Intermittent hypoxia-induced phrenic motor plasticity: role of protein kinase Cθ and mechanisms of pattern sensitivity

By

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Chapter I

Introduction

Michael J Devinney

Respiratory control has historically been viewed as fixed and immutable, primarily responding to chemosensory feedback (reviewed in Mitchell & Johnson, 2003). Negative feedback has a strong influence on many physiological systems; however, systems governed only by negative feedback are often unstable due to overreaching reflex gain, leading to catastrophic failure in specific circumstances (Doyle & Csete, 2011). Fortunately, respiratory control may represent a more complex control system. For example, during exercise ($\uparrow O_2$ demand and CO_2 production), respiratory control increases ventilation to <u>prevent</u> partial pressure of arterial O_2 (PaO_2) from decreasing and P_{aCO_2} from increasing (Forster *et al.*, 2012). Thus, chemosensory feedback signals do not drive exercise ventilation in normal adults. One possibility is that central neural responses (ie. central command) effectively shape ventilatory responses to activities (eg. exercise) or perturbations, only relying on feedback signals in extreme circumstances (Forster *et al.*, 2012).

Plasticity is one feature of respiratory control that can alter respiratory output independent of immediate chemosensory feedback (Mitchell & Johnson, 2003). Thus, plasticity may play a role in shaping ventilatory responses throughout life, providing a crucial means to adjust respiratory motor output in the face of changing conditions such as obesity, pregnancy, disease (eg. spinal cord injury) or hostile environments (eg. high altitude) (Mitchell *et al.*, 2001). The role of plasticity in stabilizing breathing is debated (Mahamed & Mitchell, 2008*a*; Mateika & Narwani, 2009); however, our understanding of plasticity in respiratory control is expanding at rapid pace. Plasticity is demonstrated at the neuromuscular (Rowley *et al.*, 2005), chemoreceptor (Prabhakar, 2011), spinal (Dale-Nagle *et al.*, 2010*a*), and brainstem levels of respiratory control (Morris *et al.*, 2003). Despite its potential therapeutic applications and significance in respiratory control, our understanding of cellular and molecular mechanisms of plasticity in respiratory control is incomplete.

This dissertation focuses on a well-known model of spinal, respiratory neuroplasticity termed phrenic long-term facilitation (pLTF). pLTF is characterized by a long-lasting increase in phrenic burst amplitude (phrenic motor facilitation; pMF, general term that includes pLTF) following exposure to modest (PaO_2 35-45 mmHg) acute intermittent hypoxia (AIH; 3, 5 min episodes separated by 5 min). Induction of pLTF does not require increased phrenic nerve activity, but instead requires episodic activation of serotonin type 2 (5-HT2) receptors (Fuller *et al.*, 2001; Baker-Herman & Mitchell, 2002; MacFarlane & Mitchell, 2009), which are abundantly found on phrenic motor neurons (Fuller *et al.*, 2005). Acute sustained hypoxia (25 min PaO_2 35-45 mmHg) does not elicit pLTF; thus pLTF is a form of pattern-sensitive spinal plasticity.

Chapters II and III of this thesis focus on canonical signaling mechanisms downstream from 5-HT$_2$ receptor activation, specifically the necessity and sufficiency of protein kinase C activation for expression of pLTF. A novel technique, intrapleural delivery of small interfering RNA, is for targeted knockdown of protein kinase C in phrenic motor neurons. This technique could help us understand the cellular specificity of respiratory motor plasticity and is a potential therapy for restoring respiratory motor function.

Chapter IV of this thesis focuses on mechanisms that underlie a hallmark feature of pLTF, specifically hypoxic pattern sensitivity. I hypothesize that pattern sensitivity is an emergent property of interactions between competing pathways, each capable of giving rise to pLTF. Recently, multiple, separate cellular cascades leading to phrenic motor facilitation were discovered. In particular, pharmacological activation of spinal adenosine 2A (A2A) receptors elicits phrenic motor facilitation (Golder *et al.*, 2008). However, selective inhibition of these receptors during modest AIH enhances pLTF (Hoffman *et al.*, 2010), suggesting that this A2A-dependent pathway constrains 5-HT2-dependent pLTF. In contrast, more severe hypoxic episodes (ie. severe AIH; PaO_2 25-35 mmHg) elicits a 5-HT2-independent, A2A-dependent pLTF (Nichols *et al.*, 2012). Thus, during hypoxia, both 5-HT2 and A2A-dependent pathways can give

rise to plasticity, but may also constrain each other such that one is dominantly activated to cause pLTF. The dominance of one pathway over the other may depend on the pattern or severity of the hypoxic stimulus. In chapter IV, I present evidence that cross-talk inhibitory interactions between the 5-HT$_2$ and A$_{2A}$-dependent pathways to pLTF confer pattern sensitivity to pLTF induced by moderate hypoxia.

Background

Neuroplasticity

Plasticity is defined as a persistent change in the neural control system based on prior experience (Mitchell & Johnson, 2003). Examples of stimuli include hypoxia, neural activity, ischemia, and pathology, etc. Importantly, the persistent change outlasts the stimulus; there is a functional long-term change in neural output following the experience. Plasticity appears to be ubiquitous within the CNS, occurring throughout the brain and spinal cord and ranging from cellular changes (ie. synaptic plasticity) to large scale remodeling (ie. cortical reorganization following injury; Ward, 2011). In this thesis, we will focus on cellular/synaptic mechanisms of plasticity.

Initial studies on plasticity demonstrated increases in synaptic transmission and firing in hippocampal regions following repetitive high frequency electrical stimulation (Bliss & Lomo, 1973; hippocampal long-term potentiation, LTP). Since then, many studies have demonstrated the complexity of molecular mechanisms underlying activity-dependent plasticity throughout the CNS (Bliss & Cooke, 2011). Stimulus pattern strongly influences expression of plasticity, as well as its dependence on signaling pathways (Lynch *et al.*, 2013; Philips *et al.*, 2013). For example, induction of hippocampal long-term potentiation in the CA1 region of the hippocampus with high frequency stimulation requires transcription while induction with theta burst stimulation does not

(Huang & Kandel, 2005). Moreover, continuous stimulation protocols fail to elicit significant plasticity (Kauer, 1999; Nguyen *et al.*, 2000; Scharf *et al.*, 2002). Such pattern sensitivity is a hallmark of neuroplasticity and will be discussed throughout this thesis.

Plasticity can also be induced by neuromodulators, such as norepinephrine (Walling & Harley, 2004), dopamine (Krishnan *et al.*, 2011), and serotonin (Mauelshagen *et al.*, 1998; MacFarlane & Mitchell, 2009). These forms of plasticity do not primarily require increased activity, but depend on neuromodulator receptor activation. Facilitation of the sensorimotor synapse in *Aplysia* is a well-studied model of serotonin-induced, pattern-sensitive plasticity. Spaced tail stimulations elicit serotonin-dependent long-term facilitation at the sensorimotor synapse while massed tail stimulations fail (Sutton *et al.*, 2002). Similarly, spaced serotonin applications elicit sensorimotor long-term facilitation while massed applications also fail (Mauelshagen *et al.*, 1998). Thus, neuromodulator-induced, activity-independent plasticity is also sensitive to pattern of stimulation.

Respiratory Neuroplasticity

Plasticity in neural control of breathing occurs at the neuromuscular junction (Rowley *et al.*, 2005), carotid body (Prabhakar, 2011), spinal cord (Dale-Nagle *et al.*, 2010*b*), and brainstem levels of control (Morris *et al.*, 2003). Distinct stimuli can elicit plasticity in respiratory control, including carotid sinus nerve stimulation (Millhorn *et al.*, 1980*a*, 1980*b*), developmental stress (Gulemetova *et al.*, 2013), exercise (Martin & Mitchell, 1993; Babb *et al.*, 2010), neural injury (Goshgarian, 2009), hypoxia (Bach & Mitchell, 1996; Nichols *et al.*, 2012), hypercapnia (Bach & Mitchell, 1998), and reduced neural activity (Mahamed *et al.*, 2011). Despite our ever-increasing knowledge of plasticity in respiratory control, its role in normal breathing or in diseases which compromise breathing (eg. spinal cord injury, ALS, or sleep apnea) is unknown (Mitchell & Johnson, 2003; Mahamed & Mitchell, 2007; Mateika & Syed, 2013). In any case, inducing

plasticity in respiratory control may represent a potential therapy for disorders which compromise breathing.

Hypoxia induces respiratory neuroplasticity

Hypoxia-induced respiratory plasticity is a well-studied form of plasticity that has several advantages, including well-characterized phases of response, quantifiable severity and duration, pathophysiological relevance, and potential therapeutic applications (Powell *et al.*, 1998; Mitchell & Johnson, 2003).

Time domains of hypoxic ventilatory responses

Hypoxic ventilatory responses differ depending on pattern, duration, and severity of exposure. Ventilatory responses to hypoxia are separated into three distinct time-domains – the acute response, short-term potentiation, post-hypoxia frequency decline, (Powell *et al.*, 1998). The acute response is the hyperbolic increase in ventilatory activity (variably respiratory frequency vs. tidal volume component) at hypoxia onset, which mirrors chemoreceptor stimulation (Bisgard & Neubauer, 1995). The acute response also includes termination of the response to hypoxia after restoration to normoxia. Short-term potentiation is the progressive increase in ventilation (mostly tidal volume) on the order of seconds following the acute response. Short-term potentiation of the hypoxic ventilatory response is a form of plasticity and can be specific to motor pools (hypoglossal vs phrenic; Jiang *et al.*, 1991), suggesting involvement of local mechanisms. It may add stability to respiratory control, allowing for hypoxia-induced increases in ventilation independent from chemoreflexes (Eldridge & Millhorn, 1986). Post-hypoxia frequency decline is the temporary overshoot or undershoot of respiratory frequency at onset or termination of hypoxia (Powell *et al.*, 1998).

If exposure to hypoxia is sustained beyond a few minutes, hypoxic ventilatory decline and ventilatory acclimatization are observed. Hypoxic ventilatory decline is a decrease in ventilation (primarily tidal volume) that occurs when moderate hypoxia lasts for 5-30 minutes, and is independent of arterial CO_2 changes or decreases in metabolism during the hypoxic stimulus (Bisgard & Neubauer, 1995; Powell *et al.*, 1998). The decline in ventilation persists throughout chronic hypoxia lasting hours to days, such as high altitude exposure (Sato *et al.*, 1992). Hypoxic ventilatory decline is thought to involve changes in both O_2 chemosensitivity and central respiratory drive and may be important for energy conservation during prolonged hypoxia (Neubauer *et al.*, 1990; Sato *et al.*, 1992; Robbins, 1995). Ventilatory acclimatization to hypoxia is an increase in ventilation during sustained hypoxic exposure lasting several hours to months (Bisgard & Neubauer, 1995). It is most often noted in humans traveling to high altitude and is a compensatory mechanism to increase O_2 delivery to tissues.   Although accompanying decreases in blood CO_2 and increases in blood pH may contribute to the development of ventilatory acclimatization, 8 h of isocapnic hypoxia elicits ventilatory acclimatization in humans (Howard & Robbins, 1995). Changes in carotid body O_2 chemosensitivity likely account for ventilatory acclimatization, although changes in processing of chemoreceptor afferent input could also play a role (Powell *et al.*, 1998). After restoration to normoxic conditions, there is persistent hyperventilation lasting hours to days; thus, ventilatory acclimatization to hypoxia is a form of plasticity, since its effects outlast the stimulus.

Intermittent exposures to hypoxia reveal two separate responses, progressive augmentation of the hypoxic response, and long-term facilitation. Progressive augmentation is the progressive increase in hypoxic ventilatory response in subsequent hypoxic exposures and is frequently reported in anesthetized preparations while recording respiratory nerve output, such as intercostal nerves during carotid sinus nerve stimulation (Fregosi & Mitchell, 1994), or phrenic nerve during exposure to severe AIH (Nichols *et al.*, 2012). The mechanism of progressive

augmentation is unknown but is unlikely to be serotonin-dependent, since it is not blocked by intravenous or intrathecal methysergide (Fregosi & Mitchell, 1994; Nichols *et al.*, 2012). Long-term facilitation refers to an increase in ventilation or respiratory motor output in minutes to hours following repeated bouts of hypoxia (ie. AIH) or episodic carotid body stimulation (Millhorn *et al.*, 1980*a*, 1980*b*). The increase in ventilation occurs after hypoxia or stimulation, when conditions have returned to baseline. Thus, long-term facilitation is an example of plasticity in respiratory motor output and ventilation. Long-term facilitation, except when induced by severe hypoxia, is dependent on activation of spinal 5-HT$_2$ receptors (Bach & Mitchell, 1998; Fuller *et al.*, 2001; Baker-Herman & Mitchell, 2002), suggesting that chemoafferent feedback during episodic hypoxia stimulates episodic release of serotonin from the raphe nucleus. Mechanisms underlying long-term facilitation of phrenic motor output will be the focus of this thesis.

Anatomical components of the hypoxic response

The carotid bodies are the peripheral chemoreceptors that primarily detect arterial O$_2$ and initiate the hypoxic response. Type-I cells (glomus cells) depolarize when P$_{aO_2}$ decreases, which stimulates neurotransmitter release and activation of sensory neurons in the petrosal ganglion (Lahiri *et al.*, 2006). These same petrosal neurons project to the caudal nucleus of the solitary tract (NTS) in the medulla for processing. NTS projects to other respiratory centers in the brainstem - the dorsal and ventral respiratory groups (Alheid *et al.*, 2011), which influence amplitude of motor output (ie. tidal volume) and respiratory frequency. Pre-motor neurons residing in the ventral respiratory group in the medulla can project directly to motor neurons within the spinal cord or brainstem which innervate respiratory pump muscle or muscles controlling upper airway patency, respectively (Feldman *et al.*, 1985; Yamada *et al.*, 1988).

Hypoxia also activates raphé nuclei

The NTS also projects to serotonergic neurons found within raphé nuclei within the brainstem (Ruggiero *et al.*, 2000). Caudal raphe nuclei, have serotoninergic projections found diffusely throughout the brainstem and spinal cord (Leanza *et al.*, 1995). During hypoxia or carotid sinus nerve stimulation, increased c-FOS expression is detected in the NTS and raphe nuclei, suggesting that these neuroanatomical pathways are activated (Erickson & Millhorn, 1991, 1994). Thus, activation of raphe neurons during hypoxia likely is responsible for serotonin release in the vicinity of respiratory motor neurons, which elicits serotonin-dependent pLTF. In support of this, increased serotonin can be detected in the phrenic motor nucleus during hypoxia (Kinkead *et al.*, 2001), and electrical activation of the raphé obscurus elicits pLTF in cats (Millhorn, 1986).

Respiratory long-term facilitation

Respiratory long-term facilitation (LTF) is a general term referring to all forms of long-term facilitation found within respiratory control, including pLTF (the focus of this thesis), ventilatory LTF, LTF of respiratory muscle EMG, and LTF in other respiratory motor pools such as hypoglossal or intercostal nerves. The mechanisms giving rise to each form of LTF are likely related; for example, pLTF mechanisms likely play a role in ventilatory LTF and diaphragmatic EMG LTF, since enhanced phrenic output could influence ventilation as well as diaphragm activity. There might be slight differences between mechanisms due to different model systems, patterns of hypoxia, contributing cellular cascades, or anatomical differences (phrenic vs. hypoglossal nerves; Baker-Herman & Strey, 2011). However, one commonality between these distinct models of long-term facilitation is their induction with various protocols of intermittent

<u>hypoxia</u>. Although sustained hypoxia can elicit LTF in specific circumstances (ie. severe ASH, chapter IV), respiratory LTF is generally pattern-sensitive.

Ventilatory long-term facilitation

Ventilatory long-term facilitation (vLTF) is an increase in ventilation following exposure to hypoxia, usually intermittent hypoxia. It was first recognized in goats exposed to ten 3-minute episodes of isocapnic hypoxia (P_aO_2 47 mmHg) separated by 5 min of normoxia (Turner & Mitchell, 1997). In contrast, 30 min or 4 h of isocapnic sustained hypoxia (ASH) failed to elicit long-term facilitation in a separate study (Dwinell *et al.*, 1997), indicating that vLTF is likely pattern-sensitive. Subsequently, vLTF was demonstrated in rats and humans (Morgan *et al.*, 1995; McEvoy *et al.*, 1996, 1997; Olson *et al.*, 2001; Xie *et al.*, 2001; Tamisier *et al.*, 2005; Querido *et al.*, 2010; Nakamura *et al.*, 2010). However, vLTF is not expressed in humans during wakefulness, unless CO_2 is elevated during intermittent hypoxia exposure (Harris *et al.*, 2006). Interestingly, elevation of CO_2 during acute sustained hypoxia (30 min) reveals vLTF in humans, suggesting that increased CO_2 also alleviates constraints of pattern sensitivity (Griffin *et al.*, 2012). vLTF in rats requires 5-HT2 receptor activation, suggesting shared mechanisms with pLTF. However it is unknown whether vLTF mechanisms are identical to pLTF, or if vLTF is positively or negatively associated with disease states that have intermittent hypoxia as a feature, such as sleep apnea (Mitchell & Terada, 2011; Mateika & Syed, 2013). For example, increased respiration following vLTF exposure may cause breathing instability by increasing gain of the chemoreflex; however this could be offset by decreasing apneic threshold to CO_2 (Mahamed & Mitchell, 2008*a*). Moreover, LTF of upper airway motor pools including hypoglossal nerves would promote upper airway patency and prevent collapse of the airway normally seen in the majority of sleep apnea patients (Mahamed & Mitchell, 2007, 2008*a*,

2008*b*). However, hypoglossal LTF is more variably expressed among rat strains than pLTF (Baker-Herman *et al.*, 2010), suggesting that it may not be robustly expressed in humans.

Non-phrenic respiratory motor pools

LTF in intercostal and hypoglossal motor pools can be elicited by similar stimuli. For example, carotid sinus nerve stimulation elicits serotonin-dependent LTF which was greater in internal intercostal nerves compared to phrenic in anesthetized cats (Fregosi & Mitchell, 1994). Intercostal LTF can also be elicited in an in vitro neonatal brainstem-spinal cord preparation by application of serotonin. Interestingly, while LTF in phrenic motor output required episodic serotonin bath application, LTF of intercostal motor output could be induced by continuous bath application (Lovett-Barr *et al.*, 2006). This suggests that differences in pattern sensitivity may exist between respiratory motor pools. Hypoglossal LTF is variably expressed and depends on rat strain, substrain, age, and sex to a greater degree than pLTF (Baker-Herman *et al.*, 2010; Baker-Herman & Strey, 2011). Systemic serotonin receptor blockade prevents expression of hypoglossal LTF, suggesting that similar mechanisms exist between hypoglossal and phrenic LTF, although much less is known about cellular mechanisms of hypoglossal LTF (Bach & Mitchell, 1996; Baker-Herman & Strey, 2011). Hypoglossal LTF is similarly pattern-sensitive compared to pLTF (Appendix A; Wilkerson et al, in preparation).

Phrenic long-term facilitation (pLTF)

pLTF was first discovered as an increase in phrenic motor output lasting up to 90 min following episodic stimulation of carotid body afferents or carotid sinus nerve in anesthetized, paralyzed and ventilated cats (Millhorn *et al.*, 1980*a*, 1980*b*). Subsequently, pLTF was demonstrated in anesthetized rats following three five-min isocapnic episodes of hypoxia (Hayashi *et al.*, 1993;

Bach & Mitchell, 1996). pLTF is also observed in rabbits (Sokołowska & Pokorski, 2006). Much progress has been made in determining the cellular/synaptic mechanisms underlying this form of respiratory neuroplasticity. We now know that pLTF induced by severe AIH elicits distinct serotonin-independent, adenosine-dependent pLTF.
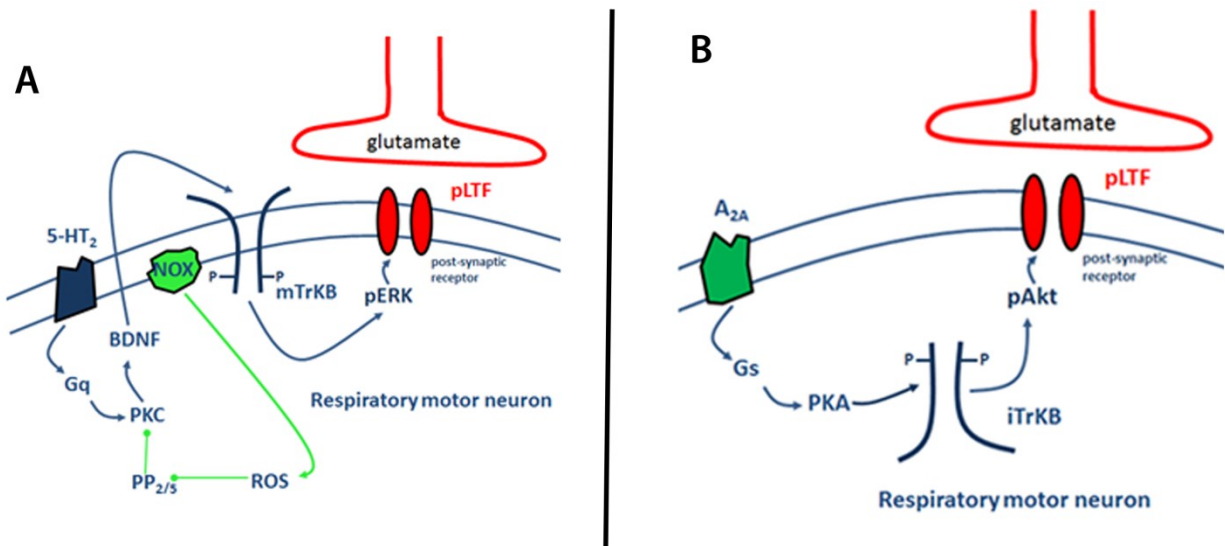

Figure 1. Working models of cellular pathways giving rise to pLTF following AIH. A. During modest AIH, activation of 5-HT2 receptor leads to protein kinase C (PKC) activation, and new synthesis of brain-derived neurotrophic factor (BDNF). BDNF activates its high-affinity receptor, TrkB, which then phosphorylates extracellular signal-related kinase (ERK). ERK activation facilitates inputs to phrenic motor neurons via an unknown mechanism, possibly including glutamate receptor trafficking, Regulation of serotonin-dependent pLTF occurs through NADPH oxidase-dependent reactive oxygen species (ROS) production. Protein phosphatases 2A and/or 5 (PP2A/5) can inhibit PKC activation or signaling, but are inhibited by ROS, thus enabling expression of pLTF. B. During severe AIH, adenosine 2A (A2A) receptor activation dominates, stimulating activity of protein kinase A (PKA), which stimulates new synthesis of an immature TrkB isoform (iTrkB). iTrkB autoactivates and phosphorylates Akt which leads to facilitation of

synaptic inputs to phrenic motor neurons via an unknown mechanism. Adapted from: Devinney MJ, Huxtable AG, Nichols NL & Mitchell GS (2013). Hypoxia-induced phrenic long-term facilitation: emergent properties. Ann N Y Acad Sci 1279, 143–153.

Cellular/Synaptic Mechanisms

Signaling cascades giving rise to pLTF following modest AIH (Figure 1)

Spinal serotonin receptor activation is necessary and sufficient for pLTF (Fuller *et al.*, 2001; MacFarlane & Mitchell, 2009). During AIH, raphe neurons are activated and likely release serotonin in the area of the phrenic motor nucleus to induce pLTF. In support of this, $5\text{-}HT_2$ receptor activation within the phrenic motor nucleus is required for pLTF during, but not following AIH (Fuller *et al.*, 2001). Additionally, episodic injections of serotonin in the area of the phrenic motor nucleus cause phrenic motor facilitation (pMF, a general term describing augmented phrenic burst amplitude that includes pLTF; MacFarlane & Mitchell, 2009). Since $5\text{-}HT_2$ receptors are abundantly expressed on phrenic motor neurons, necessary downstream cascades are likely found within motor neurons.

$5\text{-}HT_2$ receptors are G_q-coupled, canonically activating protein kinase C (PKC) through phospholipase C-mediated inositol triphosphate (IP_3) and diacylglycerol (DAG) production (Niebert *et al.*, 2011). IP_3 can release intracellular Ca^{2+} stores, supporting the activation of classical isoforms of PKC, as well other Ca^{2+}-dependent signaling. DAG binds to the C1 region of the regulatory domain of classical and novel isoforms of protein kinase C, facilitating translocation of PKC from cytosol to cell membrane, permitting its kinase activity. Thus, $5\text{-}HT_2$ receptor activation likely activates classical and/or novel isoforms of PKC, suggesting that PKC is a critical downstream molecular for pLTF expression. However, non-canonical signaling from

G_q coupled protein receptors such as Src kinase could underlie pLTF (McGarrigle & Huang, 2007). Thus, determining what downstream signaling pathway follow 5-HT$_2$ receptor activation is critical to understanding cellular/synaptic mechanisms of pLTF, and is a focus of chapter II and III of this thesis.

Brain-derived neurotrophic factor (BDNF) is another critical molecule involved in pLTF. Rats exposed to AIH exhibit increased cervical BDNF expression compared to animals not receiving hypoxia (Baker-Herman *et al.*, 2004). Additionally, intrathecal injections of small interfering RNA targeting BDNF prevent the development of pLTF (Baker-Herman *et al.*, 2004). BDNF likely activates its TrkB receptor, since the non-selective TrkB receptor antagonist K252a also blocked pLTF (Baker-Herman *et al.*, 2004). Intrathecal injections of BDNF in the area of phrenic motor nucleus are sufficient to cause phrenic motor facilitation (Baker-Herman *et al.*, 2004). Altogether, these results suggest that the new synthesis of BDNF occurs during/following AIH to cause pLTF. Interestingly, since single spinal injections of BDNF are sufficient to elicit significant pMF, mechanisms giving rise to pattern sensitivity are likely upstream from BDNF synthesis.

TrkB signaling can occur via multiple cascades, including phosphatidylinositol 3-kinase (PI3K), phospholipase C$_\gamma$ (PLC$_\gamma$), and Ras/MAPK (Huang & Reichardt, 2003; Leal *et al.*, 2013). Ras/MAPK signaling and subsequent phosphorylation of extracellular-regulated kinase (ERK) is likely the critical cascade involved in pLTF, since spinal inhibition of ERK activity prevents pLTF (Hoffman *et al.*, 2012). In contrast, inhibition of PI3K had no effect on pLTF (Hoffman *et al.*, 2012). However, the role of PLC$_\gamma$ in pLTF is unknown; further studies are needed to determine if PLC$_\gamma$ activation from TrkB activation contributes to PKC activation following AIH.

Mechanisms giving rise to pLTF likely increase phrenic motor output by increasing phrenic motor neuron excitability or by increasing synaptic strength at pre-motor or inter-neuronal synapses. Eliciting hypoglossal motor facilitation in the *in vitro* rhythmogenic medullary slice

preparation with episodic stimulation of 5-HT$_2$ receptors demonstrated increased whole-cell currents responses to local application of AMPA, suggestive of AMPA receptor insertion (Bocchiaro & Feldman, 2004). Interestingly, continuous stimulation of 5-HT$_2$ or α_1 receptors fails to elicit *in vitro* hypoglossal LTF (Feldman *et al.*, 2005; Neverova *et al.*, 2007). Studies on pLTF *in vivo* have not yet explored the role of increased AMPA receptor expression; however, NMDA receptors are necessary for the expression of pLTF (McGuire *et al.*, 2005), and maintenance of ventilatory LTF (McGuire *et al.*, 2008). However, it is unclear whether NMDA receptors have a permissive role in maintaining pLTF, or are the synaptic target responsible for increased postsynaptic transmission and/or excitability.

Cellular cascades regulating pLTF

A working model of the molecular interactions regulating pLTF expression is shown in Figure 1. Reactive oxygen species (ROS) are required for the expression of many forms of plasticity, including pLTF (MacFarlane *et al.*, 2008; Massaad & Klann, 2011). Spinal NADPH oxidase is a critical source of ROS required for the expression of pLTF (MacFarlane *et al.*, 2009), and pMF following episodic serotonin injections (MacFarlane & Mitchell, 2009). Interestingly, induction of pMF with a 5-HT$_{2B}$ receptor agonist, BW723C86, requires spinal NADPH oxidase activity while similarly induced pMF with 5-HT$_{2A}$ agonist, DOI, does not (MacFarlane *et al.*, 2011). The relative role of 5-HT2A/B receptors in pLTF induced by modest AIH is uncertain, but it seems likely that 5-HT2B play a more significant role since pLTF requires NADPH oxidase activity. ROS produced by NADPH oxidase likely inhibit okadaic-acid sensitive protein phosphatases that constrain pLTF expression (Wilkerson *et al.*, 2007), since spinal okadaic acid permits expression of pLTF when NADPH oxidase is inhibited (Macfarlane P.M. and Mitchell G.S., unpublished observations).

Protein phosphatases may inhibit PKC or ERK signaling by dephosphorylation. Inhibition of protein phosphatases (likely 2A and/or 5) with spinal okadaic acid reveals pLTF following exposure to modest acute sustained hypoxia (ASH; 25 min, PaO2 35-45 mmHg), suggesting that intermittent hypoxia relieves an inhibitory constraint to allow expression of pLTF (Wilkerson *et al.*, 2008). Spinal okadaic acid had no effect on pLTF amplitude following modest AIH, suggesting that protein phosphatases do not constrain pLTF during modest AIH. These experiments have extended our insight into mechanisms of pattern sensitivity; however, it is still unclear what mechanisms cause differential regulation of protein phosphatase activity during modest AIH vs. ASH.

Adenosinergic contributions to pLTF

Hypoxia, in addition to stimulating raphe neurons, provokes local ATP, adenosine, and/or adenine nucleotide release from glia and/or neurons (Wallman-Johansson & Fredholm, 1994; Dale *et al.*, 2000; Gourine *et al.*, 2005; Hernandez *et al.*, 2005). ATP could be degraded to adenosine by extracellular ecto-nucleotidases, contributing to increased extracellular adenosine (Parkinson *et al.*, 2005). Extracellular adenosine could activate adenosine 2A (A_{2A}) receptors and contribute to pLTF. However, because spinal blockade of A_{2A} receptors enhances serotonin-dependent pLTF during modest AIH (Hoffman *et al.*, 2010), A_{2A} receptor activation likely inhibits serotonin-dependent phrenic motor plasticity. In contrast, episodic spinal injections of the A_{2A} receptor agonist, CGS 21680 elicits pMF, by causing new synthesis of immature forms of TrkB, which are auto-activated (Golder *et al.*, 2008). Therefore adenosinergic and serotoningergic pathways interact in complex ways during hypoxia exposure (Dale-Nagle *et al.*, 2010*a*). Altogether, these studies suggest that A_{2A} receptor activation constrains serotonin-dependent signaling giving rise to pLTF, but also can give rise to a new form of A_{2A}-dependent phrenic motor plasticity.

pLTF induced by severe AIH is A_{2A}-dependent

Increasing hypoxia severity may provoke greater adenosine release/formation from neurons and glia, causing a mechanistic shift from serotonin to adenosine-dependent pLTF. Recently, Nichols *et al.* found that severe AIH exposure (P_{aO2} 25-35 mmHg) elicits serotonin-independent, A_{2A}-dependent pLTF. Interestingly, progressive augmentation of the short-term hypoxic response was observed in rats exposed to severe AIH, which was A_{2A}- and serotonin-independent. Different mechanisms to pLTF may exist to increase phrenic motor output during various patterns, duration, and severity of hypoxia that occur during respiratory challenges such as injury or disease.

Mechanisms downstream from A_{2A} receptor activation are less well understood. A_{2A} receptors are canonically coupled to protein kinase A (PKA) signaling through G_s-protein mediated activation of adenylyl cyclase isoforms. PKA may increase new synthesis of immature forms of TrkB which can signal independent of neurotrophin binding. New synthesis of TrkB is required for expression of pMF following pharmacological activation of A_{2A} receptors in the cervical spinal cord (Golder *et al.*, 2008). Immature TrkB synthesis could lead to activation of PI3K and phosphorylation of Akt, since increased phospho-Akt was found following activation A_{2A} receptors in this same study (Golder *et al.*, 2008). Importantly, spinal activation of G_s-coupled 5-HT_7 receptors with a specific agonist induces a mechanistically similar pMF which requires new synthesis of TrkB and Akt activation (Hoffman & Mitchell, 2011). The role of PKA in these pathways is unconfirmed, but spinal PKA inhibition similarly enhances pLTF following modest AIH (Hoffman & Mitchell, 2008). The downstream targets directly responsible for increasing phrenic motor output are unknown.

Emergent properties of pLTF: pattern sensitivity and metaplasticity

Pattern sensitivity of pLTF is addressed throughout this introduction, but potential mechanisms such as serotonin receptor desensitization, differential ROS production, and inhibitory interactions between serotonin and adenosine-dependent pathways are futher discussed in a review article (Appendix B; Devinney *et al.*, 2013). Furthermore, plasticity of plasticity (ie. metaplasticity) is also addressed in this article, especially pertaining to how it can arise as an emergent property of signaling cascades giving rise to plasticity (Appendix B).

Other ways to elicit phrenic motor plasticity

Spinal pharmacological activation of molecules involved in pLTF (serotonin, BDNF, A2A agonists, PKA activator) can elicit phrenic motor facilitation phenotypically similar to pLTF (pMF; a general term describing augmented phrenic burst amplitude that includes pLTF). In addition, spinal injections of vascular endothelial growth factor or erythropoietin near the phrenic motor nucleus cause pMF, which requires both spinal ERK and Akt activation (Dale-Nagle *et al.*, 2011; Dale *et al.*, 2012). Such growth factor-induced plasticity may represent plasticity for longer-term adjustments in phrenic motor output, such as responses to chronic intermittent or sustained hypoxia.

Phrenic inactivity (or reduced activity) also induces pMF (ipMF), but via distinct cellular cascades. For example, ipMF induced by sustained hypocapnia requires spinal PKCζ/ι activity during or shortly after stimulus while pLTF does not. This form of plasticity may be important to preserve phrenic motor output when synaptic inputs are disrupted (eg. spinal injury). Interestingly, ipMF induced by hypocapnia is also pattern-sensitive. One brief (7.5 min) neural apnea induced by hypocapnia caused no significant ipMF, while intermittent apneas of the same

cumulative duration (5, 1.5 min episodes, separated by 5 min) produced ipMF similar to that induced by a prolonged (30 min) neural apnea (Baertsch & Baker-Herman, 2013).

Protein kinase C: an overview

Protein kinase C (PKC) is important for many forms of plasticity (Sossin, 2007), and is postulated to play a key role in pLTF (Fuller *et al.*, 2000; Mahamed & Mitchell, 2007; Dale-Nagle *et al.*, 2010*a*; Devinney *et al.*, 2013). Chapter II and III focus on the necessity and sufficiency, respectively, of PKC activation for eliciting pLTF. Therefore, a brief overview of the PKC family of kinases is presented, with a focus on their roles in neuroplasticity.

PKC is a multigene family of serine/threonine protein kinases consisting of fifteen known isozymes (enzymes from different genes that catalyze same reaction; ie. protein isoforms) in humans. Each isoform has a highly conserved catalytic domain, and a regulatory domain containing autoinhibitory pseudosubstrate (PS) domain and membrane targeting domains C1 and C2 (Steinberg, 2008). The PS domain is present in all PKC isoforms and binds to the substrate-binding pocket in the catalytic domain to prevent kinase activity when PKC is inactive. PKC isoforms can be separated into three distinct subfamilies or classes based on their regulatory domain structure (Sossin, 2007). Classical PKC isoforms have functional C2 domains which bind anionic lipids in a Ca^{2+}-dependent manner (ie. confers Ca^{2+} sensitivity), recruiting PKC to the membrane; novel and atypical classes lack functional C2 domains and therefore are not sensitive to intracellular Ca^{2+} fluctuations. Two functional C1 domains (C1A and C1B) are present in both novel and classical but not in atypical classes of PKC, and bind lipid activators such as diacylglycerol (DAG, produced by phospholipases) or phorbol esters (tumorigenic compounds found in plants). Only one C1 domain is present in atypical PKC's, and it fails to bind DAG or phorbol esters but instead interacts with the lipids phosphatidylinositol (3,4,5)-

trisphosphate (PIP3) and ceramide. The atypical class also has a PB1 domain which interacts with scaffolding proteins (comprehensive review covering structure/function of PKC; Steinberg, 2008).
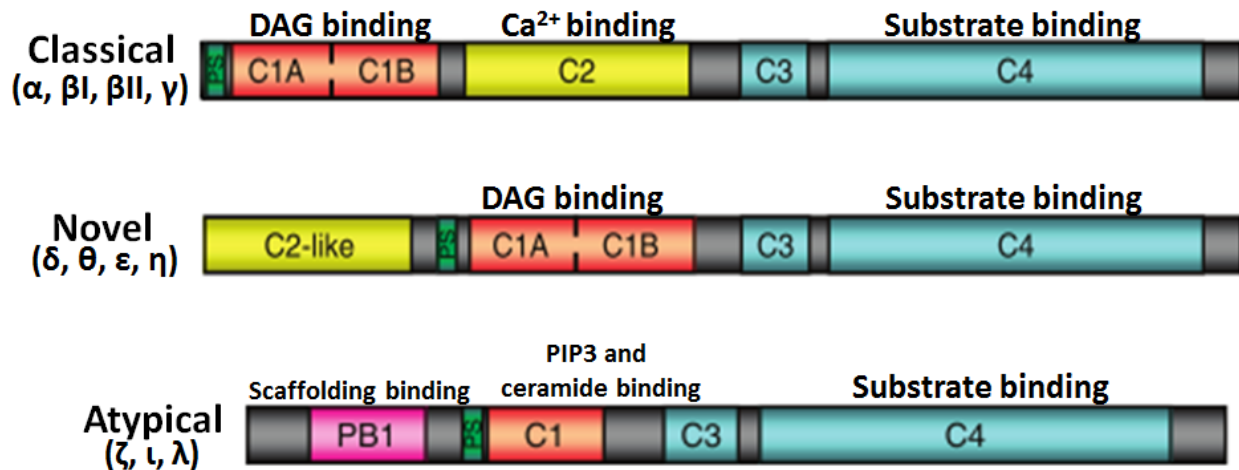

Figure 2. Structure and function different PKC classes. Classical PKC isoforms include a functional Ca^{2+} binding C2 domain in addition to the DAG binding domains C1A and C1B found on novel PKC isoforms. Atypical PKC isoforms bind scaffolding proteins with a distinct PB1 domain and also have a distinct C1 domain that binds phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and ceramide. All isoforms include a structurally distinct pseudosubstrate domain (PS) which binds at the substrate-binding region of respective isoforms to prevent catalytic activity. Domain structures adapted from: Steinberg SF (2008). Structural basis of protein kinase C isoform function. *Physiol Rev* **88,** 1341–1378.

Activation Mechanisms

Activation of PKC primarily occurs through binding of lipids such as DAG (and Ca^{2+} for classical PKC), translocation of PKC to the plasma membrane, and dissociation of the auto-inhibitory PS domain which allows for kinase activity (Steinberg, 2008). DAG is produced by phospholipase

C, coupled to G_q protein coupled-receptors such as 5-HT$_2$ and α_1 receptors or to tyrosine kinase neurotrophin receptors such as TrkB (Zirrgiebel *et al.*, 1995; Niebert *et al.*, 2011). DAG alone may not be sufficient to activate PKC, since membrane phosphatidylserine, which is produced from DAG by DAG kinase, stabilizes the regulatory domain of PKC to promote C1 domain interaction with DAG (Stahelin *et al.*, 2005). Additionally, the novel isoforms PKCθ and PKCδ can be activated by phosphotyrosine binding (ie. protein-protein interactions) due to their unusual C2 domain (Stahelin *et al.*, 2012).

PKC activity is also regulated by phosphorylation on at least three distinct sites (Keranen *et al.*, 1995). Phosphoinositide-dependent kinase (PDK) phosphorylates novel and classical isoforms at the activation loop site, usually immediately following PKC translation to allow proper folding of the kinase (Le Good *et al.*, 1998; Balendran *et al.*, 2000). In contast, PDK phosphorylation of atypical PKC is regulated by PIP3 levels, and allows for control of atypical PKC activity (Chou *et al.*, 1998). Classical and novel isoforms have a hydrophobic motif phosphorylation site which is lacking in atypical isoforms, which stabilizes the molecule and promotes kinase activity (Bornancin & Parker, 1997; Edwards & Newton, 1997). In vitro, PKC can auto-phosphorylate at this site, but it is unclear whether this occurs *in vivo* as a regulatory mechanism (Keranen *et al.*, 1995; Behn-Krappa & Newton, 1999; Sossin, 2007). Tyrosine phosphorylation at the hinge domains of PKCθ or PKCδ by Src family kinases (such as Lck) can activate these isoforms independent of DAG binding (Konishi *et al.*, 2001; Kikkawa *et al.*, 2002; Melowic *et al.*, 2007).

Reactive oxygen species can activate PKC, potentially through cysteine oxidation in the C1 domains producing conformation changes similar to DAG binding (Knapp & Klann, 2002; Sossin, 2007). Interestingly, oxidative activation of PKC produced potentiation of hippocampal synaptic transmission that was similar to LTP and occluded by high frequency stimulation (Knapp & Klann, 2002). However, high concentrations of ROS were needed to cause PKC

activation; therefore, more studies are needed to determine the role of oxidative activation of PKC in *in vivo* models of plasticity.

Proteolytic cleavage of PKC at the hinge domain produces a kinase domain separate from the regulatory domain known as PKM, which is autonomously active since is lacks PS and DAG-binding domains. This allows for persistent signaling of the PKC molecule. PKMζ has been shown to play a critical role in maintenance of rat hippocampal LTP. However, PKMζ is likely formed by alternative transcription of the PKCζ gene (Sacktor, 2012). In *Aplysia*, persistent activation of PKC API III (atypical) by proteolytic cleavage, maintains intermediate-term memory (Sutton *et al.*, 2004*). Similarly, alternative splicing of the PKCθ gene produces a persistently active PKCθ II isoform in the mouse testis, which is independent of DAG signaling or phorbol ester stimulation because it lacks C1 domains (Niino *et al.*, 2001). PKCδ can be cleaved by caspases to form PKMδ, which promotes apoptotic cascades (Kikkawa *et al.*, 2002). PKC molecules lacking a regulatory domain are unable to be blocked with inhibitors targeting the regulatory domain, such as calphostin C or NPC-15437 (Kobayashi *et al.*, 1989; Sullivan *et al.*, 1991), but are blocked by PKC inhibitors that bind the catalytic domain.

Subcellular localization of PKC signals

PKC isoforms can translocate to lipid rafts, which are sphingolipid-/cholesterol-enriched plasma membrane microdomains. Ceramide, the lipid regulator of lipid raft formation, is regulated by PKCδ activity, through phosphorylation of acid sphingomyelinase (Zeidan & Hannun, 2007). Lipid rafts may represent one potential mechanism to locally regulate signaling cascades. Another local mechanism of regulating PKC activity is diacylglycerol kinase (DGK) activity. DGK converts DAG to phosphatidylserine, and can locally deplete DAG levels to prevent PKC activity (Luo *et al.*, 2003). Some DGK activity may be necessary for PKC activation since

phosphatidylserine may play a permissive role in C1 domain binding to DAG (Huang & Huang, 1990).

Roles of PKC in plasticity

PKC has been implicated in several models of neuroplasticity, including hippocampal LTP, *Aplysia* sensorimotor facilitation, and cerebellar long-term depression (LTD; Leitges *et al.*, 2004; Sossin, 2007). In hippocampal LTP, the most notable finding is that PKMζ is required for its maintenance (Ling *et al.*, 2002). In *Aplysia,* PKC Apl I (classical isoform) is required for induction of intermediate-term facilitation, which requires synaptic activity and serotonin receptor activation. This coupling is important because PKC Apl I requires increased Ca^{2+}; serotonin alone cannot activate PKC Apl I, suggesting that PLC activation *in vivo* is insufficient to release Ca^{2+} in amounts needed to activate classical isoforms of PKC. Moreover, PKCα-dependent cerebellar LTD in Purkinje neurons is induced by coincident depolarization and metabotropic glutamate receptor 1 activation (Linden, 2003; Leitges *et al.*, 2004).

Some forms of spinal respiratory motor plasticity require PKC activation, such as phrenic motor plasticity following inactivity which requires atypical PKCζ/ι activity for its expression (Strey *et al.*, 2012). pLTF induced by modest AIH might require PKC activity. For example pMF elicited by intravenous serotonin was prevented with intravenous administration of PKC inhibitors (Liu *et al.*, 2011). Moreover, experiments presented at a conference showed that microinjections of the broad-spectrum PKC inhibitor bisindolylmaleimide I into the phrenic motor nucleus prevents pLTF induced by AIH (McGuire & Liming, 2004). Thus, spinal PKC activation is required for ipMF and possibly pLTF, although the specific isoforms likely differ since intrathecal PKCζ inhibitors had no effect on pLTF. Even though multiple PKC isoforms are often present within neurons, specific isoforms may be critical for specific forms of plasticity, such as pLTF and ipMF (Sossin, 2007).

Significance

Biological significance: Understanding mechanisms of pLTF advances basic understanding of plasticity

Plasticity is a seemingly ubiquitous property of our neural systems, yet its significance and role in neural function is barely understood. The respiratory control system was once considered fixed and immutable, but now is widely recognized to undergo significant plasticity. The role of such plasticity in stabilizing/destabilizing breathing, inducing recovery of breathing following injury, or adjusting breathing during aging or disease is unknown (Mitchell & Johnson, 2003). Understanding the molecular mechanisms and conditions giving rise to plasticity may allow us to better appreciate and understand plasticity, as well as apply it to treat disease.

Training pattern has a powerful influence on plasticity and learning/memory, but its significance and underlying mechanisms are not fully understood. Pattern sensitivity (ie. spacing effect) may enable neural systems to optimally acquire and sustain adaptive behaviors (Philips *et al.*, 2013). Single experiences may be disfavored from triggering plasticity or inducing memories to prevent adaptation to rare or uncommon experiences. In contrast, traumatic events may trigger pattern-insensitive forms of plasticity to form "flashbulb" memories (van Giezen *et al.*, 2005; Diamond *et al.*, 2007). An understanding of mechanisms giving rise to pattern sensitivity may enable optimal strategies for inducing plasticity in neural networks, such as those controlling breathing or other behaviors. It will also further our basic understanding of how emergent properties arise from complex cellular/molecular interactions within neural systems.

Clinical significance: pLTF guides therapeutic advances that restore motor function

Principles from studies on pLTF are guiding therapies aimed at restoring motor function following spinal injury. Mechanisms giving rise to pLTF extend to other motor pools; this realization lead to studies utilizing intermittent hypoxia to restore motor function in spinally hemisected rats (Golder & Mitchell, 2005; Vinit *et al.*, 2009; Lovett-Barr *et al.*, 2012). Such findings were extended to humans with long-standing spinal cord injury by a collaborative group in Chicago. In specific, subjects were found to have increased plantar flexion strength following 10 episodes of mild hypoxia (Trumbower *et al.*, 2012). Currently, studies are being done by the same group on gait and walking distance following intermittent hypoxia. Additionally, caffeine, a commonly ingested A2A receptor antagonist, is being administered to some subjects in an attempt to enhance motor plasticity following intermittent hypoxia. The principles guiding these experiments were originally demonstrated by studies on adenosinergic contributions to pLTF (Hoffman *et al.*, 2010).

Intermittent hypoxia may also benefit patients afflicted with amyotrophic lateral sclerosis (ALS). In a rat model of ALS, exposure to AIH restored phrenic motor output (Nichols *et al.*, 2013), suggesting that inducing motor plasticity with AIH restores breathing capacity and prolongs survival. The potential benefits of intermittent hypoxia exposure on sleep apnea patients require more studies. For example, upper airway patency may be protected if substantial hypoglossal motor facilitation occurs following intermittent hypoxia (Mahamed & Mitchell, 2007); however, induction of pLTF may cause an increase in gain of the respiratory response to CO_2. If this is not sufficiently offset with a decrease in CO_2 apneic threshold then breathing may become less stable (Mahamed & Mitchell, 2008*a*).

Studies on hypoxia-induced plasticity may also benefit our understanding of respiratory control in neonates. Because neonates are often hypoxic due to hypoventilation, pLTF mechanisms may be occurring to increase breathing and prevent respiratory arrest (Carroll, 2003; Gozal, 2004). Hypoxia can have detrimental effects in neonates, such as future blunting

of hypoxic response. Additionally, chronic intermittent hypoxia during development impairs pLTF, potentially through an inflammatory mechanism (Reeves *et al.*, 2006). Impaired pLTF may be a risk factor for prolonged, detrimental hypoxia in neonates.

Cellular mechanisms giving rise to pLTF may also guide pharmacological therapies aimed at restoring motor function. BDNF, VEGF, EPO, A_{2A} and 5-HT_7 agonists, PKA activators, and serotonin all induce pMF when injected intrathecally near the phrenic motor nucleus (Baker-Herman *et al.*, 2004; Golder *et al.*, 2008; Kajana & Goshgarian, 2008; Hoffman & Mitchell, 2011; Dale-Nagle *et al.*, 2011; Dale *et al.*, 2012). While these represent potential therapeutic targets, chronic spinal delivery is not feasible in human patients. Moreover, intermittent dosing is often required to induce plasticity. Therefore, targeting of specific molecules found in or near motor neurons which induce plasticity may be critical for pharmacological advances. Moreover, a better understanding of pattern sensitivity may allow us to optimize delivery patterns or circumvent the need for cumbersome intermittent dosing.

Summary of dissertation aims

My dissertation focuses on two distinct, competing mechanisms giving rise to pLTF. Moderate (P_{aO2} 45-55 mmHg) or modest (P_{aO2} 35-45 mmHg) AIH elicits pLTF which requires 5-HT_2 receptor activation. Downstream from activation of these G_q-coupled receptors, NADPH oxidase activity, new synthesis of BDNF, and ERK phosphorylation are all required for the expression of pLTF. However, it is unknown how 5-HT_2 receptor activation couples to these downstream processes. One plausible mechanism is via PKC signaling since G_q proteins activate PLC, which produces DAG, an activator of classical and novel PKC isoforms. **Chapter II** of this dissertation investigates the role of PKC in pLTF utilizing spinal administration of PKC inhibitors to determine the necessary isoform, PKCθ. In addition, specific knockdown of PKCθ with

intrapleural siRNA was performed to determine the requirement of PKCθ within phrenic motor neurons for pLTF. **Chapter III** of this dissertation investigates whether spinal PKC activation is sufficient to cause pMF, using intrathecal injections of the DAG analog, PMA. PKC inhibitors were used to block effects induced by PMA injections demonstrate requirement of PKC activation.

Severe AIH elicits a distinct form of pLTF which requires A_{2A} receptor activation (Nichols *et al.*, 2012). Activation of these G_s-coupled receptors likely activates PKA, since G_s-proteins activate adenylyl cyclase. Pharmacological activation of spinal A_{2A} receptors also elicits pMF, which requires new synthesis of TrkB. Interestingly, A_{2A} receptor activation constrains serotonin-dependent pLTF following modest AIH, since spinal A_{2A} receptor blockade enhanced this pLTF. In **Chapter IV**, I hypothesize that serotonin- and A_{2A}-dependent mechanisms interact by inhibiting each other, and that this interaction can explain fundamental properties of pLTF such as pattern sensitivity. Specifically, I hypothesize that A_{2A} receptor activation constrains pLTF during moderate ASH. In addition, I hypothesize that pLTF induced by severe hypoxia is not pattern-sensitive because of greater A_{2A} vs. $5\text{-}HT_2$ receptor activation.

Altogether, these studies increase our understanding of spinal mechanisms of respiratory motor plasticity during hypoxia. Evidence presented suggests that the specific isoform, PKCθ, is critical for the expression of pLTF; this knowledge will allow us to better understand cellular and molecular mechanisms of pLTF, including interactions between separate pathways. Specific activation of PKC may represent a pharmacological strategy to increase respiratory motor output to treat diseases or conditions that compromise breathing. Additionally, understanding mechanisms of pattern sensitivity allow us to optimally elicit plasticity and aid development of these potential therapies.

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Chapter II

Phrenic long-term facilitation is a form of PKCθ-dependent motor plasticity

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Abstract

Acute exposure to intermittent hypoxia induces spinal motor plasticity. One well known model of intermittent hypoxia-induced spinal plasticity is phrenic long-term facilitation (pLTF). Here we identify a key molecule for pLTF, a novel PKC isoform, PKCθ. Spinal administration of PKC inhibitors known to inhibit PKCθ block pLTF, whereas PKC inhibitors targeting PKC isoforms other than PKCθ do not. We demonstrate that PKCθ is highly expressed in phrenic motor neurons, and that intrapleural siRNA injections targeting PKCθ knock down ventral cervical spinal PKCθ protein levels. Whereas intrapleural PKCθ siRNAs abolish pLTF, siRNAs targeting another PKC isoform (PKCζ) do not. The novel PKC isoform, PKCθ, plays a key role in spinal, intermittent hypoxia-induced motor plasticity, and the relevant PKCθ is localized in the phrenic motor network. Intrapleural siRNA delivery has considerable potential to modulate plasticity within vital, respiratory motor networks in disease.

Introduction

Breathing is a rhythmic motor behavior that is essential for life. Although appreciated only in recent years, plasticity is a hallmark feature of this critical motor system (Eldridge & Millhorn, 1986; Mitchell, 1990; Feldman *et al.*, 2003; Mitchell & Johnson, 2003).

Plasticity in motor control systems occurs at multiple anatomical levels, including the cortex (Oudega & Perez, 2012), cerebellum (Gao *et al.*, 2012) and spinal cord (Wolpaw & Tennissen, 2001). Various forms of spinal motor plasticity include spinal operant conditioning of the H reflex (Wolpaw *et al.*, 1986), instrumental learning (Grau *et al.*, 2006) and phrenic long-term facilitation (Hayashi *et al.*, 1993; Bach & Mitchell, 1996; Mitchell *et al.*, 2001; Dale-Nagle *et al.*, 2010*a*). Our knowledge of mechanisms giving rise to spinal motor plasticity is increasing rapidly and has recently been applied to induce recovery following spinal cord injury (Trumbower *et al.*, 2012). However, the cellular/molecular determinates of spinal motor plasticity are still incompletely understood.

Spinal alpha motor neurons were once considered the equivalent of copper wire that relays information from motor centers to the relevant musculature. However, we now know that they undergo substantial plasticity, including changes in dendritic tree distribution (Inglis *et al.*, 2000), fiber type (Prakash *et al.*, 1995), and axonal sprouting (Santos & Caroni, 2003). Such anatomical plasticity suggests that spinal motor neurons undergo functional plasticity; however, no studies to date have directly linked plasticity of spinal motor neurons to increased functional motor output.

Acute intermittent hypoxia (AIH) induces a form of spinal, serotonin-dependent motor plasticity known as phrenic long-term facilitation (Fuller *et al.*, 2001*b*; Mitchell *et al.*, 2001; Feldman *et al.*, 2003; Dale-Nagle *et al.*, 2010*a*). pLTF is expressed as a prolonged enhancement of phrenic burst amplitude lasting hours after AIH (Hayashi *et al.*, 1993; Bach & Mitchell, 1996; Fuller *et al.*, 2001*b*; Baker-Herman & Mitchell, 2002). This plasticity is not limited

to phrenic motor output since AIH elicits long-term facilitation in hypoglossal (Bach & Mitchell, 1996; Baker-Herman & Strey, 2011), inspiratory intercostal (Fregosi & Mitchell, 1994), and laryngeal motor output (Bautista *et al.*, 2012). Further, AIH and repetitive AIH induce recovery of rodent forelimb (Lovett-Barr *et al.*, 2012) and human leg function (Trumbower *et al.*, 2012) after spinal cord injury. Recent research has emphasized cellular mechanisms giving rise to pLTF (Baker-Herman *et al.*, 2004; Wilkerson *et al.*, 2008; MacFarlane *et al.*, 2009; Hoffman *et al.*, 2012).. Although protein kinase C activation is hypothesized to be necessary for pLTF (Fuller *et al.*, 2000*b*; Mahamed & Mitchell, 2007; Dale-Nagle *et al.*, 2010*a*; Devinney *et al.*, 2013), this has not been demonstrated. Further, of the 15 known PKC isoforms found in mammals, the specific isoforms involved have not been identified, nor has the location of the relevant PKC been identified.

PKC is critically involved in plasticity in many neural systems (Sossin, 2007), including plasticity in the spinal dorsal horn associated with chronic pain (Peng *et al.*, 1997; Hua *et al.*, 1999). Spinal serotonin receptor activation is both necessary (Baker-Herman & Mitchell, 2002) and sufficient (MacFarlane & Mitchell, 2009), and the relevant serotonin receptors are of the 5-HT_2 subclass (Fuller *et al.*, 2001*b*; MacFarlane *et al.*, 2011). 5-HT_2 receptors activate PKC through G_q coupled signaling cascades (Farah *et al.*, 2009; Farah & Sossin, 2011). Thus, we hypothesize that PKC activation is necessary for pLTF. Activation of atypical PKC isoforms are not necessary for AIH-induced pLTF (Strey *et al.*, 2012); therefore we suspect that activation of classical or novel PKC isoforms are required for pLTF. Using spinal injections of differentially-selective PKC inhibitors, we identified a single novel PKC isoform necessary to produce pLTF following AIH, PKCθ. Further, using intrapleurally delivered siRNAs targeting PKCθ (siPKCθ) to enable delivery to phrenic motor neurons (Mantilla *et al.*, 2013), we successfully knocked down PKCθ. Targeted PKCθ knockdown blocked pLTF, whereas siRNAs targeting the atypical isoform PKCζ did not. Thus, a novel PKC isoform, PKCθ plays a previously unknown role in

AIH-induced pLTF. The relevant PKCθ appears to be localized within phrenic motor neurons, or (if siRNAs are secreted from phrenic motor neurons) in pre-motor neurons of the phrenic network.

Experimental Procedures

Animals. Experiments were performed on 3-4 month old male Sprague-Dawley rats (Harlan colony 211 or 218a) weighing from 280-480g. Rats were housed two per cage with food and water *ad libitum,* and kept in a 12 hr light/dark cycle. Experiments were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison.

Drugs

Pharmacological solutions. 1.4 mM Bisindolymalemide I (BIS, a.k.a GF109203X or Gö 6850; Tocris Biosciences), 40 mM Gö 6983 (Tocris Biosciences), 50 mM CID755673 (CID; Sigma-Aldrich), and 20 mM sotrastaurin (sotra, a.ka. AEB 071; Axon Ligands) were dissolved in DMSO and stored at -20°C. 2 µg/µl myristoylated peptide mimicking the pseudosubstrate domain of PKCθ (theta inhibitory peptide, TIP; Calbiochem) was dissolved in artificial cerebrospinal fluid (aCSF) (in mM: 120 NaCl, 3 KCl, 2 CaCl, 2 MgCl, 23 NaHCO$_3$, 10 glucose bubbled with 95% O$_2$/5% CO$_2$) and stored at -20°C. 50 mM NPC15437 (NPC; Sigma-Aldrich) in ACSF was stored at 4°C.

Intrathecal injections. Pharmacological agents were delivered at doses consistent with spinal inhibition of PKC (Yashpal *et al.*, 1995; Hua *et al.*, 1999; Ferguson *et al.*, 2008; Laferrière *et al.*, 2011). Stock solutions were diluted in aCSF to their final concentration with a DMSO concentration ranging from 0-20% (Gö 6983 20%; NPC 0%; CID 10%; BIS 20%; sotra 10%; TIP, 0%). In some cases, Gö 6983 in 100% DMSO or 100% DMSO alone (vehicle) was delivered intrathecally because of drug solubility concerns at higher doses. Dose responses were done as shown in Figure S1. A 50µl Hamilton syringe was loaded and connected to silicone catheter (2 French Access Technologies; 0.3 mm inner diameter), which was inserted sub-durally above C$_4$ spinal segment. Injections were delivered 10-15 minutes prior to AIH (12 ul total, 2µl/30s). Vehicle- treated rats received aCSF with 0-20% DMSO. There was no significant

effect on pLTF of 100% DMSO injection vs. aCSF alone (47±15% vs. 44±3% respectively, p=0.70). To estimate spinal concentration, the spinal tissue (C_2-C_6) was considered a rectangle with following dimensions 16mm x 5 mm x 4 mm for a total volume of 320 μl. The estimated concentration equals the molarity of injectate x volume of injectate divided by the sum of 320μl and the volume of the injectate.

siRNA delivery. Pools of four duplexes of Accell-modified small interfering RNAs (siRNAs) targeting PKCθ (siPKCθ), PKCζ (siPKCζ), and non-targeting (NTsiRNA) were purchased from Dharmacon, Inc (Thermoscientific). Accell-modified siRNA was chosen because this modification preferentially transfects neurons versus other cell types (Nakajima *et al.*, 2012). siPKCθ, siPKCζ, and NTsiRNA consisted of four pooled Accell modified 21-nucleotide duplexes. Each siRNA pool was suspended in Dharmacon siRNA buffer to yield a concentration of 5μM, aliquoted and stored at -20°C. Prior to intrapleural injections, 20μl of siRNA was added to 6 μl of 5X siRNA buffer (Dharmacon), 3.2μl of Oligofectamine Transfection Reagent (Invitrogen) and 0.8 μl of RNAase free H_2O (final siRNA concentration of 3.33 μM) and carefully mixed 20 minutes prior to injection, allowing the siRNA to complex with the transfection reagent.

Intrapleural injections.

Intrapleural injections of siRNAs and cholera toxin B fragment were performed similarly to other studies (Mantilla *et al.*, 2009, 2013). Rats were induced in a flow-through chamber with 5% inhaled isoflurane in 100% O_2, placed on a surgical table, and maintained with a nose cone (2% isoflurane, 100% O_2). For phrenic motor neuron back-labeling, a 25μl Hamilton syringe with attached sterile needle (6 mm) was loaded with 12.5 μl of cholera toxin B fragment (2μg/μl in sterile H_2O) per side. For intrapleural siRNA delivery, 30μl was loaded into a 50μl Hamilton syringe. The rib cage was palpated to locate the 5th intercostal space at the anterior axillary line, and the needle was inserted into the pleural space bilaterally to deliver the injectate. After

injections, isoflorane was discontinued and respiratory chest movements were monitored for signs of pneumothorax. After recovery from anesthesia, animals were monitored for 15 minutes. All rats recovered with no signs of distress.

Surgical Preparation. Rats were induced in a closed chamber with isoflurane, placed on an experimental surgical table, and maintained with a nose cone (3.5% isoflurane, 50% O_2). After performing a tracheotomy, rats were pump ventilated (Rodent Ventilator 683, Harvard Apparatus; tidal volume 2.2-2.7 ml; frequency 70-75 breaths/min) with 3.5% isoflurane in 50% O_2 (balance N_2). Small amounts of CO_2 were added to the gas mixture to maintain end-tidal CO_2 (Respironics Novametrix) at 42-45 mm Hg. Tracheal pressure was monitored to prevent over-inflation and to assure that the tracheostomy remained patent. Both vagus nerves were isolaed in the mid-cervical region and cut bilaterally to prevent ventilator entrainment. A tail vein was catheterized for continuous delivery of intravenous fluids (~1.5 ml/hr of 75% lactated Ringer's solution, 10% HCO_3, and 15% hetastarch; Hespan, 6% hetastarch in 0.9% NaCl). Intravenous fluid infusion was adjusted as needed to maintain acid-base status (-4.0 to 4.0 base excess mEq/L, ≤1.5 mEq/L change at 60 min) as determined via blood samples. Catheterization of the femoral artery was performed for blood gas sampling and blood pressure recording. The left hypoglossal and phrenic nerves were isolated using a dorsal approach, cut, desheathed and covered with saline-soaked cotton. A C_2 laminectomy was performed for intrathecal drug administration; the dura was exposed, cut dorsally, and a silicone catheter (2 Fr; Access Technologies, Skokie, IL) attached to a 50µl Hamilton syringe was advanced under the dura to C_4. Anesthesia was then slowly converted to urethane (1.8 mg/kg -2.0 mg/kg, i.v.) while isoflurane was slowly withdrawn. After sufficient time for isoflurane to wear off, rats were paralyzed with pancuronium bromide (2.5 mg/kg, i.v.). Body temperature was measured with rectal probe and kept at 37.5±1°C using the heated (perfused) surgical table. Blood pressure

was monitored throughout the experiment to ensure physiological stability (baseline: 70-150 mmHg, ≤30 mmHg change at the end of an experiment). Depth of anesthesia was assessed by toe pinch responses in blood pressure and phrenic nerve activity. At the end of each protocol, a maximal CO_2 response (end-tidal CO_2>90 mmHg) was assessed to verify adequate dyamic range for phrenic output. All rats included in the study had >30% hypoxic response during AIH and >50% maximal hypercapnic response, signifying that chemosensory inputs were not saturated and motor output was not maximal at baseline.

Electrophysiology

Desheathed phrenic and hypoglossal nerves were placed on bipolar silver electrodes and then covered with mineral oil to prevent dessication. Nerve activity was amplified (10,000X), band-pass filtered (300-10,000 Hz Model 1800, A-M Systems, Carlsborg, WA), rectified and integrated with a continuous moving averager (time constant: 50 ms; CWE Inc., MA-821 filter; Ardmore, PA). The integrated signal was digitized and analyzed with a data acquisition system (WINDAQ, DATAQ Instruments, Akron, OH).

One hour following conversion to urethane anesthesia, the CO_2 apneic and recruitment thresholds for phrenic nerve activity were determined by adjusting inspired CO_2 and/or ventilator frequency. Baseline CO_2 was maintained at 2-3 mmHg above the recruitment threshold for the duration of an experiment. Baseline nerve activity was maintained for at least 20-30 minutes, and then a blood sample was drawn to measure arterial P_{CO_2}, P_{O_2} and pH to assess "baseline" conditions. Some rats received intrathecal injections near the phrenic motor nucleus 10-15 minutes prior to experimental maneuvers, such as AIH. The rats were then exposed to either AIH or continuous baseline conditions (ie. time control). AIH consisted of 3, 5 min episodes of 10-12% inspired O_2, separated by 5 minutes of baseline oxygen conditions. During hypoxic

episodes, arterial P_{O_2} was between 35-45 mmHg, and arterial P_{CO_2} was maintained within 1.5 mmHg of baseline values. Following AIH, baseline conditions were reestablished. Blood gas analysis was performed at 15, 30 and 60 minutes post-AIH; during this time, arterial P_{O_2} was greater than 180 mmHg, P_{aCO_2} was within 1.5 mmHg of baseline, and base excess was within 1.5 mEq/L of the baseline value.

Rats treated as time controls did not receive AIH but were maintained at baseline conditions for an equal period of time. Blood samples were taken at similar time points as AIH-treated rats. Time control rats for groups treated with pharmacological agents received intrathecal injections of vehicle or drug as appropriate.

Immunoblotting.

Tissue harvesting. After electrophysiology experiments, a C_2-C_5 laminectomy was performed and the rat was partially exsanguinated. The spinal cord was quickly cut at the C_3 (mid C_3 section) and C_6 rootlets (mid C_6 section), and then placed on ice. After allowing the extracted spinal cord to cool for one minute, a cut was made at the central canal to permit separation of dorsal and ventral cord. The ventral cord was placed in a pyrex homogenizer (Corning, 1ml PTFE Pestle Tissue Grinder) filled with 250 µl of homogenization buffer (150 mM NaCl, 1% Triton X-100, 0.5% NaDeoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 with 1X Halt Phosphatase Inhibitor cocktail (Thermoscientific) and 1X Protease Inhibitor Cocktail (Sigma-Aldrich #P8340)) and homogenized by pumping 20 times. The sample was then spun for 10 minutes at 0.5 relative centrifugal force. The pellet was discarded; 12 µl of the supernatant was saved for bicinchoninic acid assay for protein; the remaining supernatant was transferred and mixed with an equal volume of 2X loading buffer (5% 2-mercaptoethanol in Laemmli buffer: 65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue). Both samples were

stored at -20°C for protein analysis and immunoblotting. Right diaphragm samples were also taken from each rat and processed in the same manner as above, except that 500 µl of homogenization buffer was used in a 3 ml tissue grinder.

Immunoblotting. Bicinchoninic acid protein assay was performed using the Thermo Scientific Pierce BCA Protein Assay Kit following manufacturer's instructions. Using the estimated protein concentration, each sample was diluted to 1 µg protein/µl with loading buffer prior to loading into 10% or 4-15% tris-HCl 18 well gels. 25µl/well of loading control (1:1 loading buffer and homogenization buffer) and 10-25µl/well of a protein ladder (1:1:1 of loading buffer, Precision Plus Protein™ Dual Color Standard (Bio-Rad), and Biotinylated Protein Ladder (Cell signaling) were also loaded on each gel. 25µl (25 µg protein) of sample was loaded into each well and the gel was run at 120 volts for 1 hr. Afterwards, wet transfer to a 0.45 µm pore Immobilon polyvinylidene difluoride membrane was performed on ice for 1 hr at 100 volts. After transfer, the blots were cut at 50 kDa to allow for concurrent separate probing of GAPDH for loading control (M.W. 37 kDa) and PKCθ (M.W. 80 kDa) or PKCζ (M.W. 75 kDa) . The blots were blocked in 5% BSA (for PKCθ antibody) or 5% milk (for PKCζ antibody or GAPDH antibody) in Tris-buffered saline (20 mM Tris, 500 mM NaCl) with 0.05% Tween (TBST) for 1 hr at room temperature. Then, antibodies were added to the blocking solution and the blots were incubated overnight at 4°C. Afterwards, the blots were washed 3 times (for 5 min) with TBST and probed with HRP-conjugated goat anti-rabbit antibodies (1:10000, Santa Cruz Biotechnology) in blocking solution for 1 hr at room temperature. Blots were then washed 5 times (for 5 min) with TBST. Signal was resolved using Pierce Super Signal West Dura Extended Duration Substrate kit (Thermoscientific). Chemiluminescence was detected with an AutoChemi Imaging system (UVP Bio-imaging systems) with Labworks 4.6. Denitometry was performed with ImageJ by following instructions found here: http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/. All relative protein comparisons are made by normalizing to

GAPDH signal for that well and then comparing the relative signal to the mean of the control values. Percent change calculation: 100 – (sample PKC signal/sample GAPDH signal)/(mean NTsiRNA samples/GAPDH) x 100)

Antibodies. Polyclonal antibodies to phospho-PKCθ (Cell Signaling, #9377) were used at 1/1000 dilution in 5% BSA TBST to assess PKCθ protein expression with immunoblotting. We utilized the phospho-PKCθ antibody instead of other PKCθ antibodies because 1) phospho-PKCθ was unaffected by AIH 2) phospho-PKCθ stains were significantly better than non-phospho antibodies and 3) the same phospho-PKCθ antibody has been used successfully in other studies to determine PKCθ expression (Jové *et al.*, 2006; Wei *et al.*, 2010).

Polyclonal antibodies to atypical PKC (Santa Cruz PKCζ/ι C-20) were used at 1/1000 dilution in 5% milk to probe for PKCζ (mol. weight ~75 kDa). GAPDH antibody (Cell Signaling 14C10, #2118) was used in 5% milk at 1/50000 to assess GAPDH protein expression. Polyclonal rabbit anti-phospho-PKCθ (1/250 Cell Signaling, #9377), polyclonal donkey anti-CtB (1/5000 EMD Millipore), conjugated goat anti-donkey Alexa Fluor 495 (1/1000 Invitrogen, Carlsbad, California) and conjugated goat anti-rabbit Alexa Fluor 488 (1/500 Invitrogen) were used in immunofluorescence treatments.

Immunofluorescence

Tissue preparation and staining. Following surgery and electrophysiology experiments, 9 rats were euthanized and perfused transcardially with 1mL/g of chilled 0.01M PBS followed by 1mL/g of chilled 4% paraformaldehyde (PFA) at a pH of 7.4.The brain and spinal cord were harvested and placed into 4% PFA for 8 hours at 4^OC. The tissues were then transferred to 20% sucrose followed by 30% sucrose until the tissues sank. 40μm transverse slices were cut using a microtome (SM2000R Leica, Buffalo Grove, IL) from C3-C5 and placed into antifreeze solution until stained. Free floating sections were washed with 0.05M Tris-buffered saline with 0.1%

Triton-X (TBS-TX) and blocked with 1.0% bovine serum albumin (BSA) for 1 hour. The tissues were stained with rabbit anti-P-PKCθ (1/250 Cell Signaling, #9377) and donkey anti-CtB (1/5000 EMD Millipore) at room temperature for 16 hours. Slices were washed with TBS-Tx and subsequently stained with conjugated goat anti-donkey Alexa Fluor 495 (1/1000 Invitrogen, Carlsbad, California) and conjugated goat anti-rabbit Alexa Fluor 488 (1/500 Invitrogen) for 2 hours at room temperature. Tissue slices were washed a final time with TBS-Tx and mounted on glass with an anti-fade solution (Prolong Gold Invitrogen). The slices were imaged on an epifluorescence confocal microscope at 20x and 40x using Nikon EZ-C1 software.

Statistical Analysis

Integrated phrenic burst amplitude and frequency was analyzed in 60s bins before, during and 15, 30 and 60 minutes after AIH or at similar points in time controls (no AIH). Average burst amplitude values were normalized to baseline; Integrated nerve burst amplitudes were expressed as a percent change from baseline. Two-way ANOVA with a repeated measures design (Sigmaplot 12) was used to compare integrated nerve burst amplitudes and frequency, as well as arterial PCO_2, PO_2, pH and base excess to detect significant treatment (AIH versus time control; drug treatment) and time effects. Individual comparisons between groups and times were made via *post-hoc* analysis with Fisher's LSD test. Values of $p<0.05$ were considered statistically significant.

Because no significant differences were found among the different time control experiments (NPC, n=3; BIS, n=3; Sotra, n=2; TIP, n=4 vehicle, n=3; p=0.59), all time control experiments were grouped for comparison with AIH-treated rats. Similarly, since differences were not observed between intrapleural siPKCθ (n=3), siPKCζ (n=3) and NTsiRNA (n=4) (p=0.43) time control experiments, all intrapleural siRNA injection time controls were grouped.

Results

pLTF requires spinal PKC activation

To test the hypothesis that spinal PKC activation is necessary for pLTF, and to narrow down the specific PKC isoform(s) involved, multiple PKC inhibitors with different pharmacological profiles were delivered intrathecally in spinal regions encompassing the phrenic motor nucleus (C_4) prior to AIH. Six different PKC inhibitors were tested; reported half-maximal inhibitory concentration (IC_{50}) values for each inhibitor at each PKC isoform are shown in Table 1. Short-term hypoxic phrenic responses were unaltered by any of the spinal PKC inhibitors used (Fig S2).

Time compressed phrenic neurograms (Fig 1A) illustrate representative responses in integrated phrenic nerve burst amplitude before, during and after AIH. In control rats (vehicle injected), phrenic nerve burst amplitude was significantly elevated above baseline 30-60 min post-AIH, an effect not observed in time control (no AIH) rats (Fig 1A bottom). This persistent elevation indicates pLTF (Bach & Mitchell, 1996; Fuller *et al.*, 2001*b*).

pLTF does not require activation of classical PKC isoforms

Gö 6983 is a broad-spectrum, PKC active-site inhibitor that potently inhibits all classical PKC isoforms (Table 1; (Gschwendt *et al.*, 1996). Rats pretreated with intrathecal Gö 6983 exhibited significant pLTF (Fig S1; 46±9%, Fig 1B) versus time controls (8±4%, no AIH; p>0.05); this pLTF was similar to that observed in rats pre-treated with vehicle (44±15%, 100% DMSO; p≥0.28). Thus, we tested inhibitors known to target novel PKC isoforms.

pLTF requires activation of a novel PKC isoform. Bisindolymalemide 1 (BIS, or GF 109203X) is a broad spectrum PKC inhibitor that potently inhibits most novel and classical isoforms (Table 1; (Martiny-Baron *et al.*, 1993; Gschwendt *et al.*, 1996; Uberall *et al.*, 1997). Figure 1A depicts a representative neurogram from rats given intrathecal BIS (280μM, 22±6%; Fig 1B), showing

significantly attenuated pLTF versus rats receiving vehicle injections (46±4%, p<0.001 vs. BIS; Fig 1B).

Since Gö 6983 and BIS are active-site inhibitors, we searched for PKC inhibitors acting by a distinct mechanism. NPC15437 (NPC) binds PKC regulatory subunits, blocking the binding of its activator, diacylglycerol. Based on *in vitro* studies, NPC inhibits most PKC isoforms, with some exceptions (Table 1; (Saraiva *et al.*, 2003; Felber *et al.*, 2007). Surprisingly, rats given 5mM NPC exhibited normal pLTF (Fig 1A), which was not significantly different from rats given vehicle injections (54±12%, p=0.40; Fig 1B). In fact, NPC did not inhibit pLTF at any dose tested (0.1-40mM, p=0.61; Fig S1). Thus, NPC does not inhibit the relevant PKC isoform for pLTF.

Since NPC has no known inhibitory effect on PKCμ or PKCθ, we tested inhibitors for these PKC isoforms (Table 1). In rats given the PKCμ inhibitor CID755673, pLTF was not significantly different from vehicle-treated rats at doses from 50μM – 5mM (12 μl injection volumes; 40±13%, p=0.26 vs. vehicle, Fig 1B and Fig S1). However, in rats given sotrastaurin, a broad-spectrum PKC inhibitor known to potently inhibits PKCθ, pLTF was significantly impaired at doses ≥2 μM (12 μl injection volumes; 17±4%, p<0.001 vs. vehicle, Fig 1B and Fig S1). Since BIS also inhibits PKCθ (Table 1), we hypothesized that spinal PKCθ is the critical PKC isoform for pLTF.

Spinal PKCθ activity is necessary for pLTF

Since BIS and sotrastaurin inhibit many PKC isoforms other than PKCθ, we sought to selectively block PKCθ activity using a myristolated peptide mimicking the pseudosubstrate region of the PKCθ protein (theta inhibitory peptide, TIP). TIP binds to the catalytic domain of PKCθ protein, blocking substrate phosphorylation (Harris *et al.*, 1996; Kilian *et al.*, 2004). pLTF was abolished in rats given intrathecal TIP (10±7% at 60 min post-AIH) versus baseline (p=0.17; Fig 1A) or time controls (8±4%, p=0.74 vs TIP; Fig 1B). In TIP-treated rats, pLTF was significantly decreased versus vehicle (47±4% at 60 min, p<0.001; Fig 1C) and NPC-treated

rats (54±12% at 60 min, p=0.001; Fig 1C). These data strengthen the argument that PKCθ activity is critical for pLTF.

pLTF with NPC was not significantly different from vehicle-treated rats at any time point (16±6% at 15 min, 31±8% at 30 min, 54±12% at 60 min, p=0.994; Fig 1C), yet NPC is an inhibitor of all PKC isoforms aside from PKCθ and PKCμ (which is covered by CID). Thus, we found no evidence that PKC isoforms other than PKCθ are necessary for AIH-induced pLTF.

PKCθ is highly expressed in phrenic motor neurons

Because spinal PKCθ activity is critical for pLTF, we sought to determine cellular expression patterns near the phrenic motor nucleus. Phrenic motor neurons were back-labeled with intrapleural cholera toxin B fragment injections (CtB; (Mantilla *et al.*, 2009); in transverse cervical sections (C_4-C_5), CtB was revealed with a CtB antibody (blue, Fig 2B and 2D). In addition, antibodies for CD11b (Fig2B, red) and GFAP (Fig 2D, red) were used to identify microglia and astrocytes, respectively.

Strong PKCθ labeling was observed in phrenic motor neurons (ie. CtB positive cells, Fig 2A, 2B, 2C, 2D) and in other, unidentified neurons (Fig 2F). At the same microscope settings, PKCθ immunolabeling was negligible in microglia (CD11b positive cells; Fig 2B) or astrocytes (GFAP positive cells; Fig 2D), suggesting that PKCθ is predominantly expressed within neurons of the ventral cervical spinal cord. High power images of phrenic motor neurons clearly demonstrate immunoreactive PKCθ protein (Fig 2E) within CtB-positive phrenic motor neurons (Fig 2F).

Targeted PKCθ knockdown prevents pLTF

To determine if delivery of siRNAs targeting PKCθ to phrenic motor neurons would block pLTF, we utilized intrapleural siRNA injections. Intrapleural siRNAs are retrogradely transported to phrenic motor neurons, where they can knockdown target mRNA and protein expression

(Mantilla *et al.*, 2013). After 3 daily intrapleural injections of non-targeting siRNA (NTsiRNA), and siRNAs targeting PKCθ (siPKCθ) or the atypical PKC isoform PKCζ (si PKCζ), which is expressed in phrenic motor neurons (Guenther *et al.*, 2010), but not required for pLTF (Strey *et al.*, 2012). Rats given NTsiRNA had normal pLTF following AIH (48±12%, p<0.001 vs. time control; Fig3A, top), demonstrating that non-specific aspects of the injection procedures, the transfection reagent or small double stranded RNAs had no significant effect on pLTF. However, siPKCθ blocked pLTF (11±13%, p=0.24 vs time control; Fig 3A, middle), demonstrating once again that PKCθ is essential for pLTF, and that a likely site for the relevant PKC is within the motor neurons themselves. In contrast, pLTF was unaffected in rats given siPKCζ (57±15%, p=0.27 vs. NTsirNA; Fig 3A, bottom), demonstrating that targeted knockdown of other PKC isoforms does not affect pLTF.

In addition, short-term hypoxic phrenic responses were unaltered by siPKCθ (83±9%) vs. NTsiRNA (83±7%, p=0.70 vs. NTsiRNA; Fig S3,); rats treated with siPKCζ appeared to have a greater short-term hypoxic phrenic response (120±24%, p=0.03 vs. siPKCθ; Fig S3), although this response was not significantly different from NTsiRNA (p=0.52). Summary data (Fig 3B) demonstrate that rats treated with NTsiRNA or siPKCζ exhibit normal pLTF (p<0.001 at 30 and 60 min vs. time control), while siPKCθ eliminates pLTF (p=0.24 vs time controls).

To confirm that intrapleural siRNA injections were not exerting unintended effects on the brainstem, we recorded hypoglossal nerve activity. Hypoglossal motor output also exhibits LTF (Baker-Herman and Strey, 2011), although its expression is highly variable (depending on age, sex and colony of Sprague Dawley rats; (Fuller *et al.*, 2000*b*, 2001*a*; Behan *et al.*, 2002). Regardless, hypoglossal LTF was unaffected by any intrapleural siRNA injection tested (Fig S4, siPKCθ and siPKCζ p>0.05 vs. NTsiRNA, *p<0.05 vs. time controls). Although there is no direct route whereby intrapleural siRNAs could be transported to hypoglossal motor neurons, these data provide some reassurance that siRNAs were not jumping synapses to brainstem pre-motor

neurons of the ventral respiratory group, which also provide respiratory related synaptic inputs to hypoglossal motor neurons (Yamada *et al.*, 1988; Woch *et al.*, 2000).

PKCθ knockdown in the ventral cervical spinal cord was confirmed in siPKCθ treated rats by harvesting the C_3 to C_5 spinal segments, discarding dorsal spinal tissues, and then performing immunoblots on homogenates with an antibody for PKCθ (Fig 4A). siPKCθ significantly decreased PKCθ protein levels (-49±18%, p=0.03 vs NTsiRNA) versus rats given NTsiRNA (Fig 4B). In contrast, siPKCζ (Fig 4C) had no effect on PKCθ protein levels (-4±14%, p=0.889 vs. NTsiRNA); Fig 4D). Thus, the siRNA pools used selectively target and knock down the PKCθ isoform. These measurements confirmed that siPKCθ caused PKCθ knockdown in the ventral cervical spinal segments associated with the phrenic motor nucleus.

Discussion

Here we demonstrate that activity of a single PKC isoform, PKCθ, is necessary for an important model of spinal motor plasticity, AIH-induced phrenic long-term facilitation (pLTF). The involvement of PKCθ in pLTF was demonstrated by: 1) spinal injections of drugs targeting different sets of PKC isoforms; 2) a peptide targeting the catalytic site of PKCθ; and 3) targeted knockdown of PKCθ in the phrenic motor network via RNA interference. No other PKC isoform was required for AIH-induced pLTF.

Although PKCθ is highly expressed in phrenic motor neurons, it was also found in unidentified neurons of the ventral cervical spinal cord; in comparison, PKCθ expression in adjacent glia appeared minimal. Partial localization of the PKCθ relevant for pLTF was achieved with intrapleural injections of siRNAs targeting PKCθ; intrapleural injections are accessible to the terminals of respiratory motor neurons (ie. neurons directly accessing the pleural space), and are retrogradely transported to phrenic motor neuron cell bodies (Mantilla et al. 2009, 2013). Although we cannot rule out trans-cellular exchange of the siRNAs targeting PKCθ, the abolition of pLTF after intrapleural siPKCθ injections suggest that the relevant PKCθ is localized in the phrenic motor network (ie. motor neurons, spinal interneurons and, possibly, brainstem pre-motor neurons). Collectively, these data provide the first evidence that PKCθ is required in any form of spinal motor plasticity, and strongly suggest the possibility that the relevant PKC is within the motor neuron itself.

PKCθ activity is required for pLTF

Using available pharmacological tools, we confirmed the requirement for PKC in AIH-induced pLTF, and demonstrated a single PKC isoform is involved in that process. Although mechanisms giving rise to AIH-induced pLTF have been studied extensively in recent years

(Dale-Nagle *et al.*, 2010*a*, 2010*b*; Devinney *et al.*, 2013)), the role of PKC had not been confirmed until now. Activation of PKCθ likely occurs downstream from spinal serotonin type 2 (5-HT$_2$) receptors. These Gq protein-coupled receptors are both necessary and sufficient for pLTF (Fuller *et al.*, 2001*b*; MacFarlane & Mitchell, 2009), are highly expressed on phrenic motor neurons (Basura *et al.*, 2001; MacFarlane *et al.*, 2011), and activate phospholipase C, which produces diacylglycerol (DAG) and insositol triphosphate (Pandey *et al.*, 1995). DAG binds to the regulatory domain (C1) of PKC, causing conformational changes and activating the catalytic domain to phosphorylate downstream targets (Steinberg, 2008). Given the data presented here, we postulate that PKCθ activity is induced by 5-HT$_2$ receptor activation and subsequent DAG production within phrenic motor neurons.

PKCθ differs from all other novel and classical PKC isoforms because it binds DAG predominantly at the C1B versus the C1A domain of the regulatory domain (Melowic *et al.*, 2007; Steinberg, 2008). Moreover, structural studies demonstrate narrowing of the phorbol ester-binding surface of C1B, in PKCθ C1B crystal structures versus the relatively similar PKCδ C1B (80% sequence homology) crystal structure (Rahman *et al.*, 2013), suggesting that NPC may not be able to bind the C1B domain. This difference may explain why NPC, which binds the DAG binding site of most PKC isoforms (Sullivan *et al.*, 1991), had no effect on pLTF. We suspect that NPC fails to block PKCθ because it cannot bind the structurally distinct C1B-DAG binding-site of PKCθ. Because of this key difference in PKCθ regulatory domain structure, NPC was an ideal pharmacological tool to rule out nearly all other PKC isoforms. Since NPC failed to block pLTF, we suggest that PKC isoforms other than PKCθ are not required for AIH-induced pLTF.

The regulation of PKCθ activity is complex. PKCθ has three phosphorylation sites: activation loop, hydrophobic motif and turn motif, which are conserved within the PKC family (Steinberg, 2008; Freeley *et al.*, 2011). Although we do not currently understand the role of phosphorylation

in PKC enzymatic activity, existing models suggest that PKC is phosphorylated when synthesized to "prime" the enzyme so that it responds to second messengers (eg. DAG) (Steinberg, 2008; Freeley *et al.*, 2011). However, some studies in T-cells also show inducible phosphorylation (Freeley *et al.*, 2005; Cheng *et al.*, 2007). In ventral cervical homogenates, we found no difference in activation loop (thr538) phosphorylation following AIH (data not shown), suggesting that inducible PKCθ phosphorylation is not responsible for increased activity, or that phosphorylation occurs at another site, such as the hydrophobic or turn motifs. PKCθ can also undergo tyrosine phosphorylation by Src family kinases, and phosphotyrosine binding (Stahelin *et al.*, 2012). Moreover, PKCθ may be activated by cleavage at hinge domain, separating catalytic domain from the regulatory domain to generate PKMθ (or PKCθ II; see (Niino *et al.*, 2001), a constitutively active PKCθ. Understanding the complex regulation of PKCθ activity might provide important insights concerning the regulation of pLTF. For example, acute systemic inflammation impairs pLTF by an unknown mechanism (Huxtable *et al.*, 2011, 2013); it is possible that changes in PKCθ phosphorylation state and activity contribute to this effect.

Cellular localization of pLTF: Targeting motor neurons with siRNA

Since 5-HT$_2$ receptors are abundantly expressed on phrenic neurons (Basura *et al.*, 2001; Fuller *et al.*, 2005), and many molecules associated with pLTF (5-HT$_2$ receptors, BDNF, TrkB, and phopho-ERK) are up-regulated in presumptive phrenic motor neurons following repetitive exposure to intermittent hypoxia (Satriotomo *et al.*, 2012), we hypothesized that mechanisms giving rise to pLTF are localized within phrenic motor neurons (Fuller *et al.*, 2000*a*; Mitchell *et al.*, 2001; Feldman *et al.*, 2003; Mahamed & Mitchell, 2008; Dale-Nagle *et al.*, 2010*b*) However, no studies to date have provided direct evidence that this is indeed the case. Here, we used a novel intrapleural siRNA technique to target phrenic motor neurons (Mantilla et al., 2013), and show that siPKCθ delivery to phrenic motor neurons abolishes pLTF. Although these results are

consistent with localization of the relevant PKCθ within phrenic motor neurons, we cannot rule out the possibility of trans-cellular siRNA exchange. Indeed, endogenous micro-RNAs are exchanged between cells (Valadi *et al.*, 2007; Montecalvo *et al.*, 2012; Mittelbrunn & Sánchez-Madrid, 2012), suggesting the possibility (not certainty) of trans-cellular siRNA exchange under the conditions explored here. Intrapleural siPKCθ is relatively unlikely to affect astrocytes or microglia in the phrenic motor nucleus given their low PKCθ expression (Fig 2B, 2D). Thus, relevant PKCθ is most likely in phrenic motor neurons, spinal phrenic interneurons (Lane *et al.*, 2008; Lane, 2011), brainstem pre-motor neurons (Feldman & Del Negro, 2006), or pre-synaptic modulatory neurons, such as the raphe serotonergic neurons the initiate and orchestrate AIH-induced pLTF (Mitchell *et al.*, 2001; Mahamed & Mitchell, 2008; Dale-Nagle *et al.*, 2010*b*) However, since intrapleural siPKCθ did not affect hypoglossal LTF, or the short-term hypoxic phrenic response, there is no evidence for transport to brainstem phrenic pre-motor neurons.

Intrapleural siRNAs likely affect other respiratory motor pools that contact the pleural space, such as the intercostal motor neurons which also show CtB labeling after intrapleural CtB injections (Mantilla *et al.*, 2009). Since intercostal motor neurons have no direct connection to phrenic motor neurons, we do not suspect that this factor influences pLTF. On the other hand, we expect that intrapleural siPKCθ would block intercostal LTF (Fregosi & Mitchell, 1994), unless there are mechanistic differences involving separate PKC isoforms or other signaling molecules downstream from serotonin receptor activation. Intrapleural siRNAs could also be retrogradely transported by the vagus nerve to the dorsal motor nucleus of the vagus or the nucleus ambiguous. While we cannot completely rule out effects from PKCθ knockdown in these areas, it highly unlikely such knockdown (if it occurs) would influence pLTF.

Targeted RNA interference in respiratory motor pools with intrapleural siRNA may lead to relatively simple and useful therapies in patients with compromised respiratory motor output, such as in amyotrophic lateral sclerosis or following spinal cord injury. For example, intrapleural

delivery of siRNAs targeting molecules that restrain motor plasticity might be used to enhance motor output. In ventilator-dependent patients, any increase in breathing capacity might enable unassisted ventilation, greatly improving quality of life (Criner, 2002; Martin, 2002). Because intrapleural siRNAs had profound effects on respiratory motor plasticity, and the technique is relatively non-invasive and well tolerated, this technique has considerable potential as a future clinical tool to implement gene therapy within respiratory motor neurons or networks.

Motor networks are plastic

Historically, spinal motor neurons have been thought of as relays that simply link the CNS to peripheral muscles. However, these critical motor neurons are dynamic, and have considerable capacity for plasticity. For example, spinal motor neurons possess extensive dendritic trees, which are influenced by developmental experiences, such as changes in gravity (Inglis *et al.*, 2000). In the respiratory system alone, we now know of at least five distinct cellular mechanisms giving rise to phrenic motor plasticity (Dale-Nagle *et al.*, 2010*a*). Here, we demonstrate that AIH elicits plasticity within motor neurons and/or their immediate network, thereby inducing pLTF. We demonstrate that a single PKC isoform (from among at least 15) is necessary for respiratory motor plasticity in the form of pLTF, and that the relevant PKC is most likely within the phrenic motor neurons per se (or in their immediate neural network). Further studies aimed at understanding motor neuron plasticity may allow us to better understand how motor systems adapt to ever-changing demands of life, such as normal development and aging, gaining or losing weight, changes in physiological conditions or the onset of disease (Mitchell & Johnson, 2003).

Motor network plasticity also has therapeutic potential in clinical disorders that compromise non-respiratory motor function. For example, intermitted hypoxia induces functional recovery of rat forelimbs (Lovett-Barr *et al.*, 2012) and human legs (Trumbower *et al.*, 2012) after chronic,

incomplete spinal cord injury. AIH-induced plasticity may also prove useful in preserving or restoring breathing capacity in patients with motor neuron disease, such as amyotrophic lateral sclerosis (Nichols *et al.*, 2013). Through a detailed understanding of mechanisms giving rise to respiratory and non-respiratory motor plasticity, we may be able to manipulate key molecules (eg. PKCθ) to better harness the inherent capacity for motor plasticity to therapeutic advantage.

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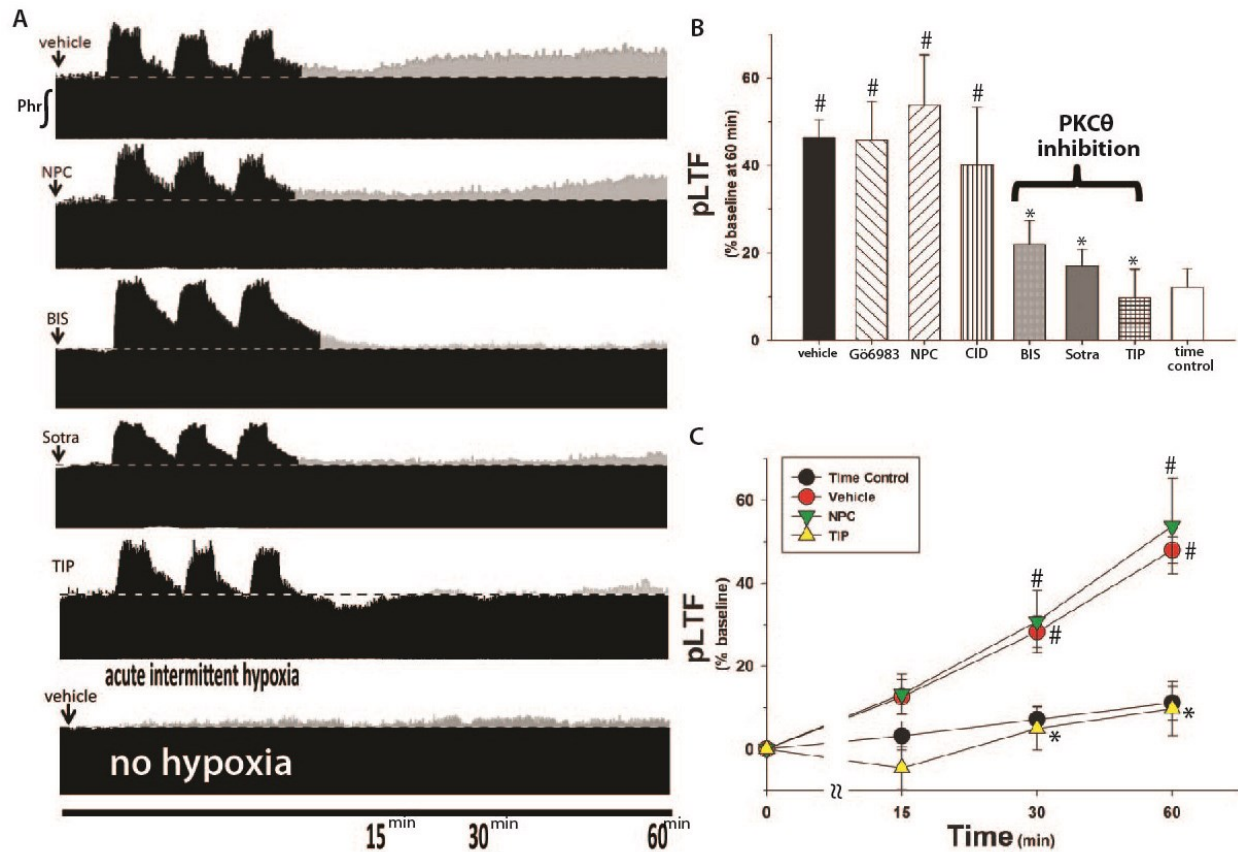
Figures

Figure 1. Spinal inhibition of PKCθ, but not other isoforms, blocks pLTF. **A**. Representative neurograms after acute intermittent hypoxia (AIH) from rats given intrathecal injections (12μl) of various PKC inhibitors. ▼Denotes intrathecal delivery of inhibitor.   Dotted line represents baseline amplitude; increases from baseline are shown in gray.   **B.** Group data from rats given intrathecal PKC inhibitors at 60 min post AIH. Delivered drug doses: 10% DMSO in artificial cerebrospinal fluid or 100% DMSO (vehicle), n = 20;   1-2 mM Gö 6983, n=8; 5 mM NPC-15437 (NPC), n=6; 280 μM Bisindolymalemide I (BIS), n=5; 2-20 μM sotrastaurin (Sotra), n=5; myristolated pseudosubstrate peptide targeting PKCθ (theta inhibitory peptide, TIP, 2μg/μl), n=5. **C.** Average time plot of pLTF following either intrathecal NPC or TIP. **#** p<0.05 indicates

significant difference from time controls (no hypoxia). *p<0.05 indicates significant difference from vehicle + AIH.

PKC inhibitor / isoform	Gö 6983	NPC	CID	BIS	Sotra
conventional alpha (α)	7 nM	+	>>10 µM	8 nM	1 nM
beta (β₁)	7 nM	+	>10 µM	20 nM	0.6 nM
gamma (γ)	6 nM	+	—	2-6 µM	—
novel delta (δ)	10 nM	+	>>7 µM	0.2 µM	2 nM
epsilon (ε)	—	+	—	0.1 µM	3 nM
eta (η)	—	++	—	—	2 nM
mu (µ) (PKD)	20 µM	—	0.2 µM	2 µM	—
theta (θ)	—	—	—	20 nM	0.2 nM
atypical iota/lamda (ι/λ)	—	—	—	—	—
zeta (ζ)	60 nM	+	—	6 µM	—
calculated spinal conc. range	3.6 µM-360 µM	3.6 µM-1.5 mM	0.36 nM-180 µM	10 µM	7.2 nM-0.7 µM

Table 1. Half maximal inhibitory concentration (IC$_{50}$) of each tested PKC inhibitor for each PKC isoform. All values were obtained from published studies on these inhibitors (citations marked with superscript for each inhibitor). For NPC, no published IC$_{50}$ values are available, but inhibitory activity is denoted with +. ++ indicates relative greater inhibitory activity. — indicates no known activity at the particular PKC isoform. The bottom row indicates estimated spinal concentration after injection of each inhibitor, for comparison with IC$_{50}$ values. Abbreviations: NPC, NPC15437; BIS, bisindolymalemide I; Sotra, sotrastaurin.
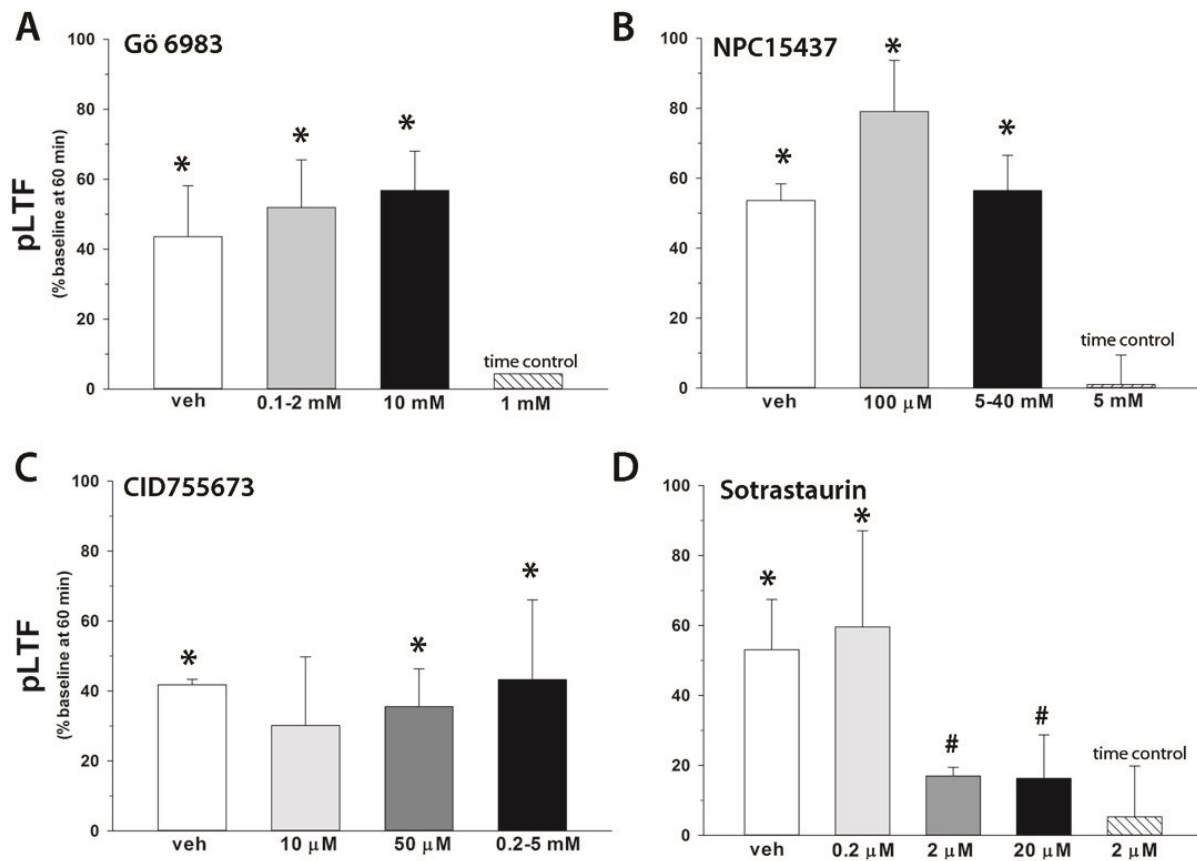
Fig. S1 Dose responses for intrathecal PKC inhibitors on pLTF. Each group received 12 µl intrathecal injections of PKC inhibitor over the phrenic motor nucleus. **A**. Gö 6983 had no effect on pLTF at any dose. Amplitude of pLTF at 60 min post-AIH in rats given injections of 0.1-2 mM gö 6983 (n=7) or 10 mM gö 6983 (n=2) compared to rats given vehicle (100% DMSO, n=7) injections. **B.** NPC had no effect on pLTF at any dose. Amplitude of pLTF at 60 min post-AIH in rats given injections of 100 µM NPC15437 (n=2) or 5-40 mM NPC15437 (n=7) compared to rats given vehicle (ACSF, n=3) injections (P>0.05). **C.** CID had no effect on pLTF at any dose tested. Amplitude of pLTF at 60 min post-AIH in rats given injections of 10 µM CID755673 (n=2), 50 µM CID755673 (n=2), or 0.2-5 mM CID755673 (n=3) compared to rats given vehicle (10-20% DMSO in ACSF, n=3) injections (P>0.05). **D.** Sotrastaurin significantly attenuated

pLTF. Amplitude of pLTF at 60 min post-AIH in rats given injections of 0.2 μM sotrastaurin (n=2), 2 μM sotrastaurin (n=4), or 20 μM sotrastaurin (n=2) compared to rats given vehicle (10% DMSO in ACSF, n=2) injections. *-denotes significant difference vs. grouped time controls (rats given vehicle or drug with no AIH, n=13). #- denotes significant difference vs. grouped vehicle AIH rats (0-20% DMSO in ACSF, 100% DMSO with AIH, n=20).
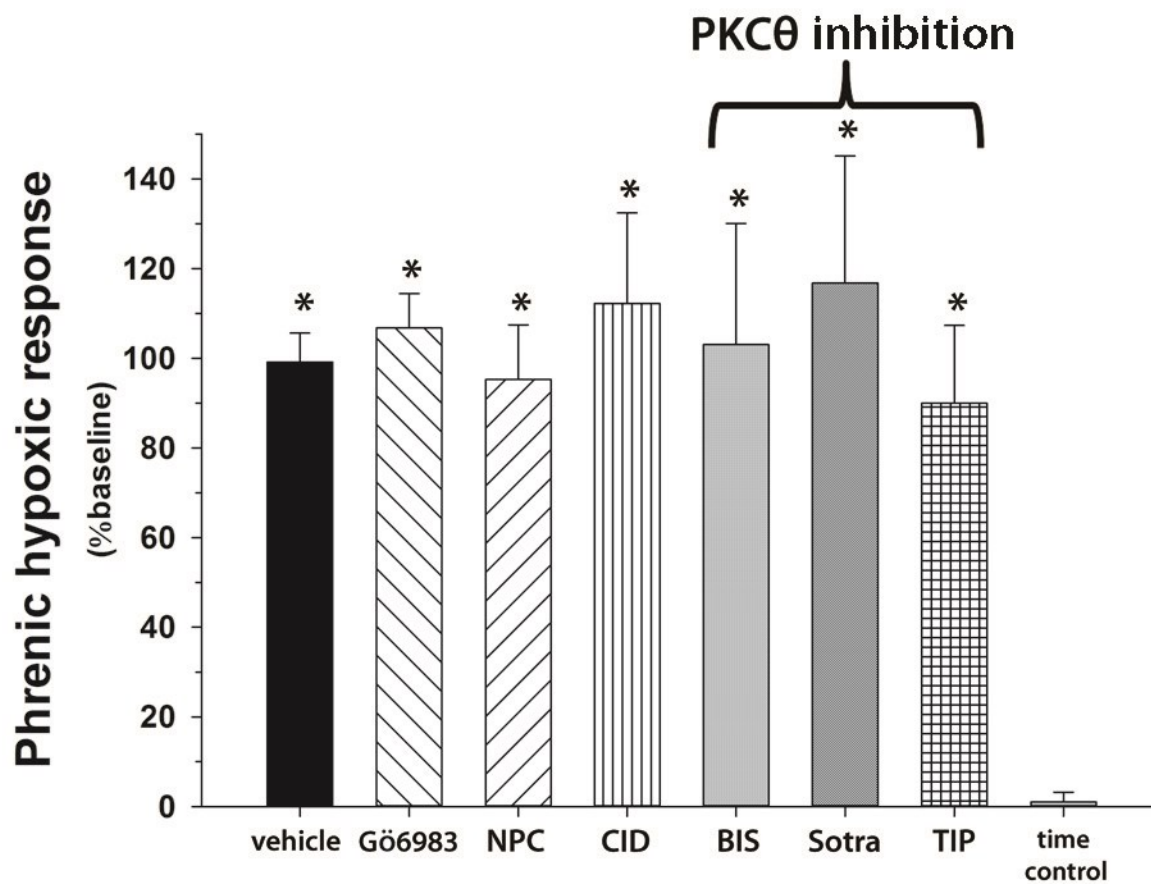
Figure S2. Spinal PKC inhibition does not alter short-term hypoxic phrenic responses.

Histogram shows average phrenic nerve amplitude (% baseline) during exposure to hypoxia

after intrathecal delivery of PKC inhibitors and compared to time control (no hypoxia) rats.

*$p < 0.05$ denotes significant difference vs. grouped time controls. Abbreviations: NPC,

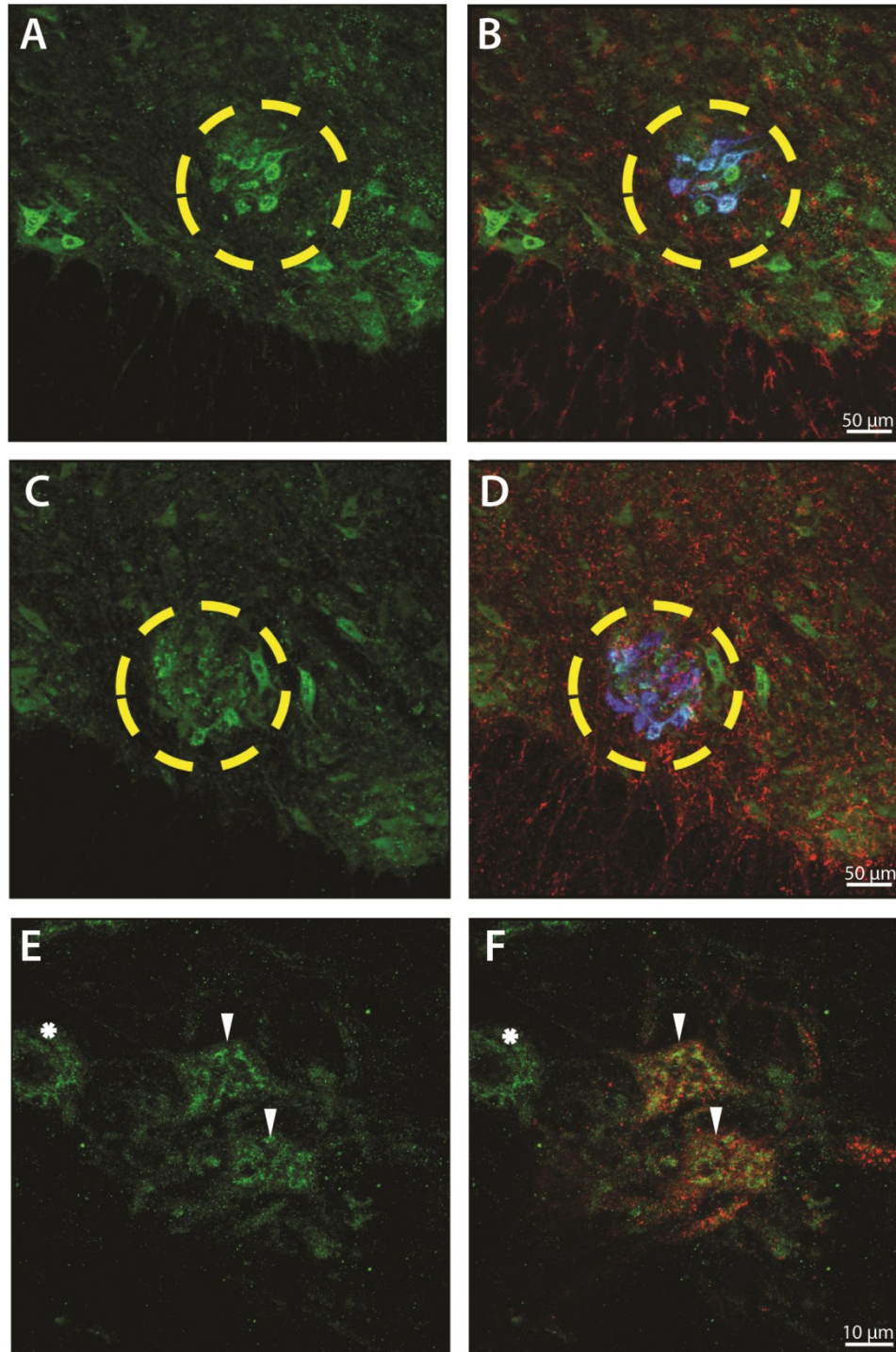NPC15437; CID, CID755673; BIS, bisindolymalemide I; Sotra, sotrastaurin.

Figure 2. PKCθ is highly expressed within phrenic motor neurons. A, C. Representative confocal fluorescence images of ventral C_4-C_5 spinal sections from rats labeling for PKCθ (Cell signaling #9377, green). B. Colabeling for phrenic motor neurons (Cholera Toxin B back-label, blue) and

microglia (CD11b, red) demonstrates colocalization of PKCθ fluorescence within identified phrenic motor neurons (CtB-positive) but not in identified microglia. D. Colabeling for phrenic motor neurons (Cholera Toxin B back-label, blue) and astrocytes (GFAP, red) shows no colocalization of PKCθ in identified astrocytes. The yellow circle outlines the phrenic motor nucleus. E. Higher magnification images of phrenic motor nucleus demonstrate distinct PKCθ immunofluorescence above background. F. PKCθ immunofluorescence is present in phrenic motor neurons (CtB-positive, ▼) but also observed in unidentified putative neurons near the phrenic motor nucleus (CtB-negative, *)
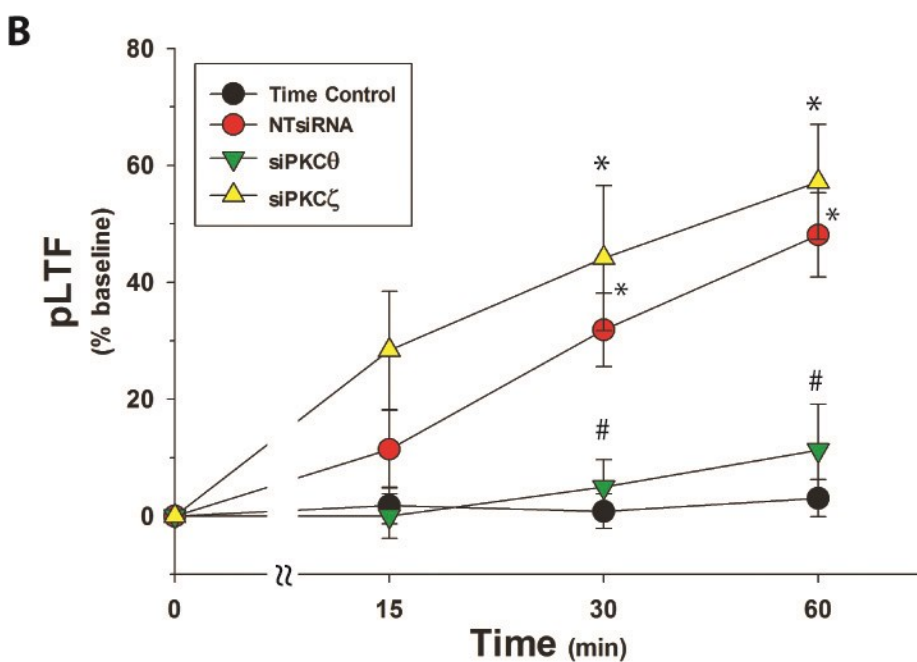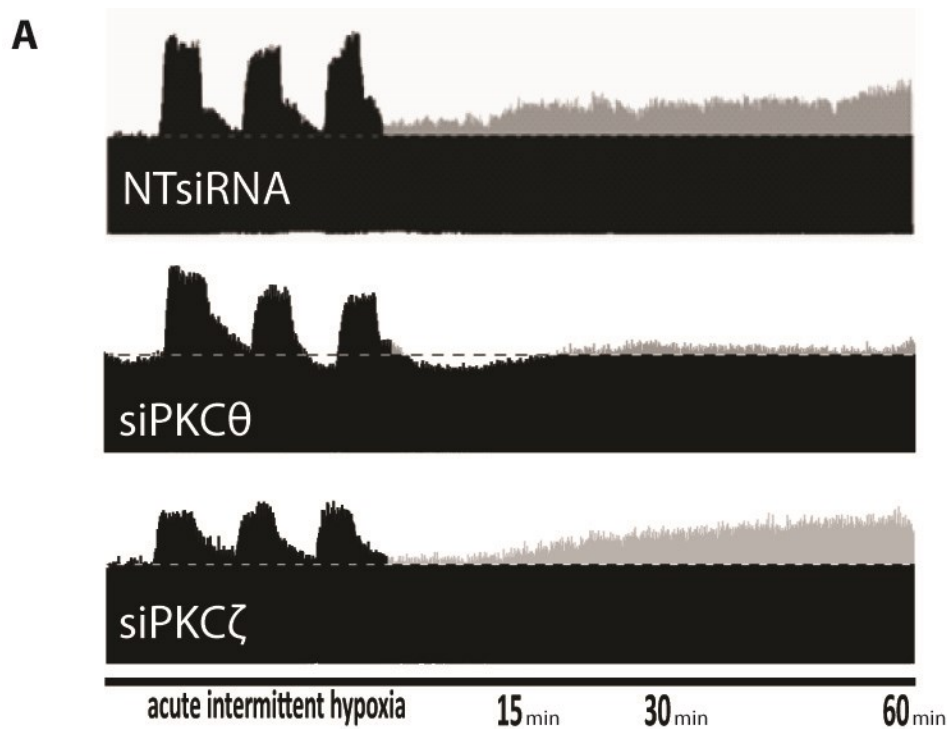
Figure 3. Intrapleural PKCθ siRNA blocks pLTF. **A.** Representative phrenic neurograms after AIH from rats given 3 daily intrapleural injections of non-targeting siRNA (NTsiRNA, n=7, top), PKCθ siRNA (siPKCθ, n=7, middle), or PKCζ siRNA (siPKCζ, n=5, bottom) and exposed to AIH.

Dotted line represents baseline amplitude; increases from baseline are shown in gray. **B.** Summary data demonstrates significant pLTF at 30 and 60 min post-AIH in NTsiRNA and siPKCζ groups but not those given siPKCθ. Time control rats given intrapleural NTsiRNA (n=4), siPKCθ (n=3), or siPKCζ (n=3) and no AIH (time controls, n=10) did not express pLTF at 60 min. *p<0.05 indicates significant difference from time controls. #-indicates significant difference (P<0.05) from siPKCζ+AIH or NTsiRNA+AIH groups.
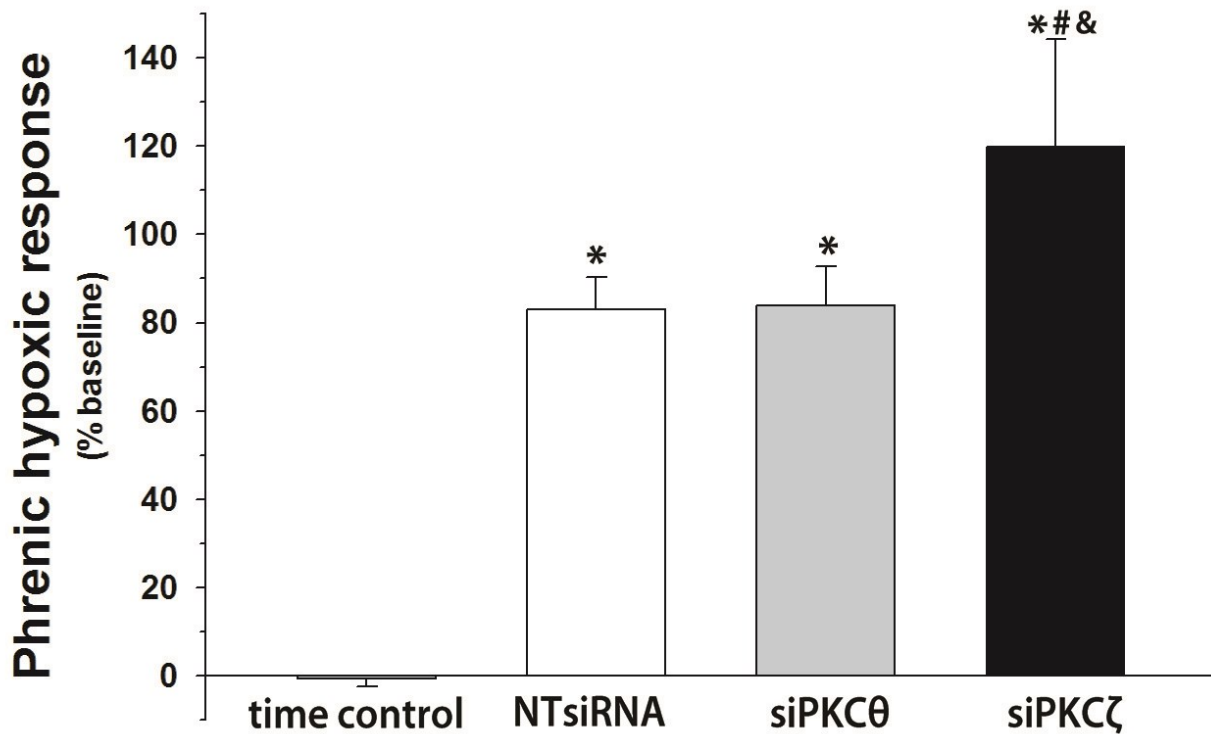
Figure S3. Hypoxic phrenic responses are unaltered by intrapleural PKCθ siRNA. Phrenic nerve amplitude (%baseline) during short-term hypoxia after pretreatment with intrapleural injections of non-targeting siRNA (NTsiRNA, n=7), PKCθ siRNA (siPKCθ, n=7), or PKCζ siRNA (siPKCζ, n=5). Time control rats (no AIH, n=10) are shown for comparison. *p<0.05 denotes significant difference vs. time controls. #p<0.05 denotes significant difference vs. intrapleural siPKCθ+AIH. & p<0.05 denotes significant difference vs. intrapleural NTsiRNA+AIH.
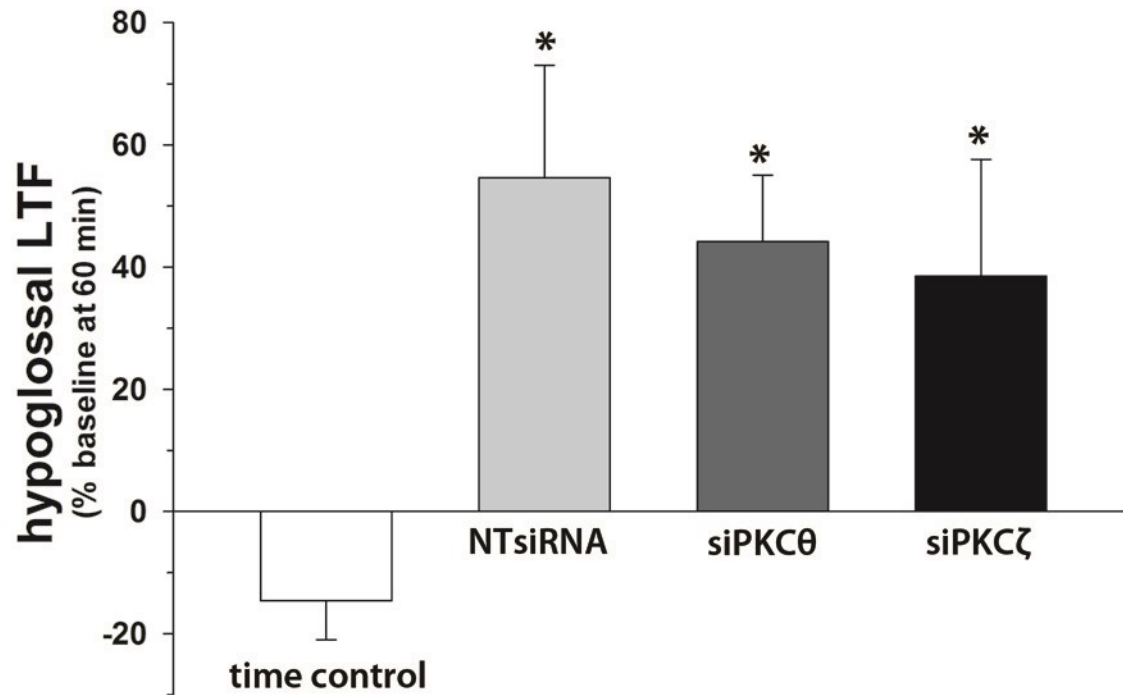
Figure S4. Hypoglossal LTF is unaffected by intrapleural siRNA. Hypoglossal nerve amplitude (% baseline) following AIH exposure after pretreatment with intrapleural injections of non-targeting siRNA (NTsiRNA, n=5), PKCθ siRNA (siPKCθ, n=3), or PKCζ siRNA (siPKCζ, n=3). All groups were significantly elevated compared to time control rats (NTsiRNA, n=3; siPKCθ, n=2; or siPKCζ, n=3 + no AIH, n=8). *p<0.05 denotes significant difference vs. time control.
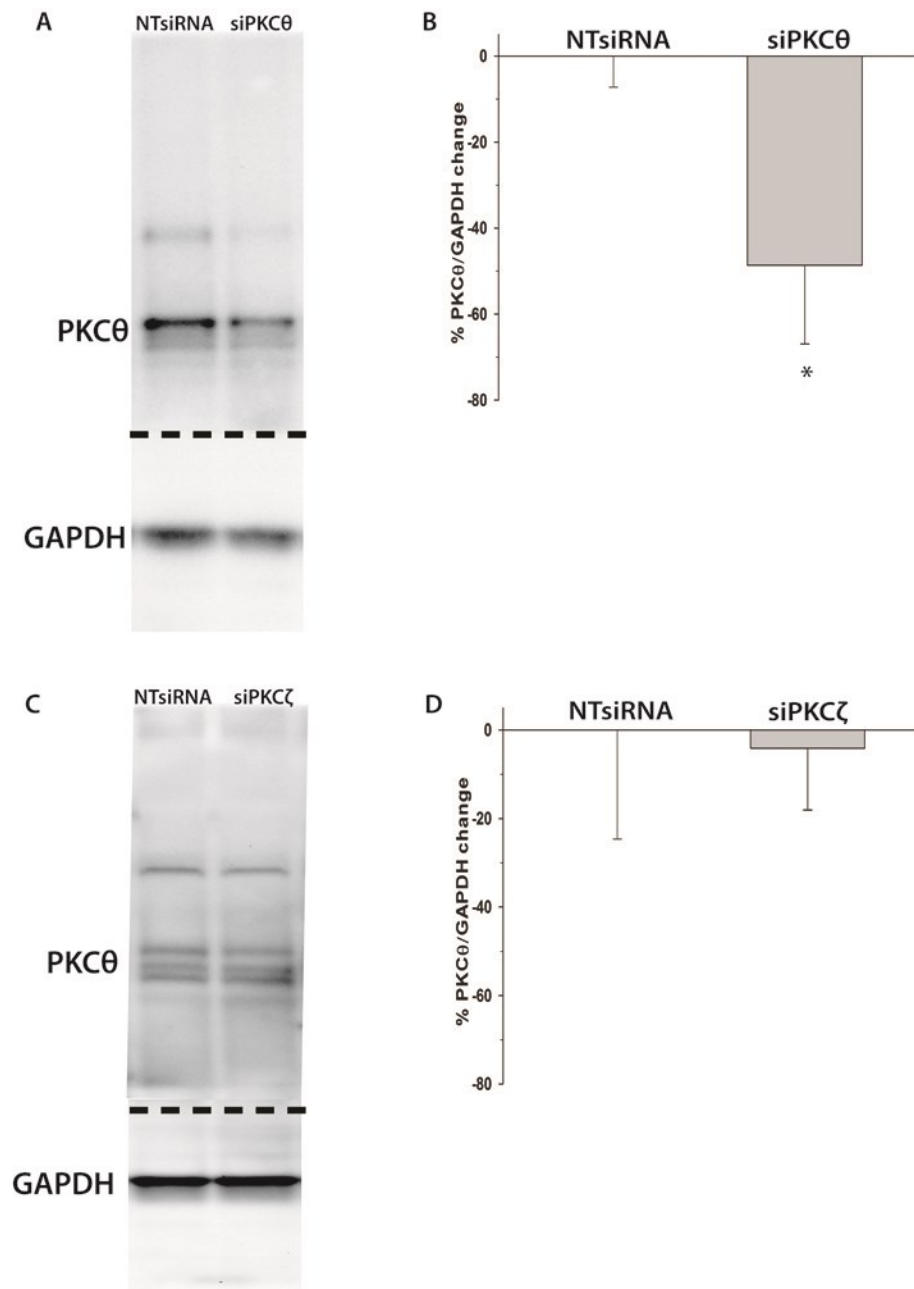
Figure 4. Intrapleural siPKCθ decreases PKCθ protein expression in ventral cervical spinal cord.

A. Representative immunoblot of ventral C_3-C_5 spinal cord from rats given three daily intrapleural injections of non-targeting siRNA (NTsiRNA, n=6) or PKCθ siRNA (siPKCθ, n=6). The immunoblot was cut (dotted line) and probed for PKCθ and GAPDH (loading control). **B.** Semi-quantitative analysis relative to GAPDH demonstrates significant knockdown of PKCθ

with intrapleural siPKCθ, compared to rats given intrapleural NTsiRNA. **C**. Representative immunoblot from ventral C_3-C_5 spinal cord for NTsiRNA (n=4) or siPKCζ (n=3). The immunoblot was cut (dotted line) and probed for PKCθ and GAPDH (loading control). **D**. Semi-quantitative analysis relative to GAPDH wit demonstrates no significant change in PKCθ protein with siPKCζ compared to NTsiRNA. *p<0.05 denotes significant difference vs. NTsiRNA.

Chapter III

Cervical spinal protein kinase C activation elicits long-lasting phrenic motor facilitation

Michael J Devinney and Gordon S Mitchell

Abstract:

The protein kinase C family of serine/threonine kinases is important for many cellular functions, including neuroplasticity in respiratory motor control. Recent evidence has implicated both novel (PKCθ) and atypical (PKCζ) PKC isoforms in distinct forms of spinal, respiratory motor plasticity known, respectively, as phrenic long-term facilitation (pLTF; induced by acute intermittent hypoxia) and inactivity-induced phrenic motor facilitation (iPMF). Thus, PKC is a critical for multiple forms of spinal respiratory motor plasticity. Here, we delivered the general PKC activator phorbol 12-myristate 13-acetate (PMA) intrathecally over the phrenic motor nucleus. Rats given PMA injections exhibited significant pMF, which was prevented by pretreatment with broad-spectrum PKC inhibitors (bisindolylmalemide 1 and NPC-15437). Because phorbol esters, including PMA, do not activate atypical PKC isoforms, activation of classical/novel PKC isoforms is sufficient to elicit pMF. These findings advance our understanding of cellular mechanisms of respiratory motor plasticity. Further, our findings suggest that spinal activation of relevant PKC isoforms is a potential therapy for disorders that impair respiratory motor output, such as amyotrophic lateral sclerosis or spinal cord injury.

Introduction

Plasticity is a key feature of the neural system controlling breathing (Feldman *et al.*, 2003; Mitchell & Johnson, 2003). Protein kinases play important roles in many forms of neuroplasticity, including hippocampal long-term potentiation (Matthies & Reymann, 1993; Sacktor *et al.*, 1993; Nayak *et al.*, 1998; Bortolotto & Collingridge, 2000), *Aplysia*

sensorimotor long-term facilitation (Chain *et al.*, 1999; Cai *et al.*, 2011), and spinal sensitization to nociceptive inputs (Coderre, 1992; Laferrière *et al.*, 2011).

Protein kinase C (PKC) is an important family of kinases that plays critical roles in multiple forms of neuroplasticity (Sossin, 2007), including spinal sensitization to nociceptive inputs after injury (Peng *et al.*, 1997; Hua *et al.*, 1999) or inactivity-induced phrenic motor facilitation (Strey *et al.*, 2012). We recently demonstrated that spinal activation of a novel protein kinase C isoform, PKC theta (PKCθ), is necessary for a well-known form of respiratory motor plasticity, phrenic long-term facilitation (pLTF) following acute intermittent hypoxia (AIH; Devinney *et al.,* ibid). pLTF is a well-studied model of spinal, respiratory motor plasticity, expressed as a prolonged increase in phrenic nerve burst amplitude lasting hours following AIH (Mitchell *et al.*, 2001; Mahamed & Mitchell, 2007; Mateika & Sandhu, 2011).

Recently multiple, distinct cellar cascades giving rise to pMF have been demonstrated (Dale-Nagle *et al.*, 2010). For example, pMF can be elicited pharmacologically by spinal injections of agonists to serotonin receptors (MacFarlane & Mitchell, 2009; Hoffman & Mitchell, 2011; MacFarlane *et al.*, 2011), adenosine 2A receptors (Golder *et al.*, 2008), or growth factors such as brain-derived neurotrophic factor (Baker-Herman *et al.*, 2004), vascular endothelial growth factor (Dale-Nagle *et al.*, 2011) and erythropoietin (Dale *et al.*, 2012). Protein kinase activation can also cause pMF; intrathecal injections of a cAMP analog over the phrenic motor nucleus elicit protein kinase A-dependent pMF (Hoffman & Mitchell, 2013) and motor recovery after spinal cord inury (Kajana & Goshgarian, 2008). We hypothesized that activation of PKC would also cause pMF.

Although PKCθ (Devinney et al., ibid) and PKCξ (Strey et al., 2012) are now known to elicit discrete forms of pMF, it is not known if other PKC isoforms are also capable of eliciting pMF. In this study, we hypothesized that cervical spinal activation of PKC isoforms other than PKCθ or PKCξ is sufficient to elicit long-lasting pMF in anesthetized rats. Thus, we delivered the non-selective classical and novel PKC activator phorbol 12-myristate 13-acetate (PMA) intrathecally in the region of the phrenic motor nucleus while recording phrenic motor output. Some rats were pretreated with intrathecal injections of the broad-spectrum PKC inhibitors bisindolylmalemide I (BIS) or NPC-15437 (NPC) to confirm effects of PMA injections were PKC-dependent. Because of the different isoform specificities of these drugs, we demonstrated that PMA elicits pMF via mechanisms that likely do not require either PKCθ or PKCξ activity.

Experimental Procedures

Animals

Adult male Sprague-Dawley rats (12-17 week old; 280-500g; Harlan Colonies 211, Houston, TX or 218a, Indianapolis, IN) were used in all experiments. Rats were housed two per cage with food and water *ad libitum*, and kept in a 12 hr light/dark cycle. The School of Veterinary Medicine Animal Care and Use Committee at the University of Wisconsin approved all experimental procedures.

Surgical preparation

Rats were anesthetized in a closed chamber via isoflurane inhalation and placed on a heated surgical table; anesthesia was maintained with nose cone (3.5% isoflurane, 50% O_2). Rats were then pump-ventilated through a tracheal tube (Rodent Ventilator 683, Harvard Apparatus; tidal volume 2.2-2.7 ml; frequency ~75 breaths/min) with 50% inspired O_2 and 3.5% isoflurane (balance N_2) for anesthetic maintenance. End-tidal CO_2 was maintained at 40-44 mmHg by adding trace CO_2 to the gas mixture as needed. A catheter was placed in a tail vein for intravenous fluid administration (~1.5 ml/hr of 75% lactated ringer's solution, 10% HCO_3, and 15% hetastarch, Hespan, 6% hetastarch in 0.9% NaCl) to maintain base excess within a range of -4 to +4 mEq/L; base excess changed less than 1.5 mEq/L from baseline throughout experiment. A catheter was placed in the femoral artery to monitor blood pressure and draw arterial blood samples for analysis (P_{aO2}, Pa_{CO2}, pH, base excess). Using a dorsal approach, the left phrenic nerve was isolated, cut, desheathed and covered with saline-soaked cotton. For intrathecal drug delivery, a C_2 laminectomy was performed to expose the dura, which was cut. A silicone catheter (2 Fr; Access Technologies, Skokie, IL) attached to a 50µl Hamilton syringe was slid under the dura over C_4. Conversion to urethane anesthesia was performed by slowly decreasing inhaled isoflurane while administering intravenous urethane (1.7-1.9 mg/kg) over 15 minutes. After ~1 hr, pancuronium bromide (2.5 mg/kg, i.v.) was administered to prevent respiratory effort. Body temperature was maintained at 37.5±1 °C (rectal thermometer; Fisher Scientific, Pittsburgh, PA). Blood pressure was monitored to ensure physiological stability (80-150 mmHg baseline, <30 mmHg change at 90 min post-intermittent hypoxia). Adequate anesthesia was

determined by the lack of responses to toe pinch in blood pressure or phrenic nerve activity.

Neurophysiology measurements

Approximately 1hr after conversion to urethane anesthesia, desheathed phrenic and hypoglossal nerves were covered with mineral oil and placed on bipolar silver electrodes. Nerve activity was amplified (10,000X) band-pass filtered (300-10,000 Hz Model 1800, A-M Systems, Carlsborg, WA), full wave rectified and integrated with a continuous moving averager (time constant: 50 ms; CWE Inc., MA-821 filter; Ardmore, PA). The integrated signal was digitized and analyzed with a data acquisition system (WINDAQ, DATAQ Instruments, Akron, OH). CO_2 apneic and recruitment thresholds were determined by lowering inspired CO_2 and/or increasing ventilator rate until phrenic nerve bursting ceased. CO_2 was then slowly raised by increasing inspired CO_2 and/or decreasing ventilator rate until phrenic nerve bursting resumed (ie, recruitment threshold). Baseline end-tidal CO_2 was set at 2-3 mmHg above this recruitment threshold. Baseline nerve activity was recorded for about 20 minutes. Throughout a protocol, arterial P_{CO_2} was maintained within ±1.5 mmHg from baseline. Following intrathecal PMA or vehicle injections, baseline conditions were maintained. Blood gas analysis at 15, 30 and 60 min post-injections confirmed that blood gas variables were within our criteria (P_{aO_2}>180 mmHg, P_{aCO_2} ±1.5 mmHg baseline, base excess ±1.5 mEq/L baseline). Maximal chemoreflex responses were elicited at the end of each experiment to verify adequate dynamic range of phrenic nerve responses and the

stability of each preparation. Rats with ≤50% maximal hypercapnic response were eliminated from analysis.

Intrathecal injections.

The PKC activator phorbol 12-myristate 13-acetate (PMA) and PKC inhibitors bisindolymalemide I (BIS) and NPC-15437 (NPC) were delivered at doses consistent with literature reports (Coderre, 1992; Yashpal *et al.*, 1995; Hua *et al.*, 1999; Ferguson *et al.*, 2008; Laferrière *et al.*, 2011). Stock solutions were diluted in aCSF (vehicle, 12μl aCSF; in mM 120 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 23 $NaHCO_3$, 10 glucose, bubbled in 95% O_2, 5% CO_2 for 15 min) to their final concentration with a DMSO concentration of 1% (v/v) for PMA, 20% for BIS and 0% for NPC. A 50μl Hamilton syringe was connected to a silicone catheter (2 French Access Technologies; 0.3 mm inner diameter) which was inserted above C_4 spinal segment. All rats received three 7μl injections (2μl bolus/30s) of either PMA (9μM) or vehicle (1% DMSO in aCSF) injections delivered in 5 minute intervals. For rats pretreated with PKC inhibitors, 12μl of either BIS (280μM) or NPC (5mM) was delivered (2μl bolus/30s) 10-15 min prior to injections of PMA or vehicle. There was no significant effect of either PKC inhibitor on phrenic nerve amplitude in vehicle-injected rats (p=0.948).

Data analysis

Integrated phrenic burst amplitude and frequency were averaged in 60 sec bins at baseline and at 15, 30, 60, and 90 min after PMA or vehicle injections. All average burst amplitude values were normalized to baseline; frequency was expressed as change

from baseline (burst/min). A two-way ANOVA with repeated measures design was used to analyze phrenic burst amplitude and frequency. *Post-hoc* comparisons were made with Fisher LSD (SigmaPlot version 12.0; Systat Software, San Jose, CA) to compare individual differences. In figure 4, vehicle-injected controls pretreated with either BIS or NPC were grouped with rats injected with vehicle alone (vehicle controls) since no significant differences were found between these three groups. Differences between groups were considered significant if $p < 0.05$. All values are expressed as mean ± 1 SEM.

Results

Spinal PMA injections elicit phrenic motor facilitation

Since phrenic long-term facilitation following acute-intermittent hypoxia requires PKC activation, we tested the hypothesis that spinal PKC activation alone is sufficient to induce similar phrenic motor facilitation. Intrathecal injections of the PKC activator PMA (black arrows, Fig 1A) near the phrenic motor nucleus significantly increased (n=6, $p < 0.001$) phrenic nerve burst amplitude (ie. pMF) at 30 (27±7%), 60 (37±5%) and 90 min (49±7%) post-injections versus vehicle (Fig 1B; 7±7% at 90 min, n=6). Vehicle injected rats exhibited no significant changes in phrenic nerve burst amplitude at 90 min versus baseline (p=0.21) in contrast to rats injected with PMA (p<0.001). No significant changes in phrenic burst frequency were found at any time in PMA or vehicle injected rats (Figure 1C, p=0.129)

PMA-induced pMF requires PKC activation

To confirm that PMA injections caused pMF through spinal PKC activation, we tested the hypothesis that spinal PKC inhibition prevents PMA-induced pMF. In rats pretreated with broad-spectrum PKC inhibitor, bisindolylmaleimide I (BIS; Fig 2A, open arrow), PMA (black arrows, Fig 2A) failed to cause significant phrenic motor facilitation ($9\pm8\%$ at 90 min, n=6) versus vehicle treated rats ($3\pm2\%$ at 90 min, n=4; p=0.321, Fig 2B). There were no significant changes in phrenic nerve amplitude at 90 min versus baseline in PMA (p=0.118) or vehicle injected rats pretreated with BIS (p=0.644). Significant changes in phrenic burst frequency were not observed at any time between PMA or vehicle injected rats pretreated with BIS (Figure 2C, p=0.129).

Since pharmacological PKC inhibition with BIS may have off-target effects, including inhibition of p90rsk (Roberts *et al.*, 2005), p70S6K (Alessi, 1997; Roberts *et al.*, 2004), and 5-HT3 receptors (Coultrap *et al.*, 1999), we utilized another non-specific PKC inhibitor, NPC-15437 (NPC). NPC inhibits PKC by preventing diacylglycerol-mediated activation of the PKC regulatory domain (Sullivan *et al.*, 1991, 1992), in contrast to BIS , which prevents phosphorylation of substrates through active-site inhibition (Gould *et al.*, 2011). In rats pretreated with NPC (Fig 3A, open arrow), PMA injections (black arrows, Fig 3A) failed to cause significant phrenic motor facilitation ($15\pm9\%$ at 90 min, n=5) versus vehicle-injected rats ($5\pm9\%$ at 90 min, n=3; p=0.343. Fig3B). However, PMA-injected rats pretreated with NPC had slightly higher phrenic nerve burst amplitudes versus baseline (p=0.017) whereas vehicle treated rats did not (p=0.484). No significant changes in phrenic burst frequency were found at any time between PMA and vehicle injected rats pretreated with NPC (Figure 3C, p=0.129).

Summary

Spinal PKC activation with intrathecal PMA injections is sufficient to elicit long-lasting pMF (Fig 4), an effect prevented by pretreatment with two distinct PKC inhibitors (BIS and NPC; Fig 4; $p<0.001$). Because Bis blocks intermittent hypoxia induced pLTF, whereas NPC does not (Devinney et al., ibid), PMA must activate unique PKC isoforms distinct from PKCθ.

Discussion

Spinal PKC activation is sufficient to cause pMF

Activation of distinct PKC isoforms are required for different forms of spinal plasticity such as PKCθ for AIH-induced pLTF (Devinney et al, *ibid*), and PKCζ for inactivity-induced pMF (Strey *et al.*, 2012). Although PKC activity is necessary for these forms of respiratory motor plasticity, it was not known if PKC activation is sufficient, nor if PKC isoforms other than PKCθ or PKCζ are capable of eliciting long-lasting pMF. Data presented here demonstrate that PKC activation is indeed sufficient to elicit long-lasting pMF, and suggest that distinct PKC isoforms are capable of doing so. The role of these isoforms in respiratory motor plasticity is not yet known, but we suggest that unique PKC isoforms contribute to plasticity elicited by novel neurotransmitter receptors, such as $\alpha 1$ adrenergic receptors (Dale-Nagle *et al.*, 2010). Activation of these adrenergic receptors elicits long-lasting, PKC-dependent facilitation of hypoglossal respiratory

activity (Neverova *et al.*, 2007) and is sufficient, but not necessary, for AIH-induced pLTF (Huxtable et al., unpublished).

Spinal PMA elicits pMF through classical/novel PKC stimulation

Phorbol esters, including PMA, are C1 domain-ligands which can activate PKC along with other proteins containing a functional C1 domain, such as chimaerins (Rac GTPase activating proteins), RasGRP1 (guanaine nucleotide exchange factor enzyme), and Munc13 (scaffolding proteins necessary for exocytosis; Kazanietz, 2002; Marland *et al.*, 2011). In this study, we ruled out involvement of other PMA activated molecules by using PKC inhibitors at doses consistent with inhibition of most classical/novel PKC isoforms (Martiny-Baron *et al.*, 1993; Gschwendt *et al.*, 1996; Uberall *et al.*, 1997; Saraiva *et al.*, 2003; Felber *et al.*, 2007). Indeed, PMA-induced pMF is prevented by two distinct PKC inhibitors, suggesting that PKC activation is necessary for the response. Atypical PKC isoforms, including PKCζ, contain C1 domains which lack affinity for DAG or phorbol esters, and are unresponsive to PMA (Kazanietz *et al.*, 1994; Colón-González & Kazanietz, 2006; Pu *et al.*, 2006). Thus, our results suggest that spinal PMA induces pMF via stimulation of a classical and/or novel PKC isoform.

Different PKC isoforms are involved in different forms of spinal respiratory motor plasticity

pLTF induced by AIH requires activation of spinal PKCθ, but not other PKC isoforms (Devinney et al, ibid). In contrast, inactivity-induced pMF requires spinal PKCζ activity (Strey *et al.*, 2012). In the current study, it is likely that PMA activates multiple

classical/novel (but not atypical) PKC isoforms. Interestingly, PKCθ activation is likely not required for PMA-induced pMF since NPC pretreatment prevents PMA-induced pMF, but does not block AIH-induced pLTF (Devinney et al., ibid), suggesting minimal inhibitory effect on PKCθ. Structural studies demonstrate that the C1 domains of PKCθ differ from all other PKC isoforms, because C1B (vs C1A) is the domain dominantly binding DAG (Melowic *et al.*, 2007; Steinberg, 2008). Moreover, structural studies demonstrate narrowing of the phorbol ester-binding surface of C1B, in PKCθ C1B crystal structures versus the relatively similar PKCδ C1B (80% sequence homology) crystal structure (Rahman *et al.*, 2013). Thus, NPC is unlikely to bind the DAG/phorbol ester binding sites on the C1B domain of PKCθ, explaining the lack of effect on AIH-induced pLTF. In contrast, NPC completely prevented PMA-induced pMF, suggesting involvement of other spinal classical/novel PKC isoforms. However, a small (15%) residual facilitation in NPC-treated rats may have resulted from less effective PMA-induced PKCθ activation. Nevertheless, most PMA-induced pMF represents a different form of phrenic motor plasticity, differing from AIH-induced pLTF because it requires activation of distinct classical/novel PKC isoform(s).

Spinal PKC activation has no effect on respiratory frequency

Phrenic motor plasticity induced by AIH (Baker-Herman & Mitchell, 2008),or spinal injections of serotonin (MacFarlane & Mitchell, 2009), BDNF (Baker-Herman *et al.*, 2004), VEGF(Dale-Nagle *et al.*, 2011), or erythropoetin (Dale *et al.*, 2012) induce small increases in phrenic burst frequency, known as frequency long-term facilitation (Baker-Herman & Mitchell, 2008). While changes in respiratory frequency are often presumed

to occur from effects on the medullary rhythm generator, these studies suggest a spinal mechanism indirectly influencing rhythmogenesis, perhaps through modulation of spinal, sensory afferent inputs to respiratory rhythm generating neurons. However, no significant frequency effects were observed after PMA, suggesting minimal role for spinal PKC activation in frequency long-term facilitation.

Therapeutic potential of PKC-dependent phrenic motor plasticity

PKC activation is critical for at least two forms of phrenic motor plasticity induced by AIH or respiratory inactivity (Devinney et al., ibid; Strey *et al.*, 2012). However, chronic intermittent hypoxia can have detrimental effects, such as systemic hypertension (Lesske *et al.*, 1997) or cognitive deficits (Row *et al.*, 2002). Further, prolonged respiratory inactivity is incompatible with life. Therefore, pharmacological strategies to induce plasticity may be useful to treat respiratory motor deficits in conditions such as amyotrophic lateral sclerosis, spinal cord injury or obstructive sleep apnea (Mahamed & Mitchell, 2007). Since PMA is a potent tumor promoter and unsuitable for human applications (Liu & Heckman, 1998), development of specific activators of specific PKC isoforms (particularly PKCθ and/or PKCζ) could benefit patients by inducing spinal plasticity as a means to restore respiratory function.

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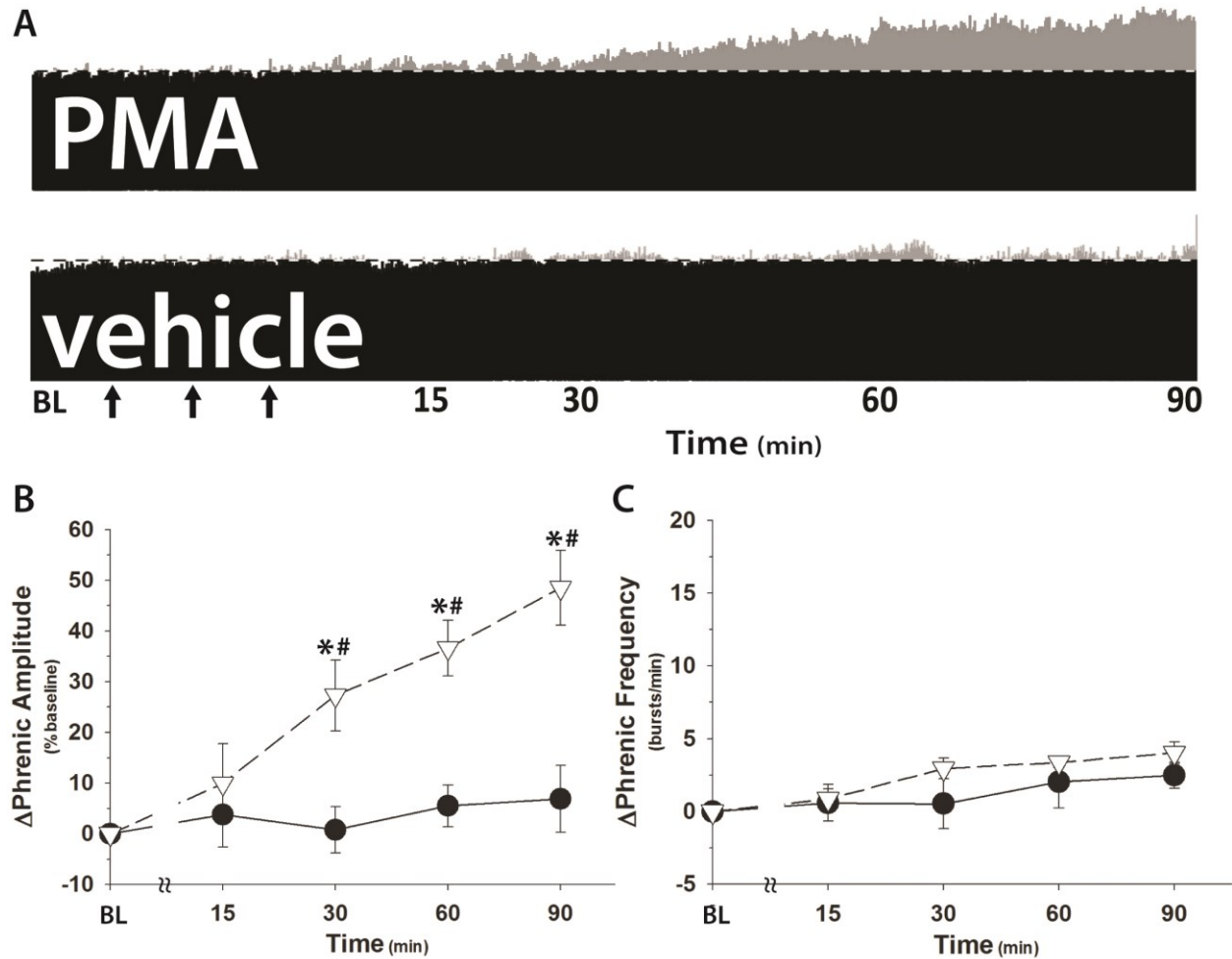
Figures

Figure 1. Spinal PKC activation with intrathecal injections of PMA causes phrenic motor

facilitation. A. Representative phrenic neurograms of rats injected (3 injections separated by 5

mins, ↑) with PMA (9µM) or vehicle (1% DMSO in ACSF). Dotted line delineates baseline;

increases from baseline (pMF) are shown in gray. B. Summary data for change in phrenic

amplitude (%baseline) following PMA (n=6, ▽) or vehicle (n=6, ●) injections. C. Summary data

for change in phrenic burst frequency (bursts/min) following PMA (▽) or vehicle (●) injections.

(*) denotes significantly increased compared to vehicle injected rats (p<0.001). (#) denotes

significantly increased compared to baseline (p<0.001)

Figure 2. Spinal PKC inhibition with BIS prevents PMA-induced phrenic motor facilitation. A. Representative phrenic neurograms from rats pretreated with BIS (12µl, 280 µM) and injected (3 injections separated by 5 mins, ↑) with PMA (9 µM) or vehicle (1% DMSO in ACSF). Dotted line delineates baseline; Increases from baseline (pMF) are shown in gray. B. Summary data for change in phrenic amplitude (%baseline) following PMA (n=6, ▽·) or vehicle (n=4, ●) injections. C. Summary data for change in phrenic burst frequency (bursts/min) following PMA (▽·) or vehicle (●) injections. (#) denotes significantly increased compared to baseline (p<0.05). No significant differences were noted in phrenic amplitude compared in rats pretreated with BIS and injected with PMA compared to vehicle-injected rats. No significant differences were noted in phrenic burst frequency at any time point.
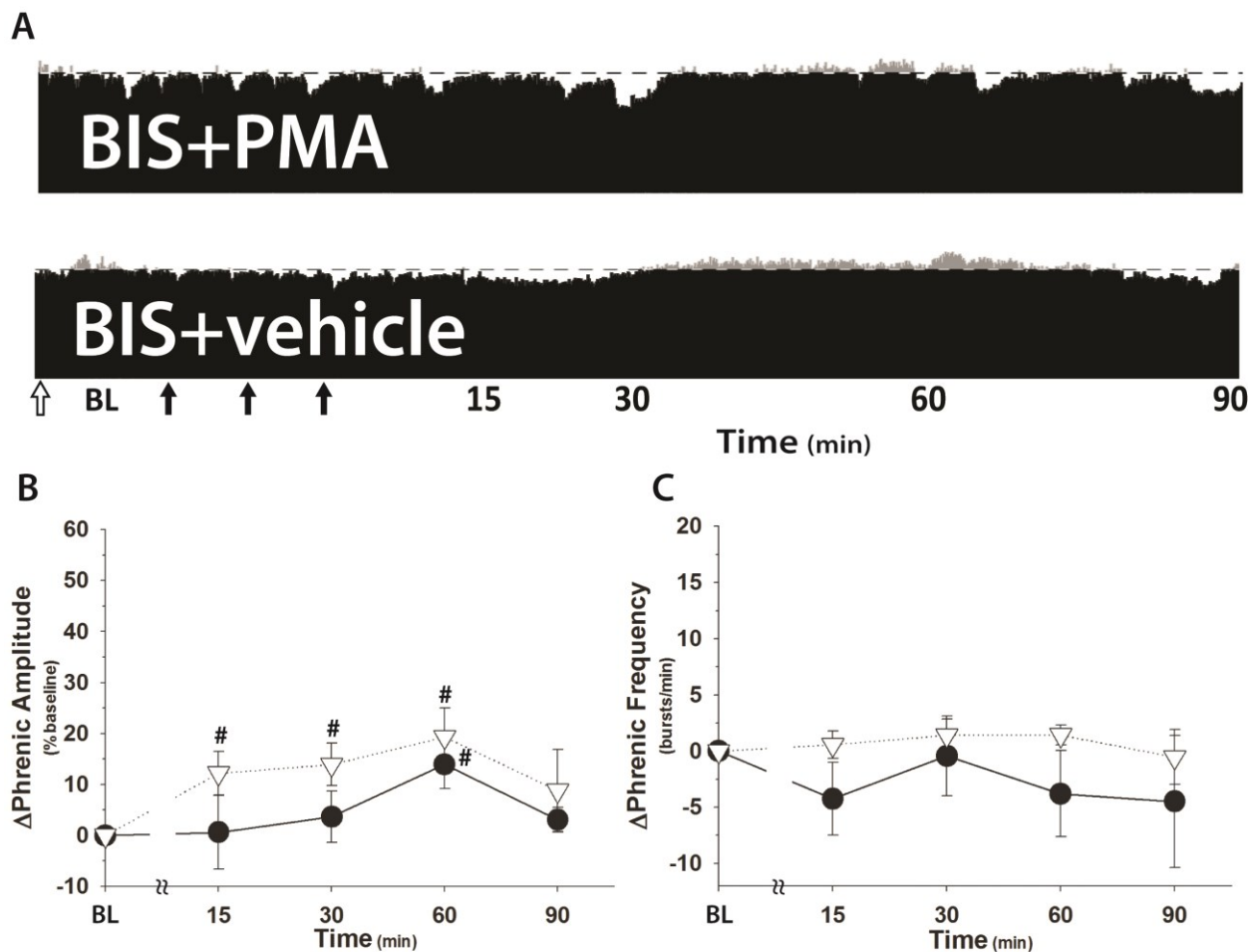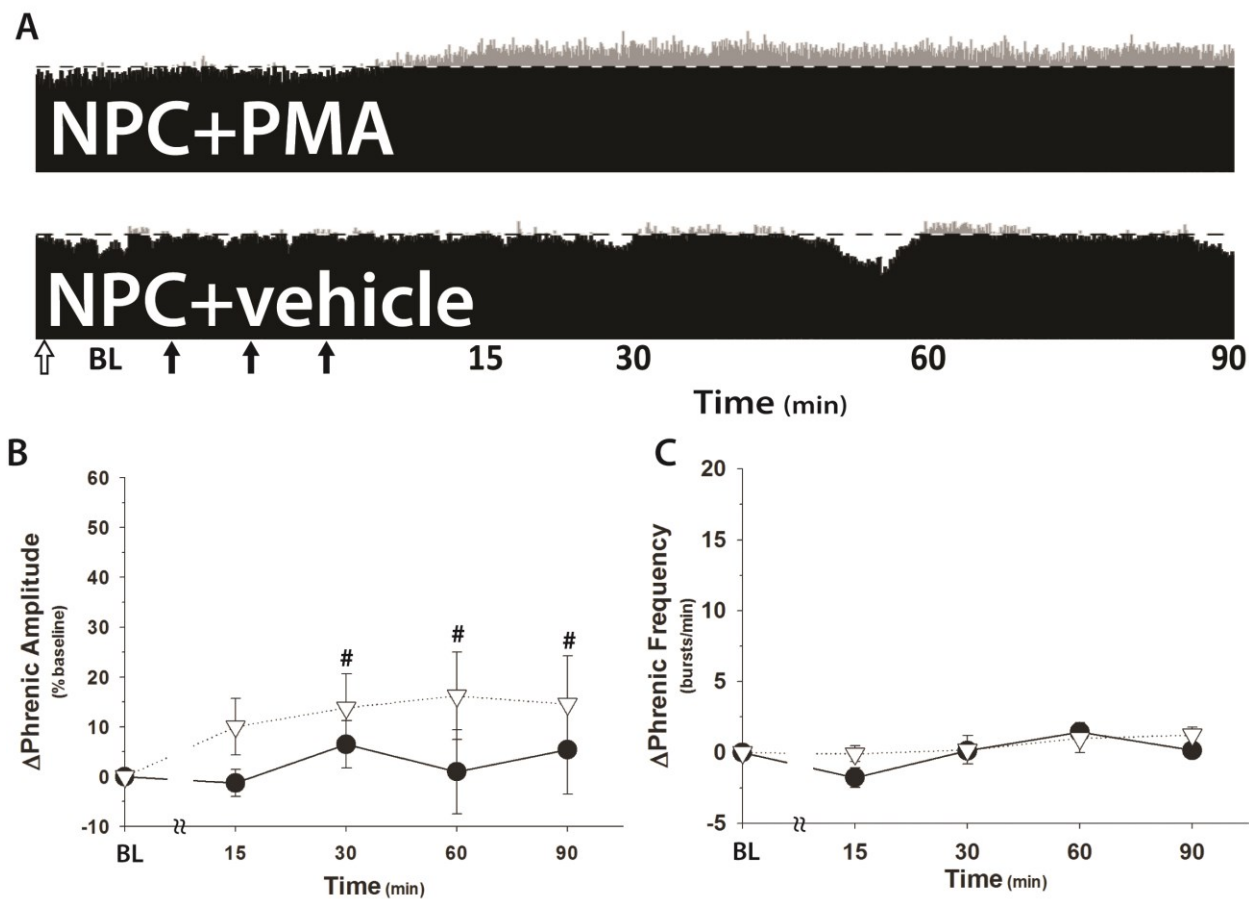
Figure 3. Spinal PKC inhibition with NPC prevents PMA-induced phrenic motor facilitation. A. Representative phrenic neurograms from rats pretreated with NPC (12μl, 5mM) and injected (3 injections separated by 5 mins, ↑) with PMA (9 μM) or vehicle (1% DMSO in ACSF). Dotted line delineates baseline; Increases from baseline (pMF) are shown in gray. B. Summary data for change in phrenic amplitude (%baseline) following PMA (n=6, ▽·) or vehicle (n=4, ●) injections. C. Summary data for change in phrenic burst frequency (bursts/min) following PMA (▽·) or vehicle (●) injections. (#) denotes significantly increased compared to baseline (p<0.05). No significant differences were noted in phrenic frequency at any time point. No significant differences were noted in phrenic amplitude compared in rats pretreated with NPC and injected

with PMA compared to vehicle-injected rats. No significant differences were noted in phrenic

burst frequency at any time point.
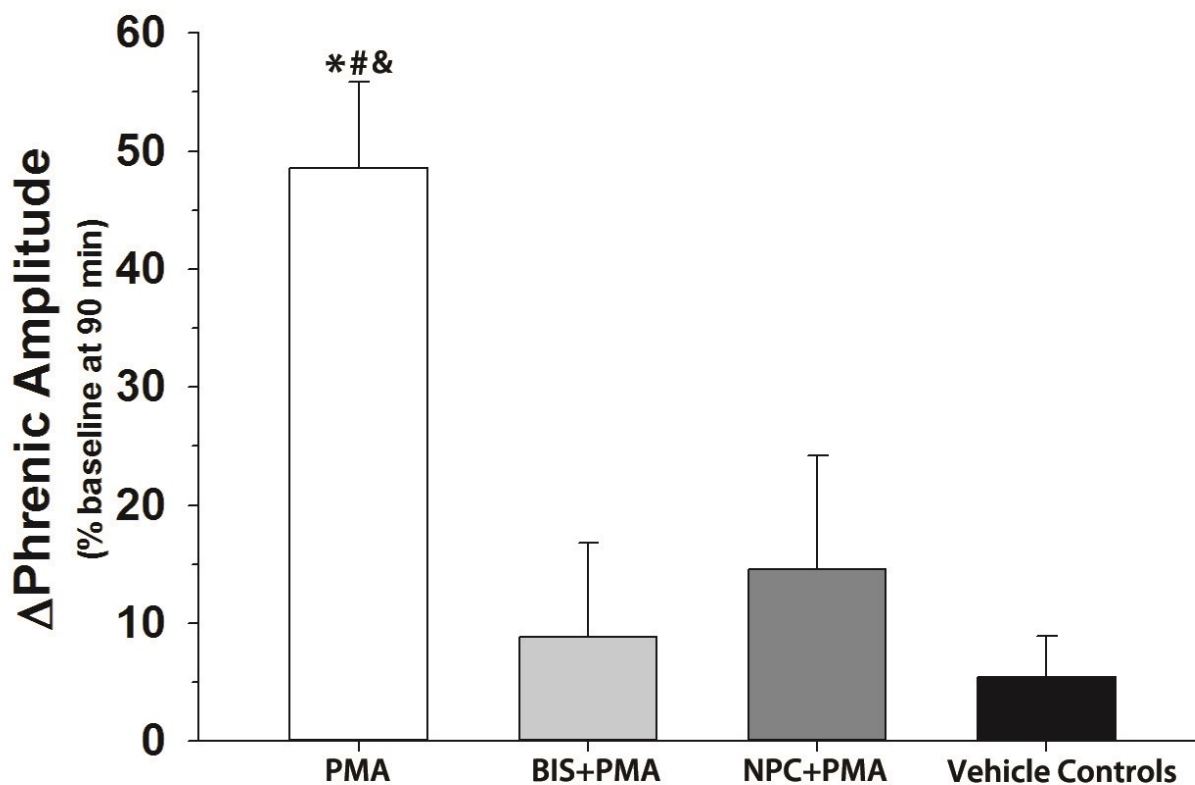
Figure 4. Spinal PMA injections cause PKC-dependent phrenic motor facilitation. Summary data

from rats given intrathecal injections of PMA or vehicle and PKC anagtonists. Rats given

intrathecal injections of PMA alone exhibit significant phrenic motor facilitation (49±7%)

compared to rats pretreated with BIS and given PMA injections (9±8%,* $p<0.001$) or rats

pretreated with NPC and given PMA injections (15±10%, # $p<0.001$) or rats given vehicle

injections instead of PMA (5±5%, & $p<0.001$).

Chapter IV

Pattern sensitivity of hypoxia-induced phrenic motor facilitation is an emergent property of competing spinal serotonin- and adenosine-dependent mechanisms

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Abstract

Pattern sensitivity is a hallmark of neuroplasticity, including spinal respiratory motor plasticity induced by intermittent hypoxia. Moderate acute intermittent hypoxia (AIH) elicits spinal, serotonin-dependent phrenic motor facilitation (pMF), known as phrenic long-term facilitation. This effect is enhanced by concomitant spinal adenosine 2A (A_{2A}) receptor inhibition. In contrast, moderate acute sustained hypoxia (ASH; 25 min) fails to elicit pMF, demonstrating pattern sensitivity. Severe AIH elicits pMF through a distinct serotonin-independent mechanism that requires spinal A_{2A} receptor activation. This shift from serotonin- to adenosine-dependent pMF may reflect greater spinal adenosine accumulation during severe hypoxia. We hypothesize that adenosine and serotonin-dependent pathways interact *via* cross-talk inhibition, and that this mutual inhibition is balanced during moderate ASH, thereby conferring pattern sensitivity. Thus, blocking spinal A_{2A} receptors during moderate ASH may reveal pMF via the opposing serotonergic mechanism. Anesthetized rats received intrathecal A_{2A} and/or serotonin receptor antagonist injections prior to moderate (PaO_2 40-50 mmHg) or severe (PaO_2 25-35 mmHg) ASH. Spinal A_{2A} receptor inhibition prior to moderate ASH revealed serotonin-dependent pMF. In contrast, severe ASH elicited A_{2A}-dependent pMF, which was enhanced by spinal serotonin receptor blockade. In summary, adenosine and serotonin-dependent pathways may compete during hypoxia *via* mutual, cross-talk inhibition; varying patterns/severities of hypoxia shifts the balance between pathways. Hypoxia-induced pMF is prevented when these distinct, competing mechanisms offset, demonstrating pattern sensitivity as an emergent property. Understanding mechanisms

of pattern sensitivity in any form of neuroplasticity is of fundamental biological significance, and may enable us to better harness spinal plasticity as a therapeutic tool.

Introduction

Patterned stimuli are often more effective at eliciting biological responses compared to continuous stimuli. This phenomenon, termed "pattern sensitivity", is quite common in neural systems. Over a century ago, Hermann Ebbinghaus reported that subjects better remember items presented intermittently vs. continuously (Ebbinghaus, 1913). Since then, pattern sensitivity has been demonstrated in models of neuroplasticity including hippocampal long-term potentiation (Scharf *et al.*, 2002), A*plysia* sensorimotor facilitation (Mauelshagen *et al.*, 1998), and long-term facilitation of phrenic motor output (Baker & Mitchell, 2000). Moreover, pattern sensitivity has been applied to enhance outcomes in education (Kerfoot, 2010), advertising (Janiszewski *et al.*, 2003), physical rehabilitation (Goverover *et al.*, 2009*a*), multiple sclerosis (Goverover *et al.*, 2009*b*), and spinal cord injury (Trumbower *et al.*, 2012). Although pattern sensitivity is a hallmark feature of neuroplasticity, the underlying mechanisms are not well understood in any system.

One interesting model of pattern-sensitive plasticity is phrenic long-term facilitation, which is a form of phrenic motor facilitation (pMF; increase in phrenic nerve burst amplitude), that persists for hours following exposure to acute intermittent hypoxia (AIH). In contrast, exposure to acute sustained hypoxia (ASH) of equal cumulative duration fails to elicit pMF, demonstrating pattern sensitivity (Baker & Mitchell, 2000). Our knowledge concerning cellular mechanisms regulating phrenic motor facilitation has rapidly increased (Dale-Nagle *et al.*, 2010), permitting conceptions of interactions generating pattern sensitivity. Intermittent activation of spinal serotonin type 2 (5-HT$_2$) receptors are necessary and sufficient for pMF following AIH ((Fuller *et al.*, 2001;

MacFarlane & Mitchell, 2009). Interestingly, okadaic acid-sensitive serine/threonine phosphatases constrain serotonin-dependent pMF following ASH, suggesting that serotonin-dependent mechanisms are activated, but constrained during ASH (Wilkerson *et al.*, 2008). Such constraints likely produce pattern sensitivity; however, it is unknown how different patterns of hypoxia modulate these constraints to produce pattern sensitivity.

Recent studies have demonstrated that multiple, distinct cellular cascades give rise to phenotypically similar pMF (Dale-Nagle *et al.*, 2010). Spinal activation of adenosine 2A (A_{2A}) receptors elicits pMF (Golder *et al.*, 2008), and this also occurs with AIH of greater severity (PaO2 25-30 mmHg(Nichols *et al.*, 2012). This shift to serotonin-independent, A_{2A}-dependent pMF may reflect greater extracellular adenine nucleotide accumulation during severe AIH. Interestingly, inhibition of A_{2A} receptors enhances serotonin-dependent pMF following modest AIH (Hoffman *et al.*, 2010), suggesting that A_{2A}-dependent mechanisms inhibit serotonin-dependent mechanisms. These interactions may explain emergent properties of hypoxia-induced pMF, such as pattern sensitivity and metaplasticity (Devinney *et al.*, 2013).

Here, we test the hypothesis that mutual inhibitory interactions between serotonin- and adenosine-induced cascades are balanced during moderate ASH (*versus* AIH), preventing pMF and producing pattern sensitivity. Moderate ASH may generate greater extracellular adenosine during the prolonged hypoxic episode, counterbalancing serotonergic mechanisms (Conde & Monteiro, 2004). Thus, we predict that 5-HT_2 or A_{2A} receptor blockade during moderate ASH reveals pMF *via* the opposing mechanism. Further, we predict that severe ASH elicits A_{2A}-dependent pMF, due to greater

adenosine accumulation. Our results provide strong evidence that pattern sensitivity of hypoxia-induced pMF is an emergent property of competing cellular mechanisms, each capable of giving rise to pMF when activated alone.

Materials and Methods

Animals: Adult (12-16 weeks; 280-500g) male Sprague-Dawley rats (Harlan Colony 211, Houston, TX; Colony 218a, Indianapolis, IN) were used. The University of Wisconsin Animal Care and Use committee approved all experimental protocols.

Intrathecal injection of drugs: Treatment groups were given intrathecal injections of 10% DMSO in artificial cerebrospinal fluid (vehicle, 12µl ACSF; in mM 120 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 23 $NaHCO_3$, 10 glucose, equilibrated with 95% O_2, 5% CO_2 for 15 min), the A_{2A} receptor antagonist MSX-3 (Sigma-Aldrich, 200 µM in 12µl of 10% DMSO/ACSF), or the broad spectrum serotonin receptor antagonist methysergide maleate (methy; Sigma-Aldrich, 20mM in 12µl ACSF). Injections were made 12 min before exposure to either moderate ASH (PaO_2 40-50 mmHg), severe ASH (PaO_2 25-35 mm Hg), or sham (ie. no hypoxia, baseline PaO_2 180-350 mmHg).

Experimental preparation: Rats were induced with isoflurane in a closed chamber and placed on a heated surgical table to maintain body temperature; anesthesia was maintained via nose cone (3.5% isoflurane, 50% O_2). Tracheotomy was performed and rats were pump-ventilated through a tracheal tube (Rodent Ventilator 683, Harvard Apparatus; tidal volume 2.2-2.7 ml; frequency 70-75 breaths/min) with a gas mixture of

50% O_2, 50% N_2, and 3.5% isoflurane. End-tidal CO_2 was maintained at 40-44 mmHg by adjusting the ventilator and/or adding CO_2 to the inspired gas mixture. Tracheal pressure was continuously monitored. A catheter was placed in the tail or femoral vein to administer intravenous fluids (1.5-3.0 ml/hr of 64-75% lactated Ringer's solution, 4-10% HCO_3, and 15-30% of 6% hetastarch in 0.9% NaCl) to maintain arterial fluid and acid base balance (base excess ±3mEq/L change at end of protocol). A catheter was placed in the femoral artery to monitor blood pressure and draw arterial blood samples for analysis (PaO_2, $PaCO_2$, pH, base excess). Using a dorsal approach, the left phrenic nerve was isolated, cut, desheathed and protected with saline-soaked cotton until nerve recordings were initiated. For intrathecal drug delivery, a C_2 laminectomy was performed to expose the dura; a silicone catheter (2 Fr; Access Technologies, Skokie, IL) attached to a 50μl Hamilton syringe was placed over the C_4 spinal segment under the dura. Conversion to urethane anesthesia was performed by slowly withdrawing inhaled isoflurane while administering intravenous urethane (1.7-1.9 mg/kg). After ~1 hr, the rat was paralyzed with pancuronium bromide (2.5 mg/kg, i.v.). Body temperature was maintained at 37.5±1 °C (rectal thermometer; Fisher Scientific, Pittsburgh, PA). Blood pressure was monitored to ensure physiological stability (80-150 mmHg baseline, <30 mmHg change at 60 min). Adequate anesthesia was tested by toe pinch induced responses in blood pressure or phrenic nerve activity. Maximal CO_2 responses were elicited at the end of each experiment to verify adequate nerve responses and preparation stability. Rats with ≤30% hypoxic response during hypoxia or ≤50% maximal hypercapnic response were eliminated from analysis.

Neurophysiological measurements: ~1hr following conversion to urethane anesthesia, the desheathed phrenic nerve was covered with mineral oil and placed on bipolar silver electrodes. Nerve activity was amplified (10,000X), band-pass filtered (300-10,000 Hz Model 1800, A-M Systems, Carlsborg, WA), rectified and integrated with a continuous moving averager (time constant: 50 ms; CWE Inc., MA-821 filter; Ardmore, PA). The integrated signal was digitized and analyzed with a data acquisition system (WINDAQ, DATAQ Instruments, Akron, OH). CO_2 apneic and recruitment thresholds were determined by lowering inspired CO_2 and/or increasing ventilator rate until phrenic nerve bursting ceased. Then, CO_2 was slowly raised by increasing inspired CO_2 and/or decreasing ventilator rate until phrenic nerve bursting resumed. Baseline end-tidal CO_2 was set at 2-3 mmHg above the recruitment threshold and baseline nerve activity was recorded for at least 20 min.

Rats then received 12 µl intrathecal injections of vehicle (10% DMSO in ACSF), 20 mM methy, or 200 µM MSX-3 near the phrenic motor nucleus delivered as 2µl boluses every 30 s. 12 min following MSX-3 or vehicle injection, a blood sample was drawn to assess baseline conditions. For methy, baseline was recorded for 15-20 min after injection, consistent with previous reports (Baker-Herman & Mitchell, 2002). In rats where both methy and MSX-3 were delivered, MSX-3 was delivered 5-8 min after methy injections. Rats were then exposed to moderate ASH (12-14% O_2; PaO_2 40-54 mmHg), severe ASH (6-8% O_2; PaO_2 25-36 mmHg), or no hypoxia (~50% O_2; baseline PaO_2, time control). Arterial PCO_2 was maintained within ±1.5 mmHg of baseline. PaO_2 was maintained in excess of 150 mmHg at baseline and post-hypoxia in all studies. Blood samples were drawn at 5 and 20 min of ASH. After ASH, baseline conditions were

restored and confirmed with blood gas analysis at 15, 30, and 60 min post-ASH ($PaO_2 > 180$ mmHg, $PaCO_2 \pm 1.5$ mmHg baseline, and base excess ± 3 mEq/L baseline).

Data Analyses: Integrated phrenic burst amplitude and frequency were averaged in 60 s bins at baseline, during ASH, and at 15, 30 and 60 min post-ASH (or similar times in time control experiments). Burst amplitude values were normalized as a percent change from baseline. Frequency was expressed as change from baseline value (burst/min). A two-way ANOVA with repeated measures design was used to analyze phrenic burst amplitude, burst frequency, $PaCO_2$, PaO_2, MAP, and base excess at baseline, during ASH, and 15, 30, and 60 min post-ASH. *Post-hoc* comparisons were made with Fisher's LSD (SigmaPlot version 12.0; Systat Software, San Jose, CA) to compare differences between individual groups. In some cases MSX-3 and methy time controls were grouped together as drug time controls because no significant differences were found between groups. For PaO_2 during hypoxia, all rats exposed to moderate ASH or severe ASH were grouped together, respectively, since no significant differences were found between drug treatment groups. All differences between groups were considered significant if $p < 0.05$. Listing all p-values for post-hoc comparisons was not possible in some cases because of the quantity of values; specific p-values are given when appropriate. All values are expressed as mean $\pm$ SEM.

Results

Experimental groups

The following experimental groups include 6-10 rats pretreated with spinal injections of vehicle, MSX-3, or methysergide (methy), or sequential injections of methy then MSX-3 prior to exposure to sham (time control; no hypoxia), moderate ASH, or severe ASH while recording phrenic nerve responses. These experimental groups with respective n values (each rat for one n) were used to generate the data in this manuscript: 1) vehicle prior to moderate ASH (veh+ mod ASH, n=10); 2) MSX-3 prior to moderate ASH (MSX-3+mod ASH, n=8); 3) methysergide (methy) prior to moderate ASH (methy+mod ASH, n=8); 4) methy prior to MSX-3 followed by moderate ASH (methy +MSX-3+mod ASH, n=6); 5) vehicle prior to severe ASH (veh+severe ASH, n=8), 6) MSX-3 prior to severe ASH (MSX-3+severe ASH, n=8); 7) methy prior to severe ASH (methy+severe ASH, n=6); 8) vehicle without hypoxia (veh time control, n=8); 9) MSX-3 without hypoxia (MSX-3 time control, n=6); and 10) methy without hypoxia (methy time control, n=7)

Blood gases, mean arterial pressure and base excess

Blood gases were similar between all groups throughout the protocol, except when inspired oxygen was purposefully adjusted during ASH. Average PaO_2 was above 200 mmHg during baseline and post-hypoxia, demonstrating that changes in PaO_2 are not influencing phrenic activity at these times. Average $PaCO_2$ was not significantly different at baseline except for vehicle-treated rats exposed to moderate or severe ASH which had small, but significant decreases in $PaCO_2$ versus rats given MSX-3 and exposed to moderate ASH (p=0.041, p=0.047 respectively; Table 1). As expected, PaO_2 during ASH is significantly decreased from baseline or time controls (p<0.001, Table 1). During moderate ASH, PaO_2 was held between 40 and 54 mmHg, significantly different from

PaO_2 during severe ASH, which was between 26 and 36 mmHg (p<0.001, Table 1). Rats given vehicle injections had significantly lower $PaCO_2$ during severe ASH (p=0.002, Table 1). However, $PaCO_2$ values in all groups were not significantly different from baseline at 60 min post-hypoxia, suggesting that blood gas differences are unable to explain observed changes in phrenic nerve burst amplitude.

Mean arterial pressure (MAP) at baseline was the same in most groups; however, for unknown reasons, it was slightly lower in vehicle time controls (p=0.045, Table 1) and rats injected with methy plus MSX-3 and exposed to moderate ASH (p=0.028, Table 1). During ASH, MAP decreased significantly from baseline (p<0.001, Table 1). All rats exposed to ASH, except for rats given methy and MSX-3 and exposed to moderate ASH or rats given methy and exposed to severe ASH, exhibited slight but significant decreases in MAP at 60 min (8-23 mmHg, p<0.05; Table 1). MSX-3 injected time control rats also exhibited a slight decrease in MAP at 60 min post-ASH (12 mmHg, p=0.005; Table1). However, these MAP changes are characteristic of this experimental preparation (Fuller et al., 2000; Baker-Herman and Mitchell, 2008). In a prior study, we demonstrated that changes in MAP of ~20 mmHg had no effect on effect on respiratory activity in this same anesthetized rat preparation (Bach & Mitchell, 1996). Overall, there is little evidence that changes in MAP from the beginning to the end of experiments, or among experimental groups were sufficient to influence the fundamental conclusions of this study.

Base excess was held within 1.5 mEq/L from baseline throughout experimental protocols, except during severe ASH when values significantly decreased from baseline (p<0.001, Table 1). This decrease in base excess most likely occurs from transient

lactic acidosis in response to severe hypoxemia. However, no groups had significantly different base excess values at 60 min post-hypoxia *versus* baseline, demonstrating that acidosis is not directly responsible for enhanced phrenic nerve activity following severe ASH. Each value is the average from multiple rats plus and minus standard error of the mean (n=10, vehicle; n=8, MSX-3; n=6, MSX-3 time control).

Short-term hypoxic responses

ASH exposure significantly increased phrenic nerve burst amplitude vs. baseline or time controls ($p \leq 0.002$, Figure 1). Vehicle, MSX-3, or methy injections had no significant effect on phrenic nerve burst amplitude in response to ASH ($p > 0.05$, Figure 1). However, rats injected with methy and MSX-3 and then exposed to moderate ASH showed a significantly decreased phrenic amplitude response versus rats injected with vehicle ($p = 0.047$) or methy ($p = 0.044$) and exposed to moderate ASH (Figure 1). Phrenic responses to severe ASH were significantly greater than responses to moderate ASH; rats injected with vehicle, MSX-3, or methy and exposed to severe ASH exhibited significantly greater amplitude response vs. the respective drug groups exposed to moderate ASH ($p \leq 0.036$, Figure 1). This result is similar to the short-term hypoxic phrenic response in a previous study comparing moderate and severe AIH (Nichols *et al.*, 2012). No significant differences in the short-term hypoxic phrenic response were found between rats injected with vehicle, MSX-3, or methy and exposed to severe ASH ($p > 0.05$ Figure 1).

Spinal A_{2A} receptor blockade reveals pMF following moderate ASH

Compressed traces of phrenic neurograms illustrate phrenic burst amplitude before, during, and after moderate ASH in rats pretreated with intrathecal injections of vehicle or MSX-3 (A_{2A} receptor antagonist; injections marked by black arrow, Fig 2A). In rats given vehicle injections and exposed to moderate ASH, there was no significant pMF *versus* vehicle time controls (15±4% vs. 10±3% respectively, p=0.40; Fig 2B) in agreement with previous reports comparing moderate AIH and ASH (Baker & Mitchell, 2000; Wilkerson *et al.*, 2008). In contrast, rats given intrathecal MSX-3 injections exhibited significant pMF following moderate ASH (39±5%), which was significant compared to rats injected with vehicle and exposed to moderate ASH (15±4%, p<0.001; Fig 2B) and MSX-3 time controls (0±3%, p<0.001; Fig 2B). There was a slight, but significant increase in phrenic burst frequency (frequency long-term facilitation) at 60 min post-ASH in rats injected with MSX-3 and exposed to moderate ASH (3.2±1.5 bursts/min) *versus* time controls (-1.4±2.8 bursts/min at 60 min, p=0.007; Fig 2C), but not compared to rats given vehicle injections and exposed to moderate ASH (0.4±1.8 bursts/min, p=0.062; Fig 2C). Small but significant changes in frequency following hypoxic exposures are consistent with findings of other studies on pMF (Baker-Herman & Mitchell, 2002, 2008; Nichols *et al.*, 2012).

Spinal serotonin receptor blockade does not reveal pMF following moderate ASH

Intrathecal injections of methy before moderate ASH revealed a slight but significant increase in phrenic burst amplitude (17±7%) *versus* methy time controls (1±3%, p=0.007; Fig 3B). However, rats injected with methy and then exposed to moderate ASH were not significantly different from rats injected with vehicle and exposed to

moderate ASH (15±4%, p=0.688), suggesting that spinal serotonin receptor blockade is not sufficient to reveal pMF following moderate ASH. There was no significant increase in phrenic burst frequency in these groups (p>0.05, Fig 3C).

pMF following A$_{2A}$ blockade and moderate ASH is serotonin-dependent

Compressed traces of phrenic neurograms illustrate phrenic burst amplitude before, during and after moderate ASH in rats pretreated with intrathecal injections of the broad-spectrum serotonin receptor antagonist, methy, MSX-3 or both (methy injections denoted by black arrow and MSX-3 injections denoted by gray arrow; Fig 3A). Pretreatment with methy in rats given MSX-3 injections and exposed to moderate ASH prevents pMF (0±6%, p<0.001 vs. MSX-3+moderate ASH and p>0.05 vs. time controls; Fig 3B), demonstrating that the moderate ASH-induced pMF revealed after A$_{2A}$ receptor inhibition is serotonin-dependent. Thus, ASH-induced pMF following spinal A$_{2A}$ blockade is similar to pMF induced by moderate AIH (Nichols *et al.*, 2012) or modest AIH (Bach & Mitchell, 1996; Fuller *et al.*, 2001; Baker-Herman & Mitchell, 2002), which both require spinal serotonin receptor activation. Frequency long-term facilitation was also prevented by pretreatment with methysergide in rats given injections of MSX-3 and exposed to moderate ASH (-1.4±1.6 bursts/min; p=0.005 vs. MSX-3+moderate ASH, p>0.05 vs. all other groups; Fig 3C), consistent with effects of methy on frequency in other studies of modest and moderate AIH (Baker-Herman & Mitchell, 2002; Nichols *et al.*, 2012)

Severe ASH causes pMF *via* an A$_{2A}$-dependent mechanism

Compressed traces of phrenic neurograms illustrate phrenic burst amplitude before, during, and after severe ASH exposure in rats pretreated with vehicle, MSX-3, or methy (denoted by black arrow, Fig 4A). Rats injected with vehicle exhibited significant pMF following exposure to severe ASH (53±12%) compared to vehicle (10±3%, p<0.001) or drug (MSX-3 or methy, 1±2%; p<0.001, Fig 4B) time controls. In contrast to rats exposed to moderate ASH, rats injected with MSX-3 and exposed to severe ASH exhibited no significant pMF *versus* vehicle (10±3%, p=0.966) or drug (MSX-3 or methy, 1±2%; p=0.394, Fig 4B) time controls. Thus, pMF induced by severe ASH requires spinal A_{2A} receptor activation, similar to severe AIH-induced pMF (Nichols *et al.*, 2012). No significant frequency long-term facilitation was observed in rats pretreated with vehicle or MSX-3 followed by severe ASH (p>0.05, Fig 4C), unlike an earlier study on severe AIH (Nichols *et al.*, 2012). This discrepancy might be due to pattern-sensitive effects of severe hypoxia on frequency long-term facilitation or rat strain differences and variability between studies, since frequency long-term facilitation is typically small and inconsistent (Baker-Herman & Mitchell, 2008)(Baker-Herman & Mitchell, 2008).

Spinal serotonin receptor blockade enhances pMF induced by severe ASH

Rats pretreated with spinal methy (denoted by black arrow, Fig 4A) exhibited significantly higher pMF (87±26%) *versus* vehicle injected rats after severe ASH (53±12%, p=0.011; Fig 4B). This enhanced pMF was present at each time point following severe ASH (15, 30, and 60 min post-ASH, p≤0.011 vs. vehicle+severe ASH), suggesting enhancement occurs during severe ASH exposure. In contrast, rats exposed to severe AIH exhibit pMF that is unaffected by spinal methy injections (Nichols *et al.*,

2012). In addition to pMF enhancement, spinal serotonin receptor blockade also revealed a slight but significant frequency long-term facilitation (6.2±2.2 bursts/min) *versus* drug time controls (MSX-3 or methy, -0.4±1.3 bursts/min; p<0.001, Fig 4C) or vehicle-injected rats exposed to severe ASH (2.6±1.1 bursts/min, p=0.049; Fig 4C).

Severe ASH elicits significant pMF while moderate ASH does not

Moderate ASH did not elicit significant pMF (15±4%) in rats injected with vehicle *versus* time controls. As predicted, severe ASH elicited significant pMF (53±12%) in vehicle treated rats *versus* vehicle time controls (10±3%, p<0.001), and compared to vehicle injected rats exposed to moderate ASH (p<0.001, Fig 5A). These results suggest that severity of the hypoxic stimulus, in addition to the stimulus pattern (intermittent vs. sustained) is a key factor regulating the induction of plasticity.

Discussion

Pattern sensitivity arises from interactions between competing pathways

The fundamental conclusion of this study is that pattern sensitivity of hypoxia-induced pMF is an emergent property, arising from complex interactions between separate competing serotonin- and adenosine-dependent pathways to pMF. Our findings are summarized in a simplified model (Figure 5). During moderate ASH, activation of serotonin and adenosine-dependent pathways is balanced, preventing pMF due to equalized cross-talk inhibition (Fig 5B). Thus, blocking spinal A_{2A} receptors during moderate ASH reveals serotonin-dependent pMF (Fig 5C); in support of this concept,

spinal 5-HT receptor blockade prevents pMF in rats treated with an A_{2A} antagonist and exposed to moderate ASH (Fig 5D). In contrast to moderate ASH, severe ASH alone elicits pMF (Fig 5E) by an adenosine-dependent mechanism (Fig 5F). Interestingly, spinal serotonin receptor blockade enhances severe ASH-induced pMF, suggesting that the serotonin-dependent pathway now inhibits the A_{2A}-dependent pathway (Fig 5G), opposite to the adenosine/serotonin balance obtained after moderate AIH (Hoffman *et al.*, 2010). Together, these results suggest that inhibitory interactions between serotonin- and A_{2A}-dependent pathways restrain pMF during moderate ASH, explaining one possible mechanism of hypoxia-induced pMF pattern sensitivity. A new finding here is that this pattern-sensitivity is also range specific, and is expressed only with moderate or modest (not severe) hypoxia. pMF induced by severe hypoxia is not completely pattern sensitive because of unbalanced activation of serotonergic and adenosinergic pathways to pMF during severe ASH, allowing A_{2A}-dependent pathway to dominate. However, we cannot rule out at least some pattern sensitivity with severe hypoxia since pMF observed after severe AIH is more robust than that found here with severe ASH (Nichols *et al.*, 2012). Without further studies to make direct comparisons, it is unknown whether pMF induced by severe hypoxia exhibits some degree of pattern sensitivity.

Serotonin receptor activation during severe ASH constrains A_{2A}-dependent pMF

While several studies have demonstrated an inhibitory effect of A_{2A} receptors on serotonin-dependent pMF induced with moderate AIH (Hoffman *et al.*, 2010; Nichols *et al.*, 2012), little knowledge is available concerning the impact of serotonin receptor activation on A_{2A}-dependent pMF. Here we demonstrate that serotonin receptor

activation constrains A_{2A}-dependent pMF during severe ASH, since pMF after severe ASH was enhanced by spinal methysergide (Fig 4). Thus, inhibitory interactions between 5-HT and A_{2A}-dependent pathways are bi-directional. Bidirectional cross-talk inhibition assures predominant serotonergic or adenosinergic pMF, with the exception of balanced stimulation where these opposing effects are equal in magnitude. We suggest that this balance is attained during moderate (not severe) ASH, giving rise to pattern sensitivity.

Suprisingly, methysergide did not reveal pMF following moderate ASH (Fig 5A). Thus, A_{2A} receptor activation alone is insufficient to elicit pMF with moderate ASH, despite the ability to inhibit the serotonin-dependent pathway. This observation suggests complex interactions between the serotonergic and adenosinergic cascades, dependent on the relative magnitude of serotonin release *versus* adenosine accumulation, the specific cell signaling molecules mediating inhibition in each direction, and the sequence of activation in the molecules mediating cross-talk inhibition *versus* phrenic motor facilitation.

The failure of methysergide to reveal pMF after moderate ASH could be due, at least in part, to methysergide inhibiting 5-HT$_7$ receptors. These receptors signal differently from 5-HT$_2$ receptors necessary for moderate AIH-induced pMF (Fuller *et al.*, 2001; MacFarlane *et al.*, 2011), but functionally similar to G_s-coupled metabotropic A_{2A} receptors. In fact, 5-HT$_7$ receptor activation elicits phrenic motor facilitation (Hoffman & Mitchell, 2011), and constrains moderate AIH-induced pMF (Hoffman & Mitchell, 2013). Since both A_{2A} and 5-HT$_7$ receptors canonically activate similar signaling cascades, such as protein kinase A (PKA), co-activation may be necessary for pMF following

hypoxia. 5-HT$_7$ receptors may contribute to A$_{2A}$-dependent pMF following moderate ASH (when opposing 5-HT$_2$-dependent cascades are blocked). Therefore, selective blockade of 5-HT$_2$ receptors during moderate ASH may in fact reveal pMF dependent on A$_{2A}$ and/or 5-HT$_7$ receptor activation.

Cross-talk interactions *via* downstream signaling molecules

Inhibitory coupling between A$_{2A}$ and 5-HT$_2$ pathway likely occurs through downstream signaling cascades, such as protein kinase C (PKC) and PKA activation. We previously found that spinal PKA activation attenuates, and PKA inhibition enhances modest AIH-induced pMF (Hoffman & Mitchell, 2013). Thus, PKA appears to be an essential molecule in the mechanism of inhibition from G$_s$ protein coupled, to G$_q$ protein coupled metabotropic receptors. Whereas PKA may be the relevant kinase for A$_{2A}$ (Golder *et al.*, 2008) or 5-HT$_7$ dependent pMF (Hoffman & Mitchell, 2011, 2013), 5-HT$_2$ dependent pMF may require protein kinase C (PKC) activation. Cross-talk interactions between PKC and PKA signaling have been demonstrated in other model systems, such as *Aplysia,* where PKC constrains PKA activity to prevent sensorimotor long-term facilitation (Farah *et al.*, 2009).

   PKA also negatively regulates PKC activity *via* modulation of reactive oxygen species formation. In cell culture models, PKA decreases ROS production by phosphorylating the NOX A1 subunit (Kim *et al.*, 2007), or *via* downregulation of the p47 subunit of NADPH oxidase (Bengis-Garber & Gruener, 1996). NADPH oxidase activity is required for pMF induced by modest AIH, most likely because reactive oxygen species inhibit constraining protein phosphatases (Wilkerson *et al.*, 2007; MacFarlane *et*

al., 2009). Multiple routes of inhibitory cross-talk interactions may occur between PKC-dependent (eg. 5-HT$_2$ receptor) and PKA-dependent (eg. A$_{2A}$ and 5-HT$_7$ receptor) signaling, allowing for multi-level control of various pathways to pMF.

Biological Significance

Patterned stimuli have a profound influence on the expression of plasticity. The benefits of "spaced" training on long-term memory formation have long been appreciated, and are being actively explored to refine therapeutic approaches to learning disorders (Ebbinghaus, 1913; Philips *et al.*, 2013*a*). However, the cellular and molecular correlates of pattern sensitivity have seldom been explored, and have been mostly studied in intervertebral model systems, such as sensorimotor long-term facilitation in *Aplysia*. Here, we present evidence for a novel mechanism of pattern sensitivity in spinal neuroplasticity: inhibitory coupling between competing 5-HT$_2$ and A$_{2A}$-induced signaling cascades. Similar to hypoxia-induced pMF, pattern sensitivity in *Aplysia* sensorimotor long-term facilitations appears to occur at multiple molecular levels, such as G-proteins (Ye *et al.*, 2008), temporal integration of PKA and MAPK signaling (Philips *et al.*, 2013*b*), and antagonism of PKA signaling by increased PKC activity (Farah *et al.*, 2009). One commonality is that pattern sensitivity is an emergent property, arising from mutual inhibitory interactions between critical signaling molecules involved in plasticity. The present study indicates that pattern sensitivity is an emergent property of inhibitory coupling between G$_q$ and G$_s$ coupled signaling cascades, most likely *via* PKC and PKA activation.

Some forms of memory and neuroplasticity lack apparent pattern sensitivity. For example, short-term memory, which lasts on the order of minutes, is not subject to the spacing effect (Xia *et al.*, 1998). Additionally, traumatic emotional memories (eg. flashbulb memory) are induced by a single event, and form long-term memories which rely on mechanisms distinct from long-term memories produced by spaced trials (Irvine *et al.*, 2005, 2006; Diamond *et al.*, 2007). Thus, pattern-insensitive mechanisms of plasticity may allow for the formation of critical memories following a single trial (eg. remembering never to touch a hot stove). In our studies, we demonstrated that severe ASH elicits pMF, which we postulate is due to greater relative activation of A_{2A} receptors (vs. 5-HT_2 receptors). This form of pattern-insensitive plasticity might serve to elevate respiratory motor output after even single, severe periods of hypoxia. Analogous to flashbulb memories after a traumatic event, plasticity following severe ASH could permit significant adaptation following deleterious events such as transient respiratory failure, severely hypoxic environments, or respiratory disease. Forms of pattern-insensitive plasticity may be ubiquitous within the nervous system to allow for more rapid adaptation following a single deleterious, life-threatening event.

The benefits of spaced training on learning are well-known, but few studies have applied these principles to plasticity in other neural systems. Historically, we have applied principles of pattern sensitivity to induce pMF by exposing animals to AIH (Hayashi *et al.*, 1993; Bach & Mitchell, 1996; Turner & Mitchell, 1997; Dwinell *et al.*, 1997; Baker & Mitchell, 2000). Here we demonstrate that pharmacological inhibition of spinal A_{2A} receptors disrupts the cross-talk inhibitory balance, thereby revealing pMF following moderate ASH, a stimulus that does not ordinarily trigger pMF (Baker &

Mitchell, 2000). We also demonstrate that severe ASH elicits A_{2A}-dependent pMF, an effect enhanced by spinal serotonin receptor inhibition. Overall, we conclude that pattern sensitivity of pMF induced by moderate hypoxia is an emergent property that results from inhibitory interactions between A_{2A} and 5-HT dependent pathways.

Detailed understanding of mechanisms giving rise to pattern sensitivity in pMF and other forms of neuroplasticity may refine or even guide future clinical applications based on the ability to elicit motor plasticity *via* hypoxia and/or small molecules that manipulate this intricate system. Such therapeutic approaches are now being developed for diverse clinical disorders that challenge respiratory (Mahamed & Mitchell, 2007; Lovett-Barr *et al.*, 2012; Nichols *et al.*, 2013) and non-respiratory motor function (Trumbower *et al.*, 2012; Hayes *et al.*, 2013) including spinal cord injury, amyotrophic lateral sclerosis or sleep apnea.

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TABLES AND FIGURES

Treatment groups	P_{aCO_2} (mmHg)			P_{aO_2} (mmHg)			MAP (mmHg)			Base Excess (mEq/L)		
	Baseline	Hypoxia	60 min	Baseline	Hypoxia	60 min	Baseline	Hypoxia	60 min	Baseline	Hypoxia	60 min
Moderate ASH												
vehicle	46.0 ± 1.1^a	46.2 ± 1.1	45.9 ± 1.2	313 ± 9	$46.2\pm1^{b,d,e}$	294 ± 8^b	108 ± 4	81 ± 6^b	100 ± 4^b	-0.2 ± 0.6	-0.6 ± 0.6	1.2 ± 0.5
MSX-3	48.6 ± 0.9	48.8 ± 1.0	49.2 ± 0.9	313 ± 5	$44.5\pm1^{b,d,e}$	295 ± 5^b	118 ± 9	77 ± 8^b	106 ± 7^b	-0.4 ± 0.5	-1.3 ± 0.5	0.7 ± 0.5
methy	46.0 ± 1.3	45.7 ± 1.2	46.4 ± 1.3	295 ± 6	$44.7\pm1^{b,d,e}$	263 ± 11^b	106 ± 4	66 ± 4^b	91 ± 3^b	-1.0 ± 0.5	-1.5 ± 0.5	0.0 ± 0.5
methy+MSX-3	46.3 ± 0.5	47.0 ± 0.6	46.4 ± 0.7	$290\pm12^{a,f}$	$45.4\pm2^{b,d,e}$	278 ± 10	$97\pm8^{\,a}$	68 ± 7^b	94 ± 5	0.3 ± 0.7	-0.3 ± 1.0	1.6 ± 1.0
Severe ASH												
vehicle	45.7 ± 0.9^a	44.2 ± 0.8^b	46.4 ± 0.8	310 ± 5	$30.4\pm1^{b,c,e}$	291 ± 9^b	110 ± 8	52 ± 9^b	94 ± 9^b	-0.9 ± 0.5	-8.9 ± 1.6^b	0.9 ± 1.0
MSX-3	46.9 ± 1.0	46.7 ± 0.9	47.0 ± 0.9	$309+4$	$31.8\pm1^{b,c,e}$	291 ± 6^b	106 ± 6	49 ± 5^b	83 ± 5^b	-0.2 ± 0.3	-7.1 ± 1.1^b	-0.1 ± 0.4
methy	48.1 ± 1.3	48.2 ± 1.0	48.5 ± 1.3	262 ± 13^g	$30.3\pm1^{b,c,e}$	238 ± 9^b	99 ± 6	40 ± 5^b	91 ± 5	1.0 ± 0.5	-8.4 ± 0.9^b	-0.5 ± 1.2
Time Control												
vehicle	46.0 ± 0.9	46.1 ± 0.8	45.3 ± 0.8	311 ± 10	310 ± 8	301 ± 7	$101\pm7^{\,a}$	99 ± 7	93 ± 7	-0.1 ± 0.7	0.0 ± 0.6	1.1 ± 0.6
MSX-3	48.3 ± 0.4	48.5 ± 0.5	48.9 ± 0.5	310 ± 7	306 ± 6	304 ± 8	112 ± 4	109 ± 4	100 ± 5^b	0.5 ± 0.6	1.1 ± 0.3	2.5 ± 0.4
methy	47.0 ± 0.9	47.1 ± 1.0	46.9 ± 0.8	299 ± 9	299 ± 11	276 ± 9^b	103 ± 6	101 ± 6	104 ± 8	-0.2 ± 0.4	0.1 ± 0.4	1.1 ± 0.2

Table 1. Arterial partial pressure of carbon dioxide (P_{aCO_2}) and oxygen (P_{aO_2}), mean arterial pressure (MAP), and base excess during baseline, hypoxia, and 60 min post-hypoxia. Values are expressed as means ± SE. Significant difference ($p<0.05$) from MSX3+mod ASH[a], baseline[b], all moderate ASH groups[c], all severe ASH groups[d], all time control groups[e], vehicle+mod ASH[f] or all groups[g].
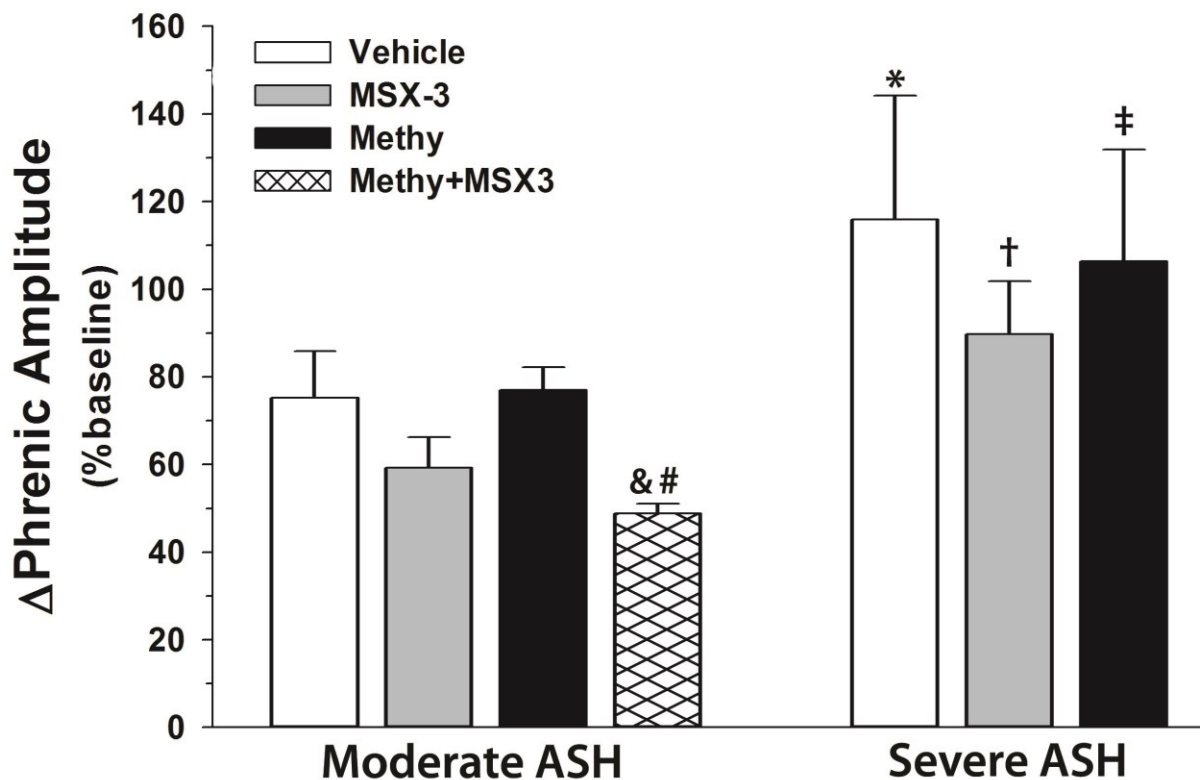
Figure 1

Figure 1. Short-term hypoxic phrenic response during moderate or severe ASH after intrathecal injections of vehicle (▢), MSX-3 (▨), methy (■), and methy+MSX-3 (⊠). All groups are significantly increased from time controls (no ASH, 2.5±1.6%; p≤0.002). (&) indicates significantly decreased vs. vehicle+mod ASH. (#) indicates significantly decreased vs. methy +mod ASH. (*) indicates significantly increased vs. vehicle+mod ASH. (†) indicates significantly increased vs. MSX-3+mod ASH. (‡) indicates significantly increased vs. methy+mod ASH. Values are means ± SE; significance is p<0.05).
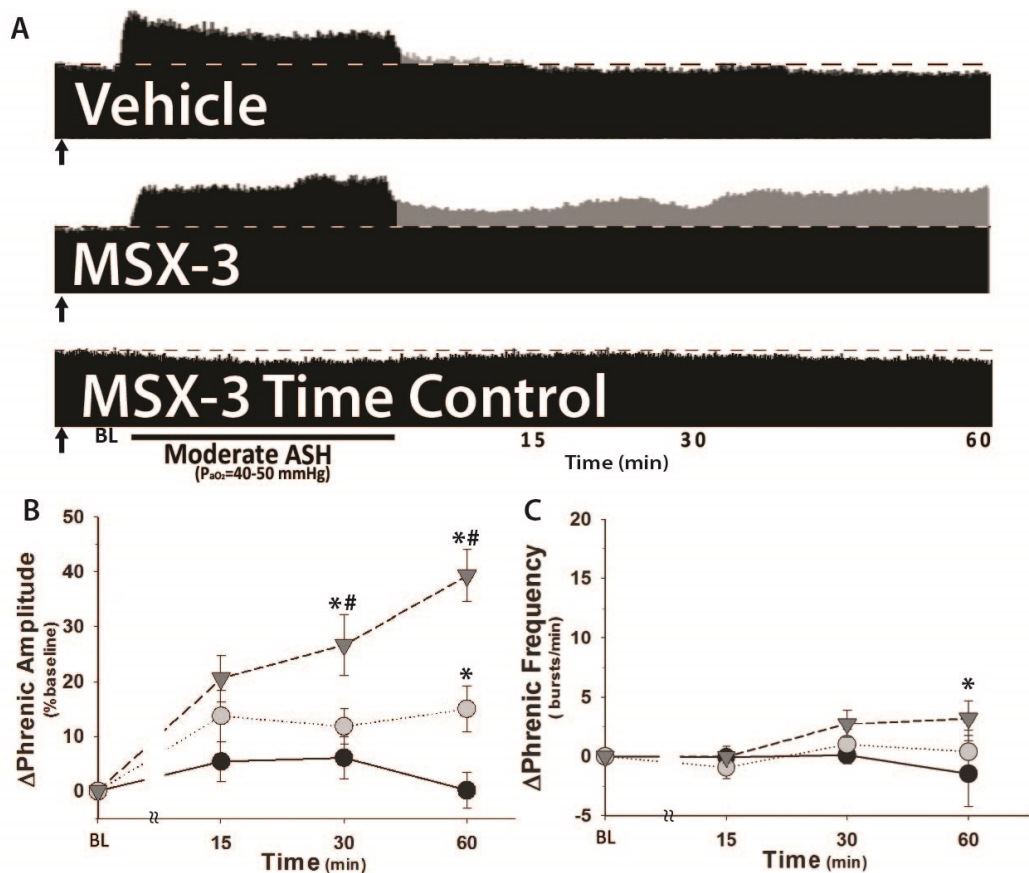
Figure 2. Pre-treatment with spinal A_{2A} receptor antagonist (200 mM MSX-3) reveals pMF following moderate ASH. A. Representative phrenic neurograms after moderate ASH (P_{aO_2}=40-50 mmHg, 25 min) from rats given intrathecal injections of vehicle or MSX-3 (↑) compared with rats treated as time controls and not exposed to ASH after MSX-3 delivery. Dotted line represents baseline; pMF is shown in gray. B. Summary data for delta phrenic amplitude following ASH exposure in rats given vehicle (▽) or MSX-3 (⊙) and exposed to ASH. MSX-3 time controls are shown for comparison (no ASH, ●) C. Summary data for Δ phrenic burst frequency following ASH exposure. n=10, vehicle; n=8, MSX-3; n=6, MSX-3 time control (no ASH). (*) denotes significantly increased from MSX-3 time control. (#) denotes significantly increased from MSX-3+mod ASH. All significant differences are $p < 0.05$.
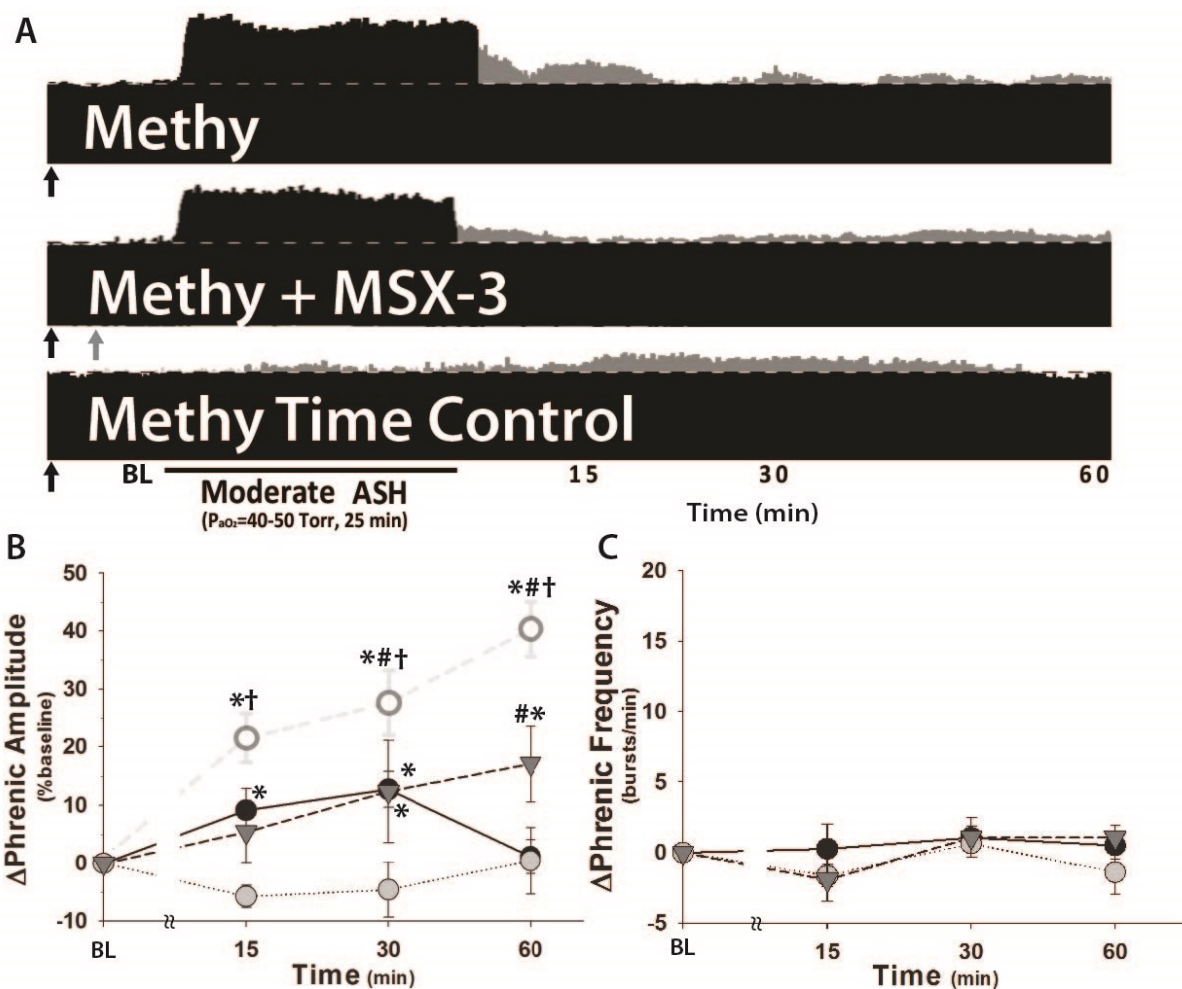
Figure 3. Pre-treatment with spinal 5-HT receptor antagonist (20 mM methysergide, 20 min

prior ASH; methy) has no effect on pMF following moderate ASH exposure, but prevents pMF in

rats injected with 200 mM MSX-3 (12 min prior ASH) and exposed to moderate ASH. A.

Representative phrenic neurograms after moderate ASH from rats given intrathecal injections of

methy (↑) or both methy and MSX-3 (↑) compared with rats treated as time controls and not

exposed to moderate ASH after methy delivery. Dotted line represents baseline; pMF is shown

in gray. B. Summary data for Delta phrenic amplitude in rats given methy (▼) or methy+MSX-3

(○) and exposed to moderate ASH or methy time controls (no ASH, ●). Rats given MSX-3

(from fig 1, ○) are shown for comparison. C. Summary data for change in phrenic burst

frequency (Δ frequency) following moderate ASH exposure in the same groups. n=8, methy;

n=7, methy+MSX-3; n=7, methy time control (no ASH). (*) indicates significant difference vs.

methy+MSX-3+mod ASH. (#) indicates significant difference vs. methy time controls (no ASH).

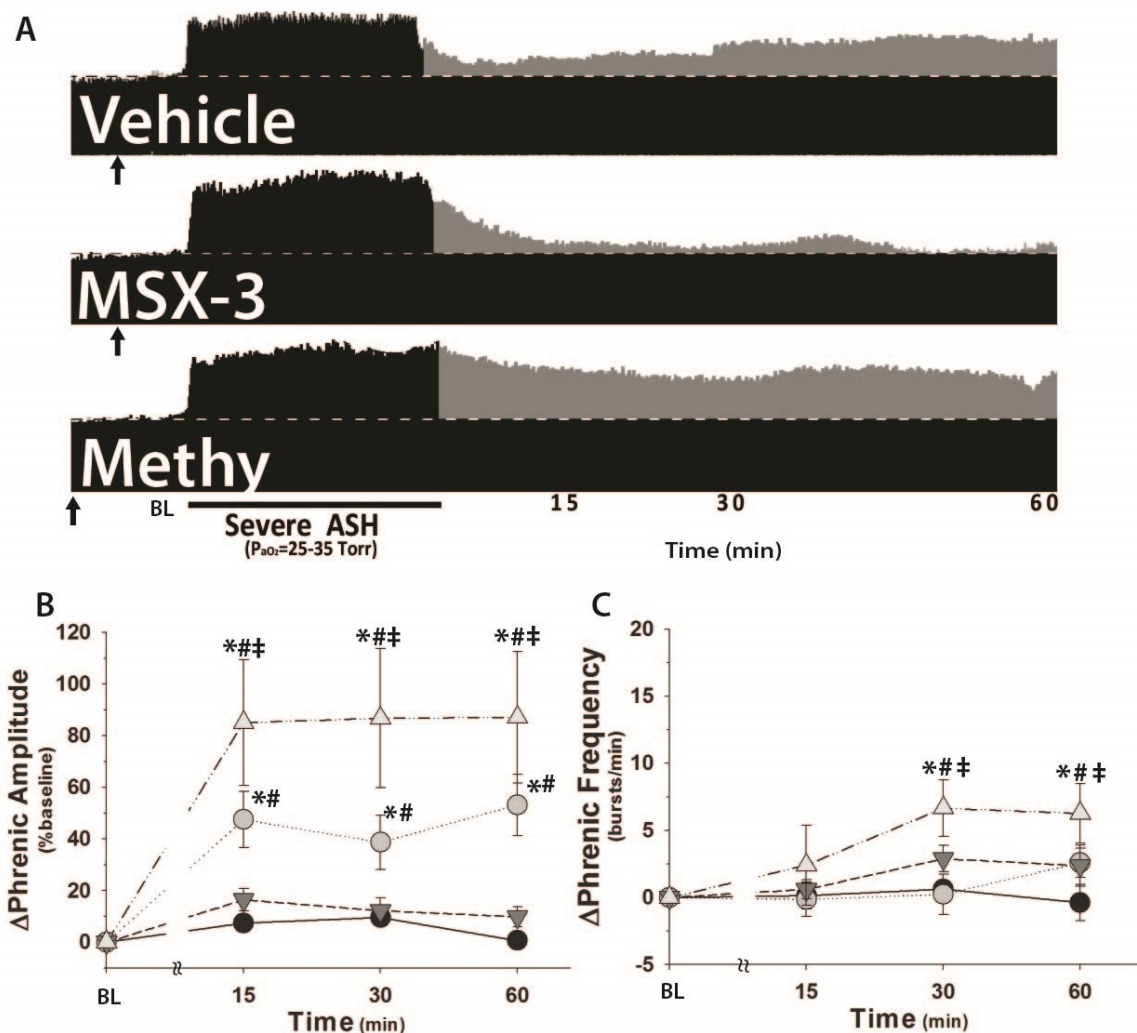(†) indicates significant difference vs. methy+mod ASH. All significant differences are p<0.05.

Figure 4. Severe ASH elicits A_{2A} receptor-dependent pMF, which is enhanced by pre-treatment with spinal 5-HT receptor blockade (20 mM methysergide, 20 min prior ASH; methy). A. Representative phrenic neurograms after severe ASH (P_{aO2}=25-35 mmHg, 25 min) from rats given intrathecal injections of vehicle, MSX-3, or methy (↑). Dotted line represents baseline; pMF is shown in gray. B. Summary data for delta phrenic amplitude in rats given vehicle (⬭), MSX-3 (▼), or methy (△) and exposed to severe ASH or no hypoxia (time controls; methy or MSX-3 injections,●). C. Summary data for change in phrenic burst frequency (Δ frequency) for

the same groups. n=8, vehicle; n=8, MSX-3; n=6, methy; n=13, time controls. (*) indicates significant difference vs. time controls. (#) indicates significant difference vs. MSX-3+severe ASH. (‡) indicates significant difference vs. vehicle+severe ASH. All significant differences are $p<0.05$.
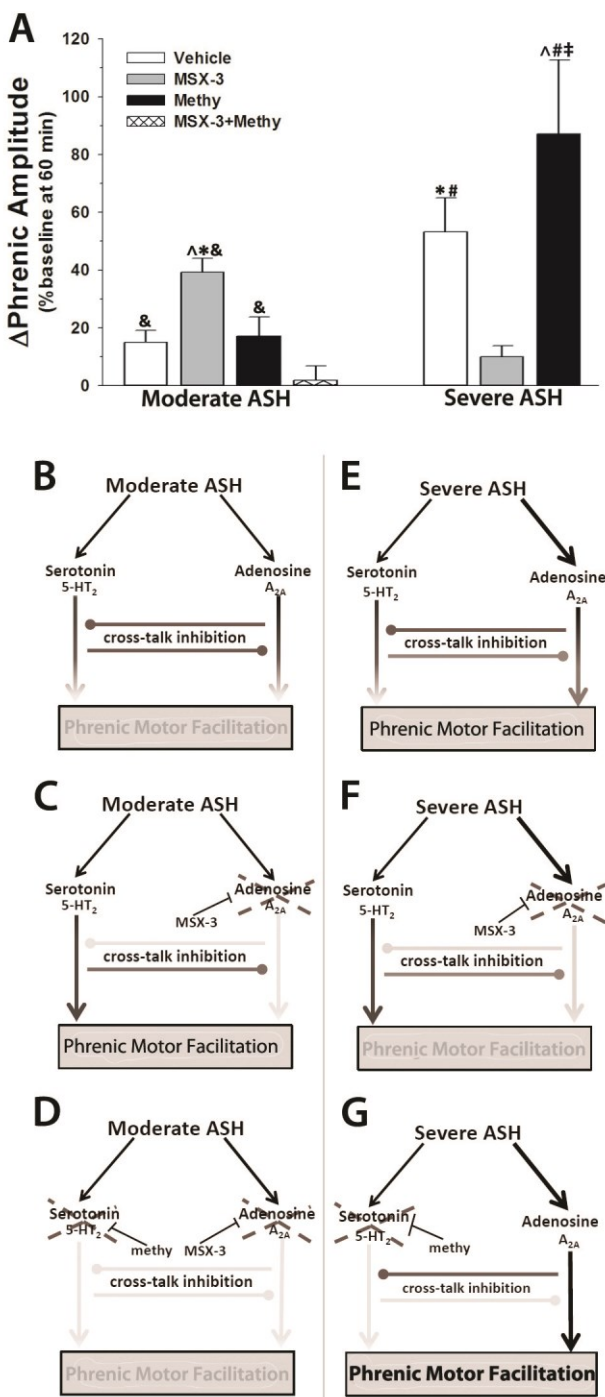
Figure 5. Competing 5-HT$_2$/A$_{2A}$-dependent mechanisms could explain pattern sensitivity of pMF

to moderate hypoxia. A. Summary of pMF following moderate or severe ASH in rats treated

with vehicle, 200 mM MSX-3, 20 mM methy, or 20 mM methy+ 200 mM MSX-3. B. Proposed 5-HT$_2$/A$_{2A}$ interactions predict magnitude of pMF. ↓ indicates activation and 🌡 indicates inhibition. ⅄ indicates blockade by intrathecal drug. During moderate ASH, balanced activation of 5-HT$_2$- and A$_{2A}$-dependent pathways allows for cross-talk inhibition to constrain expression of pMF. C. Spinal blockade of A$_{2A}$ pathway during moderate ASH prevents cross-talk inhibition of the 5-HT$_2$ pathway, revealing pMF. D. Blockade of 5-HT receptors prior to A$_{2A}$ receptor blockade and moderate ASH exposure prevents pMF, demonstrating that cross-talk inhibition from A$_{2A}$-dependent pathway restrains 5-HT$_2$-dependent pMF during moderate ASH. E. Severe ASH causes pMF because of greater relative A$_{2A}$ receptor activation allowing for cross-talk inhibition to be overcome. F. Blockade of spinal A$_{2A}$ receptors prevents expression of pMF following severe ASH, demonstrating that pMF following severe ASH is A$_{2A}$-dependent. G. Blockade of spinal 5-HT receptors prevents cross-talk inhibition of the A$_{2A}$ pathway during severe ASH, causing enhanced pMF. (*) indicates significant vs. vehicle+mod ASH. (^) indicates significant vs. methy+mod ASH. (&) indicates significant vs. methy+MSX-3+mod ASH. (#) indicates significant vs. MX3+severe ASH. (‡) indicates significant vs. vehicle+severe ASH.

Chapter V

DISCUSSION

Michael J Devinney

Overview

This thesis investigates cellular and molecular mechanisms underlying pLTF and how they give rise to pattern sensitivity. Collectively, these data demonstrate: (1) Activation of spinal PKCθ, but not other PKC isoforms, is critical for the expression of pLTF; (2) PKCθ is expressed within phrenic motor neurons but not microglia or astrocytes; (3) Relevant PKCθ is within phrenic motor neurons and/or interneurons; (4) spinal PKC activation with a phorbol ester elicits pMF which requires activation of classical/novel PKC isoform(s) distinct from PKCθ; (5) During modest to moderate ASH, A_{2A} receptor activation restrains expression of serotonin-dependent pLTF, thus conferring pattern sensitivity to pLTF and; (6) severe ASH induces A_{2A} receptor-dependent pLTF, demonstrating a mechanistic shift to relatively pattern-insensitive, adenosine-dependent plasticity during severe hypoxia. Altogether, these findings demonstrate the complexity of signaling cascades giving rise to pMF, and that the interactions between signaling cascades can give rise to pattern sensitivity as an emergent property. Within each chapter is a discussion of the findings and their significance. The discussion here will focus on future directions arising from these studies and broader significance to the field.

pLTF is a form of PKCθ-dependent motor plasticity, possibly occurring within motor neurons

Roles for PKC activation in plasticity have been known for decades, yet few studies demonstrate dependency on one specific isoform (Sossin, 2007). Here we report that pLTF depends specifically on spinal (likely motor neuronal) PKCθ activation. These findings significantly contribute to our understanding of cellular/molecular mechanisms giving rise to pLTF. Determining the specific PKC isoform required for pLTF has enabled targeted PKCθ knockdown with intrapleural siRNA, localizing cellular mechanisms of pLTF to phrenic motor neurons or nearby interneurons. Targeted inhibition or activation of PKCθ can enhance our

basic understanding of mechanisms giving rise to pLTF, and may represent potential pharmacological therapy to restore motor function after injury or disease.

Why does pLTF specifically require PKCθ activation (ie. isoform specificity)?

Isoform specificity of pLTF may arise from several factors including 1) subcellular colocalization of 5-HT$_2$ receptors and PKCθ molecules to lipid rafts (Chini & Parenti, 2004), 2) PKCθ requirement of multiple signals (ie. coincidence detection) for activation (Steinberg, 2008). For example, PKC can be activated by ROS (Knapp & Klann, 2002), DAG (Hoyer *et al.*, 2002), tyrosine phosphorylation by Src family kinases, and phosphotyrosine binding (Stahelin *et al.*, 2012). Tyrosine phosphorylation may occur from downstream Src kinase activation following TrkB receptor activation (Carreño *et al.*, 2011), or from non-canonical Src coupling to 5-HT$_2$ receptors (McGarrigle & Huang, 2007). Thus, it is very possible that PKCθ is activated via multiple signals initiated during AIH. 3) PKCθ may be activated by cleavage at hinge domain, separating catalytic domain from the regulatory domain to generate PKMθ (or PKCθ II; see Niino *et al.*, 2001), a constitutively active PKCθ. These results would explain the lack of effect of NPC on pLTF induced by modest AIH, since PKMθ lacks a regulatory domain necessary for NPC binding. PKMθ (PKCθ II) could also be transcriptionally generated, or translated locally at synapses during AIH. Identification of the role of PKCθ in pLTF allows for exciting future studies to specifically determine how PKCθ activity is regulated *in vivo*, and how this affects expression of respiratory plasticity.

How is PKCθ activity regulated?

A combination of pharmacological studies on pLTF and molecular approaches to identify PKCθ phosphorylation would further advance our understanding of PKCθ activation mechanisms in pLTF. Since PKCθ is found predominantly within spinal neurons, PKCθ protein is not heavily

diluted by surrounding glia. Thus, it may be possible to measure phosphorylation of PKCθ protein utilizing western blot of spinal homogenates. One could measure tyrosine phosphorylation of PKCθ at its C2 domain, which may be one method of inducibly activating PKCθ. Such evidence would support PKCθ as requiring multiple signals for activation. In addition, using physiological approaches with spinal pharmacology, one could rule out/in PKCθ cleavage to PKMθ. Another regulatory domain inhibitor known to block PKCθ, calphostin C (Gao *et al.*, 2004), could be utilized. If spinal calphostin C blocks pLTF, then it is unlikely that generation of a PKMθ is responsible for PKCθ activation during modest AIH.

Is PKCθ involved in pLTF induction or maintenance?

PKCθ may be involved in pLTF induction and/or maintenance. Since the present studies delivered PKC inhibitors prior to AIH, we are unable to distinguish between pLTF induction or maintenance. Spinal delivery of PKCθ inhibitors following modest AIH would determine the role of PKCθ activity for pLTF maintenance. This would suggest that PKCθ activity is maintained following AIH, perhaps through generation of a constitutively active PKCθ (Niino *et al.*, 2001). Moreover, PKCθ may elicit pLTF by phosphorylation of synaptic targets, such as NMDA or AMPA receptors (MacDonald *et al.*, 2001). Such studies would further advance our understanding of the cellular/molecular mechanisms giving rise to pLTF.

Distinct PKC isoforms elicit pMF in different circumstances

pLTF induced by modest AIH requires PKCθ activation, while ipMF induced by hypocapnia requires PKCζ activation (Strey *et al.*, 2012). Moreover, evidence from Chapter III suggests that a classical/novel PKC isoform separate from PKCθ can elicit pMF. Other PKC isoforms may be associated with receptors such as G_q-coupled α_1 receptors, which elicit pMF when pharmacologically activated, but do not elicit pLTF following modest AIH (Huxtable et al.,

personal communication). Separate PKC isoforms might represent multiple, distinct routes to achieve phrenic motor facilitation, enabling the respiratory control system to undergo plasticity in a variety of circumstances, such as during hypoxia or inactivity.

Does pLTF occur within phrenic motor neurons?

Pharmacological approaches to study pLTF demonstrate cervical spinal localization of cellular/molecular mechanisms giving rise to pLTF but do not distinguish between cell types, or dorsal vs. ventral spinal mechanisms. In chapter II, a novel intrapleural administration of siRNA targeting PKCθ was used to prevent pLTF, by knocking down PKCθ within phrenic motor neurons. This technique localized mechanisms of pLTF because intrapleural siRNA is likely restricted to phrenic, intercostal, and vagal motor pools, due to retrograde transport from the intrapleural space. While it is unlikely that siRNA is traveling trans-synaptically, we cannot rule this out. Therefore, siRNA injected intrapleurally may affect interneurons directly surrounding phrenic motor neurons and we must still consider mechanisms giving rise to pLTF as being within the phrenic network (motor neurons, interneurons, and pre-motor neurons). Because PKCθ is not detected within microglia or astrocytes via immunohistochemical stains, we doubt involvement of those cell types in inducing pLTF during modest AIH. Therefore, intrapleural siPKCθ has advanced our understanding of cell localization (ie. phrenic network) of mechanisms giving rise to pLTF.

Motor neurons remain the most plausible cellular locale in pLTF induced by modest hypoxia because 1) they express necessary 5-HT$_2$ receptors and PKCθ in abundance (Chapter II; Fuller *et al.*, 2005), and 2) pLTF induced by modest AIH is associated with short latency phrenic responses, suggesting increases in mono-synaptic drive following AIH are responsible for pLTF (Fuller *et al.*, 2002).

Interactions between 5-HT$_2$- and A$_{2A}$-dependent pLTF give rise to pattern sensitivity

Evidence presented in chapter IV of this thesis demonstrate that pattern sensitivity of pLTF induced by moderate hypoxia is an emergent property of competing A$_{2A}$ and 5-HT$_2$-dependent pathways to pLTF. During sustained periods of hypoxia, there may be greater adenosine release and/or formation, which counteract mechanisms giving rise to serotonin-dependent pLTF. No studies to date have demonstrated competing mechanisms as a potential way to generate pattern sensitivity in plasticity (Philips *et al.*, 2013). Thus, pLTF now represents one mechanisms of plasticity where we are beginning to understand the complex cellular/molecular signals giving rise to such emergent properties as pattern sensitivity. Similar models of pattern sensitivity should be explored in other forms of neuroplasticity.

Some forms of plasticity are pattern-insensitive

In chapter V, we demonstrated that a single episode of severe hypoxia elicit significant A$_{2A}$-dependent pLTF. Plasticity arising from single experiences may be important for quick adaptations to deleterious stimuli (Diamond *et al.*, 2007; Philips *et al.*, 2013). Thus, we postulate that plasticity induced by severe hypoxia or activation of A$_{2A}$ receptors may be important to increase breathing following severe challenges to respiratory control which require quick corrections to prevent catastrophes such as respiratory failure or death. Plasticity following one single episode of acute hypoxia has not been demonstrated in other studies.

Summary and significance

pLTF is a form of spinal, respiratory motor plasticity which may restore ventilatory function in patients with disorders of ventilatory control, such as sleep apnea, spinal cord injury and motor neuron disease. Moreover, this plasticity is not limited to respiratory motor pools; intermittent hypoxia induces functional motor recovery in non-respiratory pools after spinal cord injury in

both rats and humans (Golder & Mitchell, 2005; Vinit *et al.*, 2009; Trumbower *et al.*, 2012; Lovett-Barr *et al.*, 2012). Thus, pLTF has led to recent advances that represent significant potential to treat disorders that compromise motor function.

Studies of pLTF also provide insights into basic mechanisms of neuroplasticity. Our expanding knowledge of cellular/molecular mechanisms giving rise to pLTF allow us to understand fundamental features of pLTF such as cellular localization, and emergent properties including pattern sensitivity, and metaplasticity (see appendix B). We now recognize that multiple, distinct pathways can give rise to phenotypically similar pMF. Interactions between these pathways are responsible for some features of pLTF induced by moderate hypoxia, such as pattern sensitivity. Understanding and appreciating the complexity of these interactions and the emergent properties they give rise to may allow us to better understand and apply all forms of neuroplasticity.

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Appendix A

Intermittent, but not sustained, hypoxia elicits long-term facilitation of XII motor output

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Abstract

Long term facilitation (LTF) of phrenic motor output is a pattern sensitive form of serotonin-dependent plasticity that is induced by an episode of intermittent hypoxia (IH), but not by sustained hypoxia (SH). Hypoglossal LTF, induced by IH, is also serotonin-dependent but studies to date have not determined its pattern sensitivity. Because phrenic and hypoglossal LTF occur by similar mechanisms, we hypothesized that, like phrenic LTF, hypoglossal LTF is dependent on the pattern of hypoxia exposure. Integrated hypoglossal nerve activity was recorded in urethane-anesthetized, vagotomized, paralyzed, and ventilated rats exposed to either IH (three 5 min episodes, 11% O_2) or SH (one 25 min episode, 11% O_2). Isocapnia was maintained throughout the protocol. Comparable to previous studies concerning phrenic LTF and consistent with our hypothesis, hypoglossal motor output was elevated for at least 1 h following IH (50 ± 20%, $P < 0.01$) but not following SH (-6 ± 9%, $P > 0.05$). Long term facilitation of burst frequency was not observed in either condition. These results demonstrate that hypoxia induced hypoglossal LTF is sensitive to the pattern of hypoxia exposure, analogous to phrenic LTF.

Introduction

Patterned stimulation is generally more effective than continuous stimulation at inducing plasticity in the central nervous system. For example, presentations of stimuli spaced over time more effectively induce long term memory formation compared to equal number of presentations shown continuously (Ebbinghaus, 1913; Beck *et al.*, 2000; Sutton *et al.*, 2002; Cepeda *et al.*, 2006). This phenomenon, known as the spacing effect, is expressed widely throughout the animal kingdom and has been extensively studied in animal models and humans because of its potential utility in education (Kerfoot, 2010), psychology (Goverover *et al.*, 2009*b*), advertising(Janiszewski *et al.*, 2003; Appleton- Knapp *et al.*, 2005), and physical rehabilitation (Goverover *et al.*, 2009*a*). Consistent with the spacing effect in learning and memory, patterned stimulation (intermittent vs. continuous) is also more effective at eliciting synaptic plasticity in the central nervous system. For example, intermediate-term memory formation and synaptic facilitation in aplysia (Mauelshagen *et al.*, 1998; Sutton *et al.*, 2002), and hippocampal long-term potentiation in rodents (Kauer, 1999; Nguyen *et al.*, 2000; Scharf *et al.*, 2002) all exhibit apparent pattern sensitivity to the induction protocol. Despite these observations, the mechanistic basis of pattern sensitivity in neuroplasticity is not fully understood.

Respiratory neuroplasticity exhibits distinct pattern sensitivity (Baker & Mitchell, 2000; Wilkerson *et al.*, 2008). The most common model of respiratory neuroplasticity is phrenic long term facilitation (pLTF), which is a progressive increase in phrenic motor output induced by intermittent hypoxia (IH), 3 five minute episodes of hypoxia each separated by 5 minutes of normoxia(Mitchell *et al.*, 2001). In contrast, exposure to a 25 minute episode of sustained hypoxia (SH), which is of equal total duration to an episode of IH, does not elicit pLTF (Baker &

Mitchell, 2000). Serotonin type 2 (5-HT$_2$) receptor activation in or around phrenic motor neurons is necessary for IH-induced pLTF (Fuller *et al.*, 2001*b*). However, multiple spinal injections of 5-HT are required to cause phrenic motor facilitation (pMF, a general term that also includes IH-induced pLTF); single injections of 5-HT fail to elicit pMF (MacFarlane & Mitchell, 2009). This parallels findings *in vitro*; intermittent but not sustained 5-HT receptor activation is sufficient to induce pLTF in neonatal brainstem-spinal cord preparations (Lovett-Barr *et al.*, 2006). Downstream from 5-HT, reactive oxygen species derived from NADPH oxidase are necessary for the expression of pLTF (MacFarlane *et al.*, 2009) and could inhibit relevant protein phosphatases that play a role in pattern sensitivity (Wilkerson *et al.*, 2007). For example, inhibition of protein phosphatases with okadaic acid reveals pLTF after an episode of SH (Wilkerson *et al.*, 2008).

Another form of respiratory neuroplasticity is hypoglossal long term facilitation (hLTF). Similar to pLTF, hLTF is expressed as a progressive increase in hypoglossal motor output that is induced by IH (Bach & Mitchell, 1996). One important distinction is that hLTF might represent a potential mechanism to increase upper airway to maintain upper airway patency, which is a therapeutic goal in patients with sleep apnea (Fuller, 2005). Thus, determining the underlying mechanisms of hLTF could lead to potential therapeutic targets. Much less is known about hLTF, including its pattern sensitivity and cellular/synaptic mechanisms (Baker-Herman & Strey, 2011). However, hLTF, like pLTF, requires 5-HT$_2$ receptor activation (Fuller *et al.*, 2001*b*), and reactive oxygen species formation (MacFarlane & Mitchell, 2008), suggesting that similar cellular/synaptic mechanisms are responsible for hLTF. However, hLTF differs from pLTF in some key ways. For example, hLTF expression differs with rat strain (Fuller *et al.*, 2001*a*) and is more affected by age and sex than pLTF (Behan *et al.*, 2002). No studies to

date have examined *in vivo* hLTF pattern-sensitivity; one *in vitro* study using rat medullary slices showed that episodic but not continuous 5-HT$_2$ receptor activation caused increased AMPA-mediated inspiratory-related drive currents and overall hypoglossal output (Bocchiaro & Feldman, 2004), suggesting that hLTF could be pattern sensitive. However another *in vitro* study utilizing the neonatal brainstem-spinal cord preparation demonstrated that phrenic and intercostal motor outputs differ in their pattern sensitivity, suggesting that different motor pools can vary in their pattern-sensitivity (Lovett-Barr *et al.*, 2006). Thus, we sought to determine whether hLTF exhibited pattern-sensitivity similar to pLTF or lacked pattern sensitivity more comparable to thoracic (or intercostal) motor plasticity.

In the present study, because hLTF and pLTF exhibit similar mechanisms, we hypothesized that hLTF, like pLTF, is pattern sensitive. Thus, we predicted that (1) IH elicits robust hLTF and (2) SH fails to elicit significant hLTF. Collectively, our data demonstrate that hLTF (like pLTF) is pattern sensitive, such that intermittent hypoxia elicits motor facilitation but one continuous episode does not.

Materials and Methods

Experiments were performed using 3-5 month old adult, male Sprague-Dawley rats (colony PO4, Charles River Inc., Wilmington, MA). Animals were individually housed in a controlled environment (12h light/dark cycle), with food and water *ad libitum*. The University of Wisconsin's Animal Care and Use Committee approved all protocols.

Surgical Preparation and Nerve Isolation. Rats were initially anesthetized in a closed chamber containing isoflurane followed by isoflurane administration through a nose cone (3.0 - 3.5% in 50% O_2, balance N_2). The trachea was cannulated to enable pump-ventilation (tidal volume, 2 – 2.5 mL; FIO_2 = 0.50; Rodent Respirator model 682, Harvard Apparatus, South Natick, MA). A bilateral vagotomy was performed at the mid-cervical level to prevent entrainment of respiratory motor output with the ventilator. Catheters were placed into the tail vein for fluid administration (1:11 by volume $NaHCO_3$:lactated Ringer's; 2.5 mL/hr) and the femoral artery for blood pressure measurement and to draw blood samples for blood gas analysis. Body temperature was maintained at 37.5 ± 1.0 °C using a rectal probe and custom-designed heated table. The left hypoglossal nerve was isolated using a dorsal approach, cut distally, and then carefully desheathed and placed on a bipolar silver electrode. Rats were slowly converted to urethane anesthesia (1.6 g/kg, i.v.) and then paralyzed with pancuronium bromide to prevent spontaneous breathing movements (2.5 mg/kg, i.v., supplemented as necessary). End-tidal CO_2 was measured throughout the experiment using a flow-through capnograph (Capnogard, Model 1265, Novametrix; Wallingford, CT) with sufficient response time to measure expiratory gases in rats.

Hypoxic Protocols. The CO_2 apneic threshold was determined by decreasing CO_2 levels and/or increasing the pump ventilator rate until nerve activity ceased. Inspired CO_2 levels were then increased; the end-tidal CO_2 at which phrenic activity resumed was taken as the recruitment threshold (Mahamed & Mitchell, 2007). End-tidal CO_2 levels were then set at 1-2 torr above the recruitment threshold. A stable hypoglossal neurogram was established and an initial blood sample was taken to establish baseline PaO_2, $PaCO_2$, pH, and base excess values (0.3 ml in 0.5 ml heparinized glass syringe; ABL-500, Radiometer, Copenhagen, Denmark; unused blood was returned to the animal). Rats were given three 5-min episodes of hypoxia (i.e., intermittent hypoxia, FIO_2 = 0.11 ± 0.1, PaO_2 = 39 ± 1 mmHg) separated by 5 min of baseline conditions (FIO_2 = 0.5, PaO_2 > 250 mm Hg) or a single, cumulative 25-min hypoxic episode (i.e., sustained hypoxia, FIO_2 = 0.11 ± 0.1, PaO_2 = 38 ± 1 mmHg). Hypoglossal activity was monitored 60 min post-hypoxia to determine LTF magnitude. Arterial blood samples were drawn and analyzed during the final minute of the first hypoxic episode and 15, 30, and 60 min following the final hypoxic episode. Additional rats that did not receive hypoxia (time controls) were used to verify the stability of nerve output over a similar time period under equivalent conditions. Throughout the protocol, isocapnic conditions (± 1 mmHg from baseline $PaCO_2$) were maintained by adjusting ventilator frequency and/or inspired CO_2.

Electrophysiological data analysis. Hypoglossal nerve activity was amplified (x 10,000), band pass filtered (100 Hz to 10 kHz; Model 1700, A-M Systems, Inc., Carlsborg, WA),

and integrated (time constant = 50 ms, Model MA-821RSP, CWE Inc., Ardmore, PA). Integrated signals were digitized and processed with commercially available software (WINDAQ software, DATAQ Instruments, Akron, OH). Peak integrated hypoglossal burst amplitude, burst frequency, and mean arterial blood pressure were calculated over a 60 second period just prior to the first hypoxic episode (baseline), at the end of the first hypoxic episode or the equivalent time point during sustained hypoxia (short-term hypoxic response), and 30 and 60 min post-hypoxia. Data were only included in the analysis if isocapnic conditions were maintained. Amplitude data are expressed as the change in hypoglossal burst amplitude as a percent change from baseline values. Frequency data are reported as the change from baseline in bursts per minute (delta burst frequency). Data were compared using a one-way ANOVA or two-way ANOVA with repeated measures design if applicable (Fisher LSD *post-hoc* test if necessary; SigmaStat 2.03, SPSS Inc., Chicago, IL).

Results

Physiological variables

No significant differences were observed in the recruitment threshold for animals treated with hypoxia (IH: 41 ± 1 mmHg, n=9; SH: 40 ± 1 mmHg, n=14) or without hypoxia (42 ± 1 mmHg, n=14; p > 0.05).

Under baseline conditions, mean arterial blood pressure was not different between treatment groups (IH: 112 ± 5 mmHg, SH: 110 ± 4 mmHg; Table 1, p > 0.05) or rats that did not receive hypoxia: (112 ± 5 mmHg; Table 1, p > 0.05). Similar to other studies from our laboratory, mean arterial blood pressure significantly decreased during the hypoxic stimulus relative to baseline (IH: 62 ± 4 mmHg, SH: 65 ± 7 mmHg; p < 0.05), but not at an equivalent time point in time controls (112 ± 5 mmHg; Table 1, p > 0.05). We did not observe significant changes in mean arterial blood pressure at 30 min post-hypoxia in any group, however, small but significant decreases were found at the 60 min time point in the IH (105 ± 4 mmHg), SH (100 ± 3 mmHg), and time control (105 ± 5 mmHg), treatment groups (Table 1, p < 0.05). No significant treatment effect was found between the intermittent and sustained hypoxia-treated groups at any time point for mean arterial blood pressure (p > 0.05).

Pa_{CO_2} was maintained within 1 mmHg of the baseline value throughout the experimental protocol. Thus, changes in hypoglossal burst amplitude or frequency were not caused by shifts in arterial CO_2.

Long-term facilitation in hypoglossal motor output and the pattern of hypoxia.
Representative integrated hypoglossal neurograms before, during, and 60 min following IH or SH are presented in Figure 1. Figure 2 shows that hypoglossal burst amplitude and delta burst frequency significantly increased in rats exposed to intermittent hypoxia (IH, $203 \pm 31\%$ baseline and 16 ± 3 bursts/min, respectively) and sustained hypoxia (SH, $236 \pm 34\%$ baseline and 8 ± 2 bursts/min, respectively). There was no significance difference between the hypoglossal burst amplitude or frequency during SH vs. IH (Figure 2, $p > 0.05$ for both).Time control groups, which did not receive hypoxia, showed no significant difference in hypoglossal burst amplitude or delta burst frequency at the equivalent time point ($0 \pm 3\%$ baseline and -3 ± 2 bursts/min, respectively; Figure 2, $p > 0.05$).

Group data for hypoglossal burst amplitudes 60 minutes following hypoxia are presented in Figure 3. Hypoglossal burst amplitude was significantly increased from baseline 60 min ($50 \pm 20\%$ baseline, $p < 0.01$) post-intermittent hypoxia, demonstrating the development of hypoglossal LTF (Figure 3A). In contrast, hypoglossal LTF did not develop following sustained hypoxia ($-6 \pm 9\%$ baseline, $p > 0.05$, Figure 3A). Hypoglossal burst amplitude was stable over time, as animals that were not exposed to hypoxia did not show a significant change in hypoglossal burst amplitude relative to baseline (13 ± 9 %baseline, $p > 0.05$).

Frequency long term facilitation

Whereas hypoglossal burst frequency significantly increased during hypoxia (Figure 2B), no significant changes were observed in hypoglossal delta burst frequency 60 min post-hypoxia (intermittent hypoxia: 4 ± 3 bursts/min; sustained hypoxia: 1 ± 2 bursts/min; $p > 0.05$; Figure 3B). Animals that were not treated with hypoxia did not show a significant change in hypoglossal

burst frequency at the equivalent time point (2 ± 1 bursts/min; $p > 0.05$, <u>Figure 3B</u>). 60 min post-treatment, there was not a significant difference between any group ($p > 0.50$), suggesting that frequency long-term facilitation did not develop in this group of animals.

Discussion

While pLTF has been shown to be pattern sensitive (Baker & Mitchell, 2000), hLTF pattern sensitivity has not been definitively shown to date. Since it has been shown that different motor pools can vary in their pattern sensitivity (Lovett-Barr *et al.*, 2006), it is conceivable that hLTF and pLTF could differ in their pattern-sensitivity. We explored the pattern-sensitivity of hLTF, using protocols analogous to those used in pLTF studies to test the hypothesis that IH will elicit hLTF but SH will not. We report that, like pLTF, hLTF is elicited by IH, but not by an episode of SH. Thus, although hLTF differs from pLTF in some key characteristics, these data collectively demonstrate that hLTF demonstrates hypoxic pattern sensitivity.

Potential mechanisms of pattern sensitivity

In a previous study on pLTF pattern sensitivity, we demonstrated that inhibition of protein phosphatases with okadaic acid revealed pLTF following SH (Wilkerson *et al.*, 2008). The protein tyrosine phosphatase corkscrew (CSW) has been to show to regulate pattern sensitivity in *Drosophila* long term memory induction (Pagani *et al.*, 2009), suggesting that the role of phosphatases in pattern-sensitivity is not limited to respiratory neuroplasticity. We were unable to explore the roles of protein phosphatases in the current hLTF study because of the difficulty associated with pharmacological injections into the hypoglossal motor nucleus. It is likely that hLTF and pLTF exhibit similar if not identical mechanisms of pattern sensitivity. Thus, we would predict that protein phosphate inhibition in the hypoglossal motor nucleus during SH would reveal pLTF. However, although protein phosphates are likely critically involved, the mechanism of hLTF and pLTF pattern sensitivity remains to be elucidated.

Significance of pattern sensitivity in respiratory neuroplasticity

The importance of patterned stimulation in evoking plasticity is described in many different models of plasticity, yet the mechanism remains unknown. For example, intermediate-term memory formation and synaptic facilitation in aplysia (Mauelshagen *et al.*, 1998; Sutton *et al.*, 2002), habituation in the crab (Freudenthal *et al.*, 1998), olfactory memory formation in *Drosophila* (Isabel *et al.*, 2004), and hippocampal long-term potentiation (Kauer, 1999; Nguyen *et al.*, 2000; Scharf *et al.*, 2002) in rodents all exhibit apparent pattern sensitivity to the induction protocol. However, there are certain instances of pattern-insensitive plasticity. In the whole brainstem-spinal cord preparation, long term facilitation of thoracic intercostal motor output is elicited by a continuous infusion of 5-HT (Lovett-Barr *et al.*, 2006). Another example of pattern insensitive respiratory plasticity is ventilatory acclimatization to chronic hypoxia, such as occurs during exposure to high altitude (Dwinell & Powell, 1999). Thus, by describing pattern sensitivity in respiratory neuroplasticity, we may yield insights as to why pattern sensitivity exists in some motor pools, and how pattern sensitivity is determined (ie. its mechanism). Furthermore, understanding pattern-sensitive aspects of respiratory neuroplasiticity will guide its optimal utilization in strategies aiming to increase respiratory motor output through induction of plasticity.

Significance of hLTF

The physiological relevance of any form of respiratory plasticity remains unclear (Mitchell *et al.*, 2001); however, hLTF could represent a potential mechanism for increasing upper airway

tone and patency, decreasing the occurrence of upper airway collapse and apneas in patients with sleep-disordered breathing (Fuller, 2005). Furthermore hLTF might represent mechanisms that allow for long-term adaptations of hypoglossal motor output during environmental challenges, disease, or central nervous system injury (Mitchell *et al.*, 2001). Understanding mechanisms leading to hypoglossal motor facilitation will aid attempts to utilize hLTF to increase hypoglossal motor tone. Furthermore, elucidating the mechanisms responsible for hLTF that confer pattern sensitivity will increase our basic understanding of pattern sensitivity in general and could guide overall strategies to utilize plasticity to treat human disease.

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Figures and Tables

Treatment	N	Baseline	1st HX	60 min post-H
		112 ± 5 (MABP)	112 ± 5	105 ± 5
No hypoxia	14	47.7 ± 1.2 ($PaCO_2$)	47.7 ± 1.2	47.6 ± 1.2
		267.2 ± 4.0 (PaO_2)	265.9 ± 4.7	252.2 ± 8.7
		112 ± 5	68 ± 4*	105 ± 4*
Intermittent hypoxia	9	45.2 ± 1.2	44.0 ± 1.7	45.9 ± 1.1
		275.7 ± 6.5	38.9 ± 0.8*	262.0 ± 8.1
		110 ± 4	65 ± 7*	100 ± 3*
Sustained hypoxia	14	44.4 ± 1.8	43.9 ± 0.8	44.0 ± 0.9
		257.0 ± 4.9	37.9 ± 1.2*	245.4 ± 8.0

Table 1. Temporal changes in mean arterial blood pressure (MABP), $PaCO_2$, and PaO_2 in rats/ Relative to baseline, mean arterial blood pressure (MABP), arterial partial pressure of CO_2 ($PaCO_2$), and arterial partial pressure of O_2 (PaO_2) were not significantly different over time in rats that did not receive hypoxia ($p > 0.05$). As expected PaO_2 significantly decreased during hypoxia exposure ($P < 0.05$), but returned to baseline values following exposure. Rat groups treated with intermittent or sustained hypoxia showed similar significant decreases in MABP during hypoxia ($p < 0.05$) and small but significant decreases in MABP 60 min post-hypoxia compared to baseline ($p < 0.05$). Overall, there was not a significant treatment effect on MABP ($p > 0.05$). *Significantly different from baseline within treatment group
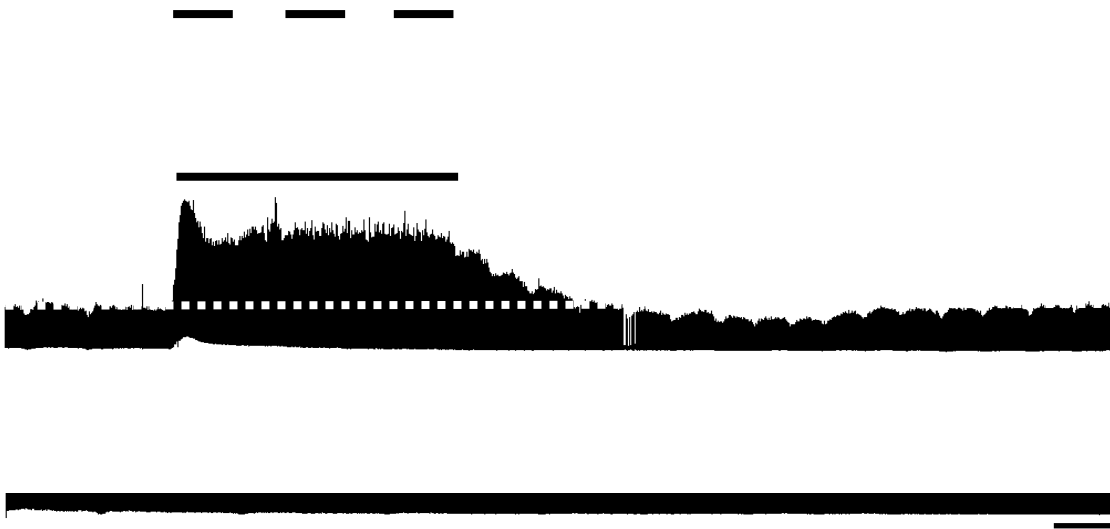
Figure 1. Representative integrated hypoglossal neurograms before, during, and 60 min following intermittent (*top*) or sustained (*middle*) hypoxia or no hypoxia (time control, *bottom*). Relative to baseline (indicated by dotted line), hypoglossal burst amplitude was increased 60 min following intermittent, but not sustained hypoxia. When hypoxia is not presented, hypoglossal motor output remains stable over time (time control). Short bar = 5 min; long bar = 25 min.
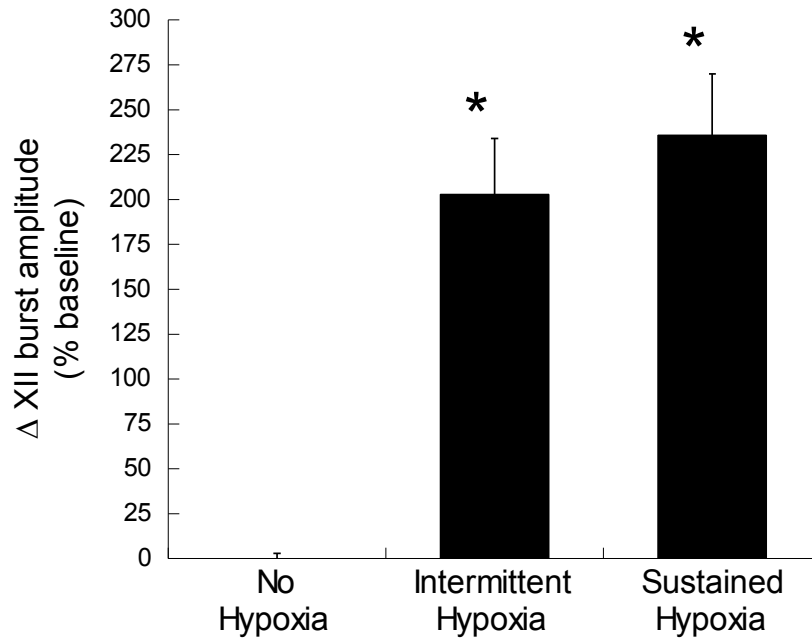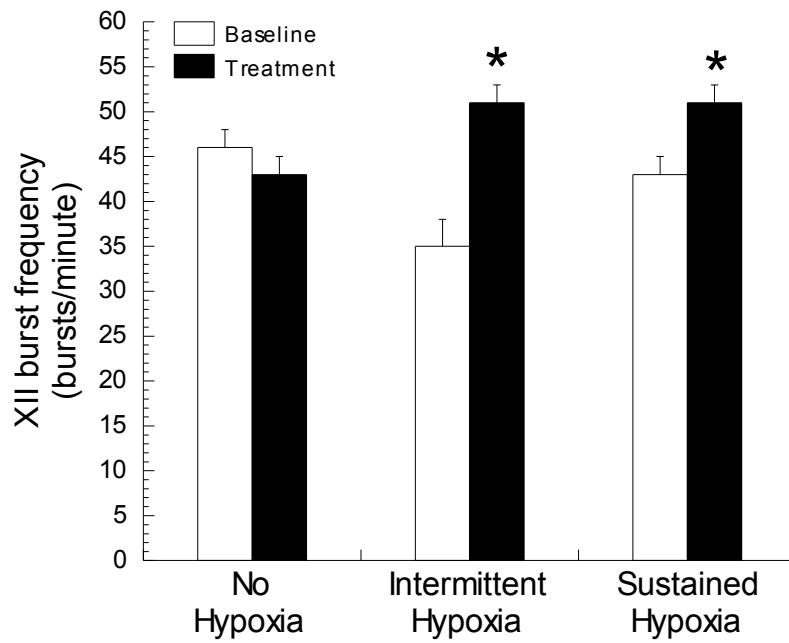
A

B

Figure 2. Short-term response of XII burst amplitude (**A**) and frequency (**B**) during intermittent or sustained hypoxia or without hypoxia. Data are presented as mean ± SEM. *Significantly increased from baseline ($p < 0.05$)
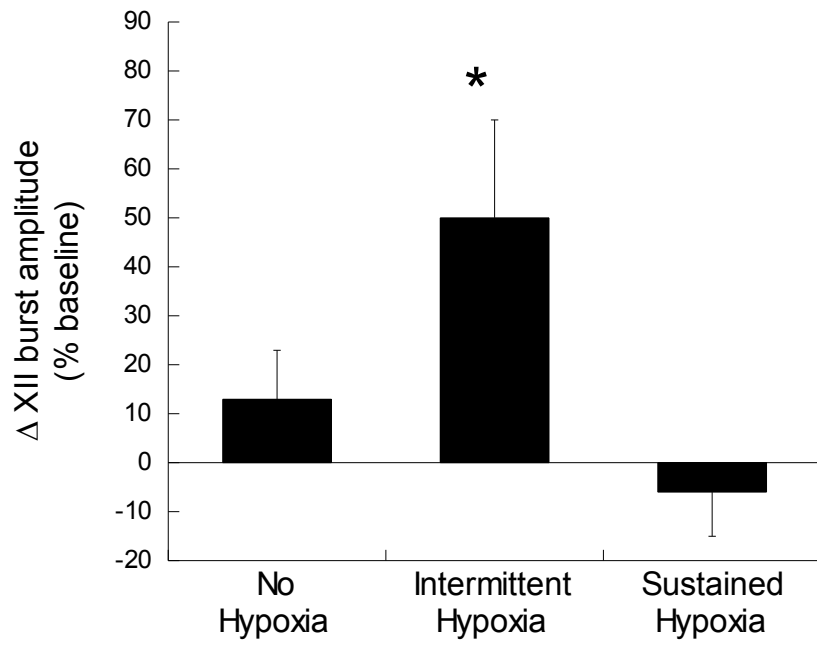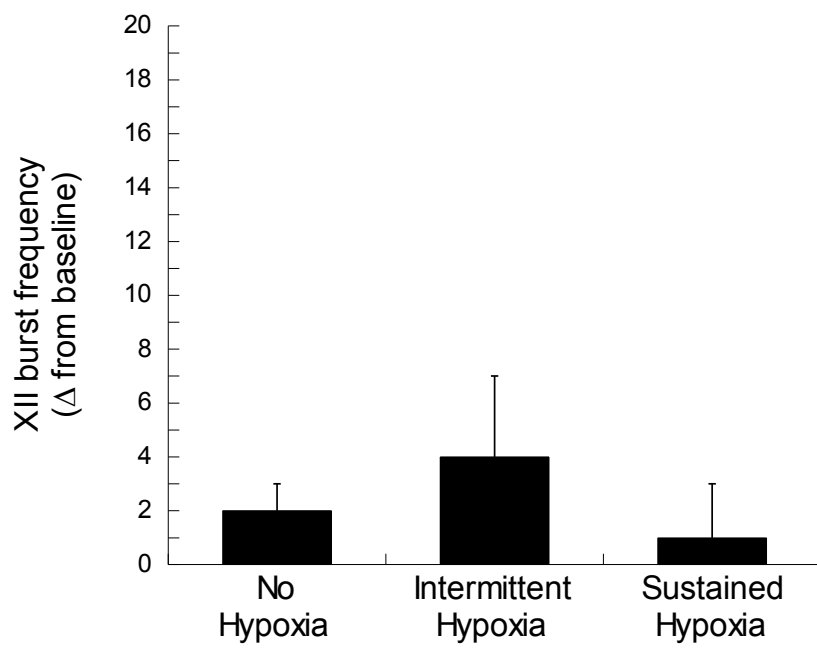
A

B

Figure 3. LTF of XII burst amplitude (**A**) but not delta burst frequency (**B**) is present 60 min following intermittent, but not sustained hypoxia. Data are presented as mean ± SEM. *Significantly increased from baseline ($p < 0.05$).

Appendix B

Hypoxia-induced phrenic long-term facilitation: emergent properties

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Abstract

Just as in other neural systems, plasticity is a hallmark of the neural system controlling breathing. One spinal mechanism of respiratory plasticity is phrenic long-term facilitation (pLTF) following acute intermittent hypoxia. Although cellular mechanisms giving rise to pLTF occur within the phrenic motor nucleus, different signaling cascades elicit pLTF in different conditions. These cascades, referred to as "Q" and "S" pathways to phrenic motor facilitation (pMF), interact via cross-talk inhibition. Whereas the Q pathway dominates pLTF after mild to moderate hypoxic episodes, the S pathway dominates after severe hypoxic episodes. The biological significance of multiple pathways to pMF is not known. We discuss the possibility that interactions between pathways confer emergent properties to pLTF, including: 1) pattern sensitivity and 2) metaplasticity. Understanding these mechanisms and their interactions may enable us to optimize intermittent hypoxia induced plasticity as a treatment for patients that suffer from ventilatory impairment or other motor deficits.

Introduction

The respiratory control system has been historically viewed as fixed and immutable, controlled primarily via negative feedback from sensory receptors.[1] This view was held in large part because breathing is an automatic, often subconscious motor behavior. However, control systems governed by negative feedback alone are frequently unstable due to inappropriate reflex gain.[2] Systems that preserve homeostasis through strong negative feedback loops are vulnerable to insults; robust control systems optimize for worst-case scenarios (ie. ventilatory failure), and incorporate mechanisms to prevent such failure when the system is challenged. Plasticity is one key property of neural systems, such as the respiratory control system, that promote robust and effective homeostatic regulation.[1] In this brief review, we use a general definition of respiratory plasticity, namely: a change in future system performance (ie. breathing or blood gas regulation) based on experience.[1]

Plasticity in the neural system controlling breathing has only been widely appreciated only for the past few decades .[1,3–5] Recently, the field of respiratory neuroplasticity has grown considerably; plasticity has been discovered at the neuromuscular,[6] chemoreceptor,[7] spinal,[8] and brainstem,[9] levels of respiratory control, and our knowledge concerning these forms of plasticity is increasing at a rapid pace. Here we focus on a single, widely studied model of spinal respiratory plasticity, phrenic long-term facilitation (pLTF). pLTF is a persistent increase in phrenic motor output lasting hours after a few brief episodes of low oxygen, or acute intermittent hypoxia (AIH; see refs 10-13). Considerable progress has been made towards an understanding of cellular and network mechanisms giving rise to pLTF.[10-11] One recent realization is that multiple distinct cellular cascades give rise to similar phenotypic plasticity.[10] An important question is: why do these multiple pathways exist? In this brief review, we will consider the potential advantages conferred by this complexity. In specific, we develop the

hypotheses that the existence of multiple interacting pathways confers two emergent properties of pLTF: pattern sensitivity and metaplasticity.

Phrenic long-term facilitation (pLTF)

Millhorn and colleagues originally demonstrated that episodic carotid sinus nerve stimulation elicits a long lasting increase in phrenic motor output in anesthetized cats.[3,4] We subsequently demonstrated a similar phenomenon in anesthetized rats following three brief hypoxic episodes, a phenomenon termed phrenic long-term facilitation (pLTF).[12,13] Following acute intermittent hypoxia (AIH), pLTF is expressed as a prolonged increase in phrenic nerve burst amplitude lasting several hours after the final hypoxic episode (**Figure 1A**). pLTF is a form of serotonin- and protein synthesis-dependent spinal plasticity.[12,14,15] pLTF induction is independent of increased phrenic nerve activity[16–17] and represents a form of neuromodulator induced plasticity[1] distinct from conventional forms of activity-dependent synaptic plasticity such as hippocampal long-term potentiation (LTP).[18]

On hallmark of pLTF is pattern sensitivity; pLTF is elicited by intermittent, but not a single period of acute sustained hypoxia (ASH) with the same cumulative duration (**Figure 1B**).[19] Although similar pattern sensitivity is shared by many forms of neuroplasticity,[20–24] we have little understanding of how such pattern sensitivity arises in any system. Another property of pLTF is its ability to express metaplasticity;[1] for example, following cervical dorsal rhizotomy[25] or chronic intermittent hypoxia,[26] subsequent responses to AIH (ie. pLTF) are amplified. We still do not fully understand mechanisms that confer metaplasticity.

Although our understanding of mechanisms giving rise to respiratory plasticity remains incomplete, considerable progress has been made.[10,11,27,28] One realization is that multiple, distinct cellular cascades exist, each capable of eliciting long-lasting phrenic motor facilitation (pMF; a general term describing augmented phrenic burst amplitude that includes pLTF).[10]

These pathways interact in interesting and complex ways, possibly increasing flexibility as the respiratory control system responds to diverse challenges throughout life.

In this review, we make the argument that interactions between distinct cellular cascades to phrenic motor facilitation confer emergent properties to respiratory plasticity, including pLTF pattern sensitivity and metaplasticity. An understanding of the diverse mechanisms giving rise to pMF and their implications will help to understand pattern sensitivity and metaplasticity in other forms of neuroplasticity, and will be essential as we begin to harness the potential of AIH-induced spinal plasticity to treat severe clinical disorders that impair breathing, such as spinal cord injury[29–31] or ALS.[32]

Multiple cellular mechanisms of pLTF: the Q and S pathways

pLTF is frequently studied in anesthetized, paralyzed and ventilated rats administered a standardized AIH protocol (3, 5 min hypoxic episodes; PaO_2 35-45 mmHg; 5 min intervals; see ref 25). We refer to this protocol as **moderate AIH**. Following moderate AIH, pLTF requires spinal serotonin type 2 ($5\text{-}HT_2$) receptor activation,[12,14,15] new synthesis of brain-derived neurotrophic factor (BDNF),[33] activation of its high-affinity receptor (TrkB),[33] and ERK MAP kinase signaling.[34] Because $5\text{-}HT_2$ receptors are G_q protein-coupled receptors,[35] we refer to this as the "Q pathway" to pMF (**Figure 2A**). Pharmacological activation of other G_q coupled receptors (such as α_1 receptors) elicit similar pMF.[10]

A distinct pathway to pMF relies on activation of G_s protein coupled metabotropic receptors, such as adenosine 2A (A_{2A})[36] and $5\text{-}HT_7$ receptors.[37] This form of pMF requires new synthesis of an immature TrkB isoform (not BDNF) and PI3 kinase/Akt signaling (not ERK).[36] We refer to this as the "S pathway" to pMF since multiple G_s protein-coupled metabotropic receptors elicit the same mechanism (**Figure 2B**).[10] Contrary to our initial expectation, the S pathway does not contribute to pLTF following moderate AIH,[38] but dominates pLTF following severe AIH.[39] In

fact, the S pathway negatively regulates pLTF with moderate AIH, demonstrating inhibitory interactions between pathways;[38] based on these findings, we proposed that the predominant interaction between pathways is "cross-talk inhibition." Although the mechanistic basis of this cross-talk inhibition is not fully understood, S to Q inhibition may require PKA activity (Hoffman and Mitchell, unpublished).

During severe AIH, the S pathway is activated to a greater extent, and dominates pLTF.[39] Rats exposed to a severe AIH exhibit pLTF phenotypically similar to moderate AIH, but via an A_{2A} receptor-dependent (serotonin-independent) mechanism (i.e. S pathway).[39] This finding suggests that cross-talk inhibition between pathways assures dominance of one or the other; the switch appears to be precipitous since PaO_2 levels above 35 mmHg elicit pLTF via the Q pathway[35] whereas PaO_2 levels of 30 mmHg or below elicit pLTF via the S pathway.[39] We suggest that this transition occurs because of relatively greater accumulation of extracellular adenosine during severe hypoxic episodes, shifting the balance towards the S pathway. Once the tipping-point is reached, we hypothesize that the now dominant S pathway suppresses the Q pathway. Although the subordinate pathway does not positively contribute to pLTF, it nevertheless modulates (inhibits) the dominant pathway.[38]

More pathways to phrenic motor facilitation

Three other stimuli elicit unique forms of pMF in anesthetized rats. Spinal injections of the growth/trophic factors vascular endothelial growth factor (VEGF) or erythropoietin (EPO) near the phrenic motor nucleus cause pMF via mechanisms that require both ERK and Akt activation for full expression.[40,41] We suspect that these hypoxia-sensitive genes enable phrenic motor plasticity in longer time domains. For example, VEGF or EPO induced pMF might play a role in longer-term adjustments of phrenic motor activity during chronic intermittent or sustained hypoxia.[40,41]

A completely different cellular cascade gives rise to pMF after brief periods of phrenic inactivity (iPMF) induced by hypocapnia, vagal stimulation and/or isoflurane.[42] Unlike pLTF, iPMF requires atypical PKC activation.[43,44] iPMF might ensure that this critical motor pool is constantly active. For example, iPMF might contribute to the preservation of adequate phrenic activity when synaptic inputs are disrupted, such as by spinal injury.

Emergent properties of pLTF

Some properties of pLTF, such as pattern sensitivity and metaplasticity, may be determined by interactions between competing pathways to pMF.[26,45] These emergent properties may determine whether pLTF is expressed or not after a given stimulus (eg. different patterns or severity of hypoxia), or its magnitude (eg. greater magnitude in response to the same AIH).

Pattern sensitivity

Pattern sensitivity is a common feature in many models of neuroplasticity, including models of serotonin-dependent synaptic facilitation.[20,21] pLTF is pattern-sensitive since it is induced by moderate AIH, but not acute sustained hypoxia (ASH) of similar severity and cumulative duration (9-25 min; **Figure 1B**).[12] Pattern sensitivity of respiratory plasticity was initially recognized in studies of ventilatory LTF (vLTF) in goats,[46,47] and subsequently observed in rat[48,49] and human vLTF.[50–55] Interestingly, vLTF in humans requires slightly elevated CO_2 during AIH.[56] It was recently reported that elevated CO_2 reveals vLTF in humans exposed to 32 minutes of ASH,[57] although the investigators found that about half of this vLTF was due to drift in ventilation caused by sustained hypercapnia alone. Griffin et al.[57] observed a persistent increase in ventilation for 20 min after AIH or ASH, but they did not measure ventilation at later time points necessary to clearly demonstrate LTF. Thus, further studies are needed to confirm that vLTF is pattern-sensitive in awake humans.

Although significant progress has been made, the mechanism of pLTF pattern sensitivity remains unknown. The optimal spacing interval has not been characterized, but is somewhere between 1 and 30 minutes.[58] pLTF is relatively insensitive to other characteristics of AIH, such as the severity or duration of hypoxic episodes. For example, pLTF is unaffected by the level of PaO_2 from 35 to 60 mmHg,[59,60] or by episode durations between 15 sec to 5 min.[61] Similar to AIH-induced pLTF, pMF induced by intraspinal 5-HT requires episodic injections, suggesting that pattern sensitivity occurs at or downstream from 5-HT receptor activation.[16,17,62] Here, we propose three possible mechanisms that could contribute to pLTF pattern-sensitivity.

Serotonin receptor desensitization (Figure 3A)

Serotonin receptors are diverse, with complex signaling and activation requirements; the $5\text{-}HT_2$ class alone includes multiple isoforms with unconventional properties, such as g-protein independent signaling[63] or constitutive activity.[64] One pertinent feature of $5\text{-}HT_2$ receptors is desensitization, which is a decrease ligand response after prolonged ligand exposure. In the brain, $5\text{-}HT_2$ receptors desensitize rapidly with persistent elevations in extracellular serotonin via mechanisms that involve receptor internalization and/or functional uncoupling.[65] Receptor internalization occurs when agonist bound receptors are internalized by clathrin coated pits into endosomes, where they are sequestered until reinserted or degraded. Functional uncoupling disrupts G-protein signaling and occurs via receptor phosphorylation, usually by G protein-coupled receptor kinases (GRKs); this phosphorylation causes the protein arrestin to bind the receptor, preventing further G protein activation.[66] *In vitro* studies demonstrate $5\text{-}HT_2$ receptor desensitization after 5-10 min of agonist exposure,[67,68] well within the time frame of a 25 minute ASH exposure. Although no studies have assessed $5\text{-}HT_2$ receptor desensitization in the spinal cord, it is a candidate to undermine pLTF during ASH.

Regulation of protein phosphatase activity (Figure 3B)

Protein phosphatases are inhibitory to many forms of CNS synaptic plasticity;[69,70] the expression of plasticity is often regulated by protein phosphatase activity during the induction phase. pLTF is constrained by constitutive okadaic acid-sensitive protein phosphatases during ASH,[45] Thus, in rats pretreated with spinal okadaic acid, ASH elicits pLTF.[45] Reactive oxygen species (ROS) inhibit protein phosphatases involved in plasticity.[71] Indeed, ROS produced by NADPH oxidase are necessary for pLTF expression,[72] and this requirement is offset by spinal protein phosphatase inhibition with okadaic acid.[73] Thus, NADPH oxidase and okadaic acid sensitive protein phosphatases (most likely PP2A) appear to constitute a "regulatory cassette" that determines if and how much pLTF will be expressed (Fig. 3B).[37,64] One likely difference between ASH and AIH in their ability to elicit pLTF could be relative ROS production (and phosphatase inhibition). While no published studies have directly compared ROS production in response to AIH vs. ASH, there is evidence that hypoxia-induced superoxide production occurs predominantly during reoxygenation[73–75] suggesting that AIH could generate greater ROS (vs. ASH) because of multiple reoxygenations. Further studies are needed to determine how AIH (vs. ASH) stimulates greater ROS production in the cervical spinal cord.

Cross-talk inhibition between Q and S pathways (Figure 3C)

Although pLTF shifts to S pathway-dependence with severe AIH,[39] it is not known how this shift occurs. We proposed that "cross-talk inhibition" between pathways (**Figure 3C**) enables one pathway to gain an "upper hand" and, thus, dominate pMF. A key factor is the strength of the initiating stimulus (5-HT_2 versus A_{2A} receptor activation). For example, during severe AIH, greater adenosine formation/accumulation may increase A_{2A} receptor activation, creating stronger S pathway activation and, subsequently, Q pathway suppression. The prevailing mechanism of pLTF may result from competition between pathways for dominance (Figure 3C).

We speculate that, at some levels of hypoxia (severity, duration, etc), inhibitory interactions could become balanced, creating an impasse where the S or Q pathways offset one another (ie. no pLTF). If so, this state represents an emergent property of competing pathways to pMF, and could underlie pLTF pattern-sensitivity. During longer hypoxic exposures, such as moderate ASH, greater adenosine formation/accumulation[76] may cause balanced activation of the Q and S pathways (**Figure 3C**), thereby obscuring pLTF. This hypothesis is consistent with the study by Griffin et al., (2012) since hypercapnia could cause greater serotonin release, shifting the balance towards the Q pathway and vLTF expression. Further studies are needed to determine the role of pathway interactions in pLTF pattern sensitivity.

Are other forms of pMF pattern-sensitive?

While many studies have focused on pLTF pattern sensitivity following moderate AIH, no studies have examined pattern sensitivity in the other forms of pMF described above (ie. S pathway, VEGF- and EPO-induced pMF, or iPMF). Studies are needed to determine if these forms of pMF are also pattern-sensitive.

Metaplasticity

Metaplasticity is loosely defined as "plastic plasticity", but more specifically as the ability of prior experience to alter subsequent plasticity.[77] Metaplasticity can be expressed as enhanced pLTF triggered by pre-conditioning of adults with repetitive intermittent hypoxia[26,78,79] or cervical sensory denervation.[25] On the other hand, developmental exposure to chronic intermittent hypoxia suppresses pLTF in adult rats.[80] pLTF enhancement from pre-conditioning is particularly interesting because it could increase the success of AIH protocols applied to improve breathing capacity or other motor functions after spinal injury.[8,27–30] Thus, metaplasticity

(and its mechanisms) are important considerations for clinical utilization of AIH-induced motor plasticity.

Enhanced pLTF after pre-conditioning with repetitive intermittent hypoxia

Rats exposed to chronic intermittent hypoxia (CIH; 10-12% O_2/air, 2-5 min intervals, 8-12 hrs/night) exhibit enhanced serotonin-dependent pLTF.[26] Other investigators have reproduced this effect with shorter hypoxic episodes (5-12% O_2, 15s episodes with 5 min intervals, 8h/day) to more closely simulate episodic hypoxia during sleep disordered breathing.[81] In this study, pLTF enhancement was blocked by antioxidant administration[81] demonstrating ROS dependence. Thus, CIH enhances pLTF, presumably by enhanced Q pathway signaling (ie. serotonin and ROS dependent). Although CIH is a potent stimulus to metaplasticity, it also elicits morbidity, including hypertension, hippocampal apoptosis and cognitive deficits, among others.[82–84] Thus, we developed more subtle protocols of repetitive intermittent hypoxia that elicit pLTF metaplasticity without detrimental effects elicited by CIH.[30,85,86]

Rats exposed to modest protocols of repetitive acute intermittent hypoxia (rAIH) exhibit enhanced pLTF[78,79] and increased respiratory and non-respiratory somatic motor recovery following cervical spinal injury.[30] Two different rAIH protocols were used in these studies. Daily AIH (10, 5 min episodes of 10.5% O_2, 5 min intervals, 7 days) improved respiratory and forelimb function and increased phrenic burst amplitude in rats with C2 spinal hemisections.[30] In Brown-Norway rats, a strain with low constitutive pLTF and no hypoglossal LTF, daily AIH enabled hypoglossal LTF, but an apparent doubling of pLTF was only marginally significant.[85] These results suggest that daily AIH is a modest inducer of metaplasticity in the phrenic motor pool of otherwise normal rats. Rats exposed to AIH 3 times per week (3xwAIH; 10, 5min episodes/day, 3 days per week for 4 weeks) exhibit enhanced pLTF,[78,79] and profound neurochemical plasticity in the phrenic motor nucleus,[86] suggesting that longer, less frequent exposures are more

effective at eliciting pLTF metaplasticity. Neither protocol caused hippocampal apoptosis, astrogliosis, or hypertension,[30,85,86] suggesting the ability to elicit respiratory metaplasticity without detectable pathology. Further studies are needed to determine optimal protocols to elicit respiratory metaplasticity, so that AIH-induced plasticity can be harnessed as a means of restoring respiratory and non-respiratory motor function in clinical disorders that challenge ventilatory control.

What mechanism underlies pLTF metaplasticity following rAIH? In animals exposed to daily AIH, increased spinal BDNF was observed,[85] suggesting Q pathway enhancement. If the Q pathway is enhanced following rAIH, what mechanism underlies this enhancement? One possibility is decreased cross-talk inhibition. For example, decreased S pathway activation (**Figure 4A**) could increase Q pathway dependent pLTF and BDNF synthesis. Alternatively, decreased inhibitory interactions between pathways would enable direct S pathway contributions to pLTF, even with moderate AIH, since the pathways would be uncoupled (**Figure 4B).** Indeed, greater phospho-Akt levels were observed after 3xwAIH for 10 weeks,[86] consistent with increased S pathway activation. Finally, pLTF metaplasticity may arise directly from a more robust Q pathway (**Figure 4C**); following 10 weeks of 3xwAIH, 5-HT$_{2A}$ receptor, BDNF, TrkB and phospho-ERK expression are all increased,[86] suggesting "hypertrophy" of this cellular cascade. Further studies are needed to determine how rAIH causes pLTF metaplasticity.

Other forms of metaplasticity in the phrenic motor system

This brief review was intended to pose rather than answer questions. It is not expansive enough to cover all potential forms of metaplasticity in respiratory motor pools. However, factors known to induce metaplasticity in other models of plasticity, such as hippocampal long-term potentiation include prior activation[77,87,88] and stress.[90] Hippocampal LTP is impaired by stress or glucocorticoids.[90] In the phrenic motor pool, we postulate that stress has two separate effects.

Initially, stress might induce pMF, due to norepinephrine release from locus coeruleus followed by activation of α_1 receptors on phrenic motor neurons.[10] On the other hand, if pLTF is similar to other models of plasticity, chronic exposure to stressful stimuli may impair pLTF, possibly through a glucocorticoid-dependent mechanism. Anecdotally, rats subject to stressful stimuli often fail to express pLTF; however, further studies are needed to determine the effects of stress on phrenic motor plasticity, and to confirm or refute these speculations.

Conclusion and Significance

Distinct pathways (ie. Q and S pathways) give rise to phenotypically similar phrenic motor facilitation. However, since the Q and S pathways interact via cross-talk inhibition, either pathway can dominate and produce pLTF; at a specific balance point, it is possible that these pathways neutralize one another, with equal and opposing inhibition of the other. Thus, cross-talk inhibition may have the ability to confer key emergent properties of pLTF, such as pattern-sensitivity. Another emergent property, enhanced pLTF (ie. metaplasticity) may arise from diminished inhibitory coupling of these pathways, so that both combine to produce a larger (enhanced) pLTF following AIH. The presence of multiple, competing pathways to pLTF confers flexibility, enabling different manifestations of plasticity as an animal responds to diverse conditions that vary in severity, pattern and/or duration of hypoxia. Greater understanding of multiple pathways giving rise to respiratory motor plasticity and their interactions could increase our understanding of physiological responses to environmental or pathological changes.

As one example, human subjects at high altitude experience chronic sustained hypoxia, giving rise to homeostatic increases in ventilation; this form of respiratory plasticity is often referred to as ventilatory acclimatization to high altitude.[90,91] What role, if any, do the Q and S pathways and their interactions play in this process? Further studies are needed to determine how pathways interact to produce changes in respiratory control appropriate for the prevailing

conditions. As another example, humans most frequently experience intermittent hypoxia during sleep, particularly in individuals suffering from obstructive sleep apnea. In such cases, does the severity and pattern of hypoxic episodes determine the type and extent of compensatory respiratory plasticity? These differences may also be of considerable relevance as we consider distinctions between ventilatory plasticity versus plasticity in respiratory-related motor output to the upper airways, which may stabilize or de-stabilize breathing depending on complex interactions between upper airway patency versus chemoreflex gain and apneic threshold. Further studies are needed to answer these questions.

An understanding of complex interactions between mechanisms of respiratory plasticity has considerable importance as we develop strategies to harness intermittent hypoxia induced motor plasticity to treat clinical disorders that impair breathing and other movements (*eg*. ref 31). While much research has been focused on characterizing new forms of plasticity, we are just beginning to understand the significance of interactions between them. These interactions, if understood and controlled, may enable us to optimize therapeutic protocols of rAIH in the treatment of spinal injury, neurodegenerative diseases and even sleep disordered breathing.[27]

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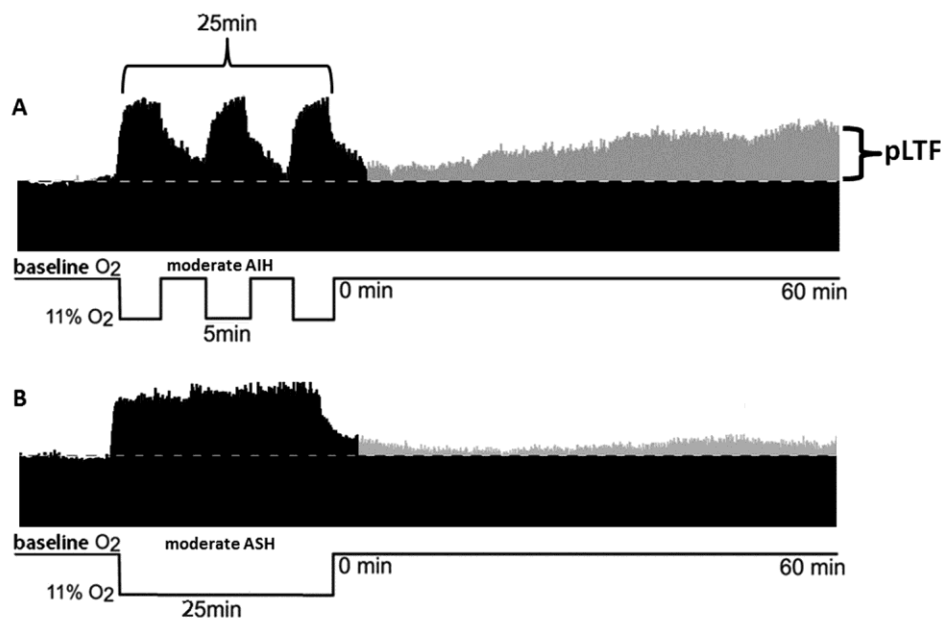
Figure Legends

Figure 1. Pattern sensitivity of phrenic long-term facilitation (pLTF). pLTF is elicited by moderate, acute intermittent hypoxia (AIH), but not by moderate, acute sustained hypoxia (ASH). **A:** Representative tracing of phrenic nerve activity from an anesthestized, vagotomized, paralyzed and pump ventilated rat exposed to moderate AIH. Following AIH, there is a progressive increase in phrenic nerve burst amplitude, indicating pLTF. **B:** Representative tracing from a rat exposed to moderate ASH. Following ASH, there is little increase in phrenic nerve burst amplitude from baseline.
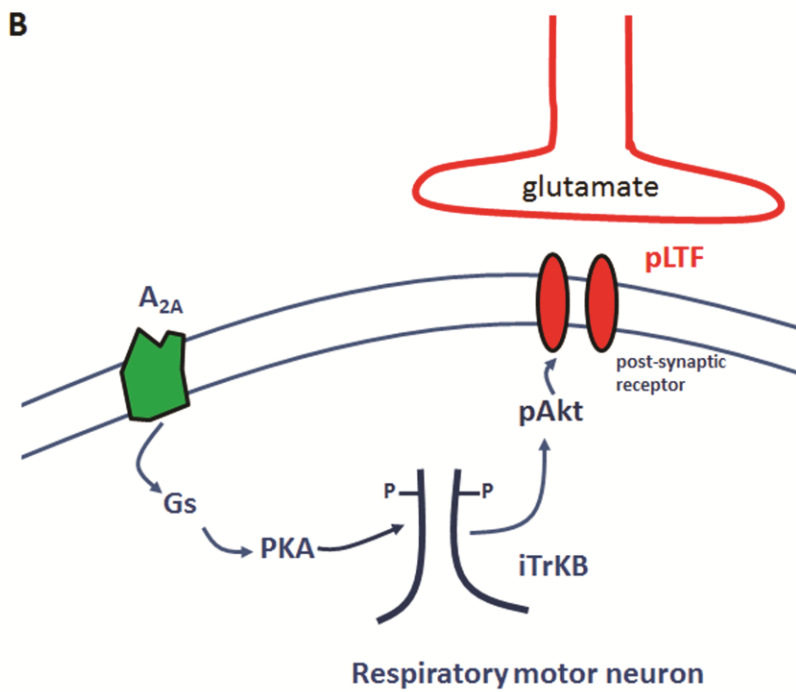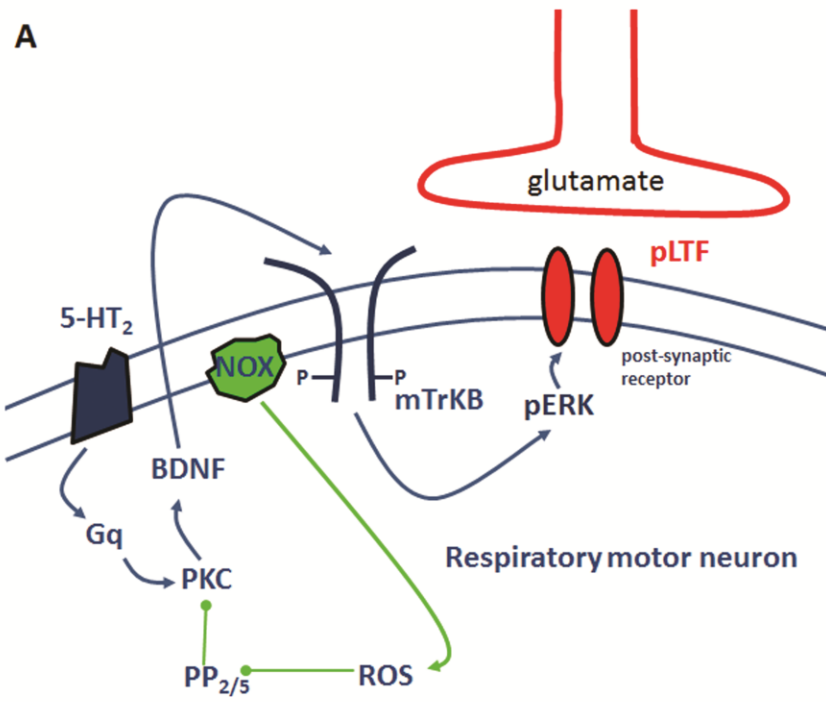
Figure 2. Working models of cellular pathways giving rise to phrenic motor facilitation following AIH. **A:**The "Q pathway" is initiated by activation of the G_q-protein coupled 5-HT_2 receptor, leading to protein kinase C (PKC) activation and new synthesis of brain-derived neurotrophic factor (BDNF). BDNF then activates its high affinity receptor, TrkB. TrkB activation phosphorylates extracellular signal-related kinase (ERK) MAP kinase, which facilitates inputs to phrenic motor neurons by an unknown mechanism (possibly glutamate receptor trafficking). The Q pathway is regulated by protein phosphatases 2A and/or 5, which can inhibit PKC activation. NADPH oxidase-induced ROS formation inhibits these phosphatases, enabling pLTF expression. Thus, NADPH oxidase and PP2A/5 constitute a "regulatory cassette" for pLTF. **B:** The "S pathway" is induced by the G_s-protein coupled adenosine 2A (A_{2A}) receptor, subsequently activating protein kinase A (PKA). PKA stimulates new synthesis of an immature TrkB isoform, which auto-activates and phosphorylates and activates Akt. Subsequent to Akt activation, synaptic inputs to phrenic motor neurons are facilitated by an unknown mechanism.
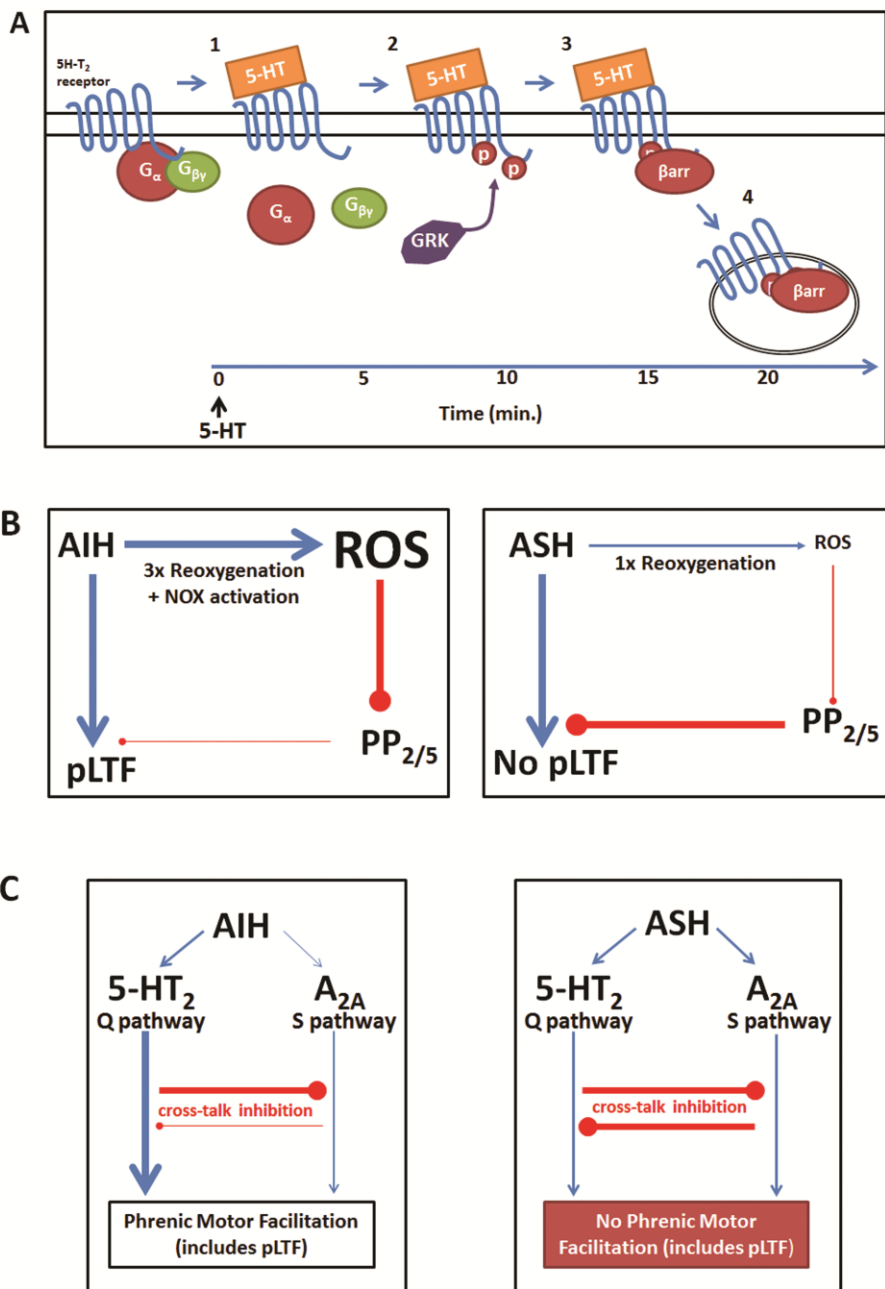
Figure 3. Possible mechanisms giving rise to pLTF pattern sensitivity. **A:** 5-HT$_2$ receptor desensitization might occur with prolonged 5-HT release, such as during ASH: 1) 5-HT binds to its receptor, releasing Gα and G$\beta\gamma$ proteins, which activate downstream second messengers; 2) after prolonged agonist exposure (~10 min), 5-HT$_2$ receptors are phosphorylated by G-protein

coupled receptor kinase (GRK), preventing subsequent activation of G-proteins (ie. functional uncoupling) and promoting β-arrestin (βarr) binding to the 5-HT$_2$ receptor; 3) β-arrestin binding prevents further 5-HT$_2$ receptor activation while promoting receptor internalization; 4) receptors are internalized via a clathrin-dependent mechanism, decreasing the number of receptors available to elicit pLTF. **B:** Differential reactive oxygen species (ROS) formation during AIH *vs*. ASH may underlie pattern sensitivity. During AIH, ROS production during repeated reoxygenation events and NADPH oxidase (NOX) activation. Increased ROS would inhibit protein phosphatases 2 and 5, disinhibiting PKC (or other kinases) and enabling forward progression of pLTF. During ASH, insufficient ROS formation due to a single reoxygenation event may not inhibit the protein phosphatases sufficient to relieve their constraint to pLTF. **C:**Inhibitory interactions between the Q and S pathways to pMF may underlie pattern sensitivity of pLTF. 1) During AIH, the Q pathway is dominant because of 5-HT2 receptor activation with relatively little adenosine accumulation. 2) During ASH, greater adenosine release/accumulation may cause sufficient S pathway activation to offset Q pathway activation. Balanced activation of both pathways may prevent any pLTF expression due to balanced cross-talk inhibition.
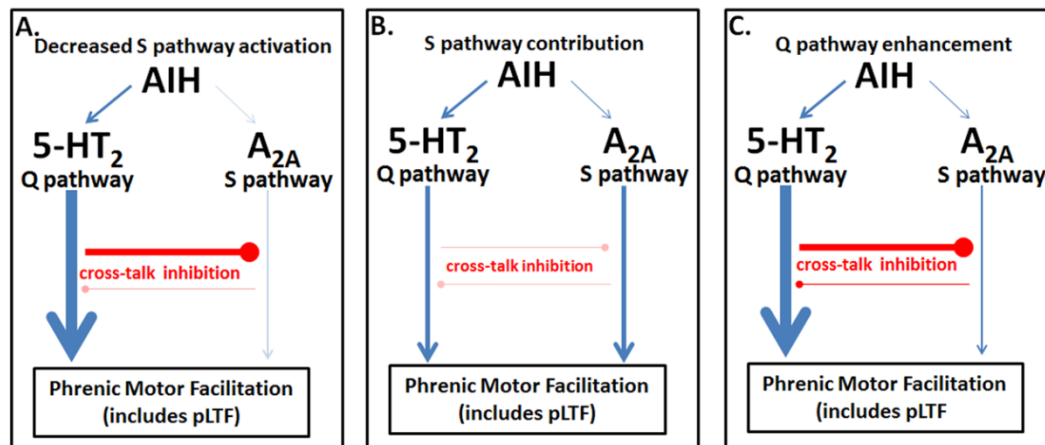
Figure 4. Possible mechanisms of metaplasticity, enhancing pLTF following repettive intermittent hypoxia. **A:** Decreased S pathway activation, thereby eliminating S to Q inhibition, could explain metaplasticity in pLTF following rAIH. **B:** Reduced cross-talk inhibition, enabling a positive S pathway contribution could enhance pLTF. **C:** Increased Q pathway signaling ("hypertrophy"), possibly involving increased 5-HT_2 receptors, ROS production, or BDNF synthesis could explain greater pLTF following rAIH.