles*). Rats pretreated with scrPKCζ-PS expressed a significant increase in ipsilateral phrenic burst amplitude at all time points compared to baseline and time controls, indicating iPMF. When pretreated with PKCζ-PS, phrenic burst amplitude was increased from baseline and time controls at 15 and 30 min; however by 60 min phrenic burst amplitude was significantly lower than scrPKCζ-PS treated rats, suggesting iPMF was blocked. Contralateral phrenic burst amplitude was increased from baseline at all time points and at 15 and 30 min relative to time controls in rats receiving scrPKCζ-PS, indicating csPMF. Similarly, following PKCζ-PS treatment rats expressed a significant increase in contralateral burst amplitude from baseline and time controls at all time points indicating csPMF does not require aPKC activity. ***D.*** Average change in phrenic burst frequency from baseline for 60 min after axon conduction recovery in rats receiving scrPKCζ-PS (*diamonds*) or PKCζ-PS (*circles*) prior to intraspinal procaine or an equivalent duration in time controls (*triangles*). No significant increases compared to time controls were detected in either group, indicating burst frequency was not elicited. The filled symbols indicate significantly different from baseline. Values presented are mean ± SEM. *significantly different from time controls; # significantly different from PKCζ-PS rats; $p<0.05$.

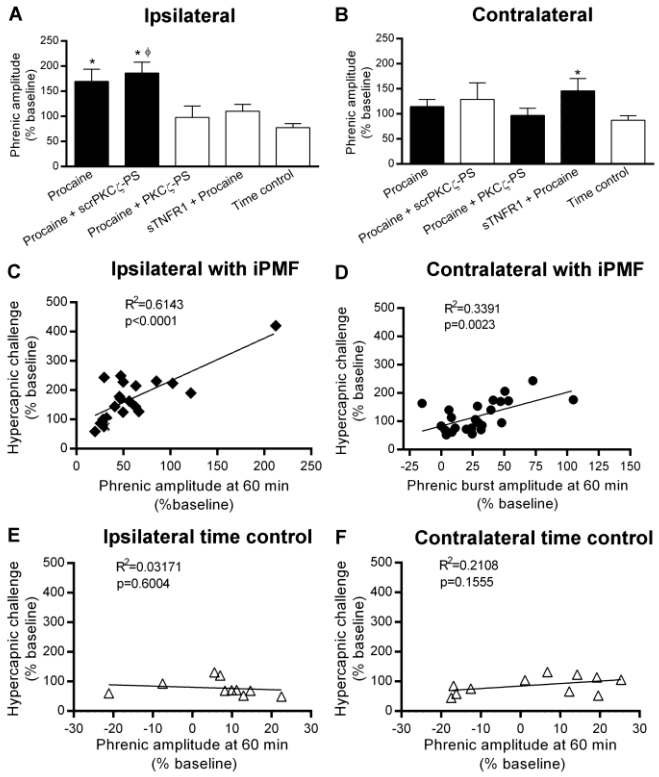

Figure 5: Ipsilateral iPMF and contralateral csPMF are associated with a proportional increase in phrenic burst amplitude during hypercapnia. Average increase in ***A.*** ipsilateral and ***B.*** contralateral phrenic burst amplitude during a high CO_2 challenge (hypercapnia) 60 min after axon conduction recovery or an equivalent duration in time controls. Shaded bars indicate significant facilitation relative to time controls at 60 min. Following C2 axon conduction block, ipsilateral phrenic amplitude was significantly increased during hypercapnia relative to time controls, suggesting an enhanced hypercapnic response was elicited. Similarly, rats pretreated with scrPKCζ-PS expressed a significant increase in ipsilateral burst amplitude during hypercapnia. However, in rats pretreated with PKCζ-PS or sTNFR1 prior to axon conduction block, ipsilateral burst amplitude was no different than time controls, indicating phrenic burst amplitude was not proportionally increased. Contralateral phrenic burst amplitude was significantly increased from time controls in rats receiving sTNFR1 and procaine however, a trend similar to ipsilateral phrenic hypercapnic response was observed in all contralateral groups, indicating a smaller but proportional increase was elicited. ***C-F.*** Linear regression analysis of ipsilateral (left) or contralateral (right) phrenic burst amplitude during hypercapnia versus phrenic bust amplitude at 60 min following axon conduction recovery or an equivalent duration in time controls. A strong positive relationship was observed in rats expressing significant ipsilateral iPMF at 60 min. A smaller, but still significant positive relationship was detected in rats expressing significant csPMF at 60 min. By contrast, no such relationship was detected in ipsilateral or contralateral time controls not receiving axon conduction block. Values presented are mean ± SEM. *significantly different from time control; ϕ significantly different from PKCζ-PS; $p<0.05$.

	PO_2	PCO_2	pH	MAP
Time control				
Baseline	318 ± 6	47.1 ± 0.7	7.33 ± 0.01	135 ± 7
Mock reduced activity	316 ± 6	47.1 ± 0.7	7.33 ± 0.01	131 ± 8
60 min	313 ± 6	47.2 ± 0.6	7.33 ± 0.01	119 ± 9
Procaine				
Baseline	323 ± 13	48.7 ± 1.1	7.32 ± 0.01	140 ± 7
Reduced activity	315 ± 16	48.5 ± 1.2	7.31 ± 0.01	125 ± 12
60 min	317 ± 14	49.0 ± 1.2	7.30 ± 0.01#	111 ± 11
Procaine + vehicle				
Baseline	310 ± 5	49.0 ± 1.7	7.35 ± 0.01	135 ± 11
Reduced activity	311 ± 5	49.0 ± 1.3	7.36 ± 0.01	118 ± 14
60 min	307 ± 7	49.6 ± 1.6	7.32 ± 0.01*	113 ± 9
Procaine + sTNFR1				
Baseline	286 ± 29	48.1 ± 1.3	7.34 ± 0.01	135 ± 6
Reduced activity	284 ± 30	48.3 ± 1.3	7.34 ± 0.01	115 ± 11
60 min	288 ± 17	48.1 ± 1.5	7.32 ± 0.01	121 ± 11
Procaine + PKCζ-PS				
Baseline	316 ± 15	48.8 ± 1.7	7.33 ± 0.01	144 ± 8
Reduced activity	294 ± 22	47.6 ± 2.1	7.32 ± 0.01	126 ± 12
60 min	296 ± 9*	48.3 ± 1.9	7.32 ± 0.02	120 ± 8
Procaine + scrPKCζ-PS				
Baseline	309 ± 12	45.2 ± 0.9	7.35 ± 0.01	136 ± 4
Reduced activity	302 ± 10	44.4 ± 1.4	7.35 ± 0.01	120 ± 9
60 min	305 ± 14	45.6 ± 1.2	7.35 ± 0.01	124 ± 8

Table 1: Average PO_2, PCO_2, pH and mean arterial pressure (MAP) during baseline, reduced activity and 60 min following the recovery of axon conduction or an equivalent duration in time control. Values presented are mean ± SEM. * significantly different than baseline; # significantly different than time controls.

	Duration (min)	Amplitude (% baseline)	
		Ipsilateral	Contralateral
Time control	0 ± 0	3 ± 1	4 ± 2
Procaine	32 ± 7*	-56 ± 7*#	-10 ± 4
Procaine + vehicle	39 ± 3*	-68 ± 6*#	6 ± 4
Procaine + sTNFR1	35 ± 4*	-58 ± 6*#	-2 ± 10
Procaine + PKCζ-PS	38 ± 3*	-46 ± 7*#$	18 ± 5*ψ
Procaine + scrPKCζ-PS	31 ± 2*	-61 ± 6*#	-8 ± 6

Table 2: Average duration of ipsilateral phrenic reduced activity, and average phrenic burst amplitude in the ipsilateral and contralateral phrenic nerve during lateralized axon conduction block or in time controls not receiving lateralized procaine. Values presented are mean ± SEM. * significantly different than time control; # significantly different than contralateral burst amplitude; $ significantly different than procaine + vehicle; ψ significantly different than procaine + scrPKCζ-PS; $p<0.05$.

CHAPTER IV

Inactivity-induced phrenic motor facilitation is differentially regulated among Sprague-Dawley rat sub-strains

K.A. Strey and T.L. Baker-Herman

In preparation for J Appl Physiol.

ABSTRACT

In ventilated Harlan Sprague-Dawley (HSD) rats, a central reduction in respiratory neural activity elicits a long-lasting, rebound increase in phrenic burst amplitude, a form of respiratory plasticity called inactivity-induced phrenic motor facilitation (iPMF). iPMF requires tumor necrosis factor alpha (TNFα) and atypical protein kinase C (aPKC) isoforms in spinal regions containing the phrenic motor nucleus. We hypothesized that similar to other forms of respiratory plasticity, the magnitude and duration of iPMF is differentially regulated among Sprague-Dawley rat sub-strains. To test this hypothesis, HSD rats and Charles River Sprague-Dawley rats (CRSD) were exposed to a reversible central neural apnea. Following the resumption of respiratory neural activity, HSD rats expressed a long-lasting (>60 min) increase in phrenic burst amplitude, indicating iPMF. By contrast, CRSD rats expressed an attenuated, transient (~15 min) increase in phrenic burst amplitude following resumption of respiratory neural activity, which slowly returned to baseline 30 min post-neural apnea, indicating an attenuated, transient iPMF. Inhibition of spinal NMDARs with APV in CRSD rats prior to or following neural apnea revealed a long-lasting iPMF following restoration of respiratory neural activity. Similar to HSD rats, long-lasting iPMF in CRSD rats receiving APV requires spinal TNFα and aPKC activity. Collectively, our results suggest that activation of spinal NMDARs restores baseline phrenic amplitude following central neural apnea, thereby constraining long-lasting iPMF in CRSD rats.

INTRODUCTION

Numerous forms of plasticity are elicited in response to stimuli including intermittent hypoxia (Peng et al., 2003; Prabhakar, 2011; Hoffman et al., 2012; Pavlinac Dodig et al., 2012), hypercapnia (Bach and Mitchell, 1998; Baker et al., 2001; Bavis et al., 2006), chemoreceptor denervation (Kinkead et al., 1998; Forster, 2003) and exercise (Babb et al., 2010). Recently, we described a form of respiratory plasticity in ventilated Harlan Sprague-Dawley (HSD) rats elicited by central neural apnea called inactivity-induced phrenic motor facilitation (iPMF;Mahamed et al., 2011; Strey et al., 2012; Baertsch and Baker-Herman, 2013). iPMF is a long-lasting (60 min) rebound increase in phrenic burst amplitude following the resumption of respiratory neural activity that requires spinal TNFα (Broytman et al., 2013) and atypical PKC (aPKC) activity (Strey et al., 2012). Since iPMF is elicited following unilateral C2 axon conduction block in the phrenic motor pool ipsilateral to conduction block (without changes in arterial blood gases), iPMF is elicited by reduced phrenic synaptic inputs *per se* (Strey and Baker-Herman, 2013).

Evidence suggests that age (Fukuda, 1992; Behan et al., 2002; Seebart et al., 2007), gender (Behan et al., 2002) and genetic background (Baker-Herman et al., 2010) impart substantial variability on the expression of respiratory plasticity. For example, the magnitude of phrenic and hypoglossal long-term facilitation following acute intermittent hypoxia (pLTF and hLTF, respectively) is greatly reduced with age in male rats (Zabka et al., 2001b), but enhanced with age in female rats (Zabka et al., 2001a). In addition, the manifestation of pLTF and/or hLTF varies considerably among male inbred rats (Baker-Herman et al., 2010), between rat sub-strains from different vendors (Fuller et al., 2001) and among rats from colonies supplied by the same vendor (Fuller et al., 2000). While factors giving rise to differential respiratory plasticity among

rats are largely unknown, they may stem from differential expression of neuromodulators (Clark et al., 1991; Clark and Proudfit, 1992) and high variability of ventilatory responses to respiratory stimuli like hypoxia (Strohl et al., 1997; Hodges et al., 2002; Golder et al., 2005; Donovan et al., 2011).

Here we tested the hypothesis that the magnitude and duration of iPMF is differentially expressed among HSD and CRSD rat sub-strains. HSD rats exposed to central neural apnea expressed a long-lasting iPMF, whereas CRSD rats expressed an attenuated and transient (~15 min) iPMF that returned to baseline 30 min post-neural apnea. HSD rats, but not CRSD rats expressed an increase in the phrenic burst amplitude response to hypercapnia. In CRSD rats, activation of spinal NMDA receptors (NMDARs) restores baseline phrenic burst amplitude following central neural apnea since blockade of spinal NMDARs with intrathecal APV enables long-lasting iPMF. Additionally, we show that following central neural apnea, long-lasting iPMF in CRSD rats receiving intrathecal APV requires spinal TNFα and aPKC activity. Collectively, our data suggest that spinal NMDARs constrain long-lasting iPMF in some rat sub-strains.

MATERIALS AND METHODS

Animals. All experiments were conducted with male (2.5-5 month-old, 230-550g) outbred Sprague-Dawley rats from two different suppliers, Harlan (Colony 217; Harlan, Indianapolis, IN) and Charles River Laboratories/Sasco Inc. (Colony P09; Portage, MI). Rats were received at similar ages (2-2.5 month-old) and housed in pairs in a controlled environment (12h light/dark cycles) with food and water *ad libitum* at the University of Wisconsin-Madison. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison.

Electrophysiology surgery and preparation. Anesthesia was induced in a closed chamber and rats were transferred to a heated table where anesthesia was maintained with 3% isoflurane (in 50% O_2:N_2 balance) through a nose cone. Core body temperature was monitored with a rectal probe (Physitemp, model 700 1H) and kept between 36-38°C. A tail vein catheter was placed for intravenous delivery of urethane and fluids. The trachea was cannulated, rats were pump-ventilated (Harvard Apparatus, Rodent Ventilator 683) and a bilaterally vagotomy was performed. End-tidal CO_2 ($ETCO_2$) was monitored (Capnogard; Respironics, Inc., Murrysville PA) and inspired gases were adjusted to maintain spontaneous respiratory neural activity throughout the surgery ($ETCO_2$ ~45-50mmHg). A femoral arterial catheter was placed to monitor blood pressure and sample blood gases throughout the protocol (ABL-500; Radiometer, Copenhagen, Denmark). Using a dorsal approach, the left phrenic nerve was isolated, cut distally and de-sheathed. The C2 spinous process was exposed and a partial C2 laminectomy was performed. A small hole in the dura was made above the exposed spinal segment and a catheter (2 French, Access Technologies) was advanced caudally (~5mm) under the dura. The catheter was connected to a Hamilton syringe filled with one of the intrathecal compounds described below.

Following surgery, rats were converted (6ml/hr; Harvard Apparatus syringe pump) to urethane anesthesia (1.7-1.8 g/kg, *i.v.*) while inspired isoflurane was slowly decreased. After rats were converted to urethane, they received an injection of the neuromuscular paralytic, pancuronium bromide (2.5 mg/kg, *i.v.*; Sicor Pharmaceuticals, CA, USA) followed by a slow infusion of a 1:1:4 solution (6% hetastarch/8.4% sodium bicarbonate/Lactated Ringers) to maintain blood pressure and fluid homeostasis. Adequate depth of anesthesia was monitored by

blood pressure responses to toe-pinch and supplemented with urethane as necessary. The left phrenic nerve was placed on a bipolar silver recording electrode and the cavity was filled with mineral oil. Compound action potentials were amplified (x10k), band-pass filtered (300 Hz to 10 kHz) and integrated (time constant: 50 ms; CWE, MA-821RSP). Raw and integrated signals were digitized and recorded with Powerlab data acquisition system (AD Instruments; Lab Chart 7.0).

Intrathecal compounds. The following compounds were dissolved in artificial cerebral spinal fluid (aCSF; in mM: 120 NaCl, 3 KCl, 2 CaCl, 2 MgCl, 23 $NaHCO_3$, 10 glucose bubbled with 95%O_2/5%CO_2; pH 7.4): DL-2-Amino-5-phosphonopentanoic acid (APV; doses below) (Sigma Aldrich), myristoylated ζ-pseudosubstrate inhibitory peptide (PKCζ-PS; 2 µg/µl; Tocris Bioscience), myristoylated scrambled ζ-pseudosubstrate peptide (scrPKCζ-PS; 2 µg/µl; Tocris Bioscience), or the soluble TNFα receptor 1 (sTNFR1; .1µg/µl; R&D Systems). Intrathecal compounds were slowly delivered over ~1-2 min, for a total volume of 10µl. An additional 3ul of sTNFR1 was delivered prior to the end of neural apnea, in sTNFR1 treated rats. Vehicle treated rats received 10µl of aCSF.

Electrophysiology protocols. One hour after isoflurane was withdrawn, the apneic threshold was determined by slowly lowering inspired CO_2 until phrenic neural activity ceased. The recruitment threshold was determined by slowly increasing inspired CO_2 until phrenic neural activity resumed. Baseline phrenic activity was established by setting the $ETCO_2$ ~2-3mmHg above the recruitment threshold. Following 15-20 min of recorded phrenic neural activity, a "baseline" arterial blood gas sample was taken. Rats were exposed to one of the following protocols: central

neural apnea or time control. A reversible central neural apnea was induced similar to finding the apneic threshold. Briefly, $PaCO_2$ was decreased below the apneic threshold by slowly reducing inspired CO_2 and/or increasing the ventilator rate. $ETCO_2$ was kept ~5mmHg below the apneic threshold throughout the neural apnea. After 30 min of neural apnea, baseline $PaCO_2$ levels were restored and phrenic neural activity resumed. To control for time dependent variations in phrenic neural output inherent to our preparation or as a consequence of intrathecal compounds (described below), separate groups of rats received a similar surgery, but were not exposed to neural apnea (time controls).

In preliminary experiments, a limited dose-response curve for APV was performed. CRSD rats received 10ul of APV at one of the following doses 20 min prior to neural apnea: 200ng/µl (n=3), 20ng/µl (n=4) and 2ng/µl (n=4). The least effective dose was determined as 20ng/µl and used for all remaining experiments. An additional four experiments were performed with 20ng/µl intrathecal APV delivered 20 min prior to neural apnea and grouped with the 20ng/µl dose response experiments and used for statistical analysis.

CRSD rats exposed to neural apnea received intrathecal: 1) vehicle 20 min prior to neural apnea (n=8), 2) APV 20 min prior to neural apnea (n=8), 3) APV 4 min prior to the end of neural apnea referred to as APV-4 (n=8), 4) sTNFR1 20 min prior to neural apnea + APV 4 min prior to the end of neural apnea and sTNFR1 (3ul) 2 min prior to the end of neural apnea (n=7), 5) APV 4 min prior to the end of neural apnea + scrPKCζ-PS 10 min after the resumption of phrenic neural activity (n=7), or 6) APV 4 min prior to the end of neural apnea + PKCζ-PS 10 min after the resumption of phrenic neural activity (n=7). Harlan rats exposed to a neural apnea received intrathecal: 1) vehicle 20 min prior to neural apnea (n=8) or APV 20 min prior to neural apnea (n=6). CRSD “time controls” underwent a similar surgery, but no neural apnea. Rats

received intrathecal: 1) vehicle (n=3); 2) APV (n=4); 3) sTNFR1 and APV (n=3); or 3) APV and PKCζ-PS (n=3). HSD time controls received intrathecal: 1) vehicle (n=3) or 2) APV (n=3).

Phrenic neural activity in all rats was monitored for 60 min post-neural apnea. Arterial blood gas samples were taken during baseline, central neural apnea, and 5 and/or 15, 30, and 60 min after respiratory neural activity resumed or an equivalent duration in controls not receiving reduced respiratory neural activity (time controls). At the end of each protocol, rats were presented with a severe hypercapnic challenge (~10% CO_2) to assure our preparation did not deteriorate and to assess the dynamic ventilatory range.

Statistical Analysis. Criteria to include data in the analysis were: mean arterial blood pressure above 70 mmHg, PaO_2 >140 mmHg, $PaCO_2$ within 1.5mmHg ± baseline and hypercapnic response >25%. Five rats were excluded from the analysis since hypercapnic responses were <25%. Integrated phrenic burst amplitude and frequency were averaged over 30-60 sec periods (immediately preceding blood gas samples) during baseline and 5, 15, 30 and 60 min post-neural apnea or an equivalent duration in time controls. Integrated phrenic amplitude was reported as a percent increase over baseline (normalized to zero) and phrenic burst frequency was expressed as an absolute change from baseline. There were no significant differences in phrenic burst amplitude or frequency between time control groups ($p>0.05$), so all groups were combined for statistical analysis (n=19). For comparison purposes, average amplitude and frequency of this combined time control group is shown in Figures 1-4, panels A and B. A two-way analysis of variance with repeated measures design was used for statistical comparisons of amplitude, frequency and blood gas parameters (Prism 6, GraphPad Software). Individual time point comparisons were determined by Fisher LSD post-hoc tests. A one-way analysis of variance was

used to detect significant differences in the maximum phrenic response to hypercapnia and individual comparisons were made using a Fisher LSD post-hoc test (Prism 6, GraphPad Software). For this analysis, CRSD rats receiving intrathecal APV prior to neural apnea or 4 min before the end of neural apnea (APV-4) were combined for clarity. For all analyses, the significance level was set to 0.05. Results are shown as means ± SEM.

RESULTS

Central respiratory neural activity was temporarily reduced in ventilated Sprague-Dawley rats supplied by Harlan and Charles River (HSD and CRSD; respectively) by reducing arterial CO_2 below the threshold for breathing (i.e. apneic threshold). Following 30 minutes of "central neural apnea," baseline arterial CO_2 was restored and respiratory neural activity was monitored for 60 min. Table 1 lists average arterial PCO_2, pH, PO_2, and mean arterial blood pressure (MAP) during baseline and 60 min post-neural apnea or an equivalent duration in time controls not receiving reduced respiratory activity. There were no significant differences in any variable from baseline to 60 min post-neural apnea within any rat group ($p>0.05$).

Slight differences in $PaCO_2$, pH and MAP were detected across groups (Table 1). CRSD rats pretreated with intrathecal sTNFR1 prior to neural apnea and receiving APV prior to the resumption of respiratory neural activity had a significantly higher $PaCO_2$ (at baseline and 60 min) and lower pH (60 min) relative to time controls and CRSD rats receiving APV only ($p<0.05$). CRSD rats receiving APV and scrPKCζ-PS following neural apnea exhibited a significantly lower pH relative to time controls ($p<0.05$). MAP was significantly lower in CRSD rats pretreated with vehicle prior to neural apnea than in time controls and HSD rats receiving vehicle ($p<0.05$). MAP was also lower in CRSD rats receiving pretreatment with APV prior to

neural apnea relative to HSD rats receiving APV ($p<0.05$). CRSD rats pretreated with sTNFR1 prior to neural apnea and receiving APV immediately prior to resumption of respiratory neural activity had a significantly lower MAP during baseline compared to time controls ($p<0.05$). Finally, CRSD rats receiving APV and PKCζ-PS following neural apnea had a significantly lower MAP at 60 min relative to time controls ($p<0.05$). These changes are not considered to have impacted our study since: 1) all observed changes were small and within normal physiological limits, and 2) no time-dependent changes were observed within any group.

iPMF is differentially regulated among Sprague-Dawley rat sub-strains

Representative compressed phrenic neurograms from vehicle treated HSD and CRSD rats during baseline, 30 min of reduced respiratory neural activity and 60 min following the resumption of respiratory neural activity are shown in Figure 1A. Average change in phrenic burst amplitude (% baseline) for 60 min post-neural apnea in HSD and CRSD rats receiving intrathecal vehicle or an equivalent duration in time controls is shown in Figure 1B. Phrenic burst amplitude in time controls was not significantly different than baseline at any time point (all $p>0.05$), suggesting that phrenic burst amplitude is stable throughout the protocol. Similar to previous findings (Strey et al., 2012; Baertsch and Baker-Herman, 2013), ventilated HSD rats exposed to central neural apnea expressed a prolonged increase in phrenic burst amplitude that was significantly increased from baseline and time controls at all time points post-neural apnea (all $p<0.05$; Fig. 1B), indicating long-lasting iPMF. By contrast, CRSD rats expressed a small, but significant increase relative to baseline and time controls at 5 and 15 min (both <0.05); however, at 30 and 60 min phrenic burst amplitude was no longer significantly increased from baseline or time controls (both $p<0.05$) suggesting an attenuated, transient iPMF. Indeed, phrenic

burst amplitude in CRSD rats was significantly lower than HSD rats at all time points post-neural apnea (all $p<0.05$; Fig. 1B). Collectively, these results suggest that Sprague-Dawley rat sub-strains exhibit a differential phrenic burst amplitude response to central neural apnea.

Average phrenic burst frequency (Δ baseline) for 60 min post-neural apnea in HSD and CRSD rats receiving intrathecal vehicle or an equivalent duration in time controls is shown in Figure 1C. Phrenic burst frequency in time control rats not exposed to central neural apnea expressed a small, but significant increase compared to baseline at 15, 30 and 60 min (all $p<0.05$; Fig 1C), consistent with previous reports that suggest small increases in phrenic burst frequency over time are inherent to our preparation (Broytman et al., 2013; Strey and Baker-Herman, 2013). In HSD rats exposed to central neural apnea, phrenic burst frequency was significantly increased relative to baseline and time controls at all time points post-neural apnea (all $p<0.05$) suggesting sustained phrenic burst frequency facilitation (Fig. 1C). CRSD rats receiving neural apnea expressed a significant increase in phrenic burst frequency relative to baseline at 5 and 15 min (both $p<0.05$), which was significantly increased compared to time controls at 5 min post-neural apnea ($p<0.05$). By 60 min, however, phrenic burst frequency in CRSD rats was significantly lower than time controls and no longer significantly increased from baseline ($p<0.05$), suggesting transient burst frequency facilitation. Phrenic burst frequency in CRSD rats was significantly lower than HSD rats at all time points (all $p<0.05$), indicating an attenuated phrenic burst frequency facilitation in CRSD rats. Together, these results demonstrate that HSD and CRSD rats express differential phrenic burst frequency facilitation following central neural apnea.

Activation of spinal NMDARs in CRSD rats impairs long-lasting iPMF

To test the hypothesis that activation of spinal NMDA receptors (NMDARs) in/near the phrenic motor nucleus in CRSD rats impairs long-lasting iPMF following central neural apnea, the competitive NMDAR antagonist APV (20ng/µl) was delivered intrathecally 20 min prior to central neural apnea. Representative compressed phrenic neurograms during baseline, 30 min of reduced respiratory neural activity and 60 min following the resumption of respiratory neural activity in CRSD and HSD rats receiving pretreatment with intrathecal APV are shown in Figure 2A. Average change (% baseline) in phrenic burst amplitude for 60 min post-neural apnea in HSD and CRSD rats pretreated with intrathecal APV or an equivalent duration in time controls is shown in Figure 2B. HSD rats receiving APV 20 min prior to neural apnea expressed a significant increase in phrenic burst amplitude relative to baseline and time controls at all time points (all $p<0.05$; Fig. 2B) that was phenotypically similar to that observed in HSD rats that did not receive APV (Fig. 1B), suggesting APV did not impair the development of long-lasting iPMF. CRSD rats receiving APV 20 min prior to neural apnea expressed a significant increase in phrenic burst amplitude relative to baseline and time controls at all time points (all $p<0.05$; Fig. 2B), indicating long-lasting iPMF. Phrenic burst amplitude in CRSD rats pretreated with APV was not significantly different than HSD rats pretreated with APV at 5 or 60 min following resumption of respiratory neural activity (both $p<0.05$), although CRSD rats expressed a small, but significantly lower phrenic burst amplitude compared to HSD rats at 15 and 30 min (both $p<0.05$). Together, these results suggest that activation of spinal NMDARs in CRSD rats constrains iPMF and restores baseline phrenic burst amplitude following central neural apnea.

Average phrenic burst frequency (Δ baseline) for 60 min post-neural apnea in HSD and CRSD rats receiving APV 20 min prior to neural apnea or an equivalent duration in time controls

is shown in Figure 2C. HSD rats pretreated with APV prior to neural apnea expressed a significant increase in phrenic burst frequency relative to baseline at all time points (both $p<0.05$) and time controls at 5 and 15 min (both $p<0.05$); however, at 30 and 60 min post-neural apnea phrenic burst frequency was no longer significantly increased from time controls (both $p>0.05$) indicating transient burst frequency facilitation (Fig. 2C). CRSD rats receiving APV prior to neural apnea expressed a significant increase in phrenic burst frequency relative to baseline at all time points (all $p<0.05$), which was significantly increased from time controls at 5, 15 and 30 min post-neural apnea (all $p<0.05$). This increase in phrenic burst frequency in CRSD rats was not significantly increased from time controls at 60 min, suggesting transient phrenic burst frequency facilitation ($p>0.05$; Fig. 2C). Collectively, these data suggest that HSD and CRSD rats receiving intrathecal APV prior to neural apnea express transient burst frequency facilitation.

Activation of spinal NMDARs following reduced neural activity in CRSD rats impairs long-lasting iPMF

To determine if activation of spinal NMDARs constrains iPMF during or following reduced respiratory neural activity, CRSD rats were given intrathecal APV (20ng/µl) prior to the resumption of respiratory neural activity (referred to as APV-4 since beginning of delivery was 4 min prior to end of neural apnea). Representative compressed phrenic neurogram during baseline, 30 min of reduced respiratory neural activity and 60 min following the resumption of respiratory neural activity in CRSD rats receiving intrathecal APV-4 is shown in Figure 3A, whereas the average responses are shown in Figure 3B. Similar to CRSD rats pretreated with APV prior to neural apnea (Fig. 2B), CRSD rats receiving APV-4 expressed a significant

increase in phrenic burst amplitude at all time points post-neural apnea, relative to baseline and time controls ($p<0.05$), suggesting that activation of spinal NMDARs during transient iPMF expression (*versus* during neural apnea) in CRSD rats restores baseline phrenic burst amplitude following central neural apnea, thereby constraining long-lasting iPMF.

Long-lasting iPMF in CRSD rats receiving APV requires spinal TNFα

Recently, we reported that spinal TNFα is necessary for late, but not early phases of iPMF (Broytman et al., 2013). To determine if TNFα is necessary for long-lasting iPMF in CRSD rats receiving APV, CRSD rats were given the soluble TNF receptor (sTNFR1) 20 min prior to neural apnea and APV prior to the end of neural apnea (APV-4). sTNFR1 is a soluble TNFα scavenger that binds endogenous TNFα and prevents/attenuates TNFα signaling. CRSD rats receiving intrathecal sTNFR1 and APV-4, expressed a significant increase in phrenic burst amplitude from baseline and time controls at 5 min (both $p<0.05$; Fig. 3B), however subsequent time points were not significantly different than baseline or time controls (all $p>0.05$), suggesting transient iPMF. Further, in CRSD rats receiving intrathecal sTNFR1 and APV-4, phrenic burst amplitude was significantly lower than CRSD receiving APV-4 alone at all time points following the resumption of respiratory neural activity (all $p<0.05$; Fig. 3B). Taken together, our results suggest that long-lasting iPMF in CRSD rats receiving APV-4 requires spinal TNFα signaling for late, but not early iPMF.

Average phrenic burst frequency (Δ baseline) for 60 min post-neural apnea in CRSD rats receiving intrathecal APV-4 or sTNFR1 and APV-4 or an equivalent duration in time controls is shown in Figure 3C. CRSD rats receiving intrathecal APV-4 expressed a significant increase in phrenic burst frequency relative to baseline and time controls at all time points (all $p<0.05$),

suggesting sustained burst frequency facilitation. By contrast, phrenic burst frequency in CRSD rats receiving intrathecal sTNFR1 and APV-4 was not significantly increased relative to time controls at any time point (all $p>0.05$), although a small increase in phrenic burst frequency compared to baseline was observed at both 5 and 60 min post-neural apnea (both $p<0.05$). In CRSD rats receiving APV-4, phrenic burst frequency was significantly increased from CRSD rats receiving intrathecal sTNFR1 and APV-4 at early time points (5, 15, 30 min; all $p<0.05$), however by 60 min post-neural apnea phrenic burst frequency was no longer significantly increased ($p<0.05$).

Long-lasting iPMF in CRSD rats receiving APV requires spinal aPKC activity

Since aPKC isoforms PKCζ and/or PKCι/λ are necessary during early phases of iPMF to transition to a long-lasting iPMF (Strey et al., 2012), we tested the hypothesis that spinal aPKC activity is necessary for long-lasting iPMF in CRSD rats receiving intrathecal APV-4. CRSD rats received intrathecal APV (20ng/µl) before the end of neural apnea (APV-4) and either the cell-permeable pseudosubstrate inhibitory peptide that inhibits all aPKC isoforms (PKCζ-PS) or the scrambled version of the peptide (scrPKCζ-PS) 10 min after phrenic neural activity was restored. Representative compressed phrenic neurograms from CRSD rats during baseline, 30min of reduced respiratory neural activity and 60min following the resumption of respiratory neural activity in rats receiving APV-4 and either scrPKCζ-PS or PKCζ-PS are shown in Figure 4A. Average change in phrenic burst amplitude (% baseline) for 60 min post-neural apnea in CRSD rats receiving APV-4 and either scrPKCζ-PS or PKCζ-PS or an equivalent duration in time controls is shown in Figure 4B. In rats receiving APV-4 and either scrPKCζ-PS or PKCζ-PS, phrenic burst amplitude was significantly increased compared to baseline and time controls

before (5 min; $p<0.05$) receiving scrPKCζ-PS or PKCζ-PS, which was not significantly different between groups ($p>0.05$), suggesting iPMF of a similar magnitude was elicited in both groups. CRSD rats receiving APV-4 and scrPKCζ-PS expressed a significant increase in phrenic burst amplitude relative to baseline and time controls at all time points post-neural apnea (all $p<0.05$; Fig. 1B), suggesting that scrPKCζ-PS had no effect on long-lasting iPMF enabled by APV-4. By contrast, in CRSD rats treated with APV-4 and PKCζ-PS, phrenic burst amplitude was significantly increased from baseline and time controls shortly after PKCζ-PS treatment (15 min; $p<0.05$); however phrenic burst amplitude slowly declined back toward baseline such that at 30 and 60 min phrenic burst amplitude was no longer significantly different than time controls (both $p>0.05$), although a small, but significant increase relative to baseline continued to be observed at 60 min (both $p<0.05$), indicating transient iPMF. Indeed, phrenic burst amplitude in rats receiving APV-4 and PKCζ-PS was significantly lower than rats treated with APV-4 and scrPKCζ-PS at 15, 30 and 60 min post-neural apnea (all $p<0.05$; Fig. 4B). Collectively, these results suggest that long-lasting iPMF in CRSD rats receiving intrathecal APV-4 requires spinal aPKC signaling.

Average phrenic burst frequency (Δ baseline) for 60 min post-neural apnea in CRSD rats receiving APV-4 and either scrPKCζ-PS or PKCζ-PS or an equivalent duration in time controls is shown in Figure 4C. Rats receiving intrathecal APV-4 and scrPKCζ-PS following the resumption or neural activity expressed a significant increase in phrenic burst frequency relative to baseline at all points (all $p<0.05$) that was significantly increased compared to time controls at 5, 15 and 60 min post-neural apnea (all $p<0.05$), suggesting sustained increase in phrenic burst frequency. Rats receiving intrathecal APV-4 and PKCζ-PS expressed a significant increase from time controls at 5 and 15 min post-neural apnea (both $p<0.05$), although a small but significant

increase in phrenic burst frequency from baseline was observed at all time points (all $p<0.05$), suggesting transient burst frequency facilitation. However, phrenic burst frequency in rats receiving scrPKCζ-PS was not significantly different than rats receiving PKCζ-PS at any time point (all $p>0.05$).

iPMF is associated with an enhanced hypercapnic response

At the end of each experimental protocol, rats were exposed to a brief hypercapnic challenge ($ETCO_2$~98). Average phrenic burst amplitude (% baseline) during the hypercapnic challenge is shown in Figure 5. Shaded bars indicate that a significant iPMF compared to time controls was expressed at 60 min. HSD rats receiving intrathecal vehicle or APV prior to neural apnea expressed a significant increase in the phrenic burst amplitude response to hypercapnia relative to the response in time controls (both $p<0.05$), indicating an enhanced hypercapnic response. By contrast, phrenic burst amplitude during hypercapnia in CRSD rats receiving vehicle prior to neural apnea was not significantly increased from time controls ($p>0.05$) and was significantly lower than the response in vehicle treated HSD rats ($p<0.05$), suggesting an enhanced hypercapnic response 60 min following neural apnea was not expressed by CRSD rats. However, in CRSD rats receiving intrathecal APV prior to or following neural apnea, a significant increase in the phrenic burst amplitude response during hypercapnia was observed relative to time controls and CRSD rats receiving intrathecal vehicle prior to neural apnea ($p<0.05$), indicating that spinal NMDA receptor inhibition in CRSD rats enabled an increased hypercapnic response. By contrast, in CRSD rats receiving sTNFR1 and APV-4, phrenic burst amplitude during the hypercapnic challenge was not significantly different than time controls or CRSD rats receiving vehicle (both $p>0.05$), indicating that inhibition of spinal TNFα signaling

impaired the enhanced hypercapnic response following neural apnea in CRSD rats with NMDA receptor inhibition. Similar to CRSD rats receiving APV, CRSD rats receiving intrathecal APV-4 and scrPKCζ-PS or PKCζ-PS expressed an enhanced hypercapnic (both $p<0.05$). With one exception (discussed below), these data suggest that rats expressing a long-lasting iPMF, but not a transient iPMF following neural apnea express an enhanced hypercapnic response.

DISCUSSION

Here we show that the magnitude and duration of iPMF and burst frequency facilitation following central neural apnea are differentially expressed among Sprague-Dawley rat sub-strains supplied by Harlan and Charles River (HSD and CRSD rats respectively). Similar to previous reports (Mahamed et al., 2011; Strey et al., 2012; Baertsch and Baker-Herman, 2013), we show that following central neural apnea, HSD rats express long-lasting iPMF, which is associated with an increased phrenic response to hypercapnia. Unlike HSD rats, CRSD rats express an attenuated, transient iPMF that returns to baseline 30 min after the resumption of respiratory neural activity and did not express an enhanced phrenic hypercapnic response 60 min post-neural apnea. Spinal NMDAR inhibition immediately prior to resumption of respiratory neural activity in CRSD rats enables a long-lasting iPMF that requires spinal TNFα and aPKC activity, suggesting that NMDAR activation during the initial phases of transient iPMF expression in CRSD rats restores baseline phrenic burst amplitude following neural apnea. Collectively, our results demonstrate that spinal NMDAR activation regulates the duration of iPMF following central neural apnea in CRSD rats.

Burst amplitude facilitation is differentially regulated among rat sub-strains

Multiple reports suggest that ventilatory responses to hypoxia and hypercapnia are differentially expressed among inbred and outbred rat strains. For instance, Brown Norway rats exhibit decreased CO_2 sensitivity and reduced minute ventilation during hypoxia relative to other inbred (e.g., Dahl salt-sensitive, Fawn-hooded hypertensive) and outbred rats (e.g., Sprague-Dawley) (Hodges et al., 2002; Subramanian et al., 2007; Donovan et al., 2011). Studies utilizing preparations that reduce the impact of metabolic rate and fluctuations in arterial blood gases (i.e. anesthetized, ventilated and temperature controlled rats) indicate that central neural mechanisms contribute to this variability (Fuller et al., 2001; Golder et al., 2005; Baker-Herman et al., 2010). Indeed, urethane anesthetized and ventilated inbred rats express a differential phrenic response to intermittent hypoxia (Golder et al., 2005).

Although the rats used in this study are supplied by two independent and geographically distinct vendors, they are not different rat "strains." Instead, they are most commonly referred to as "sub-strains" since they are descendents of the same Sprague-Dawley colony that have been isolated from one another for over 30 years. Although different rearing practices or environmental factors cannot be ruled out, a variety of studies suggest that this separation has created genetically or epigenetically distinct sub-strains (Clark et al., 1991; Clark and Proudfit, 1992; Sluka and Westlund, 1992; Graham et al., 1997; Turnbull and Rivier, 1999; Yoon et al., 1999; Fuller et al., 2001; Pecoraro et al., 2006). For example, cervical spinal segments in CRSD rats are more densely innervated by noradrenergic projections than in HSD rats (Clark et al., 1991; Clark and Proudfit, 1992). In addition, following acute intermittent hypoxia (AIH) hypoglossal long-term facilitation (hLTF), but not phrenic long-term facilitation (pLTF) is differentially expressed by Sprague-Dawley rat sub-strains. Specifically, both rats sub-strains

express pLTF of a similar magnitude, while CRSD rats, but not HSD rats express hLTF (Fuller et al., 2001). In contrast to LTF, data presented here demonstrates that HSD rats express a long-lasting iPMF, while CRSD rats express an attenuated and transient iPMF.

While HSD rats express long-lasting iPMF, previous reports suggest that central neural apnea elicits transient (~15 min) inactivity-induced hypoglossal and intercostal motor facilitation (iHMF and iIMF respectively; Baker-Herman and Strey, 2011; Strey et al., 2013). Since the duration of iPMF and iHMF/iIMF are differentially expressed within HSD rats (Baertsch and Baker-Herman, 2013; Strey et al., 2013), we suspect that mechanisms local to each motor pool dictate the magnitude and duration of burst amplitude facilitation following central neural apnea. Consistent with this idea, CRSD rats receiving intrathecal APV in spinal regions containing the phrenic motor nucleus attenuates spinal NMDAR activation and enables long-lasting iPMF expression. These results suggest that mechanisms local to the phrenic motor nucleus regulate the duration of phrenic burst amplitude following central neural apnea.

Mechanisms of long-lasting iPMF versus transient iPMF

To date, the cellular mechanisms giving rise to long-lasting iPMF have been investigated in HSD rats (Figure 6). Recent data suggests that TNFα plays a critical role in iPMF (Broytman et al., 2013). Although TNFα is typically thought of as a proinflammatory cytokine, it is also an important neuromodulator within the CNS necessary for increases in synaptic strength following reduced neural activity in cortical and hippocampal neurons (Beattie et al., 2002; Stellwagen and Malenka, 2006; Turrigiano, 2006; Kaneko et al., 2008). Following central neural apnea and unilateral reduction in phrenic synaptic inputs, spinal TNFα is necessary for the late phase of iPMF (Broytman et al., 2013; Strey and Baker-Herman, 2013). Spinal TNFα may activate

atypical PKC (aPKC) isoforms since exogenous application of TNFα to spinal regions containing the phrenic motor nucleus elicits an aPKC dependent increase in phrenic burst amplitude (Broytman et al., 2013).

The aPKC family is composed of full-length aPKC isoforms PKCζ and PKCι/λ and the truncated, constitutively active isoform PKMζ (Newton, 2001; Reyland, 2009). Our data suggest that iPMF consists of two mechanistically distinct phases: 1) an early, labile phase that requires spinal PKCζ and/or PKCι/λ activity (PKCζ/ι) to transition to 2) long-lasting iPMF. However, once established, iPMF shifts to a non-aPKC mediated pathway (~45 min post-neural apnea). Consistent with this, early, but not late iPMF is associated with an increased interaction between PKCζ/ι and the scaffolding molecule PKCζ interacting protein (p62/ZIP; Strey et al., 2012) forming an multimeric aPKCζ/ι-p62/ZIP signaling complex. The cellular molecules downstream of TNFα and the aPKCζ/ι-p62/ZIP signaling complex are currently unknown, but may involve changes in membrane bound chloride co-transporters KCC2 and NKCC1 (Gonzalez-Islas et al., 2010), AMPARs (Garcia-Bereguiain et al., 2013) and/or GABA receptors (Stellwagen et al., 2005).

Although we are beginning to understand cellular mechanisms necessary for long-lasting iPMF, little is known about the mechanisms giving rise to transient iPMF (Figure 6). Literature reports suggest that activation of NMDARs in hippocampal neurons by miniature synaptic events during global reductions in neural activity (i.e. TTX) inhibits protein synthesis and prevents synapses from being scaled-up (Sutton et al., 2004; Sutton et al., 2006). When global activity blockade is coupled with local NMDAR blockade, protein synthesis constraints are relieved and synapses are rapidly scaled (Sutton et al., 2006). Consistent with these reports, we show that pharmacological blockade of spinal NMDAR activation during neural apnea with APV in CRSD

rats enables long-lasting iPMF. To more closely examine the necessary time window for NMDA receptor activation, we delivered APV 4 min prior to the end of neural apnea (APV-4) and showed that APV-4 also enabled long-lasting iPMF in CRSD rats. Therefore, an alternative hypothesis is that the initial increase in phrenic burst amplitude observed after neural activity is restored (i.e. transient iPMF) may activate an opposing mechanism to restore baseline phrenic burst amplitude. Indeed, over-activation of NMDARs decreases synaptic strength (Colledge et al., 2003; Turrigiano, 2008) and NMDARs are necessary for homeostatic downscaling (Siddoway et al., 2013) *via* removal of membrane bound AMPA receptors (Beattie et al., 2000). This decrease in synaptic strength is prevented by pharmacological blockade of NMDARs (Carroll et al., 1999). Nevertheless, the possibility that activation of NMDARs by miniature synaptic events following the restoration of phrenic neural activity constrains local protein synthesis dependent pathways cannot be ruled out (Sutton et al., 2004). It is unknown why spinal NMDAR activation in some rats (CRSD) limits the duration of phrenic burst amplitude following central neural apnea while in other rats (HSD) NMDAR activation has little/no effect on iPMF expression.

Long-lasting iPMF is associated with an increased hypercapnic response

In addition to maintaining eupnic breathing, the respiratory control system must respond to respiratory challenges (e.g., hypercapnia and hypoxia) and participate in non-ventilatory behaviors (e.g., coughing). Since these tasks require larger diaphragmatic forces, the respiratory control system recruits specific motor units tailored to each function to mediate these behaviors. Therefore, during resting/eupnic breathing, the respiratory system operates at a fraction of its maximal dynamic range (Mantilla and Sieck, 2011). A key question is how does the respiratory

control system respond to these challenges following iPMF expression? Similar to previous reports (Baertsch and Baker-Herman, 2013), our data suggest that while all rats express an increase in phrenic burst amplitude during hypercapnia (compared to baseline), rats expressing long-lasting, but not transient iPMF have a significantly larger phrenic response to hypercapnia 60 min following resumption of respiratory neural activity compared to time controls, with one exception: CRSD rats receiving APV-4 and PKCζ-PS expressed a significant phrenic hypercapnic response compared to time controls, but did not express significant iPMF (compared to time controls) at 60 min. However, in CRSD rats receiving APV-4 and PKCζ-PS, a small increase in phrenic burst amplitude relative to baseline was still expressed at 60 min; thus, the enhanced hypercapnic response apparent in these rats may be the result of residual mechanisms that had not yet completely been completely abolished.

Although the ventilatory response to hypercapnia is initiated by central and peripheral chemoreceptors (Lahiri and Forster, 2003), we suggest that the *enhanced* hypercapnic response is due to spinal mechanisms in/near the phrenic motor pool. Indeed, in CRSD rats spinal APV application enabled long-lasting iPMF and a subsequently enhanced hypercapnic response following neural apnea. Consistent with this hypothesis, iPMF elicited following unilateral reduction in phrenic spinal synaptic inputs with no apparent change in brainstem neural activity, is associated with a proportional increase in the ipsilateral and contralateral phrenic burst amplitude response during hypercapnia (Strey and Baker-Herman, 2013). Collectively, these results demonstrate that the enhanced hypercapnic response is due to spinal mechanisms occurring locally in or near the phrenic motor pool. The specific mechanisms giving rise to this enhanced response to hypercapnia are currently unknown; however, one attractive possibility is that iPMF expression increases the excitability of phrenic motor neurons, which increases the

sensitivity to all excitatory inputs, including hypercapnia. Indeed, reduced neural activity increases spinal motor neuron excitability (Gonzalez-Islas et al., 2010).

Burst frequency facilitation

With the exception of three experimental groups, the general response to central neural apnea in both sub-strains is transient burst frequency facilitation. Similar to burst amplitude, CRSD rats have an attenuated increase in phrenic burst frequency following central neural apnea compared to HSD rats. While our data appear to suggest that in CRSD rats intrathecal application of APV-4 gives rise to sustained burst frequency facilitation and intrathecal sTNFR1 and PKCζ-PS blocks burst frequency facilitation, we refrain from making these conclusions since the magnitude of burst frequency facilitation observed following central neural apnea was small (relative to amplitude changes) and highly variable among rat groups, similar to other reports (Baker-Herman and Mitchell, 2002, 2008). Further, CRSD rats receiving sTNFR1 and APV-4 had a significantly higher $PaCO_2$ during the protocol relative to time controls and CRSD rats receiving APV-4, which may "mask" burst frequency facilitation. Future studies are necessary to fully characterize phrenic burst frequency facilitation following neural apnea in these rat sub-strains.

Significance of long-lasting and transient iPMF

Recently, we showed that a unilateral reduction in phrenic synaptic inputs *via* intraspinal procaine in HSD rats elicits long-lasting iPMF in the ipsilateral phrenic motor pool and crossed spinal phrenic motor facilitation (csPMF) within the contralateral phrenic motor pool (Strey and Baker-Herman, 2013). Since arterial blood gases were held constant during reduced phrenic

synaptic inputs, our data suggest that long-lasting iPMF is elicited by reduced neural activity *per se* (Strey and Baker-Herman, 2013). An emerging property of neural circuits is that mechanisms of homeostatic plasticity allow neurons to sense and respond to bidirectional changes in neural activity (Turrigiano, 2008). These homeostatic mechanisms are thought to offset destabilizing influences and restore stable network output following changes in synaptic input (Turrigiano, 2012). We hypothesize that iPMF may represent one mechanism similar to homeostatic plasticity to allows phrenic motor neurons to sense and respond to reduced neural activity to maintain phrenic motor output.

Currently, it is unknown if iPMF represents true "homeostatic" plasticity. On one hand, phrenic burst amplitude in CRSD rats following neural apnea returns to baseline with the approximate time course as iPMF induction. Our data suggest activation of a counter mechanism requiring NMDARs restores baseline phrenic burst amplitude, which may be similar to the pathways involved in homeostatic downscaling (Siddoway et al., 2013). On the other hand, HSD rats have features resembling homeostatic plasticity including the increase in the phrenic dynamic range (Strey and Baker-Herman, 2013). The mechanisms that prevent activity from returning to baseline in HSD are unknown, but may be related to a differential time course for hyper-activity *versus* inactivity within some rat sub-strains. Regardless of whether iPMF is truly a homeostatic response or not, we hypothesize that iPMF maintains neural activity within the phrenic motor circuit during the many situations organisms may experience reduced respiratory neural activity (Strey et al., 2013).

Acknowledgements: This work was supported by a grant from the National Institutes of Health (HL105511).

The authors declare no conflicts of interest.

REFERENCES

Babb TG, Wood HE, Mitchell GS (2010) Short- and long-term modulation of the exercise ventilatory response. Med Sci Sports Exerc 42:1681-1687.

Bach KB, Mitchell GS (1998) Hypercapnia-induced long-term depression of respiratory activity requires alpha2-adrenergic receptors. J Appl Physiol 84:2099-2105.

Baertsch NA, Baker-Herman TL (2013) Inactivity-induced phrenic and hypoglossal motor facilitation are differentially expressed following intermittent vs. sustained neural apnea. J Appl Physiol 114:1388-1395.

Baker-Herman TL, Mitchell GS (2002) Phrenic long-term facilitation requires spinal serotonin receptor activation and protein synthesis. J Neurosci 22:6239-6246.

Baker-Herman TL, Mitchell GS (2008) Determinants of frequency long-term facilitation following acute intermittent hypoxia in vagotomized rats. Respir Physiol Neurobiol 162:8-17.

Baker-Herman TL, Strey KA (2011) Similarities and differences in mechanisms of phrenic and hypoglossal motor facilitation. Respir Physiol Neurobiol 179:48-56.

Baker-Herman TL, Bavis RW, Dahlberg JM, Mitchell AZ, Wilkerson JE, Golder FJ, Macfarlane PM, Watters JJ, Behan M, Mitchell GS (2010) Differential expression of respiratory long-term facilitation among inbred rat strains. Respir Physiol Neurobiol 170:260-267.

Baker TL, Fuller DD, Zabka AG, Mitchell GS (2001) Respiratory plasticity: differential actions of continuous and episodic hypoxia and hypercapnia. Respir Physiol 129:25-35.

Bavis RW, Johnson RA, Ording KM, Otis JP, Mitchell GS (2006) Respiratory plasticity after perinatal hypercapnia in rats. Respir Physiol Neurobiol 153:78-91.

Beattie EC, Carroll RC, Yu X, Morishita W, Yasuda H, von Zastrow M, Malenka RC (2000) Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. Nat Neurosci 3:1291-1300.

Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, Von Zastrow M, Beattie MS, Malenka RC (2002) Control of synaptic strength by glial TNFalpha. Science 295:2282-2285.

Behan M, Zabka AG, Mitchell GS (2002) Age and gender effects on serotonin-dependent plasticity in respiratory motor control. Respir Physiol Neurobiol 131:65-77.

Broytman O, Baertsch NA, Baker-Herman TL (2013) Spinal TNFα is necessary for inactivity-induced phrenic motor facilitation. J Physiol.

Carroll RC, Beattie EC, Xia H, Lüscher C, Altschuler Y, Nicoll RA, Malenka RC, von Zastrow M (1999) Dynamin-dependent endocytosis of ionotropic glutamate receptors. Proc Natl Acad Sci U S A 96:14112-14117.

Clark FM, Proudfit HK (1992) Anatomical evidence for genetic differences in the innervation of the rat spinal cord by noradrenergic locus coeruleus neurons. Brain Res 591:44-53.

Clark FM, Yeomans DC, Proudfit HK (1991) The noradrenergic innervation of the spinal cord: differences between two substrains of Sprague-Dawley rats determined using retrograde tracers combined with immunocytochemistry. Neurosci Lett 125:155-158.

Colledge M, Snyder EM, Crozier RA, Soderling JA, Jin Y, Langeberg LK, Lu H, Bear MF, Scott JD (2003) Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. Neuron 40:595-607.

Donovan LM, Chai S, Gillombardo CB, Emancipator SN, Strohl KP (2011) Ventilatory behavior and carotid body morphology of Brown Norway and Sprague Dawley rats. Respir Physiol Neurobiol 178:250-255.

Forster HV (2003) Plasticity in the control of breathing following sensory denervation. J Appl Physiol 94:784-794.

Fukuda Y (1992) Changes in ventilatory response to hypoxia in the rat during growth and aging. Pflugers Archiv : European journal of physiology 421:200-203.

Fuller DD, Baker TL, Behan M, Mitchell GS (2001) Expression of hypoglossal long-term facilitation differs between substrains of Sprague-Dawley rat. Physiol Genomics 4:175-181.

Fuller DD, Bach KB, Baker TL, Kinkead R, Mitchell GS (2000) Long term facilitation of phrenic motor output. Respir Physiol 121:135-146.

Garcia-Bereguiain MA, Gonzalez-Islas C, Lindsly C, Butler E, Hill AW, Wenner P (2013) In vivo synaptic scaling is mediated by GluA2-lacking AMPA receptors in the embryonic spinal cord. J Neurosci 33:6791-6799.

Golder FJ, Zabka AG, Bavis RW, Baker-Herman T, Fuller DD, Mitchell GS (2005) Differences in time-dependent hypoxic phrenic responses among inbred rat strains. J Appl Physiol 98:838-844.

Gonzalez-Islas C, Chub N, Garcia-Bereguiain MA, Wenner P (2010) GABAergic synaptic scaling in embryonic motoneurons is mediated by a shift in the chloride reversal potential. J Neurosci 30:13016-13020.

Graham BA, Hammond DL, Proudfit HK (1997) Differences in the antinociceptive effects of alpha-2 adrenoceptor agonists in two substrains of Sprague-Dawley rats. J Pharmacol Exp Ther 283:511-519.

Hodges MR, Forster HV, Papanek PE, Dwinell MR, Hogan GE (2002) Ventilatory phenotypes among four strains of adult rats. J Appl Physiol 93:974-983.

Hoffman MS, Nichols NL, Macfarlane PM, Mitchell GS (2012) Phrenic long-term facilitation after acute intermittent hypoxia requires spinal ERK activation but not TrkB synthesis. J Appl Physiol 113:1184-1193.

Kaneko M, Stellwagen D, Malenka RC, Stryker MP (2008) Tumor necrosis factor-alpha mediates one component of competitive, experience-dependent plasticity in developing visual cortex. Neuron 58:673-680.

Kinkead R, Zhan WZ, Prakash YS, Bach KB, Sieck GC, Mitchell GS (1998) Cervical dorsal rhizotomy enhances serotonergic innervation of phrenic motoneurons and serotonin-dependent long-term facilitation of respiratory motor output in rats. J Neurosci 18:8436-8443.

Lahiri S, Forster RE, 2nd (2003) CO2/H(+) sensing: peripheral and central chemoreception. The international journal of biochemistry & cell biology 35:1413-1435.

Mahamed S, Strey KA, Mitchell GS, Baker-Herman TL (2011) Reduced respiratory neural activity elicits phrenic motor facilitation. Respir Physiol Neurobiol 175:303-309.

Mantilla CB, Sieck GC (2011) Phrenic motor unit recruitment during ventilatory and non-ventilatory behaviors. Respir Physiol Neurobiol 179:57-63.

Newton AC (2001) Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem Rev 101:2353-2364.

Pavlinac Dodig I, Pecotic R, Valic M, Dogas Z (2012) Acute intermittent hypoxia induces phrenic long-term facilitation which is modulated by 5-HT1A receptor in the caudal raphe region of the rat. J Sleep Res 21:195-203.

Pecoraro N, Ginsberg AB, Warne JP, Gomez F, la Fleur SE, Dallman MF (2006) Diverse basal and stress-related phenotypes of Sprague Dawley rats from three vendors. Physiol Behav 89:598-610.

Peng YJ, Overholt JL, Kline D, Kumar GK, Prabhakar NR (2003) Induction of sensory long-term facilitation in the carotid body by intermittent hypoxia: implications for recurrent apneas. Proc Natl Acad Sci U S A 100:10073-10078.

Prabhakar NR (2011) Sensory plasticity of the carotid body: role of reactive oxygen species and physiological significance. Respir Physiol Neurobiol 178:375-380.

Reyland ME (2009) Protein kinase C isoforms: Multi-functional regulators of cell life and death. Front Biosci 14:2386-2399.

Seebart BR, Stoffel RT, Behan M (2007) Age-related changes in the serotonin 2A receptor in the hypoglossal nucleus of male and female rats. Respir Physiol Neurobiol 158:14-21.

Siddoway B, Hou H, Xia H (2013) Molecular mechanisms of homeostatic synaptic downscaling. Neuropharmacology.

Sluka KA, Westlund KN (1992) Spinal projections of the locus coeruleus and the nucleus subcoeruleus in the Harlan and the Sasco Sprague-Dawley rat. Brain Res 579:67-73.

Stellwagen D, Malenka RC (2006) Synaptic scaling mediated by glial TNF-alpha. Nature 440:1054-1059.

Stellwagen D, Beattie EC, Seo JY, Malenka RC (2005) Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-alpha. J Neurosci 25:3219-3228.

Strey KA, Baker-Herman TL (2013) Decreased spinal synaptic inputs to phrenic motor neurons elicit localized inactivity-induced phrenic motor facilitation. Exp Neurol In Review.

Strey KA, Baertsch NA, Baker-Herman TL (2013) Inactivity-induced respiratory plasticity: Protecting the drive to breathe in disorders that reduce respiratory neural activity. Respir Physiol Neurobiol.

Strey KA, Nichols NL, Baertsch NA, Broytman O, Baker-Herman TL (2012) Spinal atypical protein kinase C activity is necessary to stabilize inactivity-induced phrenic motor facilitation. J Neurosci 32:16510-16520.

Strohl KP, Thomas AJ, St Jean P, Schlenker EH, Koletsky RJ, Schork NJ (1997) Ventilation and metabolism among rat strains. J Appl Physiol 82:317-323.

Subramanian S, Dostal J, Erokwu B, Han F, Dick TE, Strohl KP (2007) Domperidone and ventilatory behavior: Sprague-Dawley versus Brown Norway rats. Respir Physiol Neurobiol 155:22-28.

Sutton MA, Wall NR, Aakalu GN, Schuman EM (2004) Regulation of dendritic protein synthesis by miniature synaptic events. Science 304:1979-1983.

Sutton MA, Ito HT, Cressy P, Kempf C, Woo JC, Schuman EM (2006) Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. Cell 125:785-799.

Turnbull AV, Rivier CL (1999) Sprague-Dawley rats obtained from different vendors exhibit distinct adrenocorticotropin responses to inflammatory stimuli. Neuroendocrinology 70:186-195.

Turrigiano G (2012) Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function. Cold Spring Harb Perspect Biol 4:a005736.

Turrigiano GG (2006) More than a sidekick: glia and homeostatic synaptic plasticity. Trends Mol Med 12:458-460.

Turrigiano GG (2008) The self-tuning neuron: synaptic scaling of excitatory synapses. Cell 135:422-435.

Yoon YW, Lee DH, Lee BH, Chung K, Chung JM (1999) Different strains and substrains of rats show different levels of neuropathic pain behaviors. Exp Brain Res 129:167-171.

Zabka AG, Behan M, Mitchell GS (2001a) Selected contribution: Time-dependent hypoxic respiratory responses in female rats are influenced by age and by the estrus cycle. J Appl Physiol 91:2831-2838.

Zabka AG, Behan M, Mitchell GS (2001b) Long term facilitation of respiratory motor output decreases with age in male rats. J Physiol 531:509-514.

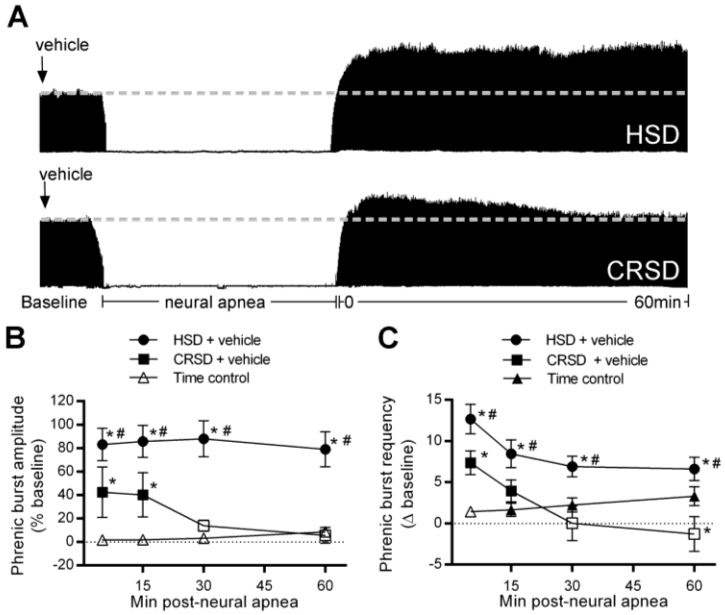

Figure 1: iPMF and burst frequency facilitation are differentially regulated among HSD and CRSD rat sub-strains. ***A.*** Representative compressed phrenic neurograms from HSD and CRSD rats receiving intrathecal vehicle depicting baseline, 30 min neural apnea and for 60 min after the resumption of phrenic neural activity. Black arrows denote intrathecal delivery of vehicle. ***B.*** Average phrenic burst amplitude (% baseline) in HSD (*circles*) and CRSD (*squares*) rats pretreated with vehicle for 60 min post-neural apnea or an equivalent duration in time control rats (*triangles*). Phrenic burst amplitude in time control rats was not significantly different from baseline at any time point. HSD rats exposed to neural apnea expressed a significant increase in phrenic burst amplitude relative to baseline and time controls at all time points, indicating long-lasting iPMF. By contrast, CRSD rats exposed to neural apnea expressed a smaller, but significant increase in phrenic burst amplitude relative to baseline and time controls at 5 and 15 min, which returned to baseline 30 min post-neural apnea, suggesting transient iPMF. ***C.*** Average phrenic burst frequency (Δ baseline) in HSD (*circles*) and CRSD (*squares*) rats pretreated with vehicle for 60 min post-neural apnea or an equivalent duration in time controls (*triangles*). Phrenic burst frequency in time control rats expressed a significant increase relative to baseline at 15, 30 and 60 min. HSD rats exposed to neural apnea expressed a significant increase in phrenic burst frequency relative to baseline and time controls at all time points indicating sustained burst frequency facilitation. CRSD rats exposed to neural apnea expressed a smaller, but significant increase relative to baseline at 5 and 15 min, which was significantly increased from time controls at 5 min; however, by 60 min phrenic burst frequency was significantly decreased from time controls, indicating transient burst frequency facilitation. Mean values $\pm$ SEM. Filled symbols indicate significantly different from baseline. *significantly different than time controls; #significantly different than CRSD rats; $p<0.05$.

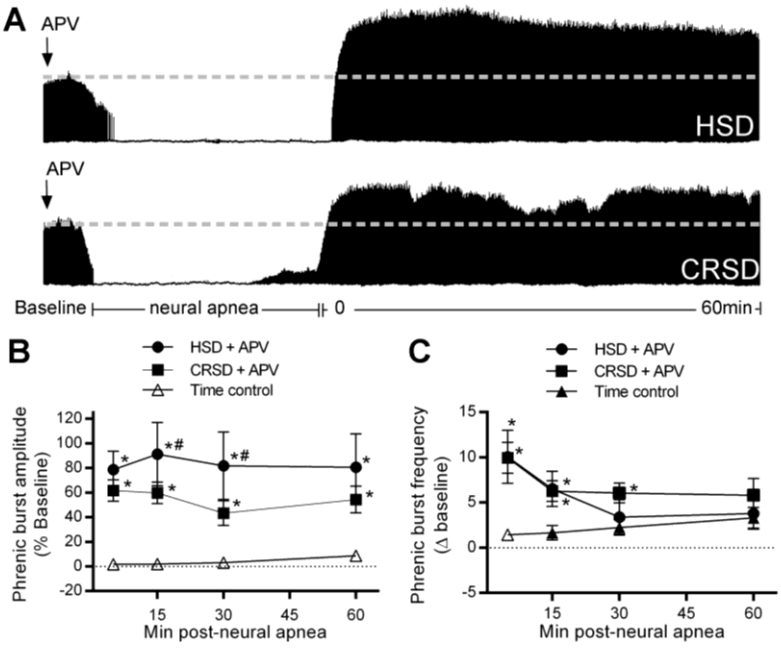

Figure 2: Activation of spinal NMDA receptors in CRSD rats, but not HSD rats prevents long-lasting iPMF. ***A.*** Representative compressed phrenic neurograms from HSD and CRSD rats pretreated with intrathecal APV depicting baseline, 30 min neural apnea and for 60 min after the resumption of phrenic neural activity. Black arrows denote intrathecal delivery of APV. ***B.*** Average phrenic burst amplitude (% baseline) in HSD (*circles*) and CRSD (*squares*) rats receiving APV prior to neural apnea for 60 min post-neural apnea or an equivalent duration in time controls (*triangles*). Both HSD and CRSD rats pretreated with APV and exposed to neural apnea expressed a significant increase compared to baseline and time controls at all time points, indicating long-lasting iPMF. This increase in CRSD rats was significantly lower than HSD at 15 and 30 min post-neural apnea. ***C.*** Average phrenic burst frequency (Δ baseline) in HSD (*circles*) and CRSD (*squares*) rats pretreated with intrathecal APV prior to neural apnea for 60 min post-neural apnea or an equivalent duration in time controls (*triangles*). HSD rats receiving APV before neural apnea expressed a significant increase in phrenic burst frequency relative to baseline at all time points and relative to time controls at 5 and 15 min, indicating transient burst frequency facilitation. CRSD rats receiving intrathecal APV prior to neural apnea expressed a significant increase from baseline at all time points and from time controls at 5, 15 and 30 min post-neural apnea, suggesting transient burst frequency facilitation. Mean values $\pm$ SEM. Filled symbols indicate significantly different from baseline. *significantly different than time controls; # significantly different than HSD rats; $p<0.05$.

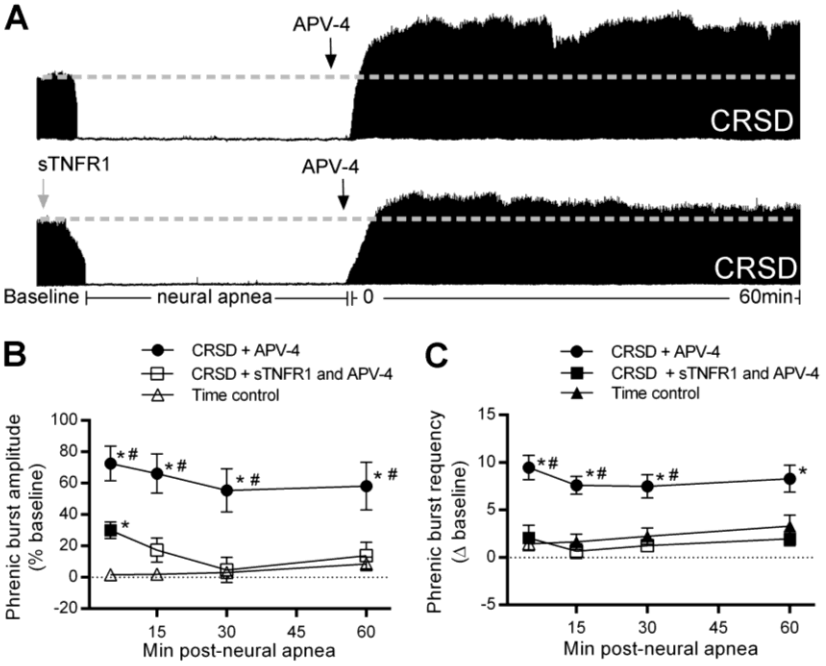

Figure 3: Long-lasting iPMF in CRSD rats receiving APV requires spinal TNFα. ***A.*** Representative compressed phrenic neurograms in CRSD rats receiving intrathecal APV-4 or sTNFR1 and APV-4 depicting baseline, 30 min neural apnea and for 60 min after the resumption of phrenic neural activity. Black arrows denote intrathecal delivery of APV-4; the gray arrow indicates intrathecal delivery of sTNFR1. ***B.*** Average phrenic burst amplitude (% baseline) in rats receiving APV-4 (*circles*) or pretreatment of sTNFR1 and APV-4 (*squares*) for 60 min post-neural apnea or an equivalent duration in time controls (*triangles*). CRSD rats receiving APV-4 and neural apnea expressed a significant increase in phrenic burst amplitude relative to baseline and time control at all time points post-neural apnea indicating long-lasting iPMF. By contrast, CRSD rats receiving sTNFR1 and APV-4 during neural apnea, expressed a small, but significant increase in phrenic burst amplitude compared to baseline and time controls at 5 min post-neural apnea; however, phrenic burst amplitude was not significantly increased from baseline or time controls at subsequent time points suggesting attenuated, transient iPMF. Indeed, phrenic burst amplitude in CRSD rats receiving sTNFR1 and APV-4 was significantly lower than CRSD rats receiving APV-4 at all time points post-neural apnea, indicating long-lasting iPMF in CRSD rats receiving APV-4 requires spinal TNFα. ***C.*** Average phrenic burst frequency (Δ baseline) in rats receiving APV-4 (*circles*) or pretreatment of sTNFR1 and APV-4 (*squares*) for 60 min post-neural apnea or an equivalent duration in time controls (*triangles*). CRSD rats receiving intrathecal APV-4 and neural apnea expressed a significant increase in phrenic burst frequency from baseline and time controls at all time points, signifying sustained burst frequency facilitation. By contrast, CRSD rats pretreated with sTNFR1 prior to neural apnea and APV-4 expressed a small, but significant increase from baseline at 5 and 60 min, however this was not significantly increased from time controls indicating no burst frequency facilitation. Indeed, phrenic burst frequency in CRSD rats pretreated with sTNFR1 prior to neural apnea and receiving APV-4 during neural apnea was significantly lower than rats receiving APV-4 during neural apnea at 5, 15 and 30 min. Mean values ± SEM. Filled symbols indicate significantly different

from baseline. *significantly different than time controls; #significantly different than sTNFR1 and APV-4; $p<0.05$.

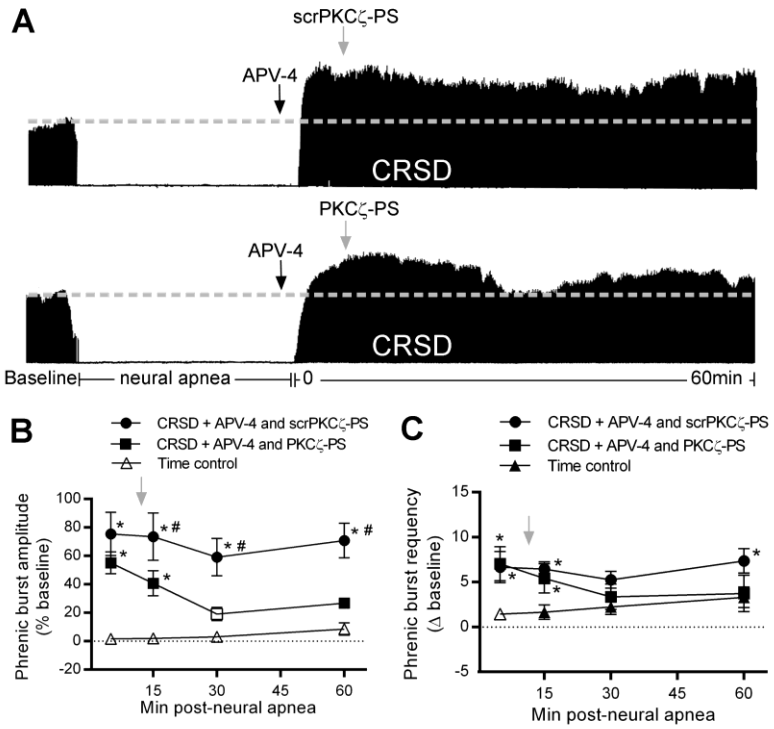

Figure 4: Long-lasting iPMF in CRSD rats receiving APV requires spinal aPKC activity. ***A.*** Representative compressed phrenic neurograms in CRSD rats receiving intrathecal APV-4 and either scrPKCζ-PS or PKCζ-PS 10 min after the resumption of neural activity depicting baseline, 30 min neural apnea and for 60 min after the resumption of phrenic neural activity. Black arrows denote intrathecal delivery of APV-4; gray arrows indicate intrathecal delivery of scrPKCζ-PS or PKCζ-PS. ***B.*** Average phrenic burst amplitude (% baseline) in rats receiving APV-4 and scrPKCζ-PS (*circles*) or PKCζ-PS (*squares*) for 60 min post-neural apnea or an equivalent duration in time controls (*triangles*). Gray arrow indicates intrathecal delivery of scrPKCζ-PS or PKCζ-PS. CRSD rats receiving APV-4 during neural apnea and scrPKCζ-PS express a significant increase in phrenic burst amplitude relative to baseline and time control at all time points post-neural apnea indicating long-lasting iPMF. By contrast, CRSD rats receiving APV-4 during neural apnea and PKCζ-PS, expressed a small but significant increase in phrenic burst amplitude compared to baseline and time controls at 5 and 15 min post-neural apnea; however, phrenic burst amplitude was not significantly increased time controls at 30 or 60 min suggesting transient iPMF. Indeed, phrenic burst amplitude in CRSD rats receiving APV-4 during neural apnea and PKCζ-PS was significantly lower than CRSD rats receiving APV-4 during neural apnea and scrPKCζ-PS, indicating long-lasting iPMF in CRSD rats requires spinal aPKC activity. ***C.*** Average phrenic burst frequency (Δ baseline) in rats receiving APV-4 during neural apnea and scrPKCζ-PS (*circles*) or PKCζ-PS (*squares*) for 60 min post-neural apnea or an equivalent duration in time controls (*triangles*). Gray arrow indicates intrathecal delivery of scrPKCζ-PS or PKCζ-PS. CRSD rats receiving intrathecal APV-4 during neural apnea and scrPKCζ-PS expressed a significant increase in phrenic burst frequency from baseline at all time points and from time controls at 5, 15 and 60 min post-neural apnea, signifying sustained burst

frequency facilitation. CRSD rats APV-4 during neural apnea and PKCζ-PS expressed a significant increase in phrenic burst frequency from baseline at all time points and from time controls at 5 and 15 min post-neural apnea, indicating transient burst frequency facilitation. Mean values ± SEM. Filled symbols indicate significantly different from baseline. *significantly different than time controls; $p<0.05$.

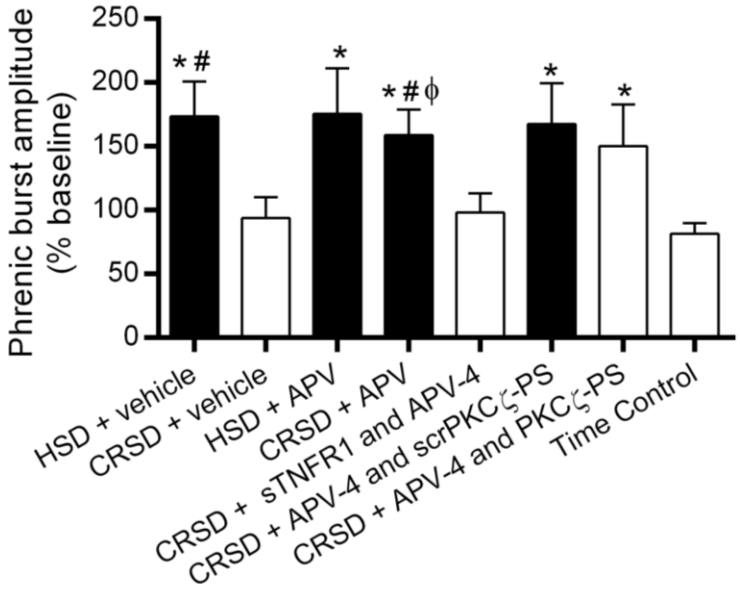

Figure 5: Long-lasting iPMF is associated with an enhanced phrenic amplitude response to hypercapnia. Average change in phrenic burst amplitude during hypercapnia (% baseline) following neural apnea or an equivalent duration in time controls. Shaded bars indicate significant iPMF at 60 min.HSD rats receiving intrathecal vehicle or APV prior to neural apnea expressed a significant increase in the phrenic burst amplitude response to hypercapnia compared to time controls, indicating an enhanced phrenic hypercapnic response. By contrast, phrenic burst amplitude during hypercapnia in CRSD rats receiving vehicle was not significantly increased compared to time controls. However, CRSD rats receiving intrathecal APV expressed a significant increase in phrenic burst amplitude during hypercapnia relative to time controls and CRSD rats receiving vehicle, suggesting an enhanced hypercapnic response is enabled by intrathecal APV. This enhanced hypercapnic response is prevented following intrathecal sTNFR1, since CRSD rats pretreated with sTNFR1 and receiving APV-4 no longer express a significant increase in the phrenic burst amplitude response. CRSD rats receiving APV-4 and scrPKCζ-PS or PKCζ-PS both expressed a significant phrenic increase during the hypercapnic response, indicating an enhanced hypercapnic response in both groups. Mean values $\pm$ SEM. *significantly different than time controls; #significantly different than CRSD + vehicle; ϕ significantly different than CRSD + sTNFR1 and APV-4; $p<0.05$.

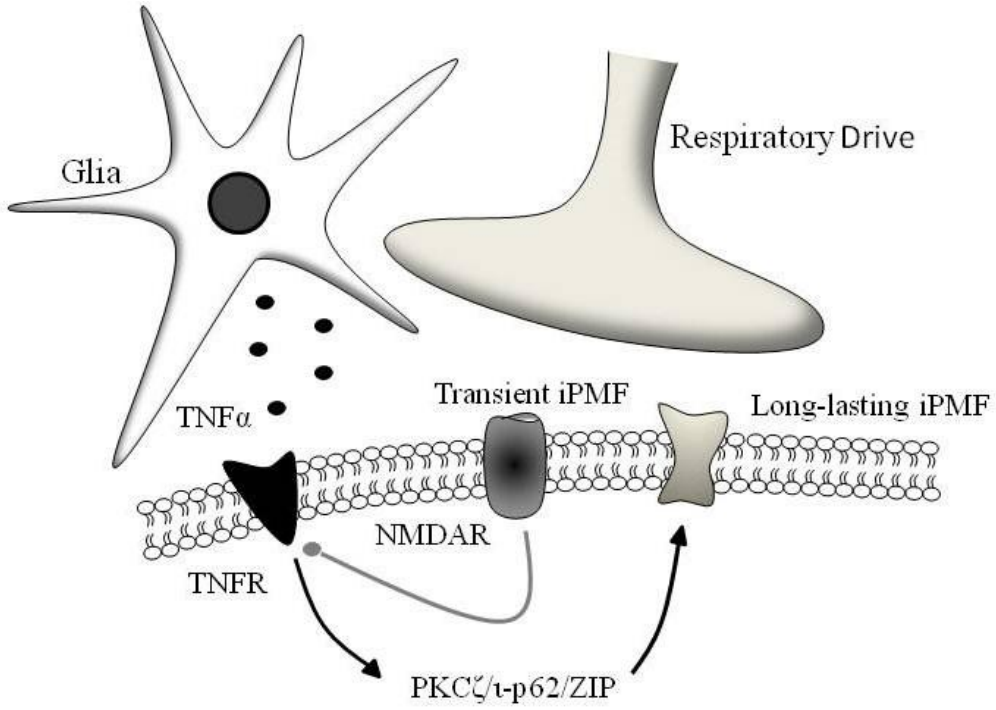

Figure 6: Working model of long-lasting (black arrows) and transient (gray) iPMF. We hypothesize that local spinal mechanisms operating within the phrenic motor pool "sense" and "respond" to reduced bulbospinal respiratory inputs to give rise to long-lasting and transient iPMF. In HSD rats, reduced respiratory neural activity increases local release of TNFα, resulting in the activation of TNFα receptors (TNFR) to promote the formation of the PKCζ/ι-p62/ZIP signaling cassette in phrenic motor neurons. This stimulus specific signaling cascade increases the synaptic strength and induces long-lasting iPMF. In CRSD rats, activation of NMDARs constrains the TNFα and aPKC-dependent pathway giving rise to transient iPMF. The mechanisms whereby long-lasting iPMF is expressed by some Sprague-Dawley rat sub-strains and not others are unknown.

		PaCO2	PaO2	pH	MAP
Time control	*Baseline*	46.2 ± 0.9	279 ± 8	7.4 ± 0.0	126 ± 4
	60 min	46.0 ± 1.0	280 ± 6	7.4 ± 0.0	111 ± 5
HSD + vehicle	*Baseline*	47.5 ± 0.8	277 ± 9	7.4 ± 0.0	129 ± 5
Neural apnea	*60 min*	47.3 ± 0.7	270 ± 8	7.4 ± 0.0	122 ± 7
CRSD + vehicle	*Baseline*	46.8 ± 1.8	274 ± 6	7.4 ± 0.0	107 ± 7*#
Neural apnea	*60 min*	46.9 ± 1.8	264 ± 5	7.4 ± 0.0	101 ± 8#
Harlan + APV	*Baseline*	46.4 ± 1.5	248 ± 9	7.3 ± 0.0	129 ± 5
Neural apnea	*60 min*	46.3 ± 1.3	251 ± 9	7.4 ± 0.0	125 ± 6
CRSD + APV	*Baseline*	48.3 ± 1.4	264 ± 4	7.4 ± 0.0	114 ± 6
Neural apnea	*60 min*	48.2 ± 1.4	259 ± 6	7.4 ± 0.0	102 ± 5φ
CRSD + APV-4	*Baseline*	46.3 ± 1.0	258 ± 5	7.4 ± 0.0	116 ± 7
Neural apnea	*60 min*	46.4 ± 0.8	253 ± 4	7.4 ± 0.0	100 ± 7
CRSD + sTNFR1 and APV-4	*Baseline*	51.1 ± 1.1*$	264 ± 15	7.3 ± 0.0	107 ±11*
Neural apnea	*60 min*	51.5 ± 1.2*$	249 ± 17	7.3 ± 0.0*$	95 ± 10
CRSD + APV-4 and PKCζ-PS	*Baseline*	48.6 ± 1.1	266 ± 6	7.3 ± 0.0	113 ± 7
Neural apnea	*60 min*	48.2 ± 1.3	264 ± 3	7.3 ± 0.0	89 ± 4*
CRSD + APV-4 and scrPKCζ-PS	*Baseline*	48.0 ± 1.1	265 ± 10	7.3 ± 0.0	120 ± 7
Neural apnea	*60 min*	48.8 ± 1.1	259 ± 11	7.3 ± 0.0*	101 ± 9

Table 1: Average arterial CO_2, pH, O_2 and mean arterial pressure (MAP) before and 60 min after central neural apnea or an equivalent duration in time control rats receiving a similar surgery, but no neural apnea. Values presented are mean ± SEM. * significantly different than time controls; # significantly different than HSD + vehicle; $ significantly different than CRSD + APV-4; ϕ significantly different than HSD + APV; ($p<0.05$).

CHAPTER V

DISCUSSION

Summary

Studies presented in this thesis highlight an exciting new avenue in respiratory control: local mechanisms sense and adjust phrenic motor output in response to reduced respiratory neural activity. The fundamental goal of my dissertation was to investigate properties and mechanisms of a novel form of respiratory plasticity called inactivity-induced phrenic motor facilitation (iPMF). The main conclusions drawn from studies presented here are the following: 1) atypical protein kinase C (aPKC) isoforms PKCζ and/or PKCι/λ form a multimeric signaling complex with the scaffolding protein p62/ZIP in or near the phrenic motor nucleus during the early phase of iPMF to transition to a long-lasting aPKC-independent phase of iPMF, 2) spinal mechanisms local to the phrenic motor circuit sense and respond to reduced synaptic inputs independent of chemoreflexes, 3) unilateral C2 axon conduction blockade gives rise to a TNFα and aPKC dependent iPMF within the ipsilateral phrenic motor pool receiving reduced phrenic synaptic inputs and a crossed spinal phrenic motor facilitation (csPMF) independent of TNFα and aPKC within the contralateral motor pool where phrenic synaptic inputs were maintained, 4) genetic/epigenetic factors regulate the magnitude and duration of iPMF since Charles River Sprague-Dawley (CRSD) rats express transient iPMF while Harlan Sprague-Dawley (HSD) rats express a long-lasting iPMF and 5) spinal NMDA receptor activation in CRSD rats inhibits long-lasting iPMF by constraining spinal TNFα and aPKC-dependent signaling, indicating that iPMF duration in CRSD rats is dictated by a spinal regulatory mechanism. In the following section I will discuss how our findings fit into a broader context of plasticity and respiratory control, expand on data presented in this thesis and offer explanations for essential questions that remain unanswered by studies presented in this thesis. Although the physiological or pathophysiological relevance of iPMF is unknown, situations in which reduced respiratory neural activity is

experienced and the possible role/s iPMF may play during these circumstances are discussed in a recent review presented in Appendix A.

Atypical PKC isoforms and iPMF

Atypical PKC isoforms PKCζ, PKCι/λ and PKMζ have been implicated in multiple forms of long-lasting synaptic plasticity within the mammalian brain and spinal cord (Ling et al., 2002; Laferriere et al., 2011; Matt and Hell, 2013; Ren et al., 2013). Most, if not all forms of plasticity requiring aPKC isoforms including hippocampal LTP (Sacktor, 2008; Ren et al., 2013), habituation of sensory neurons (Wan et al., 2012) and spinal nociceptive plasticity (Sandkuhler, 2007; Asiedu et al., 2011; Laferriere et al., 2011) are most often elicited by repeated presentations of a strong stimulus. The results presented in *Chapter 2* of this thesis extend our current understanding of the role aPKCs play in plasticity by demonstrating for the first time that PKCζ and/or PKCι/λ are necessary for plasticity elicited by reduced neural activity. However, many questions are still left unanswered by this study. For example, which aPKC isoform is necessary for iPMF or are multiple isoforms required? What is the cellular location of relevant aPKC isoform/s? What are the downstream targets of the aPKCζ/ι-p62/ZIP signaling complex giving rise to iPMF?

In this study, the pharmacological compound used to inhibit aPKC isoforms (PKCζ-PS) was delivered to cervical spinal regions (*Chapter 2*), and as such, the specific location of aPKC isoforms necessary of iPMF could not be determined. However, our data suggest that iPMF is associated with an increased interaction between PKCζ/ι and p62/ZIP in ventral spinal segments from C3-C5, indicating the relevant site of the aPKC isoforms required for iPMF may be within the ventral cervical spinal cord (Strey et al., 2012). In spinal segments, aPKC isoforms are

expressed within neurons, but not surrounding glia (Guenther et al., 2010), we therefore suspect that aPKC isoforms necessary for iPMF are located within phrenic motor neurons. Consistent with this hypothesis, preliminary data suggest that knockdown of PKCζ expression within phrenic motor neurons *via* intrapleural injection of siRNAs inhibits iPMF expression (unpublished data; Baertsch and Baker-Herman). Future studies confirming knockdown of PKCζ only occurs within phrenic motor neurons and not surrounding spinal interneurons or brainstem respiratory neurons is necessary to confirm these results. Alternatively, or additionally, PKCζ/ι and p62/ZIP could act by reducing neurotransmission within nearby inhibitory interneurons to facilitate or contribute to iPMF expression. Indeed, the response to altered activity likely requires a delicate balance between increased excitation and reduced inhibition (Turrigiano and Nelson, 2004).

Although we cannot rule out an involvement of PKMζ, our results indicate that iPMF requires PKC isoform/s containing a regulatory subunit (*Chapter 2*) (Strey et al., 2012). Further, the increased interaction between PKCζ/ι and p62/ZIP requires binding through the PB1 domain only present in full-length aPKCs, adding confidence that iPMF requires PKCζ and/or PKCι/λ (Strey et al., 2012). This finding is in contrast to most long-lasting forms of aPKC dependent-plasticity which have been shown to require the persistently active aPKC isoform PKMζ (Drier et al., 2002; Muslimov et al., 2004; Ling et al., 2006; Pastalkova et al., 2006; Asiedu et al., 2011). However, many of these conclusions are based on the finding that PKCζ is not expressed within the brain (Hernandez et al., 2003) and the assumption that PKCζ-PS is specific for PKC/PKMζ. Recent data suggest that PKCζ-PS likely inhibits PKCι/λ activity since: 1) hippocampal LTP remains intact following conventional and conditional genetic ablation of PKC/PKMζ and 2) subsequent delivery of PKCζ-PS in PKC/PKMζ knockout mice reverses LTP

(Price and Ghosh, 2013; Volk et al., 2013). One of the challenges in identifying "the" aPKC isoform/s necessary for iPMF is the similarity of the kinase across isoforms within this family. Indeed, the amino acid sequence between PKCζ and PKCι/λ is 72% conserved (Selbie et al., 1993) and the kinase domain of PKMζ and PKCι/λ are ~86% similar (Price and Ghosh, 2013). This similarity presents a technical limitation in further determining the relevant isoform necessary for iPMF at this time.

The downstream targets of the PKCζ/ι-p62/ZIP signaling complex giving rise to iPMF are unknown. Literature reports suggest that in response to specific stimuli, PKCζ/ι binds to p62/ZIP, which relocates and anchors the activated kinase in a context-specific fashion (Mochly-Rosen, 1995; Samuels et al., 2001). Since we identified a role for TNFα in long-lasting iPMF (Broytman et al., 2013), we suspect that p62/ZIP tethers TNFα receptors and PKCζ/ι (Sanz et al., 1999; Sanz et al., 2000) to downstream targets like AMPA (Jiang et al., 2009; Ren et al., 2013) $GABA_C$ (Croci et al., 2003) Trk (Geetha and Wooten, 2003) receptors or potassium channels (Croci et al., 2003; Kim et al., 2004) to encode stimulus specific response to reduced respiratory neural activity. Although, it is not clear how or if PKCζ/ι-p62/ZIP modulates these receptors and/or channels following reduced activity, PKCζ/ι-p62/ZIP has the capacity to phosphorylate and traffic receptors to the membrane, which has been shown to be an essential step in maintaining forms of long-lasting plasticity (Jiang et al., 2009; Ren et al., 2013). Studies utilizing immunoprecipitation could be performed to elucidate the interaction between aPKC and downstream receptors/channels.

Respiratory neural activity is regulated by spinal mechanisms

Previous studies suggested that reduced respiratory neural activity gives rise to compensatory increases in respiratory motor output (Castro-Moure and Goshgarian, 1996, 1997; Mahamed et al., 2011); however, these reports left fundamental questions unanswered. In each study, arterial blood gases were altered to induce (e.g., hypocapnia) or in response to reduced respiratory neural activity (e.g., nonventilated rats) and, as such, compensatory increases in respiratory motor output may be the result of chemoreflex induced increases in ventilation. In an attempt to fill this gap in knowledge, a large portion of this dissertation was devoted to testing how and if ongoing activity (or a lack there of) within phrenic motor neurons shapes respiratory neural output independent of chemoreflex induced changes in ventilation (*Chapter 3*).

Data from this study enhance our current understanding of respiratory control by demonstrating that in addition to chemoreceptor and sensory induced feedback modulation of breathing, local spinal mechanisms sense reduced phrenic synaptic inputs to activate activity-dependent mechanisms that increase phrenic neural output (i.e. iPMF and csPMF) (*Chapter 3*). A feature of iPMF that remains unresolved is: what cell type/s sense reduced respiratory neural activity and what signal are these cells sensing? Are these "sensors" also the cell types that release TNFα to elicit inactivity-induced plasticity? One possibility is that neurons sense reduced activity themselves by translating decreased intracellular [Ca^{2+}] into an activity-dependent signal (Turrigiano, 2007; Gainey et al., 2009; Turrigiano, 2012). An alternative explanation is that glia sense reduced neuronal activity *via* decreased synaptic glutamate and/or changes in extracellular [ion(s)] (Vernadakis, 1996) and secrete a critical activity-dependent factor like TNFα (Beattie et al., 2002; Stellwagen and Malenka, 2006). Although glial-derived TNFα may be part of a conserved homeostatic response to reduced activity (Beattie et al., 2002; Stellwagen and

Malenka, 2006), TNFα does not appear to be an activity signal necessary to instruct homeostatic plasticity, but rather to allow synapses to respond to activity-dependent changes (Steinmetz and Turrigiano, 2010). Therefore, glial-derived TNFα plays a permissive (*versus* instructive) role in inactivity-induced increases in neural activity. Accumulating evidence indicates that neurons sense their own firing rates encoded by decreases in somatic [Ca^{2+}], suggesting that a cell-autonomous process is used to sense and respond to decreased neural activity (Turrigiano and Nelson, 2004; Ibata et al., 2008; Turrigiano, 2012). Although, iPMF is consistent with a cell-autonomous process; experiments investigating if reduced respiratory neural activity and TNFα are additive, therefore suggesting an instructive role, have not been performed.

The unexpected observation that a TNFα and aPKC independent csPMF expressed in the contralateral motor pool that received uninterrupted synaptic inputs suggests a novel form of crossed spinal plasticity (i.e. csPMF) is elicited by unilateral reductions in phrenic synaptic inputs (*Chapter 3*). An intriguing interpretation of these data is that csPMF represents a compensatory response induced by a loss of activity within neighboring phrenic motor neurons. Although interesting, the pathways giving rise to csPMF remain largely unclear. Our results indicate that spinal mechanisms give rise to csPMF, however crossed bulbospinal projections innervating phrenic motor neurons are generally thought to be silent pathways which do not contribute to breathing under "normal" circumstances (Goshgarian and Rafols, 1984; Goshgarian et al., 1989; Sperry and Goshgarian, 1993). One explanation is that cervical spinal interneurons at or near the phrenic motor nucleus may facilitate csPMF. Indeed, cervical interneurons may contribute to the activation of crossed phrenic projections (Lane et al., 2008). Anterograde and transneuronal retrograde tracing suggest that propriospinal interneurons are located throughout the phrenic motor nucleus and are functionally coupled to phrenic motor neurons (Lane, 2011);

with a subpopulation projecting to both ipsilateral and contralateral phrenic motor neurons (Lane et al., 2008). Recording activity from individual cervical interneurons with a multichannel array inserted into the spinal tissue before, during and after unilateral axon conduction block may give insight into the potential involvement of interneurons in csPMF. Intracellular labeling of these cells and subsequent identification of their location and cellular profile would be necessary to determine if cervical interneurons contribute to csPMF.

Local spinal mechanisms regulate the duration of iPMF

Since we show a key role in the activation of spinal NMDARs in the regulation of the magnitude and duration of iPMF (*Chapter 4*), genetic/epigenetic influences of NMDARs may play a crucial part in shaping differential expression of iPMF. Indeed, genetic differences in NMDAR subunits regulate cortical excitability and lead to a differential duration of cortical LTP expression (Mori et al., 2011). Potential genetic/epigenetic differences in NMDARs among HSD and CRSD rat sub-strains may be due to altered basal spinal NMDAR subunit expression or distribution (Perez-Otano and Ehlers, 2005), activity-dependent modulation of NMDARs (Thomas and Huganir, 2013) or differential regulation of molecules downstream of NMDARs. One downstream target regulated by NMDAR activation and influencing the duration of plasticity is local dendritic protein synthesis (Sutton et al., 2004; Sutton et al., 2006). Investigations directed toward understanding if activation of NMDARs by miniature synaptic events inhibits dendritic protein synthesis to limit the duration of iPMF in CRSD rats are crucial.

In contrast to other neurally driven behaviors like locomotion, the sole respiratory CPG is located within the brainstem and supplies all respiratory motor pools with excitatory neural input. Although respiratory motor pools receive a common input from brainstem respiratory

centers, the muscles in which they innervate perform drastically different functions with regard to breathing. For example, hypoglossal motor neurons innervate the tongue to maintain upper airway patency, while phrenic motor neurons contract the diaphragm to generate negative pressure to facilitate lung inflation. Therefore, we speculate that the presence of a spinal regulatory mechanism similar to that which regulates the duration of iPMF in some rat sub-strains (*Chapter 4*) may allow for precise control of neural output tailored to the function of the respiratory motor pool. Indeed, although HSD rats express long-lasting iPMF, hypoglossal and intercostal motor facilitation in HSD rats are transient and return to baseline 30 min after reduced activity (Baker-Herman and Strey, 2011; Strey and Baker-Herman, 2013), suggesting that a regulatory mechanism similar to that regulating iPMF in CRSD rats may restore hypoglossal and intercostal motor output following central neural apnea. Future studies aimed at investigating if similar NMDAR dependent regulatory mechanisms inhibit long-lasting plasticity within other respiratory motor pools in Harlan and Charles River Sprague-Dawley rats are necessary.

Significance of inactivity-induced plasticity

The body of work presented in this thesis increases our understanding of how ongoing activity, or a lack thereof, within phrenic motor neurons shapes phrenic motor output and establishes key molecules necessary for compensatory increases in phrenic neural output following reduced phrenic synaptic inputs. Although the physiological relevance of iPMF has not been shown in any capacity, we speculate that iPMF may be one mechanism to assure appropriate activity is maintained within phrenic motor neurons throughout life.

As briefly mentioned at the beginning of this thesis, the respiratory control system is susceptible to transient and recurrent or prolonged periods of reduced respiratory neural activity

in a variety of physiological and pathophysiological situations. We suspect that the nature of reduced respiratory neural activity determines the functional contribution of inactivity-induced plasticity in the creation of a stable breathing pattern (Strey and Baker-Herman, 2013). For example, in situations where few central apneas are experienced, particularly during sleep in otherwise healthy individuals, induction of inactivity-induced plasticity may reconfigure network properties to augment respiratory motor output, thereby preventing future episodes and stabilizing breathing. However, in individuals experiencing an increase in the frequency of central apneas during sleep as occurs in certain pathological conditions (i.e. heart failure), iPMF may perpetuate central apneas and promote breathing instability. Whereas during prolonged reductions in respiratory neural activity as occurs following cervical spinal injury, inactivity-induced plasticity may be beneficial in partially restoring respiratory motor output. A complete understanding of the mechanisms giving rise to iPMF are necessary to determine how (and if) iPMF influences respiratory neural output. Since many conditions and disorders of relevance to human health are associated with reduced respiratory neural activity, an understanding of the properties and cellular pathways giving rise to iPMF is essential in order to intervene *appropriately* in situations where ventilation is compromised.

REFERENCES

Asiedu MN, Tillu DV, Melemedjian OK, Shy A, Sanoja R, Bodell B, Ghosh S, Porreca F, Price TJ (2011) Spinal protein kinase M zeta underlies the maintenance mechanism of persistent nociceptive sensitization. J Neurosci 31:6646-6653.

Baker-Herman TL, Strey KA (2011) Similarities and differences in mechanisms of phrenic and hypoglossal motor facilitation. Respir Physiol Neurobiol 179:48-56.

Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, Von Zastrow M, Beattie MS, Malenka RC (2002) Control of synaptic strength by glial TNFalpha. Science 295:2282-2285.

Broytman O, Baertsch NA, Baker-Herman TL (2013) Spinal TNFα is necessary for inactivity-induced phrenic motor facilitation. J Physiol.

Castro-Moure F, Goshgarian HG (1996) Reversible cervical hemispinalization of the rat spinal cord by a cooling device. Exp Neurol 141:102-112.

Castro-Moure F, Goshgarian HG (1997) Morphological plasticity induced in the phrenic nucleus following cervical cold block of descending respiratory drive. Exp Neurol 147:299-310.

Croci C, Brandstatter JH, Enz R (2003) ZIP3, a new splice variant of the PKC-zeta-interacting protein family, binds to GABAC receptors, PKC-zeta, and Kv beta 2. The Journal of biological chemistry 278:6128-6135.

Drier EA, Tello MK, Cowan M, Wu P, Blace N, Sacktor TC, Yin JC (2002) Memory enhancement and formation by atypical PKM activity in Drosophila melanogaster. Nat Neurosci 5:316-324.

Gainey MA, Hurvitz-Wolff JR, Lambo ME, Turrigiano GG (2009) Synaptic scaling requires the GluR2 subunit of the AMPA receptor. J Neurosci 29:6479-6489.

Geetha T, Wooten MW (2003) Association of the atypical protein kinase C-interacting protein p62/ZIP with nerve growth factor receptor TrkA regulates receptor trafficking and Erk5 signaling. The Journal of biological chemistry 278:4730-4739.

Goshgarian HG, Rafols JA (1984) The ultrastructure and synaptic architecture of phrenic motor neurons in the spinal cord of the adult rat. J Neurocytol 13:85-109.

Goshgarian HG, Yu XJ, Rafols JA (1989) Neuronal and glial changes in the rat phrenic nucleus occurring within hours after spinal cord injury. J Comp Neurol 284:519-533.

Guenther CH, Vinit S, Windelborn JA, Behan M, Mitchell GS (2010) Atypical protein kinase C expression in phrenic motor neurons of the rat. Neuroscience 169:787-793.

Hernandez AI, Blace N, Crary JF, Serrano PA, Leitges M, Libien JM, Weinstein G, Tcherapanov A, Sacktor TC (2003) Protein kinase M zeta synthesis from a brain mRNA encoding an independent protein kinase C zeta catalytic domain. Implications for the molecular mechanism of memory. The Journal of biological chemistry 278:40305-40316.

Ibata K, Sun Q, Turrigiano GG (2008) Rapid synaptic scaling induced by changes in postsynaptic firing. Neuron 57:819-826.

Jiang J, Parameshwaran K, Seibenhener ML, Kang MG, Suppiramaniam V, Huganir RL, Diaz-Meco MT, Wooten MW (2009) AMPA receptor trafficking and synaptic plasticity require SQSTM1/p62. Hippocampus 19:392-406.

Kim Y, Uhm DY, Shin J, Chung S (2004) Modulation of delayed rectifier potassium channel by protein kinase C zeta-containing signaling complex in pheochromocytoma cells. Neuroscience 125:359-368.

Laferriere A, Pitcher MH, Haldane A, Huang Y, Cornea V, Kumar N, Sacktor TC, Cervero F, Coderre TJ (2011) PKMzeta is essential for spinal plasticity underlying the maintenance of persistent pain. Molecular pain 7:99.

Lane MA (2011) Spinal respiratory motoneurons and interneurons. Respir Physiol Neurobiol 179:3-13.

Lane MA, White TE, Coutts MA, Jones AL, Sandhu MS, Bloom DC, Bolser DC, Yates BJ, Fuller DD, Reier PJ (2008) Cervical prephrenic interneurons in the normal and lesioned spinal cord of the adult rat. J Comp Neurol 511:692-709.

Ling DS, Benardo LS, Sacktor TC (2006) Protein kinase Mzeta enhances excitatory synaptic transmission by increasing the number of active postsynaptic AMPA receptors. Hippocampus 16:443-452.

Ling DS, Benardo LS, Serrano PA, Blace N, Kelly MT, Crary JF, Sacktor TC (2002) Protein kinase Mzeta is necessary and sufficient for LTP maintenance. Nat Neurosci 5:295-296.

Mahamed S, Strey KA, Mitchell GS, Baker-Herman TL (2011) Reduced respiratory neural activity elicits phrenic motor facilitation. Respir Physiol Neurobiol 175:303-309.

Matt L, Hell JW (2013) PKClambda: a new player in LTP coming to the rescue of PKCzeta's faltering role in LTP? The EMBO journal 32:1348-1349.

Mochly-Rosen D (1995) Localization of protein kinases by anchoring proteins: a theme in signal transduction. Science 268:247-251.

Mori F, Ribolsi M, Kusayanagi H, Siracusano A, Mantovani V, Marasco E, Bernardi G, Centonze D (2011) Genetic variants of the NMDA receptor influence cortical excitability and plasticity in humans. J Neurophysiol 106:1637-1643.

Muslimov IA, Nimmrich V, Hernandez AI, Tcherepanov A, Sacktor TC, Tiedge H (2004) Dendritic transport and localization of protein kinase Mzeta mRNA: implications for molecular memory consolidation. The Journal of biological chemistry 279:52613-52622.

Pastalkova E, Serrano P, Pinkhasova D, Wallace E, Fenton AA, Sacktor TC (2006) Storage of spatial information by the maintenance mechanism of LTP. Science 313:1141-1144.

Perez-Otano I, Ehlers MD (2005) Homeostatic plasticity and NMDA receptor trafficking. Trends Neurosci 28:229-238.

Price TJ, Ghosh S (2013) ZIPping to pain relief: the role (or not) of PKMzeta in chronic pain. Molecular pain 9:6.

Ren SQ, Yan JZ, Zhang XY, Bu YF, Pan WW, Yao W, Tian T, Lu W (2013) PKClambda is critical in AMPA receptor phosphorylation and synaptic incorporation during LTP. The EMBO journal 32:1365-1380.

Sacktor TC (2008) PKMzeta, LTP maintenance, and the dynamic molecular biology of memory storage. Prog Brain Res 169:27-40.

Samuels IS, Seibenhener ML, Neidigh KB, Wooten MW (2001) Nerve growth factor stimulates the interaction of ZIP/p62 with atypical protein kinase C and targets endosomal localization: evidence for regulation of nerve growth factor-induced differentiation. Journal of cellular biochemistry 82:452-466.

Sandkuhler J (2007) Understanding LTP in pain pathways. Molecular pain 3:9.

Sanz L, Diaz-Meco MT, Nakano H, Moscat J (2000) The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. The EMBO journal 19:1576-1586.

Sanz L, Sanchez P, Lallena MJ, Diaz-Meco MT, Moscat J (1999) The interaction of p62 with RIP links the atypical PKCs to NF-kappaB activation. The EMBO journal 18:3044-3053.

Selbie LA, Schmitz-Peiffer C, Sheng Y, Biden TJ (1993) Molecular cloning and characterization of PKC iota, an atypical isoform of protein kinase C derived from insulin-secreting cells. The Journal of biological chemistry 268:24296-24302.

Sperry MA, Goshgarian HG (1993) Ultrastructural changes in the rat phrenic nucleus developing within 2 h after cervical spinal cord hemisection. Exp Neurol 120:233-244.

Steinmetz CC, Turrigiano GG (2010) Tumor necrosis factor-α signaling maintains the ability of cortical synapses to express synaptic scaling. J Neurosci 30:14685-14690.

Stellwagen D, Malenka RC (2006) Synaptic scaling mediated by glial TNF-alpha. Nature 440:1054-1059.

Strey KA, Baker-Herman TL (2013) Decreased spinal synaptic inputs to phrenic motor neurons elicit localized inactivity-induced phrenic motor facilitation. Exp Neurol In Review.

Strey KA, Nichols NL, Baertsch NA, Broytman O, Baker-Herman TL (2012) Spinal atypical protein kinase C activity is necessary to stabilize inactivity-induced phrenic motor facilitation. J Neurosci 32:16510-16520.

Sutton MA, Wall NR, Aakalu GN, Schuman EM (2004) Regulation of dendritic protein synthesis by miniature synaptic events. Science 304:1979-1983.

Sutton MA, Ito HT, Cressy P, Kempf C, Woo JC, Schuman EM (2006) Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. Cell 125:785-799.

Thomas GM, Huganir RL (2013) Palmitoylation-dependent regulation of glutamate receptors and their PDZ domain-containing partners. Biochem Soc Trans 41:72-78.

Turrigiano G (2007) Homeostatic signaling: the positive side of negative feedback. Curr Opin Neurobiol 17:318-324.

Turrigiano G (2012) Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function. Cold Spring Harb Perspect Biol 4:a005736.

Turrigiano GG, Nelson SB (2004) Homeostatic plasticity in the developing nervous system. Nat Rev Neurosci 5:97-107.

Vernadakis A (1996) Glia-neuron intercommunications and synaptic plasticity. Progress in neurobiology 49:185-214.

Volk LJ, Bachman JL, Johnson R, Yu Y, Huganir RL (2013) PKM-ζ is not required for hippocampal synaptic plasticity, learning and memory. Nature 493:420-423.

Wan Q, Jiang XY, Negroiu AM, Lu SG, McKay KS, Abrams TW (2012) Protein kinase C acts as a molecular detector of firing patterns to mediate sensory gating in Aplysia. Nat Neurosci 15:1144-1152.

Appendix A

Inactivity-induced respiratory plasticity: Protecting the drive to breathe in disorders that reduce respiratory neural activity

K.A. Strey, N.A. Baertsch, T.L. Baker-Herman

Respir Physiol Neurobiol. Jun 28. 2013. Ahead of print.

ABSTRACT

Multiple forms of plasticity are activated following reduced respiratory neural activity. For example, in ventilated rats, a central neural apnea elicits a rebound increase in phrenic and hypoglossal burst amplitude upon resumption of respiratory neural activity, forms of plasticity called inactivity-induced phrenic and hypoglossal motor facilitation (iPMF and iHMF), respectively. Here, we provide a conceptual framework for plasticity following reduced respiratory neural activity to guide future investigations. We review mechanisms giving rise to iPMF and iHMF, present new data suggesting that inactivity-induced plasticity is observed in inspiratory intercostals (iIMF) and point out gaps in our knowledge. We then survey conditions relevant to human health characterized by reduced respiratory neural activity and discuss evidence that inactivity-induced plasticity is elicited during these conditions. Understanding the physiological impact and circumstances in which inactivity-induced respiratory plasticity is elicited may yield novel insights into the treatment of disorders characterized by reductions in respiratory neural activity.

INTRODUCTION

The seemingly "simple" task of maintaining ventilation throughout life is not trivial. Motor output from multiple, diverse motor neuron pools must be coordinated to activate respiratory muscles in a precise sequence to achieve efficient and adequate ventilation. In addition, the respiratory control system must produce a stable rhythmic motor output, yet remain dynamic in order to respond to respiratory challenges (e.g., exercise, hypoxia, hypercapnia) and enable non-respiratory behaviors (e.g., airway clearance, speech). Remarkably, this task is accomplished despite ever-changing conditions that occur throughout life. The processes by which the respiratory control system maintains network stability and responsiveness in the presence of changing physiological demands and network properties are not well understood, but likely involve long-lasting adjustments in system performance (i.e., plasticity; Feldman et al., 2003; Mitchell and Johnson, 2003).

The respiratory control system exhibits a range of different mechanisms that give rise to respiratory plasticity (Bach and Mitchell, 1998; Baker et al., 2001; Peng et al., 2003; Zhang et al., 2003; Mahamed and Mitchell, 2008; Tadjalli et al., 2010; Nichols et al., 2012; Strey et al., 2012; Devinney et al., 2013). For many, a common theme is that recurrent respiratory challenges, such as hypoxia or hypercapnia, drive the expression of plasticity. Although these chemoreflex-driven forms of plasticity are associated with increases in respiratory neural activity, most are activity-independent and require neuromodulators for their induction and/or maintenance (Feldman et al., 2003; Mitchell and Johnson, 2003). By contrast, reports of activity-dependent plasticity in the control of breathing are generally rare (McCrimmon et al., 1997; Zhou et al., 1997; Johnson and Mitchell, 2002), and typically describe plasticity as a result of increased activity. However, in recent years, we have begun to appreciate that decreases in

respiratory neural activity are perhaps a more potent trigger for plasticity than increases in activity (Castro-Moure and Goshgarian, 1996, 1997; Prakash et al., 1999; Zhang et al., 2003; Mantilla et al., 2007; Tadjalli et al., 2010; Mahamed et al., 2011; Strey et al., 2012; Baertsch and Baker-Herman, 2013). The apparent bias in the respiratory control network toward inactivity- (versus activity-) induced plasticity may not be surprising, since Hebbian-like plasticity may be inappropriate in a physiological system that routinely experiences recurrent increases in respiratory neural activity (e.g., during exercise) and the dire consequences that follow if the system fails to generate sufficient motor output.

One key question is: what is the physiological role of inactivity-induced plasticity? Unfortunately, the answer is not currently known. However, various conditions during health and disease are accompanied by reduced respiratory neural activity. For example, healthy individuals experience reductions in respiratory neural output during behaviors such as sleep (Uliel et al., 2004; Javaheri and Dempsey, 2013), voluntary diving (Gooden, 1994; Dutschmann and Paton, 2002) or at altitude (Berssenbrugge et al., 1984). Periods of reduced or absent respiratory motor output, may accompany a variety of other conditions, such as prematurity (Gaultier and Gallego, 2005) and aging (Ancoli-Israel et al., 1987), or may be a secondary consequence of various pathologies, including genetic disorders (Goridis et al., 2010), neurodegenerative diseases (Gaig and Iranzo, 2012) or heart failure (Yumino and Bradley, 2008). Finally, reduced respiratory neural activity is often experienced following spinal cord injury (Strakowski et al., 2007) and by patients requiring ventilatory support (Tobin, 2001; Epstein, 2011). The short- and long-term consequences of reduced neural activity in respiratory motor pools on the control of breathing in any context is not well understood.

Here, we review evidence that reduced respiratory neural activity elicits unique mechanisms of plasticity within respiratory motor circuits. To build context, we survey conditions and disorders relevant to human health that are characterized by reduced respiratory neural activity. We provide evidence that these disorders elicit endogenous mechanisms of compensatory plasticity (or whether such evidence is lacking), and if inactivity-induced plasticity may be adaptive or maladaptive in these situations. This review is not intended to be a comprehensive catalogue of conditions/disorders associated with reduced respiratory neural activity; instead we aim to identify gaps in our knowledge in basic and translational research to guide development of future lines of investigation.

2. Does-reduced respiratory neural activity elicit plasticity?

An emerging principle of neuroscience is that neural networks sense and respond to prolonged changes in activity through local homeostatic mechanisms to maintain a "set-point" level of neuronal activity (Turrigiano, 2008). As such, prolonged changes in activity that result in a deviation from the "set-point" are met with mechanisms of plasticity that attempt to restore target activity levels through a variety of negative feedback mechanisms. This homeostatic synaptic plasticity is generally bi-directional and requires hours to days of altered activity levels to induce. However, the respiratory system has unique demands that suggest a bias toward rapid induction of plasticity, particularly in response to hypoactivity, may be appropriate. In contrast to many other neural systems, it is imperative that the respiratory control system remain highly active to produce a life-sustaining, rhythmic motor output. For example, the diaphragm has a duty cycle of ~32–44% (Kong and Berger, 1986; Sieck et al., 2012), while muscles of the hind limb (e.g. soleus and extensor digitorum longus muscles) have duty cycles ranging from ~2 to

14% (Hensbergen and Kernell, 1997). Thus, the phrenic motor pool may be exquisitely tuned to a high level of activity and uniquely sensitive to a lack thereof. Here we summarize available evidence that reduced respiratory neural activity induces plasticity of inspiratory motor output and discuss the underlying cellular mechanisms. One major purpose of this review is to provide a conceptual framework in which to interpret plasticity following reduced respiratory neural activity in non-disease and disease states.

2.1. Inactivity-induced plasticity following reduced respiratory neural activity

To our knowledge, one of the first anecdotal reports of increased respiratory motor output following reduced respiratory neural activity was reported by Budzinska and colleagues (1985), who focally cooled regions of the ventral medulla to create a central apnea in anesthetized, ventilated cats. The authors remarked that upon rewarming after cold block-induced apnea, "the return of rhythmic activity after apnoea sometimes showed a 'rebound' with an enhanced [phrenic] peak inspiratory activity relative to the pre-block control breaths." The authors did not quantify the frequency of occurrence nor the magnitude of phrenic facilitation following cold block-induced apnea (Budzińska et al., 1985). In a subsequent study, Castro-Moure and Goshgarian (1996) focally cooled the ventral spinal cord at C2 to block axon conduction unilaterally in descending tracts to ipsilateral phrenic motor neurons in anesthetized rats in order to mimic disruption of descending respiratory drive associated with spinal injury. As expected, C2 cold block silenced ipsilateral diaphragm EMG activity; however, to the authors' surprise, upon reversal of the cold block and restoration of axon conduction, EMG activity in the hemidiaphragm ipsilateral (but not contralateral) to cold block was significantly increased relative to baseline and contralateral diaphragm EMG activity (Castro-Moure and Goshgarian,

1996). The duration of increased ipsilateral diaphragm EMG activity following cold block was not reported, nor could it be differentiated whether observed effects were due to central neural versus diaphragm neuromuscular junction plasticity. However, profound morphological changes were observed within the ipsilateral phrenic motor nucleus, including an increase in the number of synapses onto phrenic motor neurons (Castro-Moure and Goshgarian, 1997), consistent with the interpretation that removal of respiratory-related inputs onto phrenic motor neurons elicited local mechanisms of plasticity within the phrenic motor pool. Similar findings may have been observed by Webber and Pleschka (1984) and Martin et al. (1994). In representative traces of phrenic (Webber and Pleschka, 1984) and hypoglossal (Martin et al., 1994) motor output before, during and after inhibition of respiratory neural activity using focal cooling of the C2 spinal cord and ventral medullary surface, respectively, increased respiratory motor output was apparent following resumption of respiratory neural activity, although these authors did not remark upon nor quantify the extent of the increases in phrenic or hypoglossal burst amplitude. Nevertheless, collectively, these early reports led to the interesting suggestion that reduced respiratory-related inputs to phrenic and hypoglossal motor neurons elicits forms of plasticity that lead to enhanced respiratory motor output.

To better understand the long-lasting impact of reduced respiratory neural activity on phrenic motor output, we exposed anesthetized, ventilated rats to a prolonged (30 min) central neural apnea while monitoring phrenic neural activity (Mahamed et al., 2011). Following resumption of respiratory neural activity post-central neural apnea, a long-lasting, rebound increase in phrenic burst amplitude was apparent. Since multiple methods (with different mechanisms of action) led to similar increases in phrenic burst amplitude, we suggested that increased phrenic burst amplitude post-neural apnea was due to a common factor: reduced

respiratory neural activity (Mahamed et al., 2011), and termed this form of plasticity inactivity-induced phrenic motor facilitation (iPMF; Mahamed et al., 2011). Later studies revealed that inactivity-induced plasticity is also expressed in hypoglossal motor output (Baker-Herman and Strey, 2011), albeit with a more transient pattern of expression. Fig. 1 depicts a meta-analysis of phrenic and hypoglossal nerve responses to neural apnea from multiple studies in our lab (Baker-Herman and Strey, 2011; Mahamed et al., 2011; Strey et al., 2012; Baertsch and Baker-Herman, 2013). Following a prolonged neural apnea, iPMF manifests as a long-term (>60 min) increase in phrenic amplitude (~60–80% baseline) and is associated with a proportional increase in the phrenic burst amplitude response to a hypercapnic challenge (Baertsch and Baker-Herman, 2013). By contrast, facilitation of hypoglossal nerve burst amplitude following a prolonged central neural apnea is more modest (~30–40%) and transient, returning to baseline levels within ~30 min (Baker-Herman and Strey, 2011; Baertsch and Baker-Herman, 2013). In addition to increases in burst amplitude, central neural apnea also elicits increased respiratory burst frequency (Baker-Herman and Strey, 2011; Mahamed et al., 2011; Baertsch and Baker-Herman, 2013), albeit this inactivity-induced frequency facilitation is small and has a limited time course (~15 min).

Here, we present new data from experiments testing the response of inspiratory intercostal muscles to prolonged central neural apnea using procedures similar to those described in detail elsewhere (Mahamed et al., 2011; Strey et al., 2012; Baertsch and Baker-Herman, 2013). All experiments were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison. Briefly, Harlan Sprague Dawley rats (colony 217) were urethane anesthetized, vagotomized and mechanically ventilated. EMG electrodes were placed in

the external intercostal (T2) muscle. Stable baseline intercostal EMG activity was established at an ETCO2 ~45 mmHg and respiratory frequency ~45 bpm prior to a 30 min hyperventilation-induced central neural apnea (n = 10). Following reversal of neural apnea, intercostal EMG activity was monitored for 1 h. In a subset of rats, the cut left phrenic nerve was also recorded to ensure that iPMF was expressed under these conditions. In separate rats, baseline parameters were maintained for 90 min (no neural apnea) to control for any time-dependent changes in external intercostal EMG activity (time controls; n = 3). Blood samples were taken at baseline and 5, 15, 30, and 60 min following resumption of inspiratory intercostal activity to ensure $PaCO_2$, PaO_2, pH and SBEc post-neural apnea were maintained at baseline levels. Inspiratory intercostal EMG activity was rectified and integrated (PowerLab data acquisition and LabChart 7.0 software); the peak amplitude of integrated intercostal EMG activity post-neural apnea (or equivalent duration in time controls) was expressed as a percentage change from baseline (% baseline), whereas burst frequency was expressed as an absolute change from baseline (Δ baseline).

Following restoration of central respiratory neural activity, inspiratory-related external intercostal EMG amplitude was significantly increased for up to 15 min, relative to baseline and time controls (neural apnea: 64 ± 11; time controls: 5 ± 6% baseline; $p < 0.05$; Fig. 1), indicating inactivity-induced intercostal motor facilitation (iIMF). Although inspiratory intercostal EMG activity 30 min after restoration of respiratory neural activity was significantly increased relative to baseline (46 ± 14% baseline; $p < 0.05$), it was no longer significantly different than time controls (3 ± 12% baseline; $p > 0.05$). By 60 min following resumption of respiratory neural activity, inspiratory intercostal EMG activity was not significantly different than baseline or time controls (neural apnea: 25 ± 18, time controls: 3 ± 10% baseline; $p > 0.05$). Similar to previous

reports (Baker-Herman and Strey, 2011; Mahamed et al., 2011; Baertsch and Baker-Herman, 2013), a transient increase in intercostal EMG burst frequency was observed for up to 15 min following neural apnea compared to baseline and time controls (neural apnea: 7 ± 1; time controls: -2 ± 0 Δ baseline; $p < 0.05$). At 30 min post-resumption of respiratory neural activity, intercostal EMG burst frequency was significantly increased relative to baseline (5 ± 1 Δ baseline; $p < 0.05$), but was not significantly different than time controls (-2 ± 2 Δ baseline; $p > 0.05$). At 60 min post-resumption of respiratory neural activity, EMG burst frequency was not significantly different than baseline or time controls (neural apnea: 4 ± 2; time controls: -2 ± 1 Δ baseline; $p > 0.05$). Collectively, these data suggest that the inspiratory intercostal response to prolonged neural apnea resembles the magnitude of phrenic amplitude facilitation observed immediately after the resumption of neural activity (5 and 15 min), but has a transient time-course similar to hypoglossal nerve output (Fig. 1), and confirms the transient frequency facilitation post-neural apnea reported in previous studies (Baker-Herman and Strey, 2011; Mahamed et al., 2011; Baertsch and Baker-Herman, 2013). The mechanisms by which inactivity-induced facilitation of inspiratory burst amplitude is transient in some motor pools and long-lasting in others is unknown; however, the "activity profile" of a motor pool may be a major determinant of the rapidity of induction and duration of plasticity elicited in response to reduced respiratory neural activity. Indeed, long-lasting periods of reduced inspiratory activity may be more common for hypoglossal and some intercostal motor pools (De Troyer et al., 2005; Pagliardini et al., 2012); as such, it may be expected that inactivity-induced plasticity has a different manifestation and/or time domain in these motor pools versus the phrenic.

Clearly, a prolonged central neural apnea lasting 30 min would rarely be encountered in any but the most artificial situation (ventilated animals). As such, this model was not intended to

mimic physiological/pathophysiological situations in which a central apnea would be experienced; instead, these studies demonstrated a principle of respiratory control: Reduced central respiratory drive elicits plasticity in respiratory motor output. In order to provide a more "realistic" (albeit still somewhat artificial) context to iPMF, recent studies demonstrated that iPMF is not limited to prolonged central apnea, but is also elicited following intermittent patterns of brief central neural apnea (Baertsch and Baker-Herman, 2013). Anesthetized and ventilated rats were exposed to brief intermittent neural apnea (5, 1.5 min episodes separated by 5 min recovery); following resumption of respiratory neural activity, a sustained (>60 min) increase in phrenic burst amplitude (~60–80% baseline) was apparent, which was phenotypically similar to iPMF observed following a single prolonged neural apnea (Baertsch and Baker-Herman, 2013). In contrast, exposure to a single brief "massed" neural apnea of a similar cumulative duration (7.5 min), did not alter phrenic burst amplitude at any time point following resumption of respiratory neural activity. No changes in hypoglossal burst amplitude were observed following brief intermittent or brief massed neural apnea (Baertsch and Baker-Herman, 2013). Similarly, episodic central apneas (3, 5 min episodes separated by 5 min recovery) induced via high frequency vagal stimulation in ventilated rats elicits a phenotypically similar augmentation of phrenic burst amplitude (Zhang et al., 2003); although the authors refer to this form of plasticity as long-term facilitation, we hypothesize that it represents a variation of inactivity-induced plasticity. Collectively, these reports suggest that although a prolonged central neural apnea is sufficient to elicit iPMF (Baker-Herman and Strey, 2011; Mahamed et al., 2011; Strey et al., 2012), iPMF, but not iHMF, is more efficiently induced by recurrent (intermittent) neural apnea versus a sustained neural apnea of similar cumulative duration.

One key question is: Where in the CNS is reduced respiratory neural activity sensed and responded to? Central neural apnea results in reduced respiratory neural activity throughout the neuraxis; however, we hypothesized that local mechanisms near respiratory motor neurons sense and respond to reduced respiratory-related inputs and give rise to burst amplitude facilitation (i.e., iPMF, iHMF or iIMF in the respective motor pools), whereas mechanisms operating within brainstem respiratory rhythm generating networks give rise to neural apnea-induced increases in frequency (Mahamed et al., 2011; Strey et al., 2012; Baertsch and Baker-Herman, 2013). Supportive of this hypothesis, iPMF (but not burst frequency facilitation) is impaired by application of pharmacological inhibitors that block cellular pathways leading to iPMF to regions of the spinal cord associated with the phrenic motor nucleus (Strey et al., 2012). Further, disruption of spinal synaptic inputs to phrenic motor neurons via C2 axon conduction block (in the absence of noticeable changes in central respiratory drive) elicits iPMF (Castro-Moure and Goshgarian, 1996, 1997; Baker-Herman and Strey, 2011) and morphological plasticity within the phrenic motor pool (Castro-Moure and Goshgarian, 1997), but does not elicit frequency facilitation. Notably, a complete loss of ipsilateral phrenic motor output during axon conduction block is not required for iPMF to develop, suggesting that reductions in respiratory neural activity (versus complete inactivity) are sufficient to elicit these mechanisms (Baker-Herman and Strey, 2011).

Fig. 2 depicts our working model of cellular mechanisms giving rise to iPMF. As discussed above, we hypothesize that iPMF is the result of mechanisms operating specifically within the phrenic motor pool (Baker-Herman and Strey, 2011; Mahamed et al., 2011; Strey et al., 2012). iPMF consists of at least two mechanistically distinct phases: (1) an early, labile phase that requires activity of the atypical protein kinase C (aPKC) isoform PKCζ and/or PKC ι/λ in

spinal regions associated with the phrenic motor nucleus to transition to (2) a late, long-lasting increase in phrenic burst amplitude (Strey et al., 2012). Atypical PKCs include the isozymes PKCζ, PKC ι/λ and PKMζ, and represent one of three subfamilies of PKCs (classical, novel and atypical) that are classified based on structure and requirement for co-activators (Reyland, 2009). Consistent with a key role for spinal PKCζ and/or PKC ι/λ (referred to here as PKCζ/ι for clarity), early, but not late, iPMF is associated with an increased interaction between PKCζ/ι and the scaffolding protein ZIP (PKC ζ interacting protein)/p62 in ventral spinal regions (C3–C5) associated with the phrenic motor pool; an interaction which may confer context specificity to PKCζ/ι activity. Upstream mechanisms that promote formation of the PKCζ/ι-p62/ZIP signaling cassette are thought to include release of tumor necrosis factor alpha (TNFα) and subsequent activation of TNF receptors on phrenic motor neurons. Indeed, spinal TNFα signaling is necessary for iPMF expression, and exogenous TNFα induces an aPKC dependent increase in phrenic burst amplitude (Broytman et al., 2013). Mechanisms giving rise to inactivity-induced frequency facilitation are unknown.

2.2. Gaps in our knowledge of inactivity-induced plasticity

Although we have made considerable progress in our understanding of inactivity-induced plasticity, many questions remain unanswered. For example, what cell type/s sense reduced respiratory neural activity, and what signal are these cells sensing? Are these "sensors" also the cell types that release TNFα to elicit inactivity-induced plasticity? Does inactivity-induced plasticity reflect mechanisms occurring largely within respiratory motor neurons or as part of a cellular network within or near motor nuclei? Similarly, do all phrenic motor neurons express iPMF, or are there "sub-pools" of phrenic motor neurons particularly sensitive to reduced neural

activity (e.g., those involved in eupnic breathing vs. those recruited during respiratory challenges)? Is inactivity-induced plasticity in other respiratory motor pools mechanistically similar to iPMF? What mechanisms underlie differential expression of inactivity-induced facilitation among different motor pools? Do genetic or epigenetic factors influence the expression or absence of iPMF and related plasticity?

Important and challenging questions regarding the physiological role for inactivity-induced plasticity in the control of breathing also provide motivation for future studies. For example, what is the consequence of differential plasticity among inspiratory motor pools in the sculpting of a breath? In particular, what are the implications for airway stability with a prolonged facilitation of phrenic motor output in the absence of hypoglossal facilitation? What, if any, role does inactivity-induced plasticity play in physiological or pathophysiological conditions relevant to human health? How might an understanding of the mechanisms of inactivity-induced plasticity be used to manipulate physiological outcomes? Since virtually nothing is known concerning the applicability of iPMF to the control of breathing in health or disease, we will begin forming a necessary conceptual framework by discussing circumstances where reduced respiratory neural activity may be experienced.

3. Conditions/disorders associated with reduced respiratory neural activity

Although the respiratory control system is remarkably reliable for most individuals, it is susceptible to transient and recurrent or prolonged periods of reduced respiratory neural activity in a variety of physiological and pathophysiological situations. Here we briefly survey conditions and disorders of relevance to human health to outline situations in which reduced respiratory neural activity may be experienced.

3.1. Disruptions in brainstem respiratory neural activity

Central apnea may occur during normal physiological or pathophysiological processes, typically during sleep. Central sleep apnea (CSA) is characterized by recurrent episodes of absent or markedly reduced (hypopnea) respiratory neural output, whereas obstructive sleep apnea (OSA) is characterized by continued (futile) central neural output in the presence of a closed or reduced airway. In many cases, both CSA and OSA often co-exist ("mixed" apnea) in the same patient (Xie et al., 2011; Javaheri and Dempsey, 2013) or CSA may develop during treatment of OSA ("complex" sleep apnea; Morgenthaler et al., 2006; Dernaika et al., 2007; Lehman et al., 2007) for reasons that are not clearly understood.

During sleep, a number of physiological changes increase the propensity for ventilatory instability, even in otherwise healthy individuals (Eckert et al., 2007; Javaheri, 2010; Malhotra and Owens, 2010; Javaheri and Dempsey, 2013). For example, removal of the so-called wakefulness drive to breathe reveals a sensitive CO_2-dependent apneic threshold (Skatrud and Dempsey, 1983; Dempsey et al., 2012). Thus, periodic cessation of inspiratory efforts will occur when $PaCO_2$ drops below a critical level (Pack, 2011; Dempsey et al., 2012). Indeed, during sleep, even small decreases in $PaCO_2$ (~2–5 mmHg) can result in apnea (Skatrud and Dempsey, 1983; Henke et al., 1988; Meza et al., 1998). During sleep, $PaCO_2$ rises ~5 mmHg above wakefulness levels (Malhotra and Owens, 2010; Pack, 2011). During the transition from wakefulness to sleep in individuals with a CO_2 apneic threshold close to eupnic $PaCO_2$, a central apnea may result if the wakefulness drive to breathe is lost rapidly at sleep onset before sleep-induced reductions in ventilation occur and the establishment of the sleep $PaCO_2$ set point (Leung et al., 2012; Javaheri and Dempsey, 2013). Further, a central apnea may result following a transient arousal from sleep (Trinder et al., 1992), which represents a temporary return to

wakefulness. The sleeping $PaCO_2$ represents a relative hypercapnia to the aroused brain, which then stimulates breathing to lower $PaCO_2$ to awake eupnic levels (Eckert et al., 2007; Malhotra and Owens, 2010); if hyperventilation results in a drop of $PaCO_2$ below the apneic threshold, then upon the resumption of sleep, the relative hypocapnia to the now sleeping brain results in a central apnea. The frequency of central apnea during sleep in an otherwise healthy individual is generally minimal; indeed, a frequency of <5 CSA events/hr is considered to be within a clinically normal range (Javaheri, 2010).

The prevalence of central apnea during sleep in the general population is not entirely clear, particularly when considering the clinically normal range. Breathing pattern instability during sleep with central apneas of short duration is characteristic of the normal, healthy infant breathing pattern (Hoppenbrouwers et al., 1977; Kahn et al., 1982; Ng and Chan, 2013). Indeed, periodic breathing is apparent in 78% of infants between 0 and 2 weeks of age and declines to 29% by 39–52 weeks of age, although the apneic events are typically <10 s in duration and occupy <1% of the sleep time (Kelly et al., 1985). However, premature infants are particularly susceptible to central apnea and periodic breathing (Martin et al., 2004), with nearly 100% of preterm infants exhibiting episodes of periodic breathing that are longer in duration and more frequent than full-term infants (Henderson-Smart, 1981; Glotzbach et al., 1989). Although the frequency of central apnea decreases with increasing gestational age, central apnea is also relatively common in children (Scholle et al., 2011), with 30-40% of children experiencing a total of 1 to 7 central apneic events lasting >10 s during sleep (Marcus et al., 1992; Uliel et al., 2004). The frequency of such events appears to diminish in adolescence (Tapia et al., 2008). As an individual ages, the frequency of central apnea in sleep increases (Carskadon and Dement, 1981; Bixler et al., 1998; Chowdhuri and Badr, 2010). For example, in a large study of men aged

20-100 yr., Bixler and colleagues report that 12.1% of subjects over the age of 65 had a central apnea index ≥2.5, whereas only 1.7% of middle-aged subjects and no subjects in the young age group had a central apnea index ≥2.5 (Bixler et al., 1998).

Although central apnea may occur during sleep in healthy individuals, the frequency of such events is typically minimal and not considered to be clinically relevant (Javaheri, 2010). However, the prevalence and frequency of CSA increases during certain conditions. For example, many individuals will experience periodic breathing and frequent central apneas upon ascent to high altitude. At altitude, the ventilatory response to hypoxia (HVR) lowers $PaCO_2$ (Pack, 2011), thereby narrowing the eupnic $PaCO_2$—apneic threshold difference (i.e., "CO_2 reserve") and creating breathing instability during sleep (Berssenbrugge et al., 1984; Kohler et al., 2008; Bloch et al., 2010). Typically, individuals develop a periodic breathing pattern characterized by short crescendo–decrescendo cycles (15-30 s) of hyperventilation alternating with periods of apnea/hypopnea (Pack, 2011). At altitudes >4500 m, an apnea/hypopnea index of ~60 h^{-1}during sleep is not uncommon (Burgess et al., 2004; Nussbaumer-Ochsner et al., 2012), suggesting considerable cumulative time spent with low (or zero) respiratory neural activity. Over several days at altitude, the magnitude of CSA is reduced in many individuals (Berssenbrugge et al., 1984).

An increase in the prevalence or frequency of CSA may occur during some pathological conditions. For example, approximately ~30-40% of patients with heart failure (HF) develop frequent episodes of CSA (Javaheri et al., 1998; Sin et al., 1999; MacDonald et al., 2008). Indeed, HF is the most common cause of clinically diagnosed CSA in the general population (Javaheri, 2010; Javaheri and Dempsey, 2013). CSA in HF patients is characterized by a long (~60–90 s) crescendo-decrescendo pattern of breathing interspersed with central apneas lasting

~10-40 s, a form of periodic breathing known as Cheyne–Stokes Respiration (CSR; Hall et al., 1996; Pack, 2011). Enhanced chemosensitivity and chronic hyperventilation during sleep in HF patients reduces sleeping $PaCO_2$ levels (Tkacova et al., 1997; Xie et al., 2002; Yumino and Bradley, 2008; Javaheri, 2010), bringing eupnic PaCO2 closer to the apneic threshold and predisposing HF patients to central apnea. Although reports vary, patients with HF and CSR on average have an AHI ≥15 h−1 with more than 50% of central origin (Wang et al., 2007). Other conditions that may be associated with an increased incidence of central apnea include patients with idiopathic CSA (Bradley et al., 1986; Bradley and Phillipson, 1992), endocrine disorders (Millman et al., 1983; Grunstein et al., 1991; Rosenow et al., 1998), neuromuscular disorders (Chokroverty et al., 1978; Chokroverty et al., 1984; Ferguson et al., 1996; Labanowski et al., 1996; Santos et al., 2003; Glass et al., 2006; Gaig and Iranzo, 2012), congenital central hypoventilation syndrome (CCHS; Fleming et al., 1980; Goridis et al., 2010; Weese-Mayer et al., 2010) and Rett syndrome (Weese-Mayer et al., 2008; Katz et al., 2009).

3.2. Disruption in inputs to spinal respiratory motor neurons

Reduced respiratory neural activity may also be caused by disruption of central neural drive in transit to respiratory motor neurons as result of injuries to the spinal cord. More than half (52% since 2010) of all spinal cord injuries (SCIs) occur in the cervical spinal region while the remaining injuries are localized to thoracic, lumbar or sacral regions (NSCISC, 2013). In contrast to central neural apnea, SCIs reduce respiratory neural activity in spinal respiratory motor neurons caudal to injury, while brainstem respiratory centers continue to generate normal (or even elevated) neural drive to breathe. In addition to disrupting bulbospinal respiratory axons in white matter tracts, spinal cord injury typically disrupts gray matter continuity and damages

propriospinal interneurons and/or respiratory motor neurons in the cervical spinal cord (Lane et al., 2009). Depending on the location and severity of the injury, the level of reduced respiratory neural activity varies. For example, high cervical SCIs interrupt the descending excitatory drive to phrenic motor neurons innervating the diaphragm (Golder et al., 2011; Lane et al., 2012), while injuries below cervical regions impair breathing by damaging axons innervating accessory inspiratory muscles or muscles mainly involved in expiration (Gorini et al., 2000).

3.3. Artificial reductions in respiratory neural activity

Mechanical ventilation is a life-saving treatment for hundreds of thousands of critically ill patients each year. Most modern modes of mechanical ventilation attempt to synchronize the quantity, timing and pattern of a patient's neural drive with the ventilator, thereby avoiding deleterious effects associated with removing patient respiratory efforts altogether (Epstein, 2011). Unfortunately, attempts to achieve synchronization are often unsuccessful (Tobin, 2001; Thille et al., 2006; de Wit et al., 2009b; de Wit et al., 2009a; Epstein, 2011), often due to ineffective ventilator triggering, auto-triggering or poor correspondence in the flow or timing of the patients neural breath and the ventilator breath (Kondili et al., 2007; de Wit et al., 2009b; de Wit et al., 2009a; Mellott et al., 2009; Pierson, 2011). In addition, many ventilated patients are at risk for over-assistance, which suppresses or diminishes the patient's neural drive (Meza et al., 1998; Parthasarathy and Tobin, 2002; Colombo et al., 2008; Delisle et al., 2011). Thus, many patients receiving ventilatory support experience reduced central respiratory neural activity while on the ventilator (Younes, 2006; Kondili et al., 2007; de Wit et al., 2009b; de Wit et al., 2009a; Epstein, 2011).

In most patients, mechanical ventilation may be discontinued abruptly (Esteban et al., 1995; Esteban et al., 2000). However, up to 30% of mechanically ventilated patients have difficulty resuming breathing on their own, even after their underlying disorder has been resolved (Epstein, 2009). Indeed, up to 40% of the time spent on the ventilator is associated with the weaning process (Esteban et al., 2008). Ventilation strategies that suppress spontaneous breathing are associated with longer duration of mechanical ventilation (Chao et al., 1997; Putensen et al., 2006; Thille et al., 2006; de Wit et al., 2009b); however, even patients that maintain spontaneous breathing for the most part, but have a high level of over-assistance or ventilator asynchrony have a longer duration of weaning from mechanical ventilation than their counterparts that do not (Chao et al., 1997; Thille et al., 2006; de Wit et al., 2009b). The pathophysiology underlying weaning failure is multifactorial and likely vary from patient to patient. Much research has been focused on the impact of reduced diaphragm muscle activity during mechanical ventilation on subsequent attempts to breathe spontaneously since many weaning failure patients exhibit a reduced ability to convert central respiratory drive into an effective breath (Liu et al., 2012), in large part due to muscle weakness (Anzueto et al., 1997; Shanely et al., 2002; McClung et al., 2007; Powers et al., 2009). However, the long-lasting impact of reduced respiratory neural activity during mechanical ventilation on a patient's subsequent attempts to resume spontaneous respiratory efforts are completely unknown.

4. Is there evidence for plasticity in conditions and disorders associated with reduced respiratory neural activity?

Although recurrent or prolonged disruptions in respiratory neural activity are experienced in many conditions, little is known regarding the consequences of this reduced respiratory neural

activity. Other than in reduced animal models, little direct evidence is available concerning links between repetitive central neural apneas or prolonged reduced respiratory activity with respiratory neuroplasticity. We suggest that this lack of evidence stems from a lack of systematic investigation since the first description of inactivity-induced respiratory motor neuroplasticity was only in 2011 (Mahamed et al., 2011).

Review of the literature reveals suggestive evidence that mechanically ventilated patients exhibit respiratory behaviors consistent with inactivity-induced plasticity. For example, central respiratory drive is higher than normal in many mechanically ventilated patients, which is apparent almost immediately after being disconnected from the ventilator (Laghi, 2005; Nemer et al., 2009; Tobin et al., 2009). Central respiratory "drive" is often approximated by airway occlusion pressure during the first 0.1 s of a breath (P0.1); since it is measured at zero flow, airway occlusion pressure is independent of respiratory system compliance and resistance (Whitelaw et al., 1975). Although both weaning failure and weaning success patients exhibit increased P0.1 relative to normal values, P0.1 is often higher at the onset and increases progressively in patients that cannot resume spontaneous, independent breathing (Herrera et al., 1985; Sassoon and Mahutte, 1993; Hilbert et al., 1998; Perrigault et al., 1999; Nemer et al., 2009; Tobin et al., 2009). Although the progressive increase in P0.1 in weaning failure patients may be due to deteriorating gas exchange secondary to worsening respiratory mechanics (Jubran and Tobin, 1997a; Tobin, 2001), the initial increase in P0.1 that is apparent immediately upon the discontinuation of mechanical ventilation is often apparent prior to any measurable deterioration in respiratory mechanics (Jubran and Tobin, 1997b; Tobin, 2001). Consistent with increased central respiratory output, many mechanically ventilated patients exhibit elevated EMG activity in the diaphragm and accessory inspiratory muscles within minutes of being

disconnected from the ventilator, particularly in patients that ultimately fail the weaning trial (Dres et al., 2012; Liu et al., 2012). Although a multitude of factors may contribute to increased respiratory neuromuscular drive in mechanically ventilated patients, the potential role of central neural plasticity in reconfiguring respiratory motor output in response to prolonged reductions in respiratory neural activity experienced while on the ventilator should be considered.

To date, the best evidence that reduced respiratory neural activity elicits neuroplasticity is following SCI. Multiple reports suggest that following cervical SCI, diaphragmatic function spontaneously improves over time in humans (Axen et al., 1985; McKinley, 1996; Oo et al., 1999; Strakowski et al., 2007) and rodents (El-Bohy et al., 1998; Fuller et al., 2003; Golder and Mitchell, 2005; Baussart et al., 2006; Fuller et al., 2006; Vinit et al., 2007; Fuller et al., 2008; Lane et al., 2009; Golder et al., 2011). The recovery of phrenic output and diaphragm activity is associated with a functional recovery of breathing (Strakowski et al., 2007). Since regrowth of damaged axons across the spinal lesion is limited (Sharma et al., 2012), return of phrenic activity following SCI is likely due to endogenous mechanisms of compensatory plasticity (Goshgarian, 2003). Remodeling of spinal circuits post-injury may restore ipsilateral phrenic motor output by recruiting latent contralateral pathways (the "crossed phrenic phenomenon"; Goshgarian, 2009; Lane et al., 2009; Darlot et al., 2012) or strengthening spared ipsilateral pathways (Vinit et al., 2008; Vinit and Kastner, 2009).However, the cellular mechanisms giving rise to spontaneous functional recovery after SCI are unknown. Although spinal injury causes many changes in the spinal microenvironment, including local tissue damage, inflammation and ischemia (Hausmann, 2003), we propose that reduced synaptic inputs to the phrenic motor pool have the potential to play a prominent role in inducing spontaneous functional recovery by strengthening spared pathways via mechanisms similar to iPMF. Indeed, minutes after spinal injury, TNFα is

increased caudal to the site of injury (Wang et al., 1996; Pineau and Lacroix, 2007), triggering a rapid increase in synaptic AMPA receptor expression in motor neurons caudal to injury (Ferguson et al., 2008; Yin et al., 2012). In addition, aPKC activity within the ipsilateral phrenic motor pool is increased shortly after cervical spinal hemisection, an effect that is still observed 28 days post-SCI (Guenther et al., 2012). Consistent with a key role for withdrawal of neural inputs inducing spontaneous recovery, a 4 h disruption in descending inputs to phrenic motor neurons via unilateral cold block causes profound morphological changes within the phrenic motor pool (Castro-Moure and Goshgarian, 1997), similar to those observed 2 h after SCI (Sperry and Goshgarian, 1993). These inactivity-induced morphological changes within the phrenic motor pool are associated with enhanced ipsilateral diaphragm EMG activity following removal of cold block and restoration of axon conduction (Castro-Moure and Goshgarian, 1996). Understanding the stimulus and mechanisms for spontaneous recovery after SCI is vital to further enhance these pathways and improve ventilation following SCI.

5. Is inactivity-induced respiratory plasticity adaptive or maladaptive?

Without a clear understanding of the role for inactivity-induced plasticity in the control of breathing, it is difficult to address whether it is adaptive or maladaptive. However, we speculate that the nature of reduced respiratory neural activity determines the functional consequences of inactivity-induced plasticity in the creation of a stable breathing pattern. For example, as discussed above, even otherwise healthy individuals experience central apnea, particularly during sleep-onset or arousals, although the frequency and duration of these apneas is generally minimal (Gaultier and Gallego, 2005; Eckert et al., 2007; Javaheri, 2010; Javaheri and Dempsey, 2013). However, these infrequent and minor disruptions in respiratory neural activity may induce

mechanisms of inactivity-induced plasticity that reconfigure network properties to augment respiratory motor output, thereby preventing future episodes. In preliminary studies, we find evidence that inactivity-induced plasticity is associated with a decrease in the CO_2 apneic threshold (Baertsch and Baker-Herman, 2013), which may stabilize breathing and further protect against future apneas by increasing the CO_2 reserve. In this sense, inactivity-induced respiratory plasticity may be an important endogenous mechanism to stabilize respiratory motor output throughout life.

On the other hand, too much enhancement and ventilatory instability may result; increased inspiratory efforts may cause large swings in $PaCO_2$, perpetuating the cycle of apnea/hypopnea and predisposing an individual to periodic breathing. Such may be the case in pathological conditions associated with an increased frequency of CSA, such as patients with heart failure (although at this early stage we cannot rule out that these conditions represent a failure of inactivity-induced plasticity). Further, an imbalance between the duration and magnitude of inactivity-induced plasticity in different respiratory muscle groups may alter the recruitment patterns of inspiratory/expiratory muscles (Feroah et al., 2001), potentially impairing the coordination of a breath. Of interest, our findings in experimental models suggest that inactivity-induced plasticity may preferentially enhance inspiratory pump muscles versus those stabilizing the upper airway, which may increase the propensity for airway obstruction during breathing. Thus, in some cases, it may be desirable to reduce the magnitude of inactivity-induced plasticity to stabilize breathing.

Inactivity-induced plasticity may be beneficial in situations in which reduced respiratory neural activity is prolonged. For example, following a cervical spinal injury, induction of inactivity-induced plasticity may partially restore respiratory motor output. Thus, enhancing

inactivity-induced plasticity in patients with compromised breathing following SCI may optimize respiratory motor function and ventilation. In this case, a clear understanding of when and in which motor pool inactivity-induced plasticity should be enhanced following SCI will be critical to maximize therapeutic benefit. However, it is important to keep in mind that this restoration of activity may be double-edged since too much plasticity may result in hyperexcitability of respiratory motor neurons and contribute to respiratory discoordination and muscle spasticity (Boulenguez et al., 2010; Bos et al., 2013). Indeed, respiratory muscle spasticity compromises breathing in many SCI patients, contributing to their ventilator dependence (Silver and Lehr, 1981; Britton et al., 2005).

By contrast, induction of inactivity-induced plasticity may be inappropriate in situations where respiratory neural activity is reduced artificially, such as during mechanical ventilation. In this context, inactivity-induced plasticity may be imposed on the system, and functional adjustments may inappropriately alter system performance, such that when artificial ventilation ends, unstable breathing may result. Accordingly, preventing induction of inactivity-induced plasticity by maintaining respiratory neural activity during mechanical ventilation may be critical for the resumption of a stable breathing pattern and ventilator weaning. On the other hand, induction of inactivity-induced plasticity may serve some benefit in facilitating resumption of spontaneous breathing after mechanical ventilation by strengthening inspiratory motor output to offset weakened respiratory muscles. Clearly, the consequence of inactivity-induced plasticity in shaping future performance of the respiratory control system in any clinical context is poorly understood, and further investigation of the role for inactivity-induced plasticity in the control of breathing is warranted.

6. How do distinct forms of respiratory plasticity interact?

Reduced respiratory neural activity rarely occurs in the absence of additional stimuli that are also capable of eliciting plasticity. Indeed, during central neural apneas, intermittent reductions in neural activity are associated with intermittent hypoxia, hypocapnia, hypercapnia and diminished vagal feedback. As summarized in Fig. 3, each of these stimuli in isolation has been shown to elicit respiratory plasticity via distinct cellular mechanisms, and each differentially affects motor output in specific motor pools (Fregosi and Mitchell, 1994; Bach and Mitchell, 1996; Baker et al., 2001; Peng et al., 2003; Zhang et al., 2003; Mahamed and Mitchell, 2008; Dale-Nagle et al., 2010; Tadjalli et al., 2010; Mahamed et al., 2011; Strey et al., 2012; Baertsch and Baker-Herman, 2013; Devinney et al., 2013). For example, acute intermittent ventilator apneas in paralyzed rats (3 or 6, 25 s apneas, separated by 5 min; without disruption in central neural drive) induces a long-lasting hypoglossal and phrenic motor facilitation (Mahamed and Mitchell, 2008); similarly, hypoglossal and phrenic motor facilitation are observed following acute isocapnic intermittent hypoxia alone (Bach and Mitchell, 1996). On the other hand, diminished vagal feedback does not elicit phrenic motor facilitation, but preferentially elicits genioglossus (and presumably, hypoglossal) facilitation (Tadjalli et al., 2010). Interestingly, intermittent hypoxia in the presence of diminished vagal feedback does not appear to elicit phrenic motor facilitation (Tadjalli et al., 2010), suggesting that simultaneous induction of multiple forms of plasticity may confer unique responses.

An important question for future studies is: How do these multiple forms of plasticity interact to shape necessary long-term adaptations in the respiratory control system? Certainly, information is gained through investigations of each form of plasticity in isolation (e.g., intermittent hypoxia without reduced central drive or airway obstruction and vice versa);

however, distinct mechanisms of plasticity may interact in complex ways to give rise to the final motor output (Nichols et al., 2012; Devinney et al., 2013). Indeed, cross-talk inhibition between different forms of respiratory plasticity has been demonstrated in response to intermittent hypoxia (Dale-Nagle et al., 2010; Nichols et al., 2012; Devinney et al., 2013). A clear understanding of these mechanisms and how interactions among various forms of plasticity lend context specificity to unique respiratory challenges in order to shape ventilatory adaptations is necessary to grasp the significance of plasticity within the control of breathing.

7. Conclusions

We are beginning to grasp basic mechanisms of inactivity-induced respiratory plasticity. It is tempting to speculate that this form of plasticity provides a "boost", ensuring that the respiratory control system produces adequate motor output at all times; however, little is known regarding its functional consequences during physiological and/or pathophysiological situations. Since many conditions and disorders of relevance to human health are associated with reduced respiratory neural activity, an understanding of inactivity-induced plasticity and how (and if) it applies in these conditions is important. However, it's imperative to consider the potentially complex interactions among multiple forms of plasticity likely induced during the same event. Since our appreciation of inactivity-induced respiratory plasticity is new (Mahamed et al., 2011), we anticipate rapid increases in our understanding from here forward.

Acknowledgements: This work was supported by a grant from the National Institutes of Health (HL105511).

The authors declare no conflicts of interest.

REFERENCES

Ancoli-Israel S, Kripke DF, Mason W (1987) Characteristics of obstructive and central sleep apnea in the elderly: an interim report. Biol Psychiatry 22:741-750.

Anzueto A, Peters JI, Tobin MJ, de los Santos R, Seidenfeld JJ, Moore G, Cox WJ, Coalson JJ (1997) Effects of prolonged controlled mechanical ventilation on diaphragmatic function in healthy adult baboons. Crit Care Med 25:1187-1190.

Axen K, Pineda H, Shunfenthal I, Haas F (1985) Diaphragmatic function following cervical cord injury: neurally mediated improvement. Archives of physical medicine and rehabilitation 66:219-222.

Bach KB, Mitchell GS (1996) Hypoxia-induced long-term facilitation of respiratory activity is serotonin dependent. Respir Physiol 104:251-260.

Bach KB, Mitchell GS (1998) Hypercapnia-induced long-term depression of respiratory activity requires alpha2-adrenergic receptors. J Appl Physiol 84:2099-2105.

Baertsch NA, Baker-Herman TL (2013) Inactivity-induced phrenic and hypoglossal motor facilitation are differentially expressed following intermittent vs. sustained neural apnea. J Appl Physiol 114:1388-1395.

Baker-Herman TL, Strey KA (2011) Similarities and differences in mechanisms of phrenic and hypoglossal motor facilitation. Respir Physiol Neurobiol 179:48-56.

Baker TL, Fuller DD, Zabka AG, Mitchell GS (2001) Respiratory plasticity: differential actions of continuous and episodic hypoxia and hypercapnia. Respir Physiol 129:25-35.

Baussart B, Stamegna JC, Polentes J, Tadié M, Gauthier P (2006) A new model of upper cervical spinal contusion inducing a persistent unilateral diaphragmatic deficit in the adult rat. Neurobiol Dis 22:562-574.

Berssenbrugge AD, Dempsey JA, Skatrud JB (1984) Effects of sleep state on ventilatory acclimatization to hypoxia in humans. J Appl Physiol 57:1089-1096.

Bixler EO, Vgontzas AN, Ten Have T, Tyson K, Kales A (1998) Effects of age on sleep apnea in men: I. Prevalence and severity. Am J Respir Crit Care Med 157:144-148.

Bloch KE, Latshang TD, Turk AJ, Hess T, Hefti U, Merz TM, Bosch MM, Barthelmes D, Hefti JP, Maggiorini M, Schoch OD (2010) Nocturnal periodic breathing during acclimatization at very high altitude at Mount Muztagh Ata (7,546 m). Am J Respir Crit Care Med 182:562-568.

Bos R, Sadlaoud K, Boulenguez P, Buttigieg D, Liabeuf S, Brocard C, Haase G, Bras H, Vinay L (2013) Activation of 5-HT2A receptors upregulates the function of the neuronal K-Cl cotransporter KCC2. Proc Natl Acad Sci U S A 110:348-353.

Boulenguez P, Liabeuf S, Bos R, Bras H, Jean-Xavier C, Brocard C, Stil A, Darbon P, Cattaert D, Delpire E, Marsala M, Vinay L (2010) Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. Nat Med 16:302-307.

Bradley TD, Phillipson EA (1992) Central sleep apnea. Clin Chest Med 13:493-505.

Bradley TD, McNicholas WT, Rutherford R, Popkin J, Zamel N, Phillipson EA (1986) Clinical and physiologic heterogeneity of the central sleep apnea syndrome. Am Rev Respir Dis 134:217-221.

Britton D, Goldstein B, Jones-Redmond J, Esselman P (2005) Baclofen pump intervention for spasticity affecting pulmonary function. J Spinal Cord Med 28:343-347.

Broytman O, Baertsch NA, Baker-Herman TL (2013) Spinal TNFα is necessary for inactivity-induced phrenic motor facilitation. J Physiol.

Budzińska K, von Euler C, Kao FF, Pantaleo T, Yamamoto Y (1985) Effects of graded focal cold block in rostral areas of the medulla. Acta Physiol Scand 124:329-340.

Burgess KR, Johnson PL, Edwards N (2004) Central and obstructive sleep apnoea during ascent to high altitude. Respirology 9:222-229.

Carskadon MA, Dement WC (1981) Respiration during sleep in the aged human. Journal of gerontology 36:420-423.

Castro-Moure F, Goshgarian HG (1996) Reversible cervical hemispinalization of the rat spinal cord by a cooling device. Exp Neurol 141:102-112.

Castro-Moure F, Goshgarian HG (1997) Morphological plasticity induced in the phrenic nucleus following cervical cold block of descending respiratory drive. Exp Neurol 147:299-310.

Chao DC, Scheinhorn DJ, Stearn-Hassenpflug M (1997) Patient-ventilator trigger asynchrony in prolonged mechanical ventilation. Chest 112:1592-1599.

Chokroverty S, Sharp JT, Barron KD (1978) Periodic respiration in erect posture in Shy-Drager syndrome. J Neurol Neurosurg Psychiatry 41:980-986.

Chokroverty S, Sachdeo R, Masdeu J (1984) Autonomic dysfunction and sleep apnea in olivopontocerebellar degeneration. Arch Neurol 41:926-931.

Chowdhuri S, Badr MS (2010) Central sleep apnoea. The Indian journal of medical research 131:150-164.

Colombo D, Cammarota G, Bergamaschi V, De Lucia M, Corte FD, Navalesi P (2008) Physiologic response to varying levels of pressure support and neurally adjusted ventilatory assist in patients with acute respiratory failure. Intensive Care Med 34:2010-2018.

Dale-Nagle EA, Hoffman MS, MacFarlane PM, Mitchell GS (2010) Multiple pathways to long-lasting phrenic motor facilitation. Advances in experimental medicine and biology 669:225-230.

Darlot F, Cayetanot F, Gauthier P, Matarazzo V, Kastner A (2012) Extensive respiratory plasticity after cervical spinal cord injury in rats: axonal sprouting and rerouting of ventrolateral bulbospinal pathways. Exp Neurol 236:88-102.

De Troyer A, Kirkwood PA, Wilson TA (2005) Respiratory action of the intercostal muscles. Physiological reviews 85:717-756.

de Wit M, Pedram S, Best AM, Epstein SK (2009a) Observational study of patient-ventilator asynchrony and relationship to sedation level. J Crit Care 24:74-80.

de Wit M, Miller KB, Green DA, Ostman HE, Gennings C, Epstein SK (2009b) Ineffective triggering predicts increased duration of mechanical ventilation. Crit Care Med 37:2740-2745.

Delisle S, Ouellet P, Bellemare P, Tetrault JP, Arsenault P (2011) Sleep quality in mechanically ventilated patients: comparison between NAVA and PSV modes. Annals of intensive care 1:42.

Dempsey JA, Smith CA, Blain GM, Xie A, Gong Y, Teodorescu M (2012) Role of central/peripheral chemoreceptors and their interdependence in the pathophysiology of sleep apnea. Advances in experimental medicine and biology 758:343-349.

Dernaika T, Tawk M, Nazir S, Younis W, Kinasewitz GT (2007) The significance and outcome of continuous positive airway pressure-related central sleep apnea during split-night sleep studies. Chest 132:81-87.

Devinney MJ, Huxtable AG, Nichols NL, Mitchell GS (2013) Hypoxia-induced phrenic long-term facilitation: emergent properties. Annals of the New York Academy of Sciences 1279:143-153.

Dres M, Schmidt M, Ferre A, Mayaux J, Similowski T, Demoule A (2012) Diaphragm electromyographic activity as a predictor of weaning failure. Intensive Care Med 38:2017-2025.

Dutschmann M, Paton JF (2002) Influence of nasotrigeminal afferents on medullary respiratory neurones and upper airway patency in the rat. Pflugers Archiv : European journal of physiology 444:227-235.

Eckert DJ, Jordan AS, Merchia P, Malhotra A (2007) Central sleep apnea: Pathophysiology and treatment. Chest 131:595-607.

El-Bohy AA, Schrimsher GW, Reier PJ, Goshgarian HG (1998) Quantitative assessment of respiratory function following contusion injury of the cervical spinal cord. Exp Neurol 150:143-152.

Epstein SK (2009) Weaning from ventilatory support. Curr Opin Crit Care 15:36-43.

Epstein SK (2011) How often does patient-ventilator asynchrony occur and what are the consequences? Respir Care 56:25-38.

Esteban A, Frutos F, Tobin MJ, Alía I, Solsona JF, Valverdú I, Fernández R, de la Cal MA, Benito S, Tomás R (1995) A comparison of four methods of weaning patients from mechanical ventilation. Spanish Lung Failure Collaborative Group. N Engl J Med 332:345-350.

Esteban A, Anzueto A, Alía I, Gordo F, Apezteguía C, Pálizas F, Cide D, Goldwaser R, Soto L, Bugedo G, Rodrigo C, Pimentel J, Raimondi G, Tobin MJ (2000) How is mechanical ventilation employed in the intensive care unit? An international utilization review. Am J Respir Crit Care Med 161:1450-1458.

Esteban A et al. (2008) Evolution of mechanical ventilation in response to clinical research. Am J Respir Crit Care Med 177:170-177.

Feldman JL, Mitchell GS, Nattie EE (2003) Breathing: rhythmicity, plasticity, chemosensitivity. Annu Rev Neurosci 26:239-266.

Ferguson AR, Christensen RN, Gensel JC, Miller BA, Sun F, Beattie EC, Bresnahan JC, Beattie MS (2008) Cell death after spinal cord injury is exacerbated by rapid TNF alpha-induced trafficking of GluR2-lacking AMPARs to the plasma membrane. J Neurosci 28:11391-11400.

Ferguson KA, Strong MJ, Ahmad D, George CF (1996) Sleep-disordered breathing in amyotrophic lateral sclerosis. Chest 110:664-669.

Feroah TR, Forster HV, Pan L, Wenninger J, Martino P, Rice T (2001) Effect of slow wave and REM sleep on thyropharyngeus and stylopharyngeus activity during induced central apneas. Respir Physiol 124:129-140.

Fleming PJ, Cade D, Bryan MH, Bryan AC (1980) Congenital central hypoventilation and sleep state. Pediatrics 66:425-428.

Fregosi RF, Mitchell GS (1994) Long-term facilitation of inspiratory intercostal nerve activity following carotid sinus nerve stimulation in cats. J Physiol 477 (Pt 3):469-479.

Fuller DD, Johnson SM, Olson EB, Mitchell GS (2003) Synaptic pathways to phrenic motoneurons are enhanced by chronic intermittent hypoxia after cervical spinal cord injury. J Neurosci 23:2993-3000.

Fuller DD, Golder FJ, Olson EB, Mitchell GS (2006) Recovery of phrenic activity and ventilation after cervical spinal hemisection in rats. J Appl Physiol 100:800-806.

Fuller DD, Doperalski NJ, Dougherty BJ, Sandhu MS, Bolser DC, Reier PJ (2008) Modest spontaneous recovery of ventilation following chronic high cervical hemisection in rats. Exp Neurol 211:97-106.

Gaig C, Iranzo A (2012) Sleep-disordered breathing in neurodegenerative diseases. Curr Neurol Neurosci Rep 12:205-217.

Gaultier C, Gallego J (2005) Development of respiratory control: evolving concepts and perspectives. Respir Physiol Neurobiol 149:3-15.

Glass GA, Josephs KA, Ahlskog JE (2006) Respiratory insufficiency as the primary presenting symptom of multiple-system atrophy. Arch Neurol 63:978-981.

Glotzbach SF, Baldwin RB, Lederer NE, Tansey PA, Ariagno RL (1989) Periodic breathing in preterm infants: incidence and characteristics. Pediatrics 84:785-792.

Golder FJ, Mitchell GS (2005) Spinal synaptic enhancement with acute intermittent hypoxia improves respiratory function after chronic cervical spinal cord injury. J Neurosci 25:2925-2932.

Golder FJ, Fuller DD, Lovett-Barr MR, Vinit S, Resnick DK, Mitchell GS (2011) Breathing patterns after mid-cervical spinal contusion in rats. Exp Neurol 231:97-103.

Gooden BA (1994) Mechanism of the human diving response. Integrative physiological and behavioral science : the official journal of the Pavlovian Society 29:6-16.

Goridis C, Dubreuil V, Thoby-Brisson M, Fortin G, Brunet JF (2010) Phox2b, congenital central hypoventilation syndrome and the control of respiration. Seminars in cell & developmental biology 21:814-822.

Gorini M, Corrado A, Aito S, Ginanni R, Villella G, Lucchesi G, De Paola E (2000) Ventilatory and respiratory muscle responses to hypercapnia in patients with paraplegia. Am J Respir Crit Care Med 162:203-208.

Goshgarian HG (2003) The crossed phrenic phenomenon: a model for plasticity in the respiratory pathways following spinal cord injury. J Appl Physiol 94:795-810.

Goshgarian HG (2009) The crossed phrenic phenomenon and recovery of function following spinal cord injury. Respir Physiol Neurobiol 169:85-93.

Grunstein RR, Ho KY, Sullivan CE (1991) Sleep apnea in acromegaly. Annals of internal medicine 115:527-532.

Guenther CH, Windelborn JA, Tubon TC, Jr., Yin JC, Mitchell GS (2012) Increased atypical PKC expression and activity in the phrenic motor nucleus following cervical spinal injury. Exp Neurol 234:513-520.

Hall MJ, Xie A, Rutherford R, Ando S, Floras JS, Bradley TD (1996) Cycle length of periodic breathing in patients with and without heart failure. Am J Respir Crit Care Med 154:376-381.

Hausmann ON (2003) Post-traumatic inflammation following spinal cord injury. Spinal cord 41:369-378.

Henderson-Smart DJ (1981) The effect of gestational age on the incidence and duration of recurrent apnoea in newborn babies. Australian paediatric journal 17:273-276.

Henke KG, Arias A, Skatrud JB, Dempsey JA (1988) Inhibition of inspiratory muscle activity during sleep. Chemical and nonchemical influences. Am Rev Respir Dis 138:8-15.

Hensbergen E, Kernell D (1997) Daily durations of spontaneous activity in cat's ankle muscles. Exp Brain Res 115:325-332.

Herrera M, Blasco J, Venegas J, Barba R, Doblas A, Marquez E (1985) Mouth occlusion pressure (P0.1) in acute respiratory failure. Intensive Care Med 11:134-139.

Hilbert G, Gruson D, Portel L, Vargas F, Gbikpi-Benissan G, Cardinaud JP (1998) Airway occlusion pressure at 0.1 s (P0.1) after extubation: an early indicator of postextubation hypercapnic respiratory insufficiency. Intensive Care Med 24:1277-1282.

Hoppenbrouwers T, Hodgman JE, Harper RM, Hofmann E, Sterman MB, McGinty DJ (1977) Polygraphic studies of normal infants during the first six months of life: III. Incidence of apnea and periodic breathing. Pediatrics 60:418-425.

Javaheri S (2010) Central sleep apnea. Clin Chest Med 31:235-248.

Javaheri S, Dempsey JA (2013) Central sleep apnea. Compr Physiol 3:141-163.

Javaheri S, Parker TJ, Liming JD, Corbett WS, Nishiyama H, Wexler L, Roselle GA (1998) Sleep apnea in 81 ambulatory male patients with stable heart failure. Types and their prevalences, consequences, and presentations. Circulation 97:2154-2159.

Johnson SM, Mitchell GS (2002) Activity-dependent plasticity in descending synaptic inputs to respiratory spinal motoneurons. Respir Physiol Neurobiol 131:79-90.

Jubran A, Tobin MJ (1997a) Pathophysiologic basis of acute respiratory distress in patients who fail a trial of weaning from mechanical ventilation. Am J Respir Crit Care Med 155:906-915.

Jubran A, Tobin MJ (1997b) Passive mechanics of lung and chest wall in patients who failed or succeeded in trials of weaning. Am J Respir Crit Care Med 155:916-921.

Kahn A, Blum D, Engelman E, Waterschoot P (1982) Effects of central apneas on transcutaneous PO2 in control subjects, siblings of victims of sudden infant death syndrome, and near miss infants. Pediatrics 69:413-418.

Katz DM, Dutschmann M, Ramirez JM, Hilaire G (2009) Breathing disorders in Rett syndrome: progressive neurochemical dysfunction in the respiratory network after birth. Respir Physiol Neurobiol 168:101-108.

Kelly DH, Stellwagen LM, Kaitz E, Shannon DC (1985) Apnea and periodic breathing in normal full-term infants during the first twelve months. Pediatric pulmonology 1:215-219.

Kohler M, Kriemler S, Wilhelm EM, Brunner-LaRocca H, Zehnder M, Bloch KE (2008) Children at high altitude have less nocturnal periodic breathing than adults. The European respiratory journal 32:189-197.

Kondili E, Xirouchaki N, Georgopoulos D (2007) Modulation and treatment of patient-ventilator dyssynchrony. Curr Opin Crit Care 13:84-89.

Kong FJ, Berger AJ (1986) Firing properties and hypercapnic responses of single phrenic motor axons in the rat. J Appl Physiol 61:1999-2004.

Labanowski M, Schmidt-Nowara W, Guilleminault C (1996) Sleep and neuromuscular disease: frequency of sleep-disordered breathing in a neuromuscular disease clinic population. Neurology 47:1173-1180.

Laghi F (2005) Assessment of respiratory output in mechanically ventilated patients. Respiratory care clinics of North America 11:173-199.

Lane MA, Lee KZ, Fuller DD, Reier PJ (2009) Spinal circuitry and respiratory recovery following spinal cord injury. Respir Physiol Neurobiol 169:123-132.

Lane MA, Lee KZ, Salazar K, O'Steen BE, Bloom DC, Fuller DD, Reier PJ (2012) Respiratory function following bilateral mid-cervical contusion injury in the adult rat. Exp Neurol 235:197-210.

Lehman S, Antic NA, Thompson C, Catcheside PG, Mercer J, McEvoy RD (2007) Central sleep apnea on commencement of continuous positive airway pressure in patients with a primary diagnosis of obstructive sleep apnea-hypopnea. Journal of clinical sleep medicine : JCSM : official publication of the American Academy of Sleep Medicine 3:462-466.

Leung RS, Comondore VR, Ryan CM, Stevens D (2012) Mechanisms of sleep-disordered breathing: causes and consequences. Pflugers Archiv : European journal of physiology 463:213-230.

Liu L, Liu H, Yang Y, Huang Y, Liu S, Beck J, Slutsky AS, Sinderby C, Qiu H (2012) Neuroventilatory efficiency and extubation readiness in critically ill patients. Critical care 16:R143.

MacDonald M, Fang J, Pittman SD, White DP, Malhotra A (2008) The current prevalence of sleep disordered breathing in congestive heart failure patients treated with beta-blockers. Journal of clinical sleep medicine : JCSM : official publication of the American Academy of Sleep Medicine 4:38-42.

Mahamed S, Mitchell GS (2008) Simulated apnoeas induce serotonin-dependent respiratory long-term facilitation in rats. J Physiol 586:2171-2181.

Mahamed S, Strey KA, Mitchell GS, Baker-Herman TL (2011) Reduced respiratory neural activity elicits phrenic motor facilitation. Respir Physiol Neurobiol 175:303-309.

Malhotra A, Owens RL (2010) What is central sleep apnea? Respir Care 55:1168-1178.

Mantilla CB, Rowley KL, Zhan WZ, Fahim MA, Sieck GC (2007) Synaptic vesicle pools at diaphragm neuromuscular junctions vary with motoneuron soma, not axon terminal, inactivity. Neuroscience 146:178-189.

Marcus CL, Omlin KJ, Basinki DJ, Bailey SL, Rachal AB, Von Pechmann WS, Keens TG, Ward SL (1992) Normal polysomnographic values for children and adolescents. Am Rev Respir Dis 146:1235-1239.

Martin RJ, Abu-Shaweesh JM, Baird TM (2004) Apnoea of prematurity. Paediatric respiratory reviews 5 Suppl A:S377-382.

Martin RJ, Dreshaj IA, Miller MJ, Haxhiu MA (1994) Hypoglossal and phrenic responses to central respiratory inhibition in piglets. Respir Physiol 97:93-103.

McClung JM, Kavazis AN, DeRuisseau KC, Falk DJ, Deering MA, Lee Y, Sugiura T, Powers SK (2007) Caspase-3 regulation of diaphragm myonuclear domain during mechanical ventilation-induced atrophy. Am J Respir Crit Care Med 175:150-159.

McCrimmon DR, Zuperku EJ, Hayashi F, Dogas Z, Hinrichsen CF, Stuth EA, Tonkovic-Capin M, Krolo M, Hopp FA (1997) Modulation of the synaptic drive to respiratory premotor and motor neurons. Respir Physiol 110:161-176.

McKinley WO (1996) Late return of diaphragm function in a ventilator-dependent patient with a high cervical tetraplegia: case report, and interactive review. Spinal cord 34:626-629.

Mellott KG, Grap MJ, Munro CL, Sessler CN, Wetzel PA (2009) Patient-ventilator dyssynchrony: clinical significance and implications for practice. Critical care nurse 29:41-55 quiz 41 p following 55.

Meza S, Giannouli E, Younes M (1998) Control of breathing during sleep assessed by proportional assist ventilation. J Appl Physiol 84:3-12.

Millman RP, Bevilacqua J, Peterson DD, Pack AI (1983) Central sleep apnea in hypothyroidism. Am Rev Respir Dis 127:504-507.

Mitchell GS, Johnson SM (2003) Neuroplasticity in respiratory motor control. J Appl Physiol 94:358-374.

Morgenthaler TI, Kagramanov V, Hanak V, Decker PA (2006) Complex sleep apnea syndrome: is it a unique clinical syndrome? Sleep 29:1203-1209.

Nemer SN, Barbas CS, Caldeira JB, Carias TC, Santos RG, Almeida LC, Azeredo LM, Noe RA, Guimaraes BS, Souza PC (2009) A new integrative weaning index of discontinuation from mechanical ventilation. Critical care 13:R152.

Ng DK, Chan CH (2013) A review of normal values of infant sleep polysomnography. Pediatrics and neonatology 54:82-87.

Nichols NL, Dale EA, Mitchell GS (2012) Severe acute intermittent hypoxia elicits phrenic long-term facilitation by a novel adenosine-dependent mechanism. J Appl Physiol 112:1678-1688.

NSCISC (2013) Spinal cord injury facts and figures at a glance. J Spinal Cord Med 36:1-2.

Nussbaumer-Ochsner Y, Ursprung J, Siebenmann C, Maggiorini M, Bloch KE (2012) Effect of short-term acclimatization to high altitude on sleep and nocturnal breathing. Sleep 35:419-423.

Oo T, Watt JW, Soni BM, Sett PK (1999) Delayed diaphragm recovery in 12 patients after high cervical spinal cord injury. A retrospective review of the diaphragm status of 107 patients ventilated after acute spinal cord injury. Spinal cord 37:117-122.

Pack AI (2011) Central sleep apnea. Handbook of clinical neurology 98:411-419.

Pagliardini S, Greer JJ, Funk GD, Dickson CT (2012) State-dependent modulation of breathing in urethane-anesthetized rats. J Neurosci 32:11259-11270.

Parthasarathy S, Tobin MJ (2002) Effect of ventilator mode on sleep quality in critically ill patients. Am J Respir Crit Care Med 166:1423-1429.

Peng YJ, Overholt JL, Kline D, Kumar GK, Prabhakar NR (2003) Induction of sensory long-term facilitation in the carotid body by intermittent hypoxia: implications for recurrent apneas. Proc Natl Acad Sci U S A 100:10073-10078.

Perrigault PF, Pouzeratte YH, Jaber S, Capdevila XJ, Hayot M, Boccara G, Ramonatxo M, Colson P (1999) Changes in occlusion pressure (P0.1) and breathing pattern during pressure support ventilation. Thorax 54:119-123.

Pierson DJ (2011) Patient-ventilator interaction. Respir Care 56:214-228.

Pineau I, Lacroix S (2007) Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved. J Comp Neurol 500:267-285.

Powers SK, Kavazis AN, Levine S (2009) Prolonged mechanical ventilation alters diaphragmatic structure and function. Crit Care Med 37:S347-353.

Prakash YS, Miyata H, Zhan WZ, Sieck GC (1999) Inactivity-induced remodeling of neuromuscular junctions in rat diaphragmatic muscle. Muscle & nerve 22:307-319.

Putensen C, Muders T, Varelmann D, Wrigge H (2006) The impact of spontaneous breathing during mechanical ventilation. Curr Opin Crit Care 12:13-18.

Reyland ME (2009) Protein kinase C isoforms: Multi-functional regulators of cell life and death. Front Biosci 14:2386-2399.

Rosenow F, McCarthy V, Caruso AC (1998) Sleep apnoea in endocrine diseases. J Sleep Res 7:3-11.

Santos C, Braghiroli A, Mazzini L, Pratesi R, Oliveira LV, Mora G (2003) Sleep-related breathing disorders in amyotrophic lateral sclerosis. Monaldi archives for chest disease = Archivio Monaldi per le malattie del torace / Fondazione clinica del lavoro, IRCCS [and] Istituto di clinica tisiologica e malattie apparato respiratorio, Universita di Napoli, Secondo ateneo 59:160-165.

Sassoon CS, Mahutte CK (1993) Airway occlusion pressure and breathing pattern as predictors of weaning outcome. Am Rev Respir Dis 148:860-866.

Scholle S, Wiater A, Scholle HC (2011) Normative values of polysomnographic parameters in childhood and adolescence: cardiorespiratory parameters. Sleep medicine 12:988-996.

Shanely RA, Zergeroglu MA, Lennon SL, Sugiura T, Yimlamai T, Enns D, Belcastro A, Powers SK (2002) Mechanical ventilation-induced diaphragmatic atrophy is associated with oxidative injury and increased proteolytic activity. Am J Respir Crit Care Med 166:1369-1374.

Sharma H, Alilain WJ, Sadhu A, Silver J (2012) Treatments to restore respiratory function after spinal cord injury and their implications for regeneration, plasticity and adaptation. Exp Neurol 235:18-25.

Sieck DC, Zhan WZ, Fang YH, Ermilov LG, Sieck GC, Mantilla CB (2012) Structure-activity relationships in rodent diaphragm muscle fibers vs. neuromuscular junctions. Respir Physiol Neurobiol 180:88-96.

Silver JR, Lehr RP (1981) Dyspnoea during generalised spasms in tetraplegic patients. J Neurol Neurosurg Psychiatry 44:842-845.

Sin DD, Fitzgerald F, Parker JD, Newton G, Floras JS, Bradley TD (1999) Risk factors for central and obstructive sleep apnea in 450 men and women with congestive heart failure. Am J Respir Crit Care Med 160:1101-1106.

Skatrud JB, Dempsey JA (1983) Interaction of sleep state and chemical stimuli in sustaining rhythmic ventilation. J Appl Physiol 55:813-822.

Sperry MA, Goshgarian HG (1993) Ultrastructural changes in the rat phrenic nucleus developing within 2 h after cervical spinal cord hemisection. Exp Neurol 120:233-244.

Strakowski JA, Pease WS, Johnson EW (2007) Phrenic nerve stimulation in the evaluation of ventilator-dependent individuals with C4- and C5-level spinal cord injury. American journal of physical medicine & rehabilitation / Association of Academic Physiatrists 86:153-157.

Strey KA, Nichols NL, Baertsch NA, Broytman O, Baker-Herman TL (2012) Spinal atypical protein kinase C activity is necessary to stabilize inactivity-induced phrenic motor facilitation. J Neurosci 32:16510-16520.

Tadjalli A, Duffin J, Peever J (2010) Identification of a novel form of noradrenergic-dependent respiratory motor plasticity triggered by vagal feedback. J Neurosci 30:16886-16895.

Tapia IE, Karamessinis L, Bandla P, Huang J, Kelly A, Pepe M, Schultz B, Gallagher P, Brooks LJ, Marcus CL (2008) Polysomnographic values in children undergoing puberty: pediatric vs. adult respiratory rules in adolescents. Sleep 31:1737-1744.

Thille AW, Rodriguez P, Cabello B, Lellouche F, Brochard L (2006) Patient-ventilator asynchrony during assisted mechanical ventilation. Intensive Care Med 32:1515-1522.

Tkacova R, Hall MJ, Liu PP, Fitzgerald FS, Bradley TD (1997) Left ventricular volume in patients with heart failure and Cheyne-Stokes respiration during sleep. Am J Respir Crit Care Med 156:1549-1555.

Tobin MJ (2001) Advances in mechanical ventilation. N Engl J Med 344:1986-1996.

Tobin MJ, Laghi F, Brochard L (2009) Role of the respiratory muscles in acute respiratory failure of COPD: lessons from weaning failure. J Appl Physiol 107:962-970.

Trinder J, Whitworth F, Kay A, Wilkin P (1992) Respiratory instability during sleep onset. J Appl Physiol 73:2462-2469.

Turrigiano GG (2008) The self-tuning neuron: synaptic scaling of excitatory synapses. Cell 135:422-435.

Uliel S, Tauman R, Greenfeld M, Sivan Y (2004) Normal polysomnographic respiratory values in children and adolescents. Chest 125:872-878.

Vinit S, Kastner A (2009) Descending bulbospinal pathways and recovery of respiratory motor function following spinal cord injury. Respir Physiol Neurobiol 169:115-122.

Vinit S, Stamegna JC, Boulenguez P, Gauthier P, Kastner A (2007) Restorative respiratory pathways after partial cervical spinal cord injury: role of ipsilateral phrenic afferents. Eur J Neurosci 25:3551-3560.

Vinit S, Darlot F, Stamegna JC, Sanchez P, Gauthier P, Kastner A (2008) Long-term reorganization of respiratory pathways after partial cervical spinal cord injury. Eur J Neurosci 27:897-908.

Wang CX, Nuttin B, Heremans H, Dom R, Gybels J (1996) Production of tumor necrosis factor in spinal cord following traumatic injury in rats. Journal of neuroimmunology 69:151-156.

Wang H, Parker JD, Newton GE, Floras JS, Mak S, Chiu KL, Ruttanaumpawan P, Tomlinson G, Bradley TD (2007) Influence of obstructive sleep apnea on mortality in patients with heart failure. Journal of the American College of Cardiology 49:1625-1631.

Webber CL, Pleschka K (1984) Respiratory effects of high cervical cord cold blockade on efferent vagal and phrenic discharges in the rabbit. Pflugers Archiv : European journal of physiology 402:10-17.

Weese-Mayer DE, Lieske SP, Boothby CM, Kenny AS, Bennett HL, Ramirez JM (2008) Autonomic dysregulation in young girls with Rett Syndrome during nighttime in-home recordings. Pediatric pulmonology 43:1045-1060.

Weese-Mayer DE, Berry-Kravis EM, Ceccherini I, Keens TG, Loghmanee DA, Trang H, Subcommittee ATSCCHS (2010) An official ATS clinical policy statement: Congenital central hypoventilation syndrome: genetic basis, diagnosis, and management. Am J Respir Crit Care Med 181:626-644.

Whitelaw WA, Derenne JP, Milic-Emili J (1975) Occlusion pressure as a measure of respiratory center output in conscious man. Respir Physiol 23:181-199.

Xie A, Skatrud JB, Puleo DS, Rahko PS, Dempsey JA (2002) Apnea-hypopnea threshold for CO2 in patients with congestive heart failure. Am J Respir Crit Care Med 165:1245-1250.

Xie A, Bedekar A, Skatrud JB, Teodorescu M, Gong Y, Dempsey JA (2011) The heterogeneity of obstructive sleep apnea (predominant obstructive vs pure obstructive apnea). Sleep 34:745-750.

Yin HZ, Hsu CI, Yu S, Rao SD, Sorkin LS, Weiss JH (2012) TNF-alpha triggers rapid membrane insertion of Ca(2+) permeable AMPA receptors into adult motor neurons and enhances their susceptibility to slow excitotoxic injury. Exp Neurol 238:93-102.

Younes M (2006) Control of breathing during mechanical ventilation. . Mechanical Ventilation: Springer, Berlin-Heidelberg.

Yumino D, Bradley TD (2008) Central sleep apnea and Cheyne-Stokes respiration. Proc Am Thorac Soc 5:226-236.

Zhang Y, McGuire M, White DP, Ling L (2003) Episodic phrenic-inhibitory vagus nerve stimulation paradoxically induces phrenic long-term facilitation in rats. J Physiol 551:981-991.

Zhou Z, Champagnat J, Poon CS (1997) Phasic and long-term depression in brainstem nucleus tractus solitarius neurons: differing roles of AMPA receptor desensitization. J Neurosci 17:5349-5356.

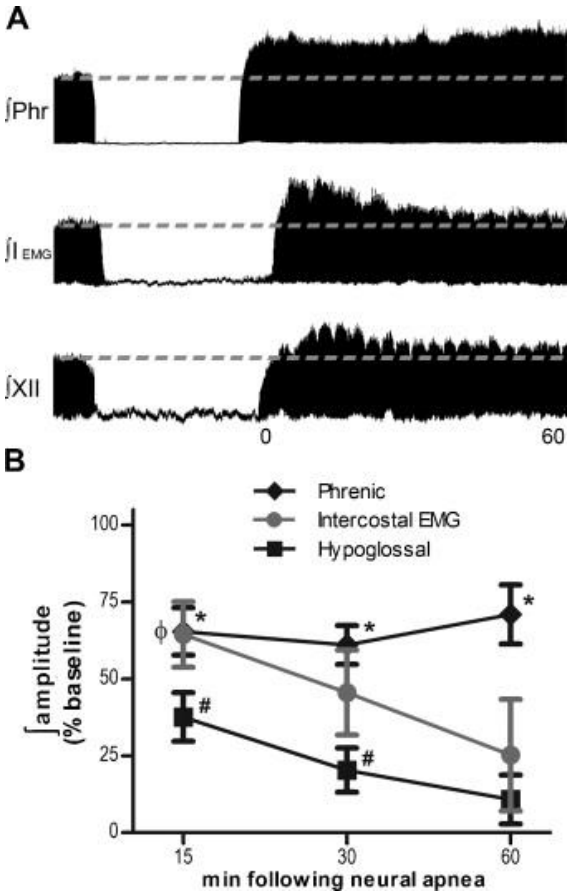

Figure 1: Differential expression of inactivity-induced respiratory plasticity. ***A.*** Representative compressed and integrated phrenic (top) and hypoglossal (bottom) neurograms and inspiratory intercostal EMG activity (middle) before, during and for 60 min following a 30 min hyperventilation-induced neural apnea, illustrating a prolonged increase in phrenic burst amplitude and a transient increase in hypoglossal burst amplitude and inspiratory intercostal EMG activity following resumption of respiratory neural activity, indicating iPMF, iHMF and iIMF, respectively. ***B.*** Average change in phrenic, hypoglossal and inspiratory intercostal EMG amplitude from baseline for 60 min following resumption of respiratory neural activity after a central neural apnea. A prolonged (>60 min) facilitation of phrenic nerve burst amplitude (diamonds) is apparent following resumption of respiratory neural activity that is significantly increased relative to phrenic time controls receiving the same surgical preparation, but no neural apnea. By contrast, hypoglossal nerve burst amplitude (squares) and intercostal EMG activity (circles) exhibit only transient (15 min) increases in inspiratory burst activity following neural apnea, relative to hypoglossal and intercostal time controls. For clarity, time controls are not shown. These data suggest that iPMF is long-lasting, whereas iHMF and iIMF are transient. * significantly increased from phrenic time controls; # significantly increased from hypoglossal time controls; Φ significantly increased from intercostal time controls ($p<0.05$).

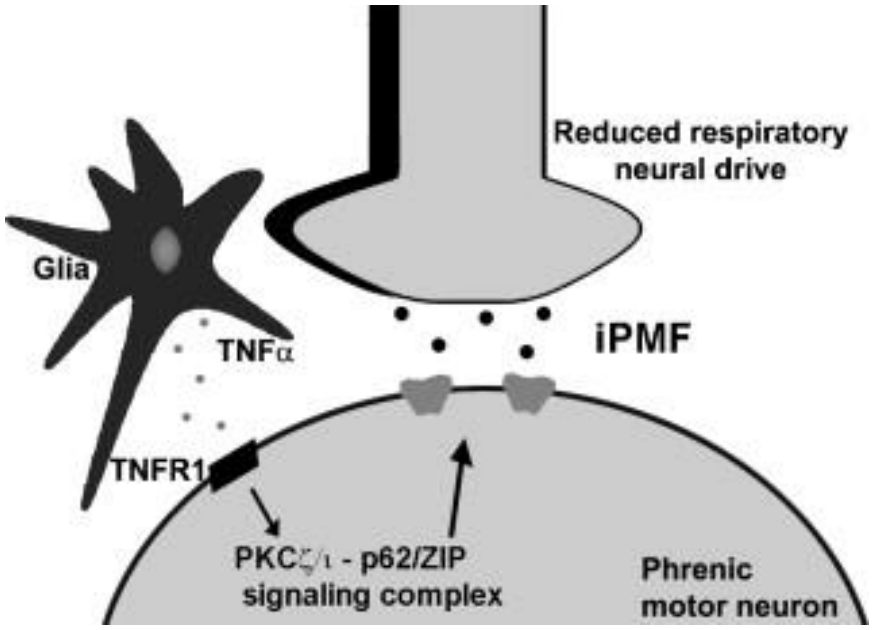

Figure 2: Current working model of iPMF. We hypothesize that local spinal mechanisms operating within the phrenic motor pool "sense" and "respond" to reduced bulbospinal respiratory inputs by local release of TNFα in/near the phrenic motor pool. Subsequent activation of TNFα receptors promotes the formation of the aPKCζ/ι-p62/ZIP signalling cassette in phrenic motor neurons. This stimulus specific signaling cascade increases the synaptic strength and induces iPMF. The pathways downstream of the aPKCζ/ι-p62/ZIP signalling cassette leading to iPMF are unknown. Similar mechanisms are proposed to occur within hypoglossal and inspiratory intercostal motor pools to give rise to iHMF and iIMF, respectively.

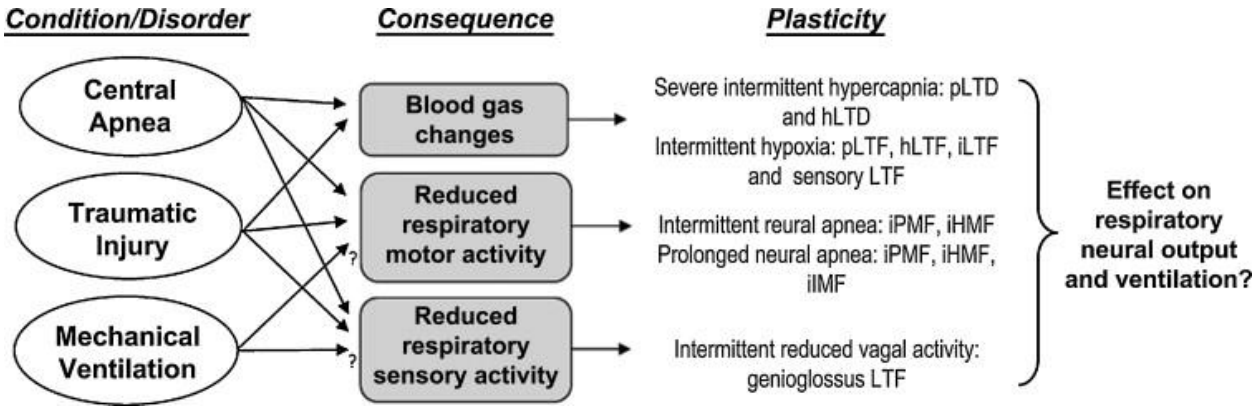

Figure 3: Reduced respiratory neural activity has the potential to elicit multiple forms of plasticity. In non-ventilated animals, central apnea results in reduced respiratory neural activity, profound changes in arterial blood gases and diminished sensory feedback. Similar effects may be observed following disruption of descending inputs to spinal motor neurons (depending on extent and location of disruption). Mechanical ventilation may be associated with reduced respiratory neural activity and/or altered sensory feedback in some patients. Animal models suggest that each of these stimuli independently elicit unique and possibly overlapping forms of plasticity. For example, acute intermittent hypoxia elicits long-term facilitation (LTF) in phrenic, hypoglossal and intercostal nerves (pLTF, hLTF and iLTF, respectively; Fregosi and Mitchell, 1994; Bach and Mitchell, 1996). Additional forms of plasticity are elicited during chronic exposures to intermittent hypoxia, specifically facilitation of carotid body afferent feedback (sensory LTF; Peng et al., 2003). Severe hypercapnia elicits long-term depression of phrenic and hypoglossal nerve activity (pLTD and hLTD; Bach and Mitchell, 1998; Baker et al., 2001). Reduced respiratory neural (motor) activity elicits long-lasting iPMF, and transient iHMF and iIMF (Baker-Herman and Strey, 2011; Mahamed et al., 2011; Baertsch and Baker-Herman, 2013), whereas reduced intermittent sensory (vagal) feedback elicits genioglossus facilitation (Tadjalli et al., 2010). To date, we lack a clear understanding regarding how these multiple forms of plasticity interact to shape necessary long-term adaptations in the respiratory control system.