Complex signaling mechanisms give rise to adaptive behaviors

in respiratory motor control

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Comparative Biomedical Sciences)

at the University of Wisconsin-Madison

2017

Date of Oral Examination: June 10th, 2016

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VI. Acknowledgements:

“It began as such things always begin - in the ooze of unnoticed swamps, in the darkness of eclipsed moons. It began with a strangled gasping for air.”

- Loren Eiseley

To the Snout.

Abstract

A hallmark feature of the neural system controlling breathing is its ability to adapt through plasticity; a persistent change in system performance following a previous experience. Two well-studied models of respiratory plasticity are inactivity-induced phrenic motor facilitation (iPMF) and hypoxia-induced phrenic long-term facilitation (pLTF). To date, these forms of plasticity have only been studied independently — however, clinically relevant reductions in respiratory neural activity often occur with concomitant hypoxia. While intermittent neural apnea and intermittent hypoxia elicit plasticity in isolation, I demonstrate that concurrent induction of iPMF and pLTF constrains plasticity. Recent advancements have defined distinct signaling pathways for iPMF and pLTF, yet little is known of how intermittent neural apnea with hypoxia limit the capacity for respiratory plasticity; a phenomenon termed “dual stimulus cross-talk inhibition”. The purpose of this thesis was to define mechanisms giving rise to dual stimulus cross-talk inhibition in the control of breathing. Specifically, I describe how complex experiences may give rise to contending signaling cascades that undermine expression of respiratory plasticity. Further, I have identified several approaches for bypassing cross-talk inhibition, thus enabling compensatory respiratory plasticity for enhanced breathing control during central sleep apnea; a disease characterized by recurrent apneas/hypopneas with concomitant hypoxia. Advancing our understanding of how distinct experiences are encoded into interactive signaling networks is an important goal as we translate basic science discoveries into novel therapeutic strategies for incurable neural disorders.

Introduction

A hallmark feature of the neural system controlling breathing is its ability to express plasticity, defined as a persistent change in system function after an experience/stimulus has ended (Mitchell and Johnson, 2003). Respiratory plasticity is characteristic of development, continues throughout life, and is of considerable importance in preserving life when confronted with clinical disorders that compromise the ability to breath (Vinit et al., 2009; Dale-Nagle et al., 2010; Nichols et al., 2013; Dale et al., 2014).

Within this thesis I investigate complex signaling interactions that shape expression of spinal respiratory plasticity. While the existence of inter-pathway communication has only recently become evident, its significance has not been determined. Thus, the primary purpose of this introduction is to demonstrate that downstream intracellular signaling interactions are critical regulators of plasticity expression. I begin by defining respiratory plasticity and related terms, continue by outlining potential sites for respiratory plasticity, and finally conclude by reviewing two well-studied examples of spinal respiratory plasticity; inactivity-induced and hypoxia-induced respiratory plasticity. This introduction will set the foundation for a series of studies demonstrating the importance of cross-talk interactions for the regulation of plasticity.

Through my work I propose an alternative perspective of plasticity signaling; rather than viewing signaling cascades as linear pathways, I propose a network perspective that acknowledges the potential for inter-pathway communication through complex intracellular interactions. I believe that a network perspective which takes into account the existence of signaling divergence, convergence, redundancy and meta-modulation will be important as we progress towards translating basic science discoveries in respiratory motor control into therapies for important clinical disorders.

Definitions: adaptations to respiratory motor control

Modulation, meta-modulation and plasticity are related but distinct features that are frequently confused. Thus, brief definitions of these terms are provided in the context of respiratory motor control (Mitchell and Johnson, 2003)

Modulation is a change in system behavior that fades rapidly (seconds to minutes) after the stimulus is removed. Neuromodulators often work through ligand sensitive ion channels that alter cell membrane excitability properties. Modulation does confer system flexibility and can initiate cellular mechanisms resulting in plasticity (Mitchell and Johnson, 2003), but the two are differentiated by what happens when the stimulating trigger is removed. For example, during a brief (5 to 30 minute) hypoxic experience, phrenic nerve activity (and breathing) increases and returns to normal seconds/minutes after hypoxia has ceased. Alternatively, 3 successive 5-minute hypoxic episodes give rise to a persistent increase in phrenic nerve activity lasting several hours after the final hypoxic episode has ended—an example of acute intermittent hypoxia (AIH; see below). In this example, modulation is the within episode (during hypoxia) augmentation of respiratory motor output, whereas plasticity is the persistence of increased activity that lasts long after the hypoxia stimulus has ended.

Meta-modulation is a reversible change in the capacity or quality of modulation (Katz, 1999; Mitchell and Johnson, 2003), and requires continued presence of the meta-modulation trigger. Meta-modulating stimuli frequently act through G protein coupled receptors (GPCRs), 2nd messengers and/or ion channels to augment the response of neurons to modulators (Katz, 1999; Mesce, 2002; Ribeiro and Sebastiao, 2010), though meta-modulation triggers have not yet been associated with long-term changes in gene expression. One interesting example of meta-modulation in respiratory control is the response of neurons in the nucleus of the solitary tract to concurrent serotonin and substance P application (Jacquin et al., 1989). Both serotonin and substance P alone positively modulate NTS neurons to enhance output of the nuclei. However, in the presence of substance P, serotonin becomes inhibitory (Jacquin et al., 1989), suppressing NTS activity below baseline, tonic levels. Thus, in these in vitro conditions, the

impact of one modulator (serotonin) is augmented by concurrent application of another (substance P).

Plasticity is a persistent change in function that outlasts the initiating stimulus (Mitchell and Johnson, 2003). Plasticity often requires new protein synthesis via translational and/or transcriptional regulation (Manahan-Vaughan et al., 2000; Alberini, 2008; Mitchell and Johnson, 2003), thus making it unique from meta-modulation. A frequent initiating stimulus in many neural systems is neuronal activity or activity-dependent synaptic plasticity (Malinow and Malenka, 2002; Wiegert and Bading, 2011); though not all forms of respiratory plasticity are activity-dependent (Mitchell and Johnson, 2003; Strey et al., 2013). Rather than the system responding directly to changes in activity, activity often serves as a stimulus to promote local release of neuromodulators that initiate necessary signaling cascades giving rise to respiratory plasticity. Thus, activity often serves as a tool for regulating neuromodulator release, but is not necessary for plasticity expression.

Potential Sites of Respiratory Plasticity

Essential processes in control of breathing include respiratory rhythm generation, burst pattern formation (Feldman and Smith, 1989; Mitchell et al., 1990), as well as sensory feedback giving rise to chemoreflexes, mechanoreflexes, neuromodulation and neuroplasticity (Feldman et al., 2003;Mitchell and Johnson, 2003). Respiratory plasticity can occur in any component of the neural system controlling breathing (Mitchell and Johnson, 2003). Of particular relevance to this thesis, plasticity occurs in spinal respiratory motor neurons (and/or interneurons), putting the final “touches” on burst pattern formation before the central nervous system relays its inspiratory command to respiratory muscles. Here, we focus on spinal respiratory motor plasticity, with less consideration given to plasticity in chemoreceptor feedback (Bisgard, 2000; Kumar and Prabhakar, 2012) or brainstem mechanisms of respiratory rhythm generation/pattern formation (Blitz and Ramirez, 2002; Feldman et al., 2003; Feldman et al., 2005); however, we appreciate

that plasticity within central/peripheral chemoreceptors may elicit functional phrenic motor plasticity that is phenotypically similar to local (spinal) motor plasticity at the motor neuron level. **Figure 1** outlines the neural network controlling breathing and identifies potential sites of respiratory neuroplasticity.

Hypoxia-induced phrenic long-term facilitation is a form of spinal respiratory plasticity

The most extensively studied model of spinal respiratory motor plasticity is acute intermittent hypoxia (AIH) induced phrenic long-term facilitation (pLTF), a persistent increase in phrenic nerve output following multiple brief experiences of moderate hypoxia (35-45 mmHg PaO2; Hayashi et al., 1993; Bach and Mitchell, 1996; Mitchell et al., 2001; Feldman et al., 2003; Mahamed and Mitchell, 2007; Dale-Nagle et al., 2010; Devinney et al., 2013). In recent years we have come to use a generic term for long-lasting enhancement of phrenic motor output—phrenic motor facilitation (pMF). Whereas pMF may arise from a variety of triggers (i.e. hypoxia, inactivity, hypercapnia, pharmaceutics), pLTF is a specific form of pMF elicited from AIH (Devinney et al. 2013).

In many instances, spinal signaling pathways that are necessary for pLTF are also independently sufficient to elicit pMF (reviewed by Dale-Nagle et al. 2010). For example, serotonin receptor antagonists applied to the C3-C5 cervical spinal cord during AIH abolishes pLTF; demonstrating that spinal serotonin receptor activation in the immediate vicinity of the phrenic motor nucleus is necessary for pLTF (Fuller et al., 2001; Baker-Herman and Mitchell, 2002). AIH-induced pLTF is also abolished by cervical spinal pre-treatment with siRNAs targeting brain derived neurotrophic factor (BDNF) mRNA (Baker-Herman et al., 2004) as well as inhibitors to protein kinase C Θ (PKC-Θ; Devinney et al., 2015), TrkB receptors (high affinity BDNF receptors; Baker-Herman et al., 2004) and neuronal nitric oxide synthase (nNOS; MacFarlane et al. 2014). Conversely, activation of cervical spinal serotonin type 2A, 2B and 7 receptors (MacFarlane and Mitchell, 2009; MacFarlane et al., 2011; Hoffman and Mitchell,

2011), TrkB receptors (Baker-Herman et al., 2004), and exogenous application of nitric oxide donors (MacFarlane et al. 2014) can all give rise to phenotypically similar pMF in the absence of hypoxia (i.e. mimicking pLTF). Collectively, these experiments reveal important aspects of the signaling pathway giving rise to AIH-induced pLTF, but also identify key spinal signaling checkpoints that are independently sufficient for pMF.

Although type-2A (5-HT2A) and type-7 (5-HT7) spinal serotonin receptors are both sufficient for pMF, they operate through distinct signaling pathways referred respectively as the Q and S pathways to pMF (Dale-Nagle et al. 2010); these pathways are named for the G proteins most often coupled to their intracellular domain of the receptors (i.e. Gq for serotonin type 2A and Gs for serotonin type 7). The Q pathway to pMF consists of: spinal Gq-linked G protein coupled receptors activation (Fuller et al., 2001; Baker-Herman and Mitchell, 2002; MacFarlane et al., 2011), protein kinase C (PKC) activation (Devinney et al., 2015), new synthesis of BDNF (Baker-Herman et al., 2002; Baker-Herman et al., 2004), TrkB receptor activation (Baker-Herman et al., 2004; Dale et al., 2016), and downstream signaling via ERK MAP kinases (Hoffman et al., 2012; Wilkerson and Mitchell, 2009). In contrast, the S pathway to pMF involves: activation of spinal Gs-linked G protein coupled receptors (Hoffman and Mitchell, 2011; Nichols et al., 2012), adenylyl cyclase activation with synthesis of cyclic AMP, new protein synthesis of an immature TrkB isoform (vs BDNF; Golder et al., 2008; Hoffman and Mitchell, 2011) and downstream signaling via Akt (vs ERK; Golder et al., 2008; Hoffman et al., 2012). While Q and S pathways require different signaling molecules, they converge onto spinal NMDA receptors (NMDAR), a necessary signaling molecule for both Q pathway and S pathway driven spinal respiratory plasticity (McGuire et al., 2005; Golder 2008). Molecular convergence suggests that NMDARs are an important effector channel for expression of hypoxia-induced pMF, though this has yet to be confirmed.

Following AIH, serotonin is released onto phrenic motor neurons, leading to dual stimulation of the Q (5-HT2A) and S (5-HT7) pathways for pMF. Dual pathway activation has

revealed unique inter-pathway interactions that we have defined as “cross-talk inhibition” (Hoffman et al., 2010; Dale et al., 2010b; Hoffman and Mitchell, 2013), inhibitory signaling constrains that occur when multiple plasticity signaling pathways are concurrently activated. Our working hypothesis is that manipulations of these cross-talk interactions during intermittent hypoxia training may underlie at least some forms of spinal respiratory metaplasticity (i.e. adaptive changes in plasticity expression). During moderate AIH-induced pLTF, serotonin release activates abundant, high affinity 5-HT2 receptors, driving the dominant Q pathway to pLTF. Although concurrent activation of Gs protein linked 5-HT7 receptors is insufficient to trigger the S pathway at these levels of hypoxia (moderate AIH-induced pLTF is exclusively Q pathway dependent plasticity), sub-threshold activation of these Gs-linked receptors constrains the Q pathway and reduces pLTF magnitude. Inhibition of spinal 5-HT7 receptors (Hoffman and Mitchell, 2013) during moderate AIH eliminates this cross-talk constraint thereby enhancing moderate AIH-induced pLTF. We suspect that all Gs-linked G protein coupled receptors have a capacity to constrain the Q pathway to pMF since cross-talk inhibition is mediated by protein kinase A (PKA), a prominent effector of downstream Gs protein/cAMP signaling. Whereas spinal PKA inhibition relieves cross-talk inhibition and enhances moderate AIH induced pLTF (to a similar extent as A2A and 5-HT7 inhibition), PKA activation suppresses moderate AIH-induced pLTF (Hoffman and Mitchell, 2013). This concept will be explored further in chapter III.

In addition, cross-talk inhibition implies the existence of bidirectional constraint with Q pathway also inhibiting S pathway pMF. Preliminary studies suggest that NADPH oxidase, potent generator of reactive oxygen species, may mediate some of the Q pathway constraint onto the S pathway pMF. Previous literature has shown that reactive oxygen species (ROS) such as hydrogen peroxide can prevent cyclic AMP generation from adenylate cyclase, a necessary step for initiation of S pathway pMF (Fields and Mitchell, 2016). Further, inhibition of NADPH during concurrent Q and S pathway induction reveals an EPAC-dependent pMF (Perim

et al., unpublished). Further work is still needed to confirm these findings and to determine if Q to S pathway inhibition can be circumvented by exogenous EPAC application.

Since Gs-linked G protein coupled receptors are commonly expressed within many CNS cell types, we cannot yet state that cross-talk inhibition is operative exclusively within spinal motor neurons. It remains possible that elements of the Q and S pathways, and their interactions, arise from interneuron, astrocyte and/or microglia. The cellular localization of processes involved in spinal respiratory plasticity is an area that requires and warrants additional research. This topic and the implications for rehabilitative therapies is discussed further within chapter III.

Inactivity-induced phrenic motor facilitation is a form of spinal respiratory plasticity

The neural respiratory control system exhibits a remarkable capacity to produce continuous, rhythmic motor activity. Yet, the neural respiratory control system must be dynamic as it readily adapts to changes in demand during development, aging, everyday activities such as exercise, and importantly, during disease states that compromise neural control of breathing. Thus, two hallmark features of the neural system controlling breathing are stability and flexibility. While seemingly oxymoronic, I propose that within respiratory motor control, stability and flexibility are in fact complementary; with stability ensuring basal function during severe perturbations, and flexibility enabling acute adjustments to meet changing demands (Turrigiano, 2012). Thus, homeostatic plasticity (i.e. the capacity for neurons to regulate their excitability relative to network activity; Chen et al., 2014) may be a critical property of the neural respiratory control network (Bragelmann et al., 2017), ensuring continuous firing activity within a malleable system, while also enabling flexible adjustments to acute changes in demand.

Much like hypoxia-induced plasticity, inactivity-induced plasticity can be expressed within many motor systems, including the phrenic (Mahamed et al., 2010), hypoglossal (XII); Strey et al., 2011) and intercostal motor pools of respiration (Strey et al., 2013), non-respiratory

neuromuscular junctions (Turrigiano, 2008) and non-motor regions such as the hippocampus (Sutton et al., 2006; Aoto et al., 2008; Wang et al., 2011) and visual region of the cortex (Huang et al., 2012). To better understand the role of local (spinal) mechanisms in sensing and responding to reductions in respiratory neural activity, Strey et al. developed a novel model whereby synaptic inputs to one phrenic motor pool were reduced while brainstem respiratory neural activity and contralateral phrenic nerve activity were left intact (Strey and Baker-Herman, 2014). Procaine was micro-injected into the C2 ventrolateral funiculus on one side of the spinal cord to reversibly block axon conduction of descending bulbospinal tracts providing respiratory-related synaptic inputs to ipsilateral phrenic motor neurons. Upon resumption of phrenic activity, ipsilateral phrenic motor output was significantly increased relative to pre-activity deprivation levels, indicating that localized (spinal) activity deprivation elicited a localized iPMF. Importantly, complete “inactivity” was not necessary to induce iPMF, and the magnitude of iPMF was directly proportional to the degree to which phrenic neural activity was reduced (Braegelmann et al., 2017); these data suggest that even moderate reductions in respiratory neural activity elicit enhancements in phrenic inspiratory output that compensate for the degree of activity deprivation. If these results can be extended to other respiratory motor pools, these data suggest that local mechanisms sense and respond to a reduction in synaptic inputs for a compensatory increase in inspiratory motor output. The “sensors” and “responders” for this form of plasticity are currently unknown, but include any combination of motor neurons, glia, or interneurons.

Precise cellular mechanisms enabling inactivity-induced plasticity differ depending on the pattern of respiratory neural inactivity (prolonged vs intermittent; Broytman et al., 2013; Strey et al., 2012; Baertsch and Baker-Herman, 2013; Baertsch and Baker-Herman, in revision). For example, in response to prolonged reductions in respiratory neural activity, tumor necrosis factor alpha (TNF-α) increases activation of an atypical protein kinase C (aPKC) isoform known as protein kinase C-ζ (PKC-ζ). PKC-ζ stabilizes early, transient increases in phrenic burst

amplitude to elicit long-lasting plasticity (Broytman et la. 2013; Strey et al., 2012). NMDA receptor activation during transient iPMF expression prevents TNF-α-induced PKC-ζ activation (Streeter and Baker-Herman, 2014), likely by activating a counter-mechanism that down regulates phrenic burst amplitude and restores baseline phrenic activity (i.e., hyperactivity-induced reversal of iPMF). By contrast, inactivity-induced plasticity following intermittent reductions in respiratory neural activity requires retinoic acid synthesis to activate PKC-ζ and elicit long-lasting iPMF (Baertsch and Baker-Herman, unpublished). Unlike iPMF following prolonged neural apnea, iPMF following intermittent neural apnea does not appear to be under NMDA receptor-mediated constraint. This differential requirement for NMDA receptor activation is not understood, but is investigated in Chapter IV.

Concurrent iPMF and pLTF induction mechanistically constrains plasticity

Much like respiratory plasticity induced by reduced respiratory neural activity, activity deprivation elicits plasticity within other systems that can be categorized into one of two signaling categories; retinoic acid (RA)-dependent and RA-independent (Chen et al. 2014). Whereas suppression of action potential firing alone is sufficient for RA-independent plasticity, RA-dependent plasticity is only expressed when blockade of neuronal firing is accompanied by inhibition of post-synaptic activity (e.g., via inhibition of calcium-permeable glutamate receptors, such as NMDAR; Aoto et al. 2008). Mechanisms whereby NMDAR inhibition (with APV) in combination with suppressed neural activity enables RA-dependent plasticity are not fully understood, but is hypothesized to be due to a release of calcium constrained RA synthesizing enzymes (Wang et al., 2011). While neuronal firing inhibition significantly attenuates NMDAR stimulation to decrease calcium influx, APV inhibits spontaneously evoked NMDAR activation (i.e., “minis”), suppressing activity further. Thus, RA-dependent plasticity requires NMDAR inhibition in addition to neural inactivity (Aoto et al. 2008).

While intermittent iPMF does not require APV pretreatment, it is RA-dependent, suggesting that intermittent neural apnea inherently inhibits NMDARs. How then do intermittent reductions in respiratory neural activity enable RA-dependent plasticity? Though intermittent neural apnea is not intermittent stimulation per se, the periods separating individual inactivity episodes mimic intermittent stimulation and could lead to similar molecular adaptions. Through negative feedback, intermittent stimulation of NMDARs promote their removal from the membrane (Joshi et al. 2007; Thiagarajan et al. 2002), decreasing NMDAR mediated calcium influx, effectively mimicking pharmacological inhibition with APV.

This hypothesis of contending roles for spinal NMDAR potentially explains why concurrent hypoxia abolishes intermittent iPMF, as I will show in chapter IV (**Figure 2**). For example, while intermittent neural apnea may remove relevant NMDARs from the membrane, intermittent hypoxia would counter this through PKC-θ dependent phosphorylation of NR2B subunits. Enhanced NMDAR activity through NR2B phosphorylation maintains calcium influx, preventing RA synthesis to abolish iPMF. This concept will be explored further in chapter IV. Alternatively, IH-induced upregulation of NMDAR may augment the “counter” mechanisms that returns elevated phrenic motor output induced by respiratory neural inactivity back to baseline (Streeter and Baker-Herman, 2014). Clearly, further work is needed to identify if and how regulation of NMDA receptor activity may undermine hypoxia-induced plasticity.

Conclusions

The neural system controlling breathing exhibits a profound capacity to adapt through plasticity. With continuous rhythmic breathing activity being essential for life, enhanced motor output through respiratory plasticity may be an important tool for preserving breathing function within incurable neural disorders. As we begin to translate our findings into novel therapeutic strategies it is important that we appreciate the existence of signaling “networks” for regulating respiratory motor activity; complex inter-pathway interactions that regulate expression of

respiratory motor plasticity. A full understanding of these discrete interactions may reveal unique approaches for treating breathing control disorders.

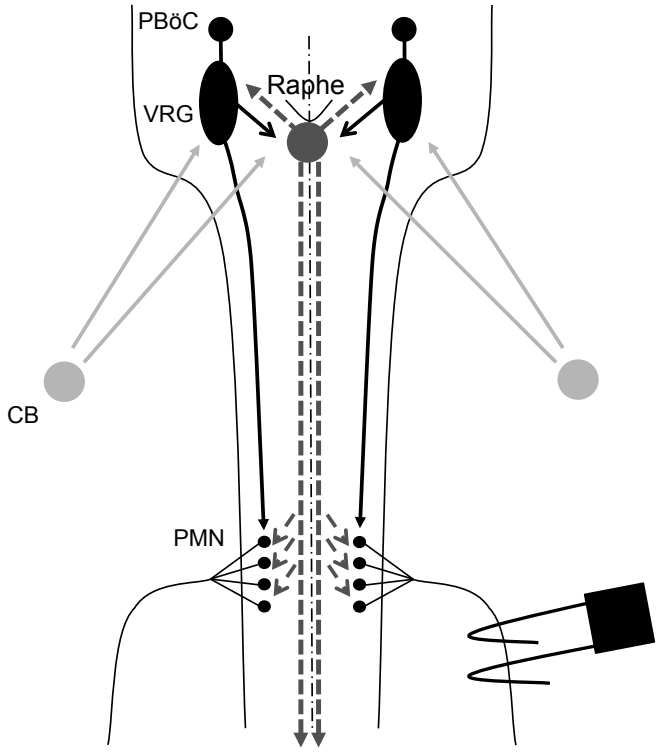

Figure 1: Representation of brainstem and spinal cord regions critical for respiratory motor control. Respiratory rhythm generation requires a small region of the medulla known as the pre-Bötzinger complex (PBöC). The central rhythm is transmitted to brainstem respiratory pre-motor neurons of the ventral respiratory group (VRG). VRG pre-motor neurons subsequently relay respiratory drive projections to different respiratory motor neuron pools, including phrenic motor neurons (PMN). Sensory input to the respiratory system during episodic hypoxia is provided by the carotid body chemoreceptors (CB) in the neck, which project via chemoafferent neurons to the medullary nucleus of the solitary tract (not shown). These second order sensory neurons subsequently project (directly or indirectly) to multiple structures of importance in ventilatory control, including the PBöC, VRG and serotonergic neurons in the medullary raphe (raphe). Raphe serotonergic neurons play a key role in phrenic long-term facilitation following acute intermittent hypoxia and, presumably, in metaplasticity of pLTF.

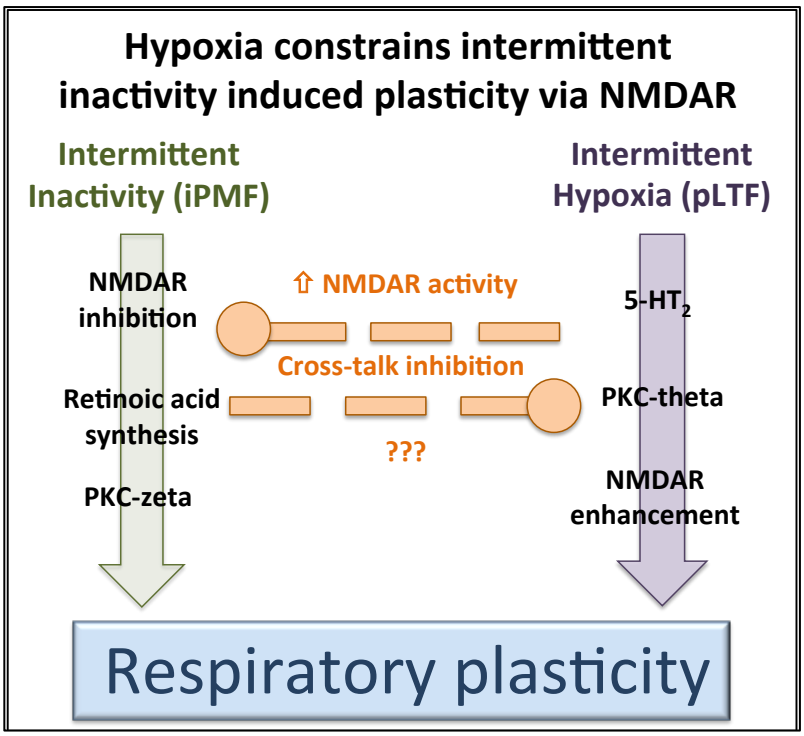

Figure 2: Concurrent hypoxia constrains inactivity-induced plasticity via NMDA receptors. While intermittent reductions in respiratory neural activity limit spinal NMDA receptor (NMDAR) activation to enable local retinoic acid synthesis necessary for iPMF, intermittent hypoxia enhances spinal NMDAR activity for pLTF. Contrasting regulation of spinal NMDARs may constrain expression of respiratory plasticity during concurrent reductions in respiratory neural activity with hypoxia.

Bibliography:

Abbott S.M., and Videnovic A. (2016). Chronic sleep disturbance and neural injury: links to neurodegenerative disease. Nat Sci Sleep 25, 55-61.

Amann M., Blain G.M., Proctor L.T., Sebranek J.J., Pegelow D.F., Dempsey J.A. (2010). Group III and IV muscle afferents contribute to ventilatory and cardiovascular response to rhythmic exercise in humans. J Appl Physiol *109*, 966-976.

Aoto J., Nam C.L., Poon M.M., Ting, P., and Chen L. (2008). Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. Neuron *60*, 308-320.

Bach, K.B., and Mitchell, G.S. (1996). Hypoxia-induced long-term facilitation of respiratory activity is serotonin dependent. Respir Physiol 104, 251-260.

Baertsch N.A., and Baker-Herman T.L. (2013). Inactivity-induced phrenic and hypoglossal motor facilitation are differentially expressed following intermittent vs. sustained neural apnea. J Apply Physiol *114*, 1388-1395.

Baertsch N.A., and Baker-Herman T.L. (2013). Inactivity-induced phrenic and hypoglossal motor facilitation are differentially expressed following intermittent vs. sustained neural apnea. J Appl Physiol *114*, 1388-1395.

Baertsch N.A., and Baker-Herman T.L. (2015). Intermittent reductions in respiratory neural activity elicit spinal TNF-α-independent, atypical PKC-dependent inactivity-induced phrenic motor facilitation. Am J Physiol Regul Integr Comp Physiol *308*, R700-R707.

Baker-Herman T.L., and Mitchell G.S. (2002). Phrenic long-term facilitation requires spinal serotonin receptor activation and protein synthesis. J Neurosci *22*, 6239-6246.

Broytman O., Baertsch N.A., and Baker-Herman, T.L. (2013). Spinal TNFa is necessary for inactivity phrenic motor facilitation. J. Physiol *591*, 5585-98.

Butler J.E., Hudson A.L., and Gandevia S.C. (2014). The neural control of human inspiratory muscles. Prog Brain Res *209*, 295-308.

Chen L., Lau A.G., and Sarti F. (2014). Synaptic retinoic acid signaling and homeostatic synaptic plasticity. Neuropharm *78*, 3-12.

Chowdhuri S., Quan S.F., Almeida F., Ayappa I., Batool-Anwar S., Budhiraja R., Cruse P.E., Drager L.F., Griss B., Marshall N., Patel S.R., Patil S., Knight S.L., Rowley J.A., and Slyman A. (2016). An Official American Thoracic Society Research Statement: Impact of Mild Obstructive Sleep Apnea in Adults. Am J Respir Crit Care Med *193*, e37-54.

Dale, EA, Mabrouk F., and Mitchell G.S. (2014). Unexpected benefits of intermittent hypoxia: enhanced respiratory and nonrespiratory motor function. Physiology (Bethesda) *29*, 39-48.

Devinney M.J., Huxtable A.G., Nichols N.L., and Mitchell G.S. (2013). Hypoxia-induced phrenic long-term facilitation: emergent properties. Ann. N Y Acad. Sci. *1279*, 143-153.

Fields D.P., and Mitchell G.S. (2015). Spinal metaplasticity in respiratory motor control. Front Neural Circuits *11*, 2.

Fields D.P., and Mitchell G.S. (2016). Divergent cAMP signaling differentially regulates serotonin-induced spinal motor plasticity. *J Neuropharm 113,* 82-88.

Fuller D.D., Bach K.B., Baker T.L., Kinkead R., and Mitchell G.S. (2000). Long-term facilitation of phrenic motor output. Respir Physiol *121*, 135-146.

Garcia A.J., Zanella S., Dashevskiy T., Khan S.A., Khuu M.A., Prabhakar N.R., and Ramirez J.M. (2016). Chronic Intermittent Hypoxia Alters Local Respiratory Circuit Function at the Level of the preBötzinger Complex. Front Neurosci 10, 4.

Golder F.J. (2008). Spinal NMDA receptor activation is necessary for de novo, but not the maintenance of, A2a receptor-mediated phrenic motor facilitation. J Appl Physiol *107*, 217-223.

Guimarães L., Domínguez-del-Toro E., Chatonnet F., Wrobel L., Pujades C., Monteiro L.S., and Champagnat J. (2007). Exposure to retinoic acid at the onset of hindbrain segmentation induces episodic breathing in mice. Eur J Neurosci *25*, 3526-3536.

Heinzer R., Vat S., Marques-Vidal P., Marti-Soler H., Andries D., Tobback N., Mooser V., Preisig M., Malhotra A., Waeber G., Vollenweider P., Tafti M., Haba-Rubio J. (2015). Prevalence of sleep-disordered breathing in the general population: the HypnoLaus study. The Lancet Respir Med *3*, 310-318.

Hoffman M., and Schulman D.A. (2012). The appearance of central sleep apnea after treatment of obstructive sleep apnea. Chest *142*, 517–522.

Joshi, I., Yang, Y.M., and Wang, L.Y. (2007). Coincident activation of metabotropic glutamate receptors and NMDA receptors (NMDARs) downregulates perisynaptic / extrasynaptic NMDARs and enhances high-fidelity neurotransmission at the developing calyx of Held synapse. J Neurosci *27*, 9989-99.

Leung R.S. (2009). Sleep-disordered breathing: autonomic mechanisms and arrhythmias. Prog Cardiovasc Dis *51*, 324-338.

Mahamed S., and Mitchell G.S. (2008). Simulated apneas induce serotonin-dependent respiratory long-term facilitation in rats. J Physiol *586*, 2171-2181.

Mahamed S., Strey K.A., Mitchell G.S., and Baker-Herman T.L. (2011). Reduced respiratory neural activity elicits phrenic motor facilitation. Respir Physiol Neurobiol 175, 303-309.

McGuire M., Liu C., Cao Y., and Ling L. (2008). Formation and maintenance of ventilatory long-term facilitation require NMDA but not non-NMDA receptors in awake rats. J Appl Physiol *105*, 942-950.

McGuire M., Zhang Y., White D.P. and Ling L. (2005). Phrenic long-term facilitation requires NMDA receptors in the phrenic motonucleus in rats. J Physiol *567*, 599-611.

McKay L.C., and Feldman J.L. (2008). Unilateral ablation of pre-Botzinger complex disrupts breathing during sleep but not wakefulness. Am J Respir Crit Care Med *178*, 89-95.

Mehra R., and Redline S. (2014). Arrhythmia risk associated with sleep disordered breathing in chronic heart failure. Curr Heart Fail Rep *11*, 88-97.

Rice A., Fuglevand A.J., Laine C.M., and Fregosi R.F. (2011). Synchronization of presynaptic input to motor units of tongue, inspiratory intercostal, and diaphragm muscles. J Neurophysiol *105*, 2330-2336.

Streeter K.A., and Baker-Herman T.L. (2014). Spinal NMDA receptor activation constrains inactivity-induced phrenic motor facilitation in Charles River Sprague-Dawley rats. J Appl Physiol *117*, 682-693.

Strey K.A., Baertsch N.A., and Baker-Herman T.L. (2013). Inactivity-induced respiratory plasticity: protecting the drive to breathe in disorders that reduce respiratory neural activity. Respir Phyiol Neurobiol *189*, 384-394.

Strey K.A., Nichols N.L., Baertsch N.A., Broytman O., and Baker-Herman T.L. (2012). Spinal atypical protein kinase C activity is necessary to stabilize inactivity-induced phrenic motor facilitation. J Neurosci *32*, 16510-20.

Thiagarajan, T.C., Piedras-Renteria, E.S., and Tsien, R.W., (2002). Alpha and beta CaMKII: inverse regulation by neuronal activity and opposing effects on synaptic strength. Neuron *36*, 1103-14.

Torvaldsson S., Grote L., Peker Y., Basun H., and Hedner J. (2005). A randomized placebo-controlled trial of an NMDA receptor antagonist in sleep-disordered breathing. J Sleep Res *14*, 149-155.

Valic M., Pecotic R., Pavlinac I., Valic Z., Stipica I., and Dogas Z. (2016). Intermittent hypercapnia-induced phrenic long-term depression is revealed after serotonin receptor blockade with methysergide in anaesthetized rats. Exp Phyiol *101*, 319-331.

Wang H.L., Zhang Z., Hintze M., and Chen L. (2011). Decrease in calcium concentration triggers neuronal retinoic acid synthesis during homeostatic synaptic plasticity. J Neurosci *31*, 17764-17771.

Westhoff M., Arzt M., and Litterst P. (2012). Prevalence and treatment of central sleep apnoea emerging after initiation of continuous positive airway pressure in patients with obstructive sleep apnoea without evidence of heart failure. Sleep Breath *16*, 71-78.

Wheatley J.R., Mezzanotte W.S., Tangel D.J., and White D.P. (1993). Influence of sleep on genioglossus muscle activation by negative pressure in normal men. Am Rev Respir Dis *148*, 597-605.

Wilkinson V., Malhotra A., Nicholas C.L., Worsnop C., Jordan A.S., Butler J.E., Saboisky J.P., Gandevia S.C., White D.P., and Trinder J. (2008). Discharge patterns of human genioglossus motor units during sleep onset. Sleep *31*, 525-533.

Xu F., Plummer M.R., Len G.W., Nakazawa T., Yamamoto T., Black I.B., and Wu K. (2006). Brain-derived neurotrophic factor rapidly increases NMDA receptor channel activity through Fyn-mediated phosphorylation. Brain Res *1121*, 22-34.

Young T., Palta, M., Dempsey, J., Skatrud, J., Weber, S., and Badr, S. (1993). The occurrence of sleep-disordered breathing among middle-aged adults. N Eng J Med *328*, 1230-1235.

Spinal 5-HT7 receptors induce phrenic motor facilitation via EPAC-mTORC1 signaling

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Acknowledgements: Funded by NIH grants HL80209 and HL69064. DPF was supported by an Advanced Opportunity Fellowship at the University of Wisconsin and the Medical Scientist Training Program (NIH T32 GM008692).

Key words: motor neuron, phrenic, spinal cord, respiratory plasticity, neuroplasticity, 5-HT7, receptor, exchange protein activated by cAMP, protein kinase A, rapamycin, mTOR

Fields, D.P., Springborn, S.R., and Mitchell G.S. (2015). 5HT7 receptor induced phrenic motor facilitation requires EPAC and mTORC1, but not PKA activity. J Neurophys 114, 2015-2022.

Abstract:

Spinal serotonin type-7 (5-HT7) receptors elicit complex effects on motor activity. Whereas 5-HT7 receptor activation gives rise to long-lasting phrenic motor facilitation (pMF), it also constrains 5-HT2 receptor-induced pMF via "cross-talk inhibition." We hypothesized that divergent cAMP-dependent signaling pathways give rise to these distinct 5-HT7 receptor actions. Specifically, we hypothesized that protein kinase A (PKA) mediates cross-talk inhibition of 5-HT2 receptor-induced pMF whereas 5-HT7 receptor-induced pMF results from exchange protein activated by cAMP (EPAC) signaling. Anesthetized, paralyzed and ventilated rats receiving intrathecal (C4) 5-HT7 receptor agonist (AS-19) injections expressed pMF for > 90 minutes, an effect abolished by pretreatment with a selective EPAC inhibitor (ESI-05) but not a selective PKA inhibitor (KT-5720). Further, intrathecal injections of a selective EPAC activator (8-pCPT-2'-Me-cAMP) were sufficient to elicit pMF. Finally, spinal mTORC1 inhibition via intrathecal rapamycin abolished 5-HT7 receptor- and EPAC-induced pMF, demonstrating that spinal 5-HT7 receptors elicit pMF by an EPAC-mTORC1 signaling pathway. Thus, 5-HT7 receptors elicit and constrain spinal phrenic motor plasticity via distinct signaling mechanisms that diverge at cAMP (EPAC vs. PKA). Selective manipulation of these molecules may enable refined regulation of serotonin-dependent spinal motor plasticity for therapeutic advantage.

Introduction

Serotonin plays a key role in important forms of sensory-motor plasticity, including sensitization of the gill withdrawal reflex in *Aplysia* (reviewed in Kandel, 2012). For example, episodic serotonin presentations enhance sensory motor synaptic transmission giving rise to the gill withdrawal reflex (Brunelli et al., 1976). This well studied form of plasticity in an invertebrate model system relies on multiple serotonin receptor subtypes, each activating unique kinase signaling cascades (Barbas et al., 2003).

In ways similar to sensory motor facilitation in *Aplysia*, episodic serotonin receptor activation is necessary and sufficient for important forms of spinal respiratory motor plasticity, such as long-lasting (> 90min) phrenic motor facilitation (pMF) following acute intermittent hypoxia (AIH; reviewed by Mahamed and Mitchell, 2007; Dale-Nagle et al. 2010; Devinney et al., 2013) or direct injections of serotonin or serotonin receptor agonists into the cervical spinal cord of rats (MacFarlane et al., 2009, 2011; Hoffman et al., 2011). Indeed, multiple serotonin receptor subtypes elicit pMF via mechanistically distinct signaling cascades named for the G proteins most often coupled with the initiating metabotropic serotonin receptor (Dale-Nagle et al., 2010). Specifically, Gs protein-coupled 5-HT7 receptors give rise to pMF through the “S pathway” (Dale-Nagle et al., 2010; Hoffman and Mitchell, 2011), whereas Gq protein-coupled 5-HT2 receptors induce the “Q pathway” to pMF (Dale-Nagle et al., 2010; MacFarlane et al., 2011).

While the S and Q pathways elicit pMF through distinct signaling cascades, they interact via mutual, cross-talk inhibition (Dale-Nagle et al., 2010; Hoffman and Mitchell, 2013; Devinney et al., 2013). One manifestation of cross-talk inhibition is the bell-shaped dose-response curve of pMF in response to intermittent, intrathecal serotonin injections (Macfarlane and Mitchell, 2009). Although low serotonin doses elicit pMF by activating spinal 5-HT2 receptors (Fuller et al., 2001; MacFarlane et al., 2009; 2011), high doses do not unless spinal 5-HT7 receptors are inhibited. Thus, when activated alone, 5-HT2 (MacFarlane et al., 2011) and 5-HT7 receptors (Hoffman and Mitchell, 2011) each give rise to distinct forms of pMF. When activated concurrently, they

effectively cancel each other due to balanced cross-talk inhibition (MacFarlene and Mitchell, 2009; Hoffman and Mitchell, 2013; Devinney et al., 2013). Cross-talk inhibition from 5-HT7 receptors to Q pathway induced pMF is mediated by protein kinase A (PKA) activity (Hoffman and Mitchell, 2013); however, it is not known how 5-HT7 receptor activation initiates pMF when acting alone (Hoffman and Mitchell, 2011).

An alternative, but only recently appreciated cAMP dependent signaling cascade operating through exchange protein activated by cAMP (EPAC; de Rooij et al., 1998; Kawasaki et al., 1998) contributes to at least some forms of cAMP dependent synaptic plasticity (Ster et al. 2007; Woolfrey et al., 2009; Fernades et al. 2015). A key downstream molecule differentiating the PKA vs EPAC signaling pathway is protein kinase B or Akt, a molecule necessary for 5-HT7 induced pMF (Hoffman et al. 2011). Whereas PKA inhibits neuronal Akt activity, EPAC stabilizes Akt's association with scaffolding proteins to enhance its kinase function (Nijholt et al. 2008). Thus, we hypothesized that EPAC (not PKA) is both necessary and sufficient for 5-HT7 receptor-induced pMF, whereas PKA mediates cross-talk inhibition of the Q pathway to pMF. Further, Akt may elicit plasticity by increasing mammalian target of rapamycin complex-1 (mTORC1) activity, a major regulator of new protein synthesis (Ma and Blenis, 2009). Thus, we tested the hypotheses that spinal 5-HT7 receptors give rise to pMF through an EPAC-mTORC1 signaling pathway.

We found that spinal EPAC activation is necessary and sufficient for 5-HT7 receptor-induced pMF, and that these effects require mTORC1 signaling. We confirm that PKA is not necessary for 5-HT7 receptor-induced pMF, and conclude that divergent cAMP signaling pathways underlie the distinct functions of 5-HT7 receptors as they elicit (EPAC) and constrain (PKA) spinal serotonin-dependent respiratory motor plasticity.

Methods

Experiments were performed on anesthetized 300-400g male Lewis rats (Colony 202c, Harlan,

Indianapolis, IN, USA). The Animal Care and Use Committee at the University of Wisconsin, Madison approved all procedures.

Immunohistochemistry

Although PKA constrains 5-HT2 receptor-dependent pMF through cross-talk inhibition (Hoffman and Mitchell, 2013), several lines of evidence suggest that EPAC (not PKA) mediates 5-HT7 receptor-induced pMF. However, no study has attempted to localize these proteins in spinal respiratory neurons in general, or phrenic motor neurons (PMNs) in specific. Thus, we investigated 5-HT7 receptor, PKA and EPAC protein expression within PMNs.

To identify PMNs, 4 untreated rats received intrapleural injections of the retrograde tracer cholera toxin B fragment (CtB, List BioLogicals, Campbell, CA, USA; Mantilla et al. 2009). Briefly, each rat was anesthetized with isoflurane (1–1.5% in 100% O_2), and a 50µL Hamilton syringe with a sterile, slightly beveled 27G, needle was used to inject 12.5µL of 0.2% CtB in sterile saline. Bilateral injections were made at the 5th intercostal space (25µL of 0.2% CtB total). After injections rats were carefully monitored for signs of respiratory distress associated with potential pneumothorax as isoflurane was discontinued (15min). Seven days post-injection rats were again anesthetized with isoflurane, euthanized and transcardially perfused with 0.1M phosphate buffered saline (pH 7.4), followed by 4.0% PFA (freshly made in 0.1M phosphate buffer, pH 7.4). The cervical spinal cord was removed and placed in 4.0% PFA overnight at 4 °C, then transferred to 20% and 30% sucrose in PBS buffer until sinking. Serial coronal sections (40µm) of cervical C3-C5 were prepared using a microtome (Leica SM2000R) before storing sections in a cryoprotectant solution (30% ethylene glycol, 30% glycerol in PBS 0.1M).

Free-floating sections were washed with PBS 0.1M and incubated in a blocking solution (2% normal donkey serum, 0.1% triton in PBS) for 60min. Afterwards, tissues were co-stained for CtB:EPAC, CtB:PKA or CtB:5-HT7 using the following antibodies: Goat anti-CtB (1:5000,

Calbiochem), rabbit anti-EPAC (1:100, Santa Cruz), rabbit anti-PKA (1:100 Abcam) and rabbit anti 5-HT7 (1:100, Abcam). Unbound primary antibodies were removed with several PBS (0.1M, pH 7.4) washes prior to incubation with secondary antibodies (Alexa 594 anti-goat 1:1000 for CtB; Alexa 488 anti-rabbit 1:500 for EPAC and 5-HT7; Alexa 488 anti-rabbit 1-1000 for PKA, Invitrogen) for 2 hours at room temperature. Afterwards, sections were washed in PBS and mounted on charged slides using an anti-fade solution (Prolong Gold antifade reagent, Invitrogen, OR, USA). Control staining with each primary antibody (without secondary antibodies) and all secondary antibodies (without primary antibodies) ruled out non-specific, off-target staining (supportive Figure 1).

Slides were examined with a confocal microscope (Eclipse TE 2000-U, Nikon, Japan) using EZ-C1 software (Nikon, Japan). Z stacks were taken (26μm thick, 2μm steps) with 100x magnification objective. Images were rendered and finished using EZ-C1 software.

Experimental preparation

Rat anesthesia was induced with isoflurane (3.5%) before placing on a heated stainless steel surgical table. The inspired oxygen concentration was continuously monitored (TED 60T, Teledyne Analytical Instruments, USA) with adjustments made as necessary by changing nitrogen and/or oxygen flow rates. A tail vein catheter (24gauge, Surflo, Elkton, MD, USA) was inserted, and an infusion pump (Cole–Palmer, Vernon Hills, IL, USA) was used to deliver intravenous fluids throughout the experiment; the solution used was 9:1 Lactated Ringers (Baxter, IL, USA): sodium bicarbonate solution (8.4% Hospira Inc., IL, USA; 1.5-2mL/h). Rats were tracheotomized and bilaterally vagotomized through a ventral midline incision. A polyethylene catheter (PE50, ID/OD-0.58mm/0.965mm; Intramedic MD, USA) was inserted into the femoral artery to monitor blood pressure (Gould Pressure Transducer, P23, USA) and allow for arterial blood sampling (ABL 500, Radiometer, Copenhagen). Dorsal lamenectomy and durotomy (C1/C2) were performed to enable intrathecal drug delivery. Muscles overlying the

cervical spinal cord were separated to expose the C1-C2 cervical vertebrae; after C2 laminectomy, a silicone catheter (OD 0.6mm; Access Technologies, IL, USA; primed with drug/vehicle) was inserted through a small hole in the dura and advanced caudally (3mm) resting just over spinal regions C3-C4. This catheter was not placed in position until the end of surgical preparations to minimize unintended drug diffusion from the catheter. The left phrenic and hypoglossal (XII) nerves were dissected via a dorsal approach, de-sheathed and covered with saline soaked cotton to prevent desiccation. The rat was then slowly converted to urethane anesthesia (1.8g/kg) while simultaneously weaned from isoflurane (over 15-20min). During anesthetic conversion, a rectal thermometer (Fisher Scientific, USA) was inserted and body temperature was maintained at 37.5 ± 1.0°C throughout the experiment by adjusting temperature of the surgical table. After conversion to urethane anesthesia, the rat was allowed at least 1h for stabilization before starting experimental protocols.

During the stabilization period the intrathecal catheter was inserted while both nerves were placed on bipolar silver recording electrodes and submerged in mineral oil. Once adequacy of anesthesia was confirmed (absent blood pressure spike in response to toe pinch) and respiratory nerve activity was detected, the rat was paralyzed with pancuronium bromide (3mg/kg; Sicor Pharmaceuticals, CA, USA); 20min was allowed for further stabilization. Nerve activity was amplified (gain, 10,000; A-M Systems, Everett, WA, USA), bandpass-filtered (100Hz to 10kHz), and integrated (CWE 821 filter; Paynter, Ardmore, PA, USA; time constant, 50 ms). The signal was digitized and recorded using a WINDAQ data acquisition system (DATAQ Instruments, Akron, OH, USA). The resulting signal was analyzed using custom-designed software on a Lab-View platform.

Throughout surgery and experimental protocols rats were ventilated with 60% inspired oxygen. End-tidal CO_2 was monitored using a flow through capnoguard with sufficient response time to detect end-tidal CO_2 levels in a rat (Novametrix, Wallingford, CT, USA). Rats were

ventilated at a frequency of 70breaths/min and a tidal volume of 2.5ml or less (rodent ventilator model 683; Harvard Apparatus, South Natick, MA, USA). This ventilation level caused hypocapnia (reduced end-tidal CO_2); thus, inspired CO_2 was increased until the desired end-tidal value was attained. The CO_2 apneic threshold was determined by progressively lowering inspired CO_2 until respiratory nerve activity ceased; the recruitment threshold was subsequently determined by progressively raising inspired CO_2 until nerve activity resumed. To establish baseline levels, end-tidal CO_2 was set 2–3mmHg above the CO_2 recruitment threshold as done previously (Bach and Mitchell, 1996). $PaCO_2$ was maintained within 1mmHg of baseline values throughout the experiment by manipulating inspired CO_2 and monitoring arterial blood gases. Base excess values more negative than -3.0mEq/L were corrected with I.V. sodium bicarbonate (8.4% Hospira Inc., IL, USA) prior to baseline measurements. Blood was sampled (0.3mL using a heparinized capillary) once baseline nerve recordings had stabilized, and then again at 30, 60 and 90min post-injection of intrathecal drugs (see below). Measurements of nerve activity (burst amplitude and frequency) were monitored continuously and evaluated immediately prior to blood samples in 1min bins. At the end of experiments rats were euthanized by urethane overdose.

Physiological variables for respective groups are summarized within Table 1. While several groups showed a significant (*) increase in firing frequency, this was mild relative to phrenic nerve amplitude changes. Because of the inconsistency and faintness of this difference we did not pursue this further.

Drugs and Vehicles

The following drugs were obtained from Santa Cruz (Dallas, TX, USA): AS-19 (5-HT7 receptor agonist), 8-pCPT-2'-Me-cAMP (8-pCPT; EPAC selective activator; EPACa) and KT-5720 (PKA selective inhibitor; PKAi). Rapamycin (mTORC1 inhibitor) was obtained from Thermo-Fisher

(Waltham, MA, USA), while ESI-05 (EPAC selective inhibitor; EPACi) was obtained from BioLog Life Science Institute (Germany). All drugs were initially dissolved in dimethylsulfoxide (DMSO) before diluting with vehicle (20% DMSO in sterile saline) before use. Aliquots of stock solutions remained viable for up to one week if stored frozen (-20°C) in 100% DMSO; after this time unused drug solutions were discarded. Prior studies confirm that EPACa is a selective EPAC activator (Christensen et al. 2003; Poppe et al. 2008); conversely, EPACi, PKAi and rapamycin are regarded as selective inhibitors of EPAC (Tsalkova et al. 2012; Rehman 2013), PKA (Davies et al., 2000) and mTORC1 (Davies et al. 2000), respectively.

Experimental protocols

Following stabilization of nerve signals a baseline blood sample was drawn followed by a control intrathecal injection of vehicle (12µl), a 15min gap, and three consecutive intrathecal injections (C4) of 5-HT7 receptor agonist (3 x 5µl; 100µM; 5min intervals) or a single injection of EPACa (10µL; 100µM). The 5-HT7 receptor agonist dose was determined from a previous study using the same experimental protocol (Hoffman and Mitchell, 2011), while a limited dose-response curve was completed for EPACa (data not shown).

Whereas the 5-HT7 receptor agonist gave rise to pMF when injected intermittently (not as a single bolus), intrathecal EPACa gave rise to pMF when given as a single bolus (not intermittently). This requirement for intermittent 5-HT7 receptor activation is consistent with previous studies demonstrating pattern sensitivity of both serotonin-induced and serotonin dependent pMF (Baker and Mitchell, 2000; MacFarlane and Mitchell, 2009). In contrast, single (vs intermittent) injection requirements for 8-pCPT (EPACa) are consistent with previous studies of EPAC-induced plasticity (Ster et al. 2007).

To identify molecules necessary for 5-HT7 receptor- and EPAC-induced pMF, additional groups received intrathecal injections of selective inhibitors prior to the 5-HT7 receptor agonist or EPAC activator. All inhibitors were given intrathecally via a second catheter (over a period of

2min) 15min prior to 5-HT7 receptor agonist or EPACa injections. To determine if PKA is necessary for 5-HT7 receptor-induced pMF, KT-5720, a PKA selective inhibitor (PKAi; N = 6; 12μL, 100μM) was given prior to 5-HT7 receptor agonist injections. We previously demonstrated that KT-5720 at this dose prevents PKA mediated constraint of 5-HT2A receptor dependent, AIH-induced pMF (Hoffman and Mitchell, 2013). In addition, this dose prevents PKA, but not EPAC, dependent signaling within cell cultures (Davies et al. 2000). Using an EPAC selective inhibitor (EPACi; 12μL, 2mM), ESI-05, we tested if EPAC is necessary for 5-HT7 receptor (N = 7) and EPACa-induced (N = 6) pMF. Finally, by pretreating with the highly selective inhibitor rapamycin (12μL, 100μM) we determined if mTORC1 was necessary for 5-HT7 receptor (N = 6) or EPACa-induced (N = 6) pMF.

Additional control groups were completed for vehicle (N = 6), PKAi (N = 5), EPACi (N = 5) and rapamycin (N = 4) in which the drug was given followed by 3 x 5μL injections of vehicle 15 minutes later. None of the control groups effected phrenic nerve activity and they were not significantly different from each other; thus these groups were assembled into a single control group (N = 20; $p \geq 0.26$).

Statistical analyses

Peak integrated amplitude and frequency of phrenic and XII nerves were averaged in 1 min bins at baseline (pre-injection), and then 30, 60 and 90min following intrathecal injections. Amplitude values were expressed as a % change from baseline, while frequency was expressed as change from baseline in bursts/min. Statistical comparisons were made for control and experimental groups with a two-way repeated measures ANOVA; a Tukey *post hoc* test was used to identify pair-wise differences. Significance was accepted as $p < 0.05$. All values are expressed as means ± 1 SEM.

Results

Phrenic motor neuron (PMN) expression of EPAC, PKA and 5-HT7 receptors

PMNs were identified via intrapleural CtB injections. Antibodies targeting EPAC2, PKA and 5-HT7 receptors all revealed immunofluoresence within/near CtB identified PMNs. EPAC2 immunoreactivity was cytoplasmic with the densest staining in the peri-nuclear region (Figure 1: A-C). PKA immunoreactivity was observed within the cytoplasm of CtB co-labeled PMNs (Figure 1: D-F). Immunofluorescence for 5-HT7 receptors was localized to the neuropil with relatively limited cytoplasmic or nuclear staining (Figure 1: G-I). Thus, PMNs can directly respond to 5-HT7 receptor agonists via EPAC and/or PKA signaling, though we cannot rule out additional contributions to pMF from surrounding cells; glia, presynaptic neurons or interneurons.

EPAC, but not PKA, is necessary for 5-HT7 induced pMF

Intermittent intrathecal injections of 5-HT7 receptor agonist (3 x 5μL, 100μM), AS-19, elicited long lasting pMF; expressed as a progressive enhancement in phrenic nerve amplitude (>90min: 88.8 ± 7.2%; Figure 2: A and Bii). The dose/selectivity of this same 5-HT7 receptor agonist was determined within a previous study using the same experimental protocol (Hoffman and Mitchell 2011).

Pretreatment with intrathecal EPAC selective inhibitor (EPACi; 10μL, 2mM), ESI-05, abolished 5-HT7 receptor induced pMF (90min: 15.1 ± 10.5%, $p = 0.0002$; Figure 2: A and Biii) demonstrating EPAC as necessary for 5-HT7 receptor induced pMF. In contrast, pretreating with a PKA selective inhibitor (PKAi; 10μL, 100μM), KT-5720, did not significantly reduce pMF relative to the vehicle pretreated 5-HT7 receptor agonist group (81.8 ± 10.0%, $p = 0.61$; Figure 2: A and Biv). The KT-5720 dose is based on previous work using the same in-vivo protocol (Hoffman and Mitchell, 2013) and PKA cell culture activity assays (Davies et al. 2000). Cervical spinal injections of the 5-HT7 receptor agonist did not affect hypoglossal nerve (XII) activity ($N = 4$; 90min: -0.1 ± 13.1%; $p = 0.95$), suggesting 5-HT7 receptor agonist induced pMF requires

activation of spinal (vs braintem) receptors (Baker-Herman and Mitchell, 2002; MacFarlane and Mitchell, 2009)

Spinal EPAC activation elicits pMF

Intrathecal injection of an EPAC selective activator (EPACa; 10μL; 100μM), 8-pCPT, elicited robust pMF lasting at least 90min (104.4 ± 7.8%, p = 0.0000040; Figure 3, A and Bii). Pretreatment with intrathecal EPAC inhibitor (EPACi; 10μL; 2mM), ESI-05, significantly attenuated EPACa induced pMF (90min: 24.5 ± 7.8%, p = 0.00064; Figure 3, A and Biii), confirming the selectivity of EPACa. Although cervical spinal 5-HT7 receptor agonist injections did not cause XII facilitation, similar EPACa injections did elicit significant XII burst amplitude facilitation (data not shown) at 10μM (N = 2; 90min: 59.5 ± 8.2%) and 100μM (N = 4; 90min: 77.2 ± 23.1%); this effect that was blocked by intrathecal EPACi pretreatment (N = 3; 90min: 7.9 ± 11.6%; p = 0.00064). These differential 5-HT7 versus EPAC effects on XII activity are difficult to explain and we did not pursue this issue further in the present study.

mTORC1 is necessary for 5-HT7 and EPAC induced pMF

Pretreatment with the mTORC1 inhibitor rapamycin (10μL; 100μM) blocked both 5-HT7 receptor (11.2 ± 9.2%, p = 0.00007) and EPACa (9.9 ± 10.0%, p = 0.000039) induced pMF (Figure 4, A and B), demonstrating that mTORC1 is necessary for both processes.

Discussion

Although cervical spinal 5-HT7 receptor activation elicits pMF, it also constrains 5-HT2 receptor-induced pMF via cross-talk inhibition (MacFarlane and Mitchell, 2009; Hoffman and Mitchell, 2013). Here we show that these diverse actions result from divergent cAMP signaling. Specifically, 5-HT7 receptors constrain 5-HT2 receptor-induced pMF through cAMP/PKA signaling (Hoffman et al., 2013), but elicit pMF via a mechanistically distinct cAMP/EPAC

pathway. Since 5-HT7 receptor- and EPAC-induced pMF are rapamycin sensitive, this pathway requires mTORC1 signaling. We conclude that serotonin elicits spinal plasticity via diverse mechanisms. First, serotonin elicits phrenic motor plasticity via unique mechanisms associated with distinct serotonin receptor subtypes (e.g. 5-HT7 versus 5-HT2 receptors). Second, 5-HT7 receptors elicit heterogeneous effects (presumably) within the same cell via divergent cAMP signaling (PKA and EPAC). Understanding distinct serotonin and cAMP functions may be critical as we attempt to harness serotonin-dependent plasticity as a treatment for severe neuro-motor disorders that compromise breathing, such as spinal injury and amyotrophic lateral sclerosis (Mitchell, 2007; Dale et al., 2014).

While EPAC activation is necessary and sufficient for some forms of cAMP dependent plasticity, selective PKA activation (EPAC independent) also gives rise to plasticity in some model systems (Castellucci et al., 1980). Thus, EPAC and PKA may give rise to alternate, parallel signaling cascades for cAMP-dependent plasticity. Though PKA dependent plasticity has been well-studied, considerably less is known concerning how EPAC activation elicits plasticity. Here we demonstrate that EPAC dependent, 5-HT7 receptor-induced pMF is mechanistically distinct from the serotonin-induced PKA-ERK-BDNF signaling pathway described in invertebrate model systems (for review, see Kandel, 2012). Based on available evidence concerning 5-HT7 receptor-induced pMF, 5-HT7 receptors elicit pMF via EPAC-Akt-mTORC1 signaling, resulting in new synthesis of an immature TrkB isoform (Hoffman and Mitchell, 2011; Golder et al., 2008). This mechanism is independent of PKA, ERK signaling or new BDNF synthesis (Hoffman and Mitchell, 2011).

We are not aware of any pervious studies connecting EPAC or mTORC1 signaling with serotonin-induced plasticity. By showing that the canonical pathway of PKA-ERK-BDNF does not play a role in 5-HT7 receptor induced spinal respiratory motor plasticity we have uncovered an interesting question: what purpose do parallel plasticity pathways operating downstream from a single receptor serve? We suggest that mechanistic heterogeneity in spinal motor

plasticity signaling is important to maintain appropriate network activity throughout maturation, changing environmental conditions and during severe disease states (Dale et al., 2014). Although PKA signaling does not contribute to 5-HT7 induced pMF, it may serve as a “reserve” pathway when EPAC dependent signaling is impaired. For example, while wild-type mice express EPAC- dependent (PKA-independent) mossy fiber plasticity, EPAC2 knockout mice retain certain forms of mossy fiber plasticity by shifting to PKA dependent signaling (Fernandes et al. 2015). Thus, in some conditions PKA activity is sufficient to induce plasticity during compromised EPAC signaling.

Finally, since PKA does not normally contribute to 5-HT7 receptor-induced pMF our findings suggest that reduced PKA signaling may prevent cross-talk inhibition without compromising 5-HT7 receptor-induced pMF. By minimizing cross-talk inhibition with PKA selective inhibitors it may be possible for both 5-HT2 and 5-HT7 receptors to independently contribute to an enhanced form of pMF (i.e. metaplasticity; Fields and Mitchell, 2015). In agreement, whereas PKA mediates cross-talk inhibition (Hoffman and Mitchell, 2013), EPAC enables concurrent activation of signaling pathways operating downstream from 5-HT2 and 5-HT7 receptors (Johnson-Farley et al. 2005). The respective contributions of reduced PKA and/or enhanced EPAC activity to enhanced AIH-induced pMF following intermittent hypoxia preconditioning (Fields and Mitchell, 2015) remain to be explored.

Although we are only beginning to understand inter-pathway interactions in serotonin receptor-induced spinal motor plasticity, the ability of non-essential accessory signaling pathways to regulate plasticity has considerable significance, both from a biological and a therapeutic perspective.

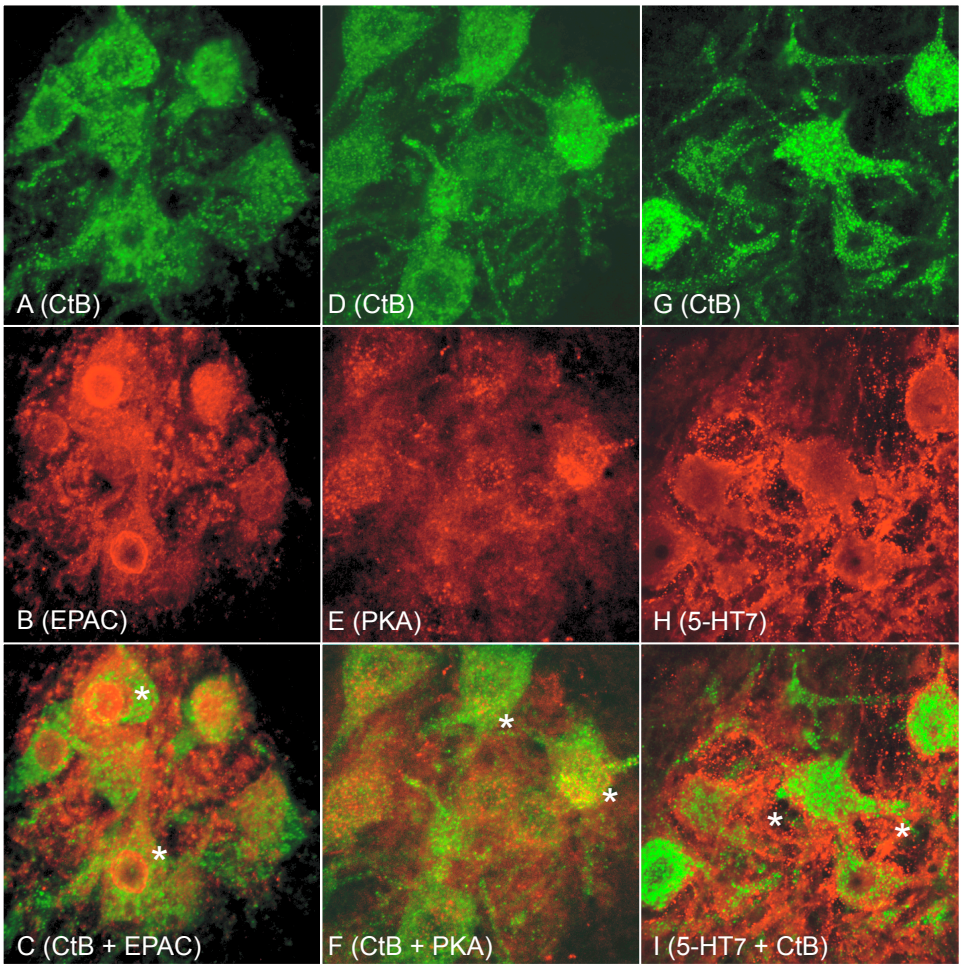

Figure 1: Identified phrenic motor neurons (PMNs) express EPAC, PKA and 5-HT7 receptors. Immunofluorescence images (100x) from three rats (left to right) for EPAC (A-C), PKA (D-F) and 5-HT7 receptor (G-I) protein (red), with retrograde cholera toxin B fragment (CtB; green). **A-C:** EPAC + CtB staining of PMNs reveal peri-nucluear EPAC distribution (*). **D-F:** PKA + CtB staining of PMNs exhibits diffuse cytoplasmic PKA distribution (*). **G-I:** 5-HT7 receptor + CtB staining show abundant neuropil (*), but limited cytoplasmic/cell-body 5-HT7 receptor distribution. CtB staining was consistent in all groups, with prominent cytoplasmic staining and limited immunofluorescence in the nucleus or neuropil.

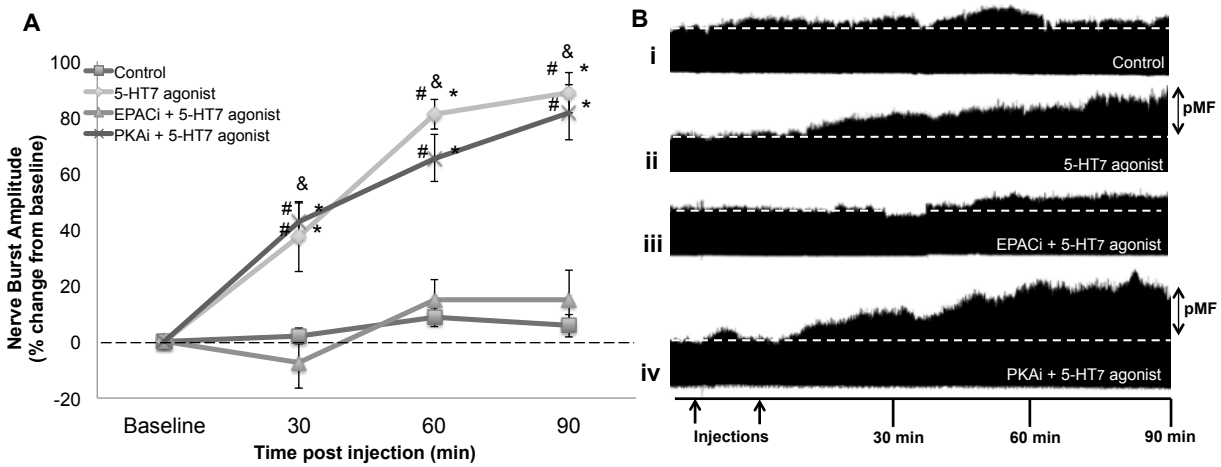

Figure 2: 5-HT7 receptor-induced pMF requires EPAC, but not PKA activity. **A:** Average change in phrenic burst amplitude from baseline to 90min post injections. Lines are from rats with intrathecal injections of the 5-HT7 receptor agonist (AS-19; 3 x 5μL, 100μM) with saline (◆; N = 7; 10μL), EPACi (▲; N = 7; 10μL, 2mM), or PKAi (**X**; N = 5; 10μL, 100μM) pretreatment, or control rats that did not receive the 5-HT7 receptor agonist (■; N = 20; 10μL). Data represent mean values ± 1 SEM. Significant differences from baseline (**#**), control (*), or PKAi + 5-HT7 agonist (**&**); all $p \leq 0.05$. **B:** On the right, representative traces of phrenic neurograms are shown before, during and after intrathecal injections of vehicle + 5-HT7 receptor agonist, EPACi + 5-HT7 receptors agonist or PKAi + 5-HT7 receptor agonist. The first arrow (below bottom trace) represents the pretreatment injection; the second arrow represents the first of three 5-HT7 receptor agonist injections. Data from control and 5-HT7 agonist groups are repeated within multiple figures.

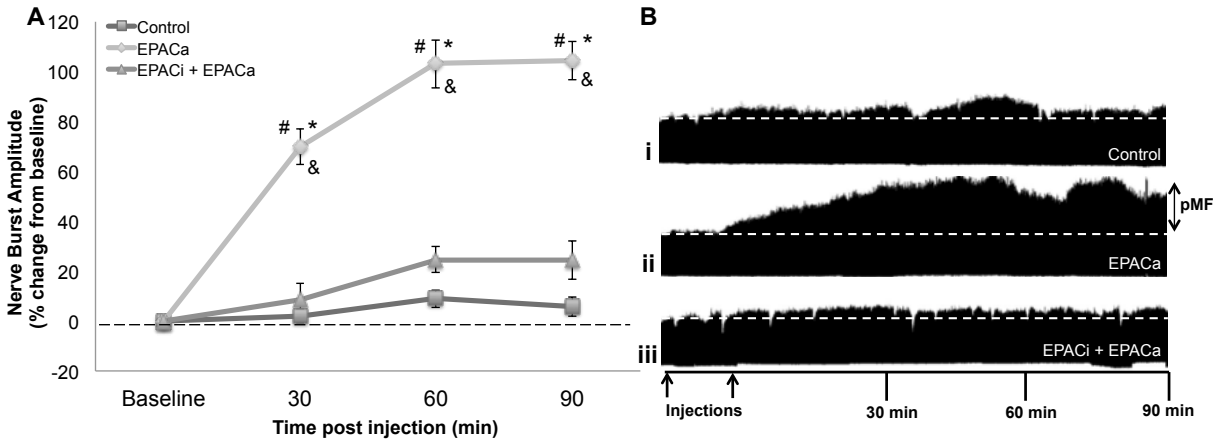

Figure 3: Spinal EPAC activation is sufficient to elicit pMF. **A:** Average change in phrenic burst amplitude from baseline. Individual curves represent EPACa treated rats (10μL, 100μM) with saline (◆; N = 7; 10μL), with EPACi pretreatment (▲; N = 6; 10μL, 2mM), or control injections alone (■; N = 20; 10 μL). Data represent mean values ± 1 SEM. Significant differences from baseline (**#**), control (*) or EPACi + 5-HT7 receptor agonist (**&**); all $p \leq 0.05$. **B:** Representative phrenic neurograms are on the right; time control, vehicle + EPACa or EPACi + EPACa. First arrow represents pretreatment injection; second arrow represents single EPACa injection. Data from control and EPACa groups are shown within multiple figures.

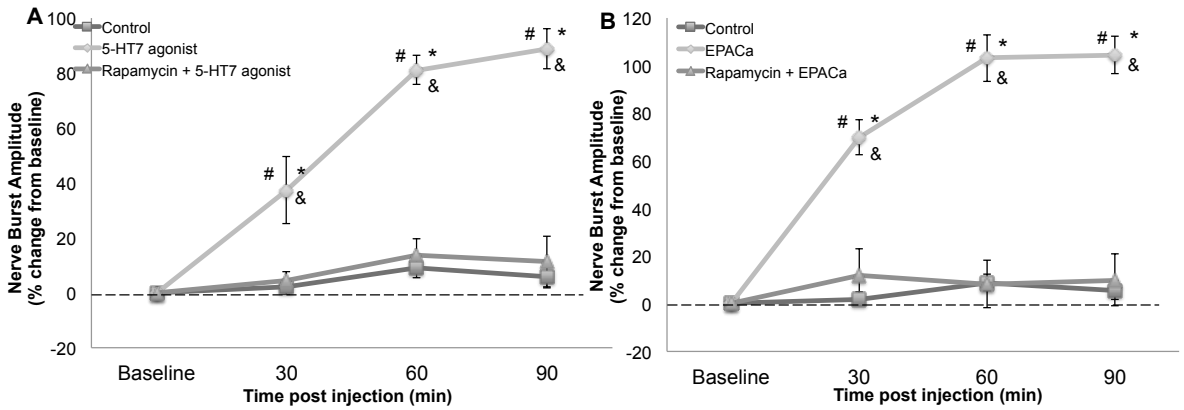

Figure 4: mTORC1 activity is necessary for 5-HT7 receptor and EPAC induced pMF. **A:** Average change in phrenic burst amplitude from baseline for 5-HT7 receptor agonist (10μL, 100μM) with saline (◆; N = 7; 10μL) or rapamycin (▲; N = 6; 10μL, 100μM) pretreatment, or control injection alone (■; N = 20; 10 μL). Data represent mean values ± 1 SEM. Significant differences from baseline: (**#**), controls (*****), and groups with rapamycin pretreatment (**&**); all $p \leq 0.05$. Data from control, 5-HT7 agonist and EPACa groups are shown in multiple figures.

	Time (min)	Control	5-HT7 agonist	EPACi + 5-HT7 agonist	Rapamycin + 5-HT7 agonist	PKAi + 5-HT7 agonist	EPACa	EPACi + EPACa	Rapamycin + EPACa
PaCO2 (mmHg)	Baseline	49.8 ± 1.5	47.2 ± 1.5	52.3 ± 2.0	52.6 ± 0.9	50.5 ± 1.7	52.7 ± 0.9	55.5 ± 0.6	52.6 ± 0.9
	30	48.7 ± 2.4	48.2 ± 1.7	52.5 ± 1.9	52.2 ± 1.2	50.6 ± 1.7	53.4 ± 1.1	55.5 ± 0.9	52.2 ± 1.2
	60	50.7 ± 1.5	47.5 ± 1.6	54.0 ± 2.1	52.9 ± 1.5	50.5 ± 1.5	53.0 ±0.8	55.4 ± 0.7	52.9 ± 1.5
	90	50.6 ± 1.9	47.0 ± 1.5	51.9 ± 1.9	52.5 ± 1.4	50.7 ± 1.8	52.8 ± 1.0	55.7 ± 0.8	52.5 ± 1.4
PaO2 (mmHg)	Baseline	310 ± 27	301 ± 11	306 ± 17	341 ± 25	310 ± 8	322 ± 12	369 ± 18	309 ± 23
	30	291 ± 29	303 ± 18	297 ± 22	337 ± 26	296 ± 6.0	302 ± 11	347 ± 22	299 ± 16
	60	289 ± 26	289 ± 9.9	295 ± 25	359 ± 26	291 ± 7.5	304 ± 14	344 ± 25	302 ± 15
	90	291 ± 29	283 ± 11	289 ± 30	364 ± 27	305 ± 15	288 ± 7.7	344 ± 27	305 ± 15
MAP (mmHg)	Baseline	50.7 ± 3.0	49.8 ± 4.6	73.4 ± 14	58.5 ± 3.6	54.1 ± 4.8	44.5 ± 2.6	49.3 ± 3.3	39.8 ± 3.5
	30	50.0 ± 3.4	48.5 ± 3.8	69.5 ± 12	47.9 ± 3.9	50.5 ± 4.8	40.4 ± 2.3	41.1 ± 2.9	31.7 ± 3.4
	60	42.5 ± 3.4	44.6 ± 3.6	67.4 ± 11	45.9 ± 7.3	45.3 ± 3.0	37.5 ± 2.5	41.1 ± 2.5	28.5 ± 3.0
	90	41.9 ± 2.5	44.9 ± 2.4	66.0 ± 12	43.7 ± 6.5	42.3 2.8	31.1 ± 3.4	40.9 ± 3.8	24.5 ± 3.4
Phrenic Burst Frequency	Baseline	55.8 ± 8.4	44.8 ± 1.39	49.3 ± 0.5	47.9 ± 1.1	50.0 ± 0.8	44.4 ± 0.8	41.0 ± 0.9	45.1 ± 1.1
	30	54.9 ± 8.5	47.4 ± 1.3	48.3 ± 1.8	48.3 ± 1.1	49.4 ± 0.9	47.1 ± 1.0	41.9 ± 1.2	44.5 ± 1.5
	60	55.6 ± 9.5	48.6 ± 1.4 *	48.8 ± 1.4	49.3 ± 2.1	51.0 ± 1.1	47.5 ± 1.1 *	43.3 ± 0.8	43.4 ± 1.5
	90	56.3 ± 10.2	48.8 ± 1.5 *	47.1 ± 0.5	50.0 ± 1.8	49.6 ± 1.9	46.3 ± 1.2	44.6 ± 0.8 *	43.0 ± 1.5

Table 1: Physiological variables at baseline, 30, 60 and 90 min post final injection. All values are expressed as means ± 1 SEM. * denotes significantly different from baseline within same group, $p \leq 0.05$.

References:

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

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

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

Barbas D, DesGroseillers L, Castellucci VF, Carew TJ, Marinesco S. Multiple Serotonergic Mechanisms Contributing to Sensitization in Aplysia: Evidence of Diverse Serotonin Receptor Subtypes. Learn and Mem 10: 373-386, 2003.

Brunelli M, Castellucci V, Kandel ER. Synaptic facilitation and behavioral sensitization in Aplysia: Possible role of serotonin and cyclic AMP. *Science* **194**:1178-1181, 1976

Christensen AE, Selheim F, de Rooij J, Dremier S, Schwede F, Dao KK, Martinez A, Maenhaut C, Bos JL, Genieser HG, Døskeland SO. cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. *J Biol Chem* **278**: 35394-35402, 2003.

Castellucci VF, Kandel ER, Schwartz JH, Wilson FD, Nairn AC, Greengard P. Intracellular injection of the catalytic subunit of cyclic AMP-dependent protein kinase simulates facilitation of transmitter release underlying behavioral sensitization in Aplysia. Proc Natl Acad Sci USA **77**:7492–7496, 1980

Dale EA, Ben Mabrouk F, Mitchell GS. Unexpected benefits of intermittent hypoxia: enhanced respiratory and nonrespiratory motor function. *J Physiol* **29**: 39–48 2014.

Dale-Nagle EA, Hoffman MS, MacFarlane PM, Mitchell GS. Multiple pathways to long-lasting phrenic motor facilitation. *Adv Exp Med Biol* **669**: 225–230, 2010.

Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351 Pt1**: 95-105, 2000.

de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* **396**: 474-477, 1998.

Devinney MJ, Huxtable AG, Nichols NL, Mitchell GS. Hypoxia-induced phrenic long-term facilitation: emergent properties. *Ann NY Acad Sci* **1279**: 143–153, 2013.

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

Fields DP, Mitchell GS. Spinal Metaplasticity in Respiratory Motor Control. *J Neural Circuits* **9**: 2, 2015

Fuller DD, Zabka AG, Baker TL, Mitchell GS. Phrenic long-term facilitation requires 5-HT receptor activation during but not following episodic hypoxia. J App Physiol **90**: 2001-2006, 2001

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

Hoeffer CA, Klann E. mTOR signaling: at the crossroads of plasticity, memory and disease. *Trends Neurosci* **33**: 67-75, 2010.

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

Hoffman MS, Mitchell GS. Spinal 5-HT7 receptors and protein kinase A constrain intermittent hypoxia-induced phrenic long-term facilitation. *Neuroscience* **250**: 632–643, 2013.

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

Johnson-Farley NN, Kertesy SB, Dubyak GR, Cowen DS. Enhanced activation of Akt and extracellular-regulated kinase pathways by simultaneous occupancy of Gq-coupled 5-HT2A receptors and Gs-coupled 5-HT7A receptors in PC12 cells. *J Neurochem* **92**: 72-82, 2005.

Kandel ER. The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. *Mol Brain* **5**: 14, 2012.

Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM. A family of cAMP-binding proteins that directly activate Rap1. *Science* **282**: 2275-2279, 1998.

Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* **10**: 307-318, 2009.

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

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

Mahamed S, Mitchell GS. Is there a link between intermittent hypoxia-induced respiratory plasticity and obstructive sleep apnoea? *Exp Physiol* **92**: 27-37, 2007.

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

Mironov SL, Skorova EY. Stimulation of bursting in pre-Bötzinger neurons by Epac through calcium release and modulation of TRPM4 and K-ATP channels. *J Neurochem* **117**: 295-308, 2011.

Mironov SL, Skorova EY, Kügler S. Epac-mediated cAMP-signalling in the mouse model of Rett Syndrome. *Neuropharm* **60**: 869-877, 2011.

Mitchell GS. Respiratory plasticity following intermittent hypoxia: a guide for novel therapeutic approaches to ventilatory control disorders. *In: Genetic Basis for Respiratory Control Disorders (Gaultier C, ed)*, 2007.

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

Murray AJ, Shewan DA. Epac mediates cyclic AMP-dependent axon growth, guidance and regeneration. *Mol Cell Neurosci* **38**: 578-588, 2008.

Murray AJ, Tucker SJ, Shewan DA. cAMP-dependent axon guidance is distinctly regulated by Epac and protein kinase A. *J Neurosci* **29**: 15434-15444, 2009.

Navarrette-Opaza A, Mitchell GS. Therapeutic potential of intermittent hypoxia: a matter of dose. Am J Physiol Regul Integr Comp Physiol **307**: R1181-R1197, 2014

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

Nijholt IM, Dolga AM, Ostroveanu A, Luiten PG, Schmidt M, Eisel UL. Neuronal AKAP150 coordinates PKA and Epac-mediated PKB/Akt phosphorylation. *Cell Signal* **20**: 1715-1724, 2008.

Poppe H, Rybalkin SD, Rehmann H, Hinds TR, Tang XB, Christensen AE, Schwede F, Genieser HG, Bos JL, Doskeland SO, Beavo JA, Butt E. Cyclic nucleotide analogs as probes of signaling pathways. *Nat Methods* **5**: 277-278, 2008.

Rehmann H. Epac-inhibitors: facts and artifacts. *Sci Rep* **3**: 3032, 2013.

Srivastava DP, Jones KA, Woolfrey KM, Burgdorf J, Russell TA, Kalmbach A, Lee H, Yang C, Bradberry MM, Wokosin D, Moskal JR, Casanova MF, Waters J, Penzes P. Social, communication, and cortical structural impairments in Epac2-deficient mice. *J Neurosci* **32**: 11864-11878, 2012.

Ster J, De Bock F, Guérineau NC, Janossy A, Barrère-Lemaire S, Bos JL, Bockaert J, Fagni L. Exchange protein activated by cAMP (Epac) mediates cAMP activation of p38 MAPK and modulation of Ca2+-dependent K+ channels in cerebellar neurons. *Proc Natl Acad Sci USA* **104**: 2519-2524, 2007.

Tsalkova T, Mei FC, Li S, Chepurny OG, Leech CA, Liu T, Holz GG, Woods VL Jr, Cheng X. Isoform-specific antagonists of exchange proteins directly activated by cAMP. *Proc Natl Acad Sci USA* **109**: 18613-18618, 2012.

Woolfrey KM, Srivastava DP, Photowala H, Yamashita M, Barbolina MV, Cahill ME, Xie Z, Jones KA, Quilliam LA, Prakriya M, Penzes P. Epac2 induces synapse remodeling and depression and its disease-associated forms alter spines. *Nat Neurosci* **12**: 1275-1284, 2009.

Yang Y, Shu X, Liu D, Shang Y, Wu Y, Pei L, Xu X, Tian Q, Zhang J, Qian K, Wang YX, Petralia RS, Tu W, Zhu LQ, Wang JZ, Lu Y. EPAC null mutation impairs learning and social interactions via aberrant regulation of miR-124 and Zif268 translation. *Neuron* **73**: 774-788, 2012.

Divergent cAMP signaling differentially regulates serotonin-induced spinal motor plasticity

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Acknowledgements: We thank Drs. BJ Dougherty and AG Huxtable for helpful critiques of this manuscript. Funded by NIH grants RO1 HL80209 and RO1 HL69064. DPF supported by Advanced Opportunity Fellowship at the University of Wisconsin, a NIH NRSA (F30 HL126351) and the University of Wisconsin Medical Scientist Training Program (NIH T32 GM008692).

Key words: motor neuron, phrenic, spinal cord, respiratory plasticity, 5-HT7 receptor, 5-HT2 receptor, exchange protein activated by cAMP, protein kinase A, EPAC, PKA.

Fields, D.P.,* and Mitchell, G.S. 2016. Divergent cAMP signaling differentially regulates serotonin-induced spinal motor plasticity. J Neuropharm (in press).

Abstract

Spinal metabotropic serotonin receptors encode transient experiences into long-lasting changes in motor behavior (i.e. motor plasticity). While cross-talk interactions between serotonin receptor subtypes are known to regulate plasticity, the significance of divergence in downstream intracellular signaling is not well understood. Here we tested the hypothesis that distinct cAMP dependent signaling pathways differentially regulate serotonin-induced phrenic motor facilitation (pMF). Specifically, we studied the capacity of cAMP-dependent protein kinase A (PKA) and exchange protein activated by cAMP (EPAC) to regulate 5-HT2A receptor-induced pMF within mature male rats. Although spinal PKA, EPAC and 5-HT2A each elicit pMF when activated alone, concurrent PKA and 5-HT2A activation interact via mutual cross-talk inhibition thereby blocking pMF expression. Conversely, concurrent EPAC and 5-HT2A activation enhance pMF expression reflecting additive contributions from both mechanisms. Thus, we demonstrate that distinct downstream cAMP signaling pathways enable differential regulation of 5-HT2A-induced spinal motor plasticity. Conditional activation of divergent intracellular signaling mechanisms may explain experience amendable changes in plasticity expression (i.e. metaplasticity), an emerging concept thought to enable flexible motor control within the adult central nervous system.

Significance Statement: Through distinct downstream signaling mechanisms, cAMP constrains and additively enhances plasticity elicited by Gq protein coupled serotonin receptors. These findings provide unique insight concerning the significance of intracellular signaling divergence for regulating plasticity.

Introduction

Serotonin elicits long-lasting motor plasticity via G protein-coupled receptors (GPCRs; Brunelli et al., 1976; Randić et al., 1993; Clark and Kandel, 1993), with different receptor subtypes giving rise to plasticity via independent signaling pathways (reviewed in Barbas et al., 2003).

Concurrent activation of multiple serotonin receptor subtypes reveals inhibitory inter-receptor cross-talk interactions thereby regulating serotonin-induced plasticity (Seol et al., 2007; Treviño et al., 2012; Hoffman et al., 2013; MacFarlane et al., 2014). Although intracellular signaling pathways downstream from individual GPCRs are known to diverge, it is not known how intracellular signaling divergence differentially impacts serotonin-induced plasticity.

Serotonin-induced motor plasticity is a major feature of the neural system controlling breathing (Mitchell and Johnson, 2003; Feldman et al., 2003). For example, serotonin elicits plasticity in respiratory defense reflexes of gastropod mollusks (Glanzman et al., 1989; Macket et al., 1989; Levy and Susswein, 1993), and enhances spinal respiratory motor control in mammals (Bach and Mitchell, 1996; Baker-Herman and Mitchell, 2002). In rats, selective activation of spinal Gq-coupled serotonin 2A receptors (5-HT2A; MacFarlane et al., 2011) or Gs-coupled serotonin 7 receptors (5-HT7; Hoffman and Mitchell, 2011) elicits long-lasting phrenic motor facilitation (pMF). When multiple spinal serotonin receptors are stimulated with non-specific serotonin, pMF expression exhibits a bell-shaped dose response curve; low serotonin doses elicit pMF through Gq associated 5-HT2 receptors, but high serotonin doses elicit pMF only when spinal Gs associated 5-HT7 receptors are blocked (Macfarlane and Mitchell, 2009). Thus, there is a complex interplay between Gq and Gs-coupled serotonin receptors regulating the expression of serotonin-induced pMF. Whereas each elicits pMF when activated alone (MacFarlane et al., 2011; Hoffman an Mitchell, 2011), poorly understood mechanisms of cross-talk inhibition prevent concurrent contributions from Gq and Gs serotonin receptors (MacFarlane and Mitchell, 2009; Hoffman et al., 2013).

Due to differences in cAMP binding affinity (Dostmann and Taylor, 1991; Ponsioen et al., 2004) and sub-cellular distribution (Seino and Shibasaki, 2005), cAMP can independently activate cAMP-dependent protein kinase A (PKA) versus exchange protein activated by cAMP (EPAC), thus enabling distinct functional outcomes from Gs-coupled receptor signaling. For example, netrin-1 receptors differentially activate PKA and EPAC to dynamically regulate spinal

axonal growth (Murray et al., 2009). While netrin-1 induced, cAMP-dependent, EPAC signaling promotes growth cone extension early in development, PKA signaling predominates later in development switching netrin-1/cAMP effects to growth cone repulsion. Thus, EPAC and PKA underlie contrasting time-specific and context-specific functions within the developing nervous system.

Here, we tested the hypothesis that EPAC and PKA differentially regulate serotonin-induced pMF. Using recently available, highly selective, drugs to manipulate spinal cAMP signaling (**Table 1**), we investigated the functional significance of divergent cAMP signaling on 5-HT2A induced pMF. We demonstrate that whereas PKA constrains 5-HT2A induced pMF, EPAC and 5-HT2A co-activation exert additive effects, enhancing pMF expression. Thus, divergent cAMP signaling differentially regulates serotonin-induced pMF. This is the first demonstration that downstream signaling from a single intracellular molecule enables differential regulation of plasticity in the adult nervous system. Flexible signaling through distinct PKA vs EPAC mechanisms may explain a number of emergent properties of serotonin-induced neuroplasticity, including metaplasticity (Huang et al., 1992; Kirkwood et al., 1995; Abraham and Bear, 1996; Fischer et al., 1997; Mitchell and Johnson, 2003).

Materials/Methods

Animals

Adult male Sprague-Dawley rats (2-5 months old; colony 218A, Harlan; Indianapolis, IN) were doubly housed, with food and water *ad libitum*, a 12h light/dark cycle, and controlled humidity/temperature. The University of Wisconsin Institutional Animal Care and Use Committee approved all animal procedures.

Neurophysiology experiments

Anesthesia was induced with isoflurane in a closed chamber and then maintained via nose cone (3.5% isoflurane in 50% O2, balance N2). Rats were tracheotomized and pump ventilated (2.5ml per breath; frequency adjusted to regulate end-tidal PCO_2 between 40-50mmHg; Rodent Ventilator, model 683; Harvard Apparatus; South Natick, MA, USA) with an inspiratory mixture of 50% O_2; 2% CO_2; balanced N_2 Followed by bilateral vagotomy in the mid-cervical region to eliminate ventilator entrainment of breathing efforts. An arterial catheter was placed into the right femoral artery to enable blood sampling for blood-gas analysis during protocols. To enable intrathecal drug delivery, a dorsal lamenectomy and durotomy (C1/C2) was performed, a silicone catheter (OD 0.6mm; Access Technologies, IL, USA; primed with drug/vehicle) was inserted through a small hole in the dura and advanced caudally (~3mm) until resting at the C3-C4 spinal region. To minimize unintended drug diffusion from the catheter it was not placed until the stabilization period at the end of surgical preparations. The left phrenic and left hypoglossal (XII) nerves were isolated via a dorsal approach, cut distally, de-sheathed, submerged in mineral oil and then placed on bipolar silver wire electrode. After nerve dissection, rats were slowly converted to urethane anesthesia (1.8 g/kg, i.v. via tail vein catheter). Rectal body temperature (Traceable™, Fisher Scientific; Pittsburgh, PA, USA) was maintained within ± 1.0 of 37.5 ^oC using a custom temperature-controlled surgical table. A flow-through capnoguard with sufficient response time to measure exhaled CO_2 in rats (Capnoguard, Novametrix; Wallingford, CT; USA) was used to monitor and control end-tidal CO_2 (via adjustments to ventilator frequency). A heparinized plastic capillary tube (250x125 µl cut in half) was used to sample arterial blood to measure gas tensions (PaO_2, $PaCO_2$), pH and base excess (ABL 800Flex, Radiometer; Copenhagen, Denmark). Intravenous fluid infusions at a rate of 1mL/Hr (1:10:5 by volume of $NaHCO_3$/Lactated Ringer's/Hetastarch) were used to maintain blood pressure, acid/base and fluid balance from induction with isoflurane to euthanasia (overdose with urethane) following neurophysiology recordings.

Phrenic nerve activity was amplified (x10,000: A-M Systems, Everett, WA), band-pass filtered (100Hz to 10kHz), full-wave rectified, processed with a moving averager (CWE 821 filter; Paynter, Ardmore, PA: time constant, 50ms) and analyzed using a WINDAQ data-acquisition system (DATAQ Instruments, Akron, OH). Peak integrated phrenic burst frequency, amplitude, and mean arterial blood pressure (MAP) were analyzed in 60sec bins prior to blood samples. Data were included only if $PaCO_2$ was maintained within ± 1.5mmHg of baseline (set by recruitment threshold; approx. 45mmHg), base excess was within ± 3mEq/L of 0mEq/L, MAP had decreased less than 30mmHg of baseline values (approx. 120mmHg), and PaO_2 decreased less than 50mmHg from baseline (approx. 300mmHg) while remaining above 150mmHg for the entire protocol. There was no significant drift tendency in any of the physiological variables as assessed via 2-way ANOVA (**Table 2**).

One hour after conversion to urethane adequate levels of anesthesia were confirmed by an absence of response (movement, arterial blood pressure, phrenic nerve activity) to toe pinch. Rats were then paralyzed with pancuronium bromide (2.5mg/kg, i.v.) and baseline end-tidal CO_2 levels were set 2-3mmHg above the recruitment threshold for each individual rat (described in Bach and Mitchell, 1996). After 20min of stable nerve recordings a blood sample was drawn to establish baseline blood gas values. Rats then received the first of two series of intrathecal injections. Fifteen minutes after completion of the first injection series, rats received the second series as outlined below.

Drugs

The following drugs were obtained from Santa Cruz (Dallas, TX, USA): 6-Bnz-cAMP (PKA selective activator; PKAa), 8-pCPT-2'-Me-O-cAMP (EPAC selective activator; EPACa) and Rp-8-Br-cAMP (PKA selective inhibitor; PKAi). 2,5-Dimethoxy-4-iodoamphetamine (DOI; 5-HT2A receptor agonist; 5HT2a) was ordered from Sigma-Aldrich (St. Louis, MO) while ESI-05 (EPAC selective inhibitor; EPACi) was obtained from BioLog Life Science Institute (Germany). All drugs

were initially dissolved in dimethylsulfoxide (DMSO) and then diluted with saline (maximum DMSO concentration of 20%) before use. Aliquots of stock solutions remained viable for up to one week if stored frozen (-20^{O} C) in 100% DMSO; after this time unused drug solutions were discarded. Prior studies using a similar protocol confirmed that EPACa is a selective EPAC activator (Fields et al. 2015) and DOI is a selective 5-HT2A receptor agonist (MacFarlane et al. 2011). Separate studies using cell culture assays have shown that ESI-05 and Rp-8-Br-cAMP are selective inhibitors of EPAC (Tsalkova et al. 2012; Rehmann 2013) and PKA (Poppe et al. 2008) respectively (**Table 1**). In addition, cross-over control studies were done to confirm selectivity of the cAMP analogue drugs within our *in-vivo* model.

Experimental groups

All drugs were delivered as a single injection bolus over a 2min period with the exception of the 5-HT2A agonist, which was delivered as 3 smaller injections of 5μL over a 1min period, each separated by 5min intervals to establish intermittent receptor activation. Previous studies have shown that intermittent 5-HT2A agonist injections are required to elicit this form of pMF (MacFarlane et al 2011), whereas single injections (not intermittent) are necessary for PKA and EPAC induced pMF (Fields et al. 2015). To confirm individual molecules are sufficient to elicit pMF, intrathecal 5-HT2A receptor agonist (3x6μL, 100μM), PKAa (10μL, 100μM) or EPACa (10μL, 100μM) injections were given intrathecally. To maintain volume consistency vehicle injections were given in each of these groups via a second intrathecal catheter. Dosing for 5-HT2A (MacFarlane et al., 2011) and EPACa (Fields et al., 2015) were determined from previous studies; a limited dose response curve was completed for PKAa (data not shown). Intrathecal injections of the 5-HT2A agonist, PKAa, or EPACa, gave rise to pMF without affecting hypoglossal (XII) nerve activity. XII nerve activity serves as an internal control to confirm pMF is due to spinal mechanisms and not drug diffusion to brainstem respiratory centers which would elicit motor facilitation in both phrenic *and* XII nerves (Baker-Herman and Mitchell, 2001). For

cAMP cross-talk groups either PKAa or EPACa were given via a second catheter 15min prior to 5-HT2A receptor agonist injections at the same dose sufficient to elicit pMF when given alone. When PKAa and EPACa were given alone, intermittent vehicle injections (3x6μL) were given as a sham for 5-HT2A agonist injections.

To better understand signaling pathways necessary for pMF, additional rat groups were pretreated with PKAi (10μL, 1mM) or EPACi (10μL, 2mM) 15min prior to 5-HT2A agonist (3x6μL, 100μM), PKAa (10μL, 100μM), or EPACa (10μL, 100μM) injections. All inhibitors were given intrathecally via a second catheter over 2min. "Time post injection" was started after the final injection of the second series.

Statistical analyses

Peak amplitude and frequency of integrated phrenic bursts were averaged in 60sec bins at baseline (pre-injection), and at 30, 60 and 90min after the final intrathecal injection. Amplitude is expressed as a percent change from baseline in each rat; frequency is expressed as change from baseline in bursts/min. Phrenic nerve burst frequency did not change significantly in any group (**Table 2**). Statistical comparisons were made for experimental, vehicle and drug control groups using two-way repeated measures ANOVA with Tukey *post hoc* test to identify statistically significant pair-wise differences. All values are expressed as means ± 1SEM. Significance was accepted as $p \leq 0.05$. p values are relative to baseline phrenic nerve amplitude for the respective group unless otherwise noted. Since none of the control groups exhibited significant pMF, and since there were no significant differences between any of the control groups (vehicle + vehicle, n = 5; EPACi + vehicle, n = 4; PKAi + vehicle, n = 4), they were combined into a single, master control group (n = 13). Individual group data from 5-HT2A agonist and control groups are repeated in figures 1 and 2. Group numbers are defined in the figure legends and in **Table 2**.

Results

PKA activation elicits pMF, but constrains 5-HT2A induced pMF

Intermittent, intrathecal 5-HT2A agonist injections (2,5-Dimethoxy-4-iodoamphetamine; 3 x 6μL, 100μM) elicited pMF (**Fig. 1, A**; 50.6 ± 3.1% 90 min post-injection; n = 6; p < 0.001). 5-HT2A induced pMF was not affected by pretreatment with the selective PKA inhibitor, Rp-8-Br-cAMP (PKAi; 10μL, 1mM; 67.2 ± 4.9% 90 min post-injection; n = 5; p = 0.1 versus 5-HT2A agonist alone), confirming that PKA activity is not necessary for 5-HT2A induced pMF.

6-Bnz-cAMP is a cell permeable cAMP analogue that preferentially activates PKA (PKAa) versus EPAC (**Table 1**). Intrathecal 6-Bnz-cAMP injections (10μL, 100μM) elicited progressive increases in phrenic nerve burst amplitude (**Fig. 1, B**; 58.9 ± 8.6% at 90 min post-injection; n = 7; p < 0.001), demonstrating PKA activity is sufficient to elicit pMF. Although PKAa-induced pMF was attenuated by PKAi (**Fig. 1, B**; 7.3 ± 6.7%; n = 6; p < 0.001 vs. PKAa alone), it was unaffected by EPAC inhibition (EPACi) with the selective inhibitor, ESI-05 (10μL, 2mM; **Fig. 1, B**; 69.1 ± 7.3%; n = 4; p = 0.819 vs PKAa alone). Thus, we confirm PKAa-induced pMF requires PKA, but not EPAC activation.

Although 5-HT2A and PKA activation each elicit pMF alone, concurrent PKAa and 5-HT2A receptor activation prevented pMF expression (**Fig. 1, C**; 30min post-injection: 12.9 ± 5.4%; 60min: 9.9 ± 3.4%; 90min: 18.6 ± 6.5%; n = 7; p < 0.001 vs. 5-HT2A or PKAa-induced pMF). Thus, concurrent PKA and 5-HT2A activation are mutually inhibitory, disenabling pMF.

EPAC activation elicits pMF, and enhances 5-HT2A induced pMF

Pre-treatment with an EPAC inhibitor (ESI-05; 10μL, 2mM) had no effect on 5-HT2A induced pMF (**Fig. 2, A**; 52.4 ± 6.1% 90 min post-injection; n = 5; p < 0.001 vs. 5-HT2A agonist induced pMF), demonstrating EPAC activity plays no role in 5-HT2A-induced pMF.

8-pCPT-2'-Me-O-cAMP is a cAMP analogue with high relative selectivity for EPAC (EPACa) versus PKA activation (**Table 1**). Intrathecal EPACa (10μL, 100μM) elicited pMF (**Fig. 2, B**; 58.9

± 8.2% 90 min post-injection; n = 6; p < 0.001) similar to our previous report (Fields et al., 2015). EPACa induced pMF was attenuated by EPACi (**Fig. 2, B**; 6.2 ± 16.7%; n = 4; p < 0.001 vs. EPACa alone), but not PKAi (**Fig. 2, B**; 55.2 ± 2.5%; n = 4; p = 0.979 vs. EPACa alone). Thus, EPACa induced pMF requires EPAC, not PKA activity.

Concurrent spinal EPAC and 5-HT2A activation gave rise to pMF greater than that elicited by either drug alone (**Fig. 2, C**; 110.9 ± 10.0% 90 min post-injection; n = 6; p < 0.001 vs. EPACa or 5-HT2A agonist alone). Combined EPACa + 5-HT2A agonist-induced pMF was additive (i.e. equal to the sum of pMF induced by each drug alone (**Fig. 2, E**; p = 0.999 vs. EPACa plus 5-HT2A agonist induced pMF). Thus, EPAC and 5-HT2A make independent pMF contributions, much in contrast to the mutual inhibition observed with concurrent PKA and 5-HT2A activation.

Discussion

Serotonin elicits multiple forms of sensory-motor plasticity through diverse GPCR subtypes (Brunelli et al., 1976; Randić et al., 1993; Clark and Kandel, 1993). However, the functional implications of serotonin receptor co-activation have seldom been explored. In spinal pMF, Gq-coupled 5-HT2A and Gs-coupled (cAMP-linked) 5-HT7 receptors give rise to pMF through mechanistically distinct signaling cascades (MacFarlane et al., 2011; Hoffman and Mitchell, 2011; Fields et al., 2015). Although each receptor is sufficient to elicit pMF when stimulated alone, intracellular, inter-receptor, cross-talk inhibition limits pMF when they are co-activated (MacFarlane et al., 2009). Here we provide the first evidence that divergent cAMP signaling enables differential regulation of serotonin Gq receptor-induced spinal motor plasticity. Whereas PKA activity attenuates 5-HT2A receptor-induced pMF, EPAC enhances pMF by combining (additively) with 5-HT2A receptor-induced pMF. We propose that a shift in cAMP signaling from PKA to EPAC predominance may relieve cross-talk constraints, potentially enabling independent contributions of 5-HT2A and 5-HT7 receptors for enhanced spinal motor plasticity.

Whereas PKA is activated at nanomolar cAMP levels (Dostmann and Taylor, 1991), EPAC activation requires cAMP levels in the micromolar range (Ponsioen et al., 2002). Different cAMP affinities suggest that stimulation of Gs protein coupled receptors will initially activate PKA with EPAC signaling following only with greater/stronger receptor activation. However, certain growth/trophic factors can change the relative cAMP sensitivity of PKA and EPAC, shifting activation thresholds in favor of EPAC signaling (Vasko et al., 2014). For example, although nerve growth factor-1 (NGF-1) does not affect plasticity expression in a well-studied model of sensory hypersensitivity, it does convert the plasticity from PKA- to EPAC-dependence (Vasko et al., 2014). Similar effects could shift the PKA/EPAC balance downstream from 5-HT7 receptors, enable 5-HT7 receptors to contribute rather than constrain serotonin-induced plasticity, potentially explaining enhanced serotonin-dependent plasticity observed with preconditioning experiences known to increase growth/trophic factor expression (Kinkead et al., 1998; Johnson et al., 2000; Ling et al., 2001; Wilkerson and Mitchell, 2009).

Alternatively, direct manipulation of cAMP signaling with selective drugs may enable modulation of cross-talk interactions to enhance serotonin-induced pMF for experimental or therapeutic advantage. For example, serotonin-induced, 5-HT2A-dependent, spinal motor plasticity may be enhanced via PKA inhibition to relieve inhibitory cross-talk constraints; conversely, selective EPAC activation will contribute to serotonin induced, 5-HT2A-dependent, pMF via additive contributions from the mechanistically distinct Gs associated pathway (Fields et al., 2015). By enhancing respiratory control through spinal motor plasticity we may restore lost breathing capacity in severe clinical disorders such as cervical spinal injury (Lovett-Barr et al., 2012) or motor neuron disease (Nichols et al., 2013).

Here we utilized recently available, highly selective, drugs to independently manipulate PKA and EPAC activity. While rodent knockout models are often used to assure target selectivity (vs drugs), a recent study demonstrated that plasticity investigations are sometimes hampered in EPAC knockout mice due to compensatory signaling responses to gene deletions. For example,

although PKA does not contribute to forskolin-induced, cAMP dependent, mossy fiber plasticity in wild-type mice, PKA inhibition suppresses mossy fiber plasticity in EPAC knockout mice, suggesting a compensatory/supportive role for PKA revealed only when EPAC signaling is impaired during development (Fernandes et al., 2015). Furthermore, despite the potential for off-target effects when using pharmacological approaches, literature supports the selectivity of the drugs used here (**Table 1**), and we have confirmed selectivity within our conditions with carefully designed control experiments cross-comparing EPAC and PKA activators/inhibitors.

Although contrasting roles for EPAC and PKA have been emphasized in embryonic model systems (Murray et al., 2009), the present data are the first to confirm differential actions in the fully mature adult nervous system. This is an important advancement as it supports the idea that spinal motor networks retain their capacity to adapt important motor behaviors long after maturation. Due to the limited capacity of spinal motor neurons to replicate, functional flexibility through plasticity represents an important target for therapeutic intervention in neural disorders that compromise essential motor behaviors; such as breathing. These possibilities await further exploration.

Figures:

Drug (volume:conc)	EPAC Ka	PKA Ka	PKA Ki	EPAC Ki
6-Bnz-cAMP[a] (10µL:100µM)	NSa	2.7µM	-	-
8-pCPT-2'-O-Me-cAMP[a] (10µL:100µM)	1.8µM	190µM	-	-
Rp-8-Br-cAMP[a] (10µL:1mM)	-	-	8.5µM	NSi
ESI-05[b] (10µL:2M)	-	-	NSi	0.43µM

Ka concentration for half of maximum cAMP induced response
Ki concentration for inhibition of half maximum cAMP induced response
NSa non-significant activating effect; ≥100 fold Ka difference
NSi non-significant inhibitory effect; ≥100 fold Ki difference
[a] Poppe et al. 2008; [b] Tsalkova et al. 2012

Table 1: Published selectivity of PKA/EPAC activators and inhibitors from cell culture assays. The volume:conc (concentration) values listed in column 1 were concentration and volume used for intrathecal injections in the present *in-vivo* study.

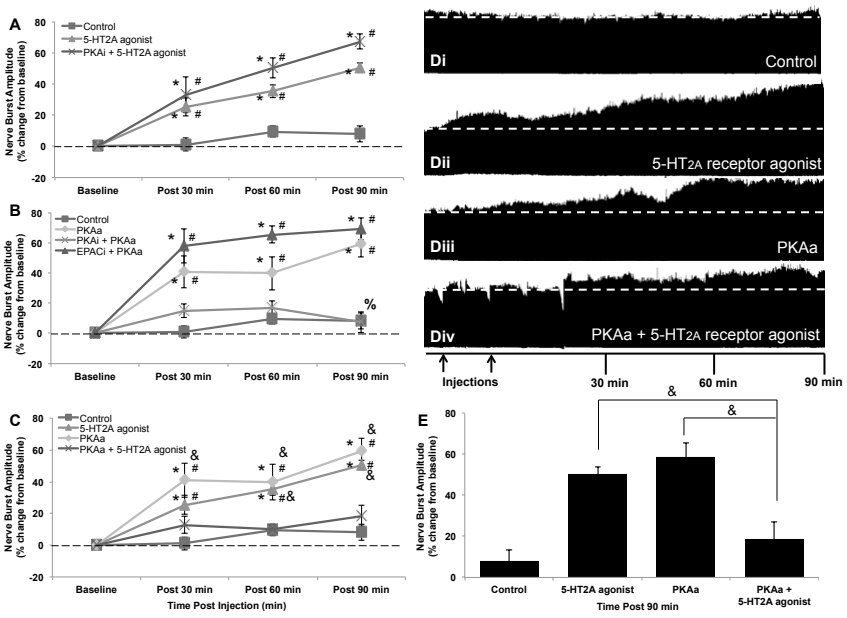

Figure 1: PKA constrains 5-HT2A receptor-induced phrenic motor facilitation. A) intermittent intrathecal injections of 5-HT2A receptor agonist (3x6μL, 100μM) elicited pMF (90min: 50.6 ± 3.1%; n = 6; $p < 0.001$) were not affected by PKAi (10μL, 1mM; 90min: 67.2 ± 4.9%; n = 5; $p = 0.098$ relative to 5-HT2A agonist induced pMF). **B)** Intrathecal injections of PKAa (10μL, 100μM) elicited pMF (90min: 58.9 ± 8.6%; n = 7; $p < 0.001$), an effect that was undermined by PKAi (90min: 7.3 ± 6.7%; n = 6; $p < 0.001$), but not EPACi pretreatment (90min: 69.1 ± 7.3%; n = 4; $p = 0.819$). **C)** Concurrent application of PKAa and 5-HT2A receptor agonist limited the capacity for either to elicit pMF (90min: 18.6 ± 6.5%; n = 7; $p < 0.001$ relative to PKAa or 5-HT2A receptor agonist-induced pMF). **D)** Representative phrenic neurograms; **i)** vehicle control, **ii)** vehicle + 5-HT2A receptor agonist, **iii)** vehicle + PKAa and **iv)** PKAa + 5-HT2A receptor agonist. First arrow represents pretreatment injection; second arrow represents either start of intermittent 5-HT2A agonist injections or start of single PKAa injection. **E)** Summary of data from A-C at 90min post-final injection. Data represent mean values ± 1 SEM. Significant differences from baseline (**#**), control (*****), PKAa (**%**), or PKAa + 5-HT2A receptor agonist (**&**).

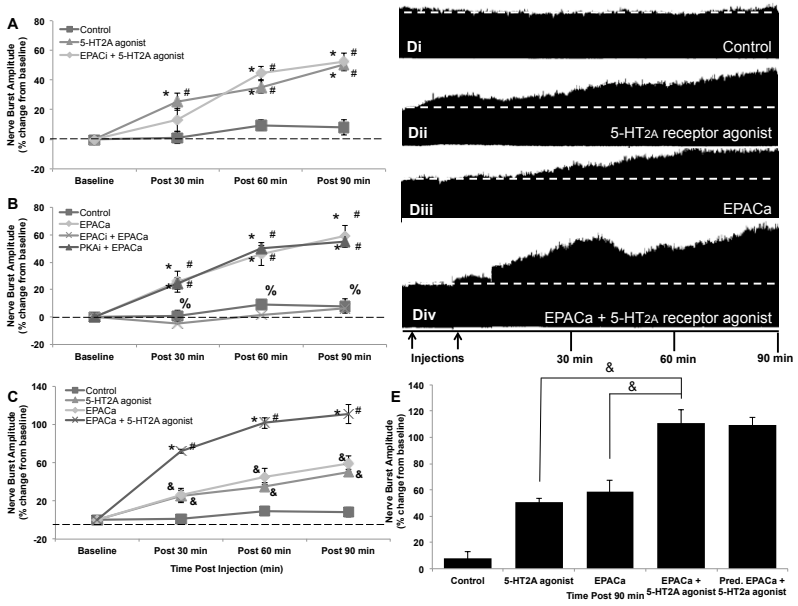

Figure 2: EPAC additively enhances 5-HT2A receptor-induced phrenic motor facilitation. A) Intermittent intrathecal injections of 5-HT2A receptor agonist (3x6μL, 100μM) elicited pMF (90min: 50.6 ± 3.1%; n = 6; p < 0.001); this pMF was not affected by EPACi pretreatment (10μL, 2mM; 90min: 52.4 ± 6.1%; n = 5; p < 0.001 versus 5-HT2A-induced pMF). **B)** Intrathecal EPACa injections (10μL, 100μM) elicited pMF (90min: 58.9 ± 8.2%; n = 6; p < 0.001), an effect constrained by EPACi (90min: 6.2 ± 16.7%; n = 4; p < 0.001 versus EPACa induced pMF), but not PKAi (10μL, 1mM; 90min: 55.2 ± 2.5%; n = 4; p = 0.979 versus EPACa induced pMF). **C)** Concurrent EPACa and 5-HT2A agonist injections elicited an enhanced pMF significantly greater than EPACa or 5-HT2A-induced pMF alone (90min: 110.9 ± 10.0%; n = 6; p < 0.001 versus EPACa or 5-HT2A agonist-induced pMF). **D)** Representative phrenic neurograms; **i)** vehicle control, **ii)** vehicle + 5HT2A receptor agonist, **iii)** vehicle + EPACa and **iv)** EPACa + 5-HT2A receptor agonist. First arrow represents pretreatment injection; second arrow represents either start of intermittent 5-HT2A agonist injections or start of single EPACa injection. **E)** Summary of data from A-C; the actual pMF from combined EPACa and 5-HT2A activation was not different from predicted pMF resulting from additive contributions from the pMF elicited when each molecule is activated alone. Data represent mean values ± 1 SEM. Significant differences from baseline (**#**), control (*), EPACa (**%**), or EPACa + 5-HT2A agonist (**&**).

	Time (min)	Vehicle Control (N = 13)	Veh + 5-HT2A agonist (N = 6)	EPACi + 5-HT2A agonist (N = 5)	EPACa + 5-HT2A agonist (N = 6)	PKAi + 5-HT2A agonist (N = 5)	PKAa + 5HT2A agonist (N = 7)
$PaCO_2$ (mmHg)	Baseline	48.6 ± 0.9	48.5 ± 1.1	48.3 ± 0.8	48.0 ± 1.6	48.0 ± 0.6	48.4 ± 0.6
	30	48.7 ± 0.9	49.3 ± 0.9	47.5 ± 0.8	48.0 ± 1.9	48.1 ± 0.6	48.0 ± 0.9
	60	48.3 ± 0.9	49.3 ± 1.0	48.7 ± 0.7	48.2 ± 1.8	48.8 ± 0.6	48.5 ± 0.6
	90	48.4 ± 1.0	48.2 ± 1.5	48.6 ± 0.8	48.3 ± 1.6	47.8 ± 0.6	48.7 ± 0.6
PaO_2 (mmHg)	Baseline	316.2 ± 10.4	296.3 ± 16.4	340.6 ± 12.1	349.7 ± 8.5	333.0 ± 13.1	324.1 ± 16.8
	30	321 ± 7.6	302.7 ± 15.0	339.6 ± 8.0	343.3 ± 5.4	343.4 ± 6.2	344.0 ± 14.2
	60	323.9 ± 8.0	314.7 ± 7.3	329.2 ± 8.4	342.7 ± 7.0	332.8 ± 6.8	348.7 ± 14.5
	90	325.5 ± 7.3	312.8 ± 10.9	328.0 ± 5.3	339.7 ± 7.2	321.6 ± 5.4	347.6 ± 13.6
MAP (mmHg)	Baseline	120.6 ± 6.0	106.1 ± 10.1	124.2 ± 5.1	106.7 ± 11.8	107.1 ± 6.3	111.9 ± 11.9
	30	110.3 ± 6.5	104.9 ± 10.8	114.1 ± 10.5	97.8 ± 11.6	102.4 ± 6.7	94.1 ± 9.6
	60	102.9 ± 6.2	94.9 ± 8.8	108.3 ± 4.0	96.5 ± 10.6	97.9 ± 6.5	89.6 ± 8.3
	90	103.2 ± 6.2	95.6 ± 9.8	100.1 ± 10.1	84.8 ± 7.6	83.0 ± 7.2	77.4 ± 9.2
Phrenic Burst Frequency (Burst/min)	Baseline	49.0 ± 1.2	49.1 ± 2.9	49.3 ± 2.2	49.3 ± 2.1	47.8 ± 2.2	45.7 ± 2.0
	30	47.9 ± 1.7	48.9 ± 2.5	48.4 ± 2.3	50.3 ± 1.2	48.9 ± 1.9	46.5 ± 2.3
	60	46.6 ± 2.0	48.8 ± 1.7	49.6 ± 2.8	50.2 ± 0.6	48.7 ± 1.9	45.5 ± 2.5
	90	47.7 ± 1.8	48.3 ± 2.2	49.8 ± 1.8	51.0 ± 0.9	49.6 ± 2.2	45.8 ± 2.3

	Time (min)	Veh + EPACa (N = 6)	EPACi + EPACa (N = 4)	PKAi + EPACa (N = 4)	Veh + PKAa (N = 7)	PKAi + PKAa (N = 6)	EPACi + PKAa (N = 4)
$PaCO_2$ (mmHg)	Baseline	50.4 ± 1.7	48.7 ± 1.4	48.0 ± 1.8	49.4 ± 1.2	48.4 ± 0.8	50.8 ± 1.0
	30	50.1 ± 1.7	49.5 ± 1.3	48.0 ± 1.9	50.2 ± 1.0	48.2 ± 1.0	51.3 ± 1.2
	60	50.9 ± 1.6	48.2 ± 1.1	48.5 ± 1.0	49.8 ± 1.4	48.9 ± 0.8	51.1 ± 1.6
	90	50.2 ± 1.7	48.3 ± 1.4	48.6 ± 1.3	49.5 ± 1.2	48.8 ± 0.9	50.3 ± 1.2
PaO_2 (mmHg)	Baseline	311.5 ± 10.9	329.0 ± 15.1	274.0 ± 39.6	316.7 ± 17.5	315.7 ± 14.0	291.2 ± 25.1
	30	320.0 ± 6.9	322.3 ± 16.2	330.3 ± 12.9	311.9 ± 12.7	307.2 ± 16.3	306.7 ± 10.8
	60	315.8 ± 8.5	310.0 ± 14.6	333.7 ± 14.3	267.5 ± 44.2	309.8 ± 14.6	305.2 ± 13.3
	90	315.8 ± 9.3	310.5 ± 13.6	336.7 ± 10.9	273.0 ± 40.0**	306.4 ± 10.9	311.2 ± 3.8
MAP (mmHg)	Baseline	121.4 ± 5.8	118.9 ± 10.1	130.9 ± 10.6	113.3 ± 5.3	103.6 ± 6.9	116.3 ± 7.4
	30	109.9 ± 2.9	99.5 ± 11.1	110.5 ± 13.3	102.5 ± 5.5	91.7 ± 1.7	92.8 ± 7.9
	60	108.2 ± 2.8	100.5 ± 7.7	106.4 ± 8.6	100.4 ± 7.4	91.2 ± 5.9	89.9 ± 10.0
	90	98.8 ± 2.7	86.7 ± 12.7	104.3 ± 9.7	89.7 ± 6.5	84.6 ± 3.0	89.8 ± 10.1
Phrenic Burst Frequency (Burst/min)	Baseline	50.2 ± 2.8	54.5 ± 2.5	51.7 ± 1.1	45.8 ± 1.5	41.7 ± 3.3	56.0 ± 1.3*
	30	50.8 ± 2.2	52.4 ± 1.5	50.6 ± 0.5	47.7 ± 1.7	48.4 ± 4.9	58.7 ± 1.1*
	60	51.8 ± 2.1	53.6 ± 2.2	51.1 ± 0.5	47.2 ± 1.9	48.2 ± 3.3	57.2 ± 1.1*
	90	51.3 ± 2.0	54.4 ± 1.3	50.3 ± 1.2	46.8 ± 1.9	44.3 ± 3.7	58.3 ± 1.3*

Table 2: Physiology variables. There were no consistent differences of $PaCO_2$, PaO_2, mean arterial pressure (MAP) or phrenic burst frequency within any individual group or amongst different groups. Differences between groups at a given time point (*) and differences from baseline within individual groups (**) are denoted within the table; $p < 0.05$. Values expressed as means ± SEM.

References

Abraham, W.C., and Bear, M.F. (1996). Metaplasticity: the plasticity of synaptic plasticity. Trends Neurosci *19*, 126-130.

Bach, K.B., and Mitchell, G.S. (1996). Hypoxia-induced long-term facilitation of respiratory activity is serotonin dependent. Respir Physiol *104*, 251-260.

Barbas, D., DesGroseillers, L., Castellucci, V.F., Carew, T.J., and Marinesco, S. (2003). Multiple serotonergic mechanisms contributing to sensitization in aplysia: evidence of diverse serotonin receptor subtypes. Learn Mem *10*, 373-386.

Brunelli, M., Castellucci, V., and Kandel, E.R. (1976). Synaptic facilitation and behavioral sensitization in Aplysia: possible role of serotonin and cyclic AMP. Science *194*, 1178-1181.

Clark, G.A., and Kandel, E.R. (1993). Induction of long-term facilitation in aplysia sensory neurons by local application of serotonin to remote synapses. PNAS *90*, 11411-11415.

Dostmann, W.R., and Taylor, S.S. (1991). Identifying the molecular switches that determine whether (Rp)-cAMPS functions as an antagonist or an agonist in the activation of cAMP-dependent protein kinase I. Biochemistry *30*, 8710-8716.

Fernandes, H.B., Riordan, S., Nomura, T., Remmers, C.L., Kraniotis, S., Marshall, J.J., Kukreja, L., Vassar, R., and Contractor, A. (2015). Epac2 mediates cAMP-Dependent Potentiation of Neurotransmission in the Hippocampus. J Neurosci *35*, 6544-6553.

Fields, D.P., Springborn, S.R., and Mitchell, G.S. (2015). Spinal 5-HT7 receptors induce phrenic motor facilitation via EPAC-mTORC1 signaling. J Neurophys *114*, 2015-2022.

Fischer, T.M., Blazis, D.E.J., Priver, N.A., and Carew, T.J. (1997). Metaplasticity at identified inhibitory synapses in Aplysia. Nature *389,* 860-865.

Glanzman, D.L., Mackey, S.L., Hawkins, R.D., Dyke, A.M., Llyod, P.E., and Kandel, E.R. (1989). Depletion of serotonin in the nervous system of Aplysia reduces the behavioral enhancement of gill withdrawal as well as the heterosynaptic facilitation produced by tail shock. J Neurosci *9*, 4200-4213.

Hoffman, M.S., and Mitchell, G.S. (2011). Spinal 5-HT7 receptor activation elicits long-lasting phrenic motor facilitation. J Physiol *589*, 1397-1407.

Hoffman, M.S., and Mitchell, G.S. (2013). Spinal 5-HT7 receptors and protein kinase A constrain intermittent hypoxia-induced phrenic long-term facilitation. Neurosci *250*, 632-643.

Huang, Y.Y., Colino, A., Selig, D.K., and Malenka, R.C. (1992). The influence of prior synaptic activity on the induction of long-term potentiation. Science *255*, 750-753

Johnson, R.A., Okragly, A.J., Haak-Frendscho, M., and Mitchell, G.S. (2000). Cervical dorsal rhizotomy increases brain-derived neurotrophic factor and neurotrophin-3 expression in the ventral spinal cord. J Neurosci *20*, RC77.

Kinkead, R., Zhan, W.Z., Prakash, Y.S., Bach, K.B., Sieck, G.C., and Mitchell, G.S. (1998). Cervical dorsal rhizotomy enhances serotonergic innervation of phrenic motoneurons and serotonin-dependent long-term facilitation of respiratory motor output in rats. J Neurosci *18*, 8436-8443.

Kirkwood, A., Lee, H.K., and Bear, M. (1995). Co-regulation of long-term potentiation and experience-dependent synaptic plasticity in visual cortex by age and experience. Nature *375*, 328-331.

Levy, M., and Susswein, A.J. (1993). Separate neural pathways respond to different noxious stimuli affecting respiratory pump frequency in Aplysia fasciata. Brain Res *616*, 218-229.

MacFarlane, P.M., and Mitchell, G.S. (2009). Episodic spinal serotonin receptor activation elicits long-lasting phrenic motor facilitation by an NADPH oxidase-dependent mechanism. J Physiol *587*, 5469-5481.

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

Mackey, S.L., Kandel, E.R., and Hawkins, R.D. (1989). Identified serotonergic neurons LCB1 and RCB1 in the cerebral ganglia of Aplysia produce presynaptic facilitation of siphon sensory neurons. J Neurosci *9*, 4227-4235.

Murray, A.J., Tucker, S.J, and Shewan, D.A. (2009). cAMP-dependent axon guidance is distinctly regulated by Epac and protein kinase A. J Neurosci *29*, 15434-15444.

Ponsioen, B., Zhao, J., Riedl, J., Zwartkruis, F., van der Krogt, G., Zaccolo, M., Moolenaar, W.H., Bos, J.L., and Jalink, K. (2004). Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. EMBO Rep *5*, 1176-1180.

Poppe, H., Rybalkin, S.D., Rehmann, H., Hinds, T.R., Tang, X.B., Christensen, A.E., Schwede, F., Genieser, H.G., Bos, J.L., Doskeland, S.O., Beavo, J.A., and Butt, E. (2008). Cyclic nucleotide analogs as probes of signaling pathways. Nat Methods *5*, 277-278.

Randić, M., Jiang, M.C., and Cerne, R. (1993). Long-term potentiation and long-term depression of primary afferent neurotransmission in the rat spinal cord. J Neursci *13*, 5228-5241.

Rehmann, H. (2013). EPAC-inhibitors: facts and artefacts. Sci Rep *3*, 3032.

Seino, S., and Shibasaki, T. (2005). PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. Physiol Rev *85*, 1303-1342.

Seol G.H., Ziburkus, J., Huang, S., Song, L., Kim, I.T., Takamiya, K., Huganir, R., Lee, H.K., and Kirkwood, A. (2007). Neuromodulators Control the Polarity of Spike-Timing-Dependent Synaptic Plasticity. Neuron *55*, 919-929.

Treviño, M., Huang, S., He, K., Ardiles, A., Pasquale, R., Guo, Y., Palacios, A., Huganir, R., Kirkwood, A. (2012). Push-pull neuromoulation of LTP and LTD enables bidirectional experience-induced synaptic scaling in visual cortex. Neuron *73*, 497-510.

Tsalkova, T., Mei, F.C., Li, S., Chepurny, O.G., Leech, C.A., Liu, T., Holz, G.G., Woods, V.L. Jr, Cheng, X. (2012). Isoform-specific antagonists of exchange proteins directly activated by cAMP. PNAS *109*, 18613-18618.

Vasko, M.R., Habashy-Malty, R., Guo, C., Duarte, D.B., Zhang, Y., Nicol, G.D. (2014). Nerve growth factor mediates a switch in intracellular signaling for PGE2-induced sensitization of sensory neurons from protein kinase A to Epac. PloS One *9*, e104529.

Wilkerson, J.E., and Mitchell, G.S. (2009). Daily intermittent hypoxia augments spinal BDNF levels, ERK phosphorylation and respiratory long-term facilitation. Exp Neurol *217*, 116-123.

Moderate intermittent hypoxia impairs plasticity induced by reductions in respiratory neural activity by an NMDA receptor-dependent mechanism

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Acknowledgements: This work was funded by NIH HL105511. DPF is supported by NIH F30HL126351, the University of Wisconsin Medical Scientist Training Program (T32GM008692), and a UNCF/Merck graduate research fellowship.

Abstract

Sleep apnea is classically defined by its persistent pattern of unstable breathing control, with recurrent apneas and acute hypoxic episodes intermittently occurring during sleep. A poor understanding of the etiology underlying sleep apnea has hindered development of drug therapies. Despite the prevalence of sleep apnea, the neural system controlling breathing exhibits an intrinsic capacity to sense and auto-correct perturbations in breathing control (i.e. compensatory respiratory plasticity), suggesting breathing instability in sleep apnea may be the result of a failure to elicit compensatory plasticity mechanisms. One well-characterized form of compensatory respiratory plasticity is inactivity-induced phrenic motor facilitation (iPMF); a prolonged enhancement of inspiratory motor output following episodes of neural inactivity (i.e. neural apnea). Since clinically relevant apneas rarely occur without concomitant hypoxia, we sought to determine the impact of concurrent hypoxia on iPMF. In anesthetized, paralyzed, and mechanically ventilated adult male rats we demonstrate that while isolated neural apneas give rise to iPMF, concurrent exposure to moderate (but not mild) hypoxia abolishes iPMF expression. Specifically, we show that hypoxia enhances spinal NR2B-containing NMDA receptors (NMDARs) to constrain signaling processes necessary for iPMF. Further, we demonstrate that spinal application of trans-retinoic acid bypasses NMDAR constraints, thereby rescuing expression of respiratory plasticity. This study provides important mechanistic insight into understanding how concurrent reductions in respiratory neural activity with hypoxia may undermine compensatory forms of respiratory plasticity. In addition, we identify retinoic acid as a novel pharmacological approach for improving breathing control during sleep apnea and related breathing control disorders.

Introduction

Sleep apnea is an insidious disease estimated to affect 1 in 15 Americans, with prevalence peaking in middle-aged individuals to 1 in 12 women and 1 in 4 men (Young et al., 1993; Heinzer et al., 2015). While development of sleep apnea often goes unnoticed, the long-term complications impact many organ systems as patients experience recurrent breathing disruptions that fragment sleep, decrease blood oxygenation, and promote hyperactivity of the sympathetic nervous system (Leung, 2009). Thus, sleep apnea is a dangerous precipitator of acute complications in patients with neural and cardiovascular disease (Mehra and Redline, 2014; Abbott and Videnovic, 2016; Chowdhuri et al., 2016). Sleep apnea represents a subpopulation of periodic breathing disorders with at least two diagnostically distinct subclasses: obstructive sleep apnea (OSA) and central sleep apnea (CSA). OSA is characterized by an anatomical narrowing of the upper airway that impedes airflow during attempted breaths (Wheatley et al., 1993). Conversely, CSA is characterized by insufficient neural motor output to the diaphragm, undermining breathing efforts despite significant hypoxemia (reviewed in Hernandez and Patil, 2016). Recent evidence suggests that these two conditions may not be as distinct as previously believed. In a large cohort of patients treated for OSA with continuous positive air pressure (CPAP), once OSA was resolved, previously unidentified CSA became apparent (Hoffman and Schulman, 2012; Westhoff et al., 2012); suggesting central apneas may be a precipitating factor for development of OSA. Alternatively, CSA and OSA may originate from a common derivation related to impaired motor output within the neural respiratory network (Babcock and Badr, 1998; Aboubakr et al., 2001; Malhotra and White, 2002).

Despite the prevalence of sleep apnea, the neural respiratory system exhibits a profound capacity for adapting through compensatory respiratory plasticity, many of which are activated following perceived failures (i.e. neural apneas/inactivity, hypoxia or hypercapnia; Mahamed et al., 2007; Bach and Mitchell, 1996; Mahamed and Mitchell, 2008; Valic et al., 2016). Phenotypically, compensatory respiratory plasticity presents in several ways: 1) enhanced

phrenic motor output (i.e. phrenic motor facilitation; **Figure 1A**), strengthening inspiratory efforts (Bach and Mitchell, 1996), 2) enhanced hypoglossal (XII) motor output (i.e. XII motor facilitation; **Figure 1B**), improving upper airway patency (Malhotra and White, 2002; Wilkinson et al., 2008), and 3) a reduction in apneic threshold (**Figure 1C**), reducing susceptibility to future apneas (Baertsch and Baker, in review; Khoo et al., 1991; Carley et al., 1988). While aberrant firing of the respiratory rhythm generator, the PreBotzinger Complex (PBC), is thought to be the primary cause of many breathing disorders (McKay and Feldman, 2008), poor neural transmission of inspiratory effort from the PBC is emerging as an important contributor to unstable breathing patterns. For example, prolonged exposure to hypoxia disrupts PBC inspiratory transmission to respiratory motor nuclei without directly affecting the firing pattern of the PBC (Garcia et al., 2016). This results in non-synchronous firing patterns of the XII motor nuclei, a primary controller of upper airway patency. In patients, inspiratory transmission failure from the PBC to the XII or spinal phrenic motor pools could respectively lead to OSA (Wheatley et al., 1993) and CSA (Dale et al., 2014). Thus, therapies improving transmission fidelity of the respiratory control network (i.e. respiratory plasticity) may be one method for treating OSA, CSA and related breathing disorders.

Two well-studied models of compensatory respiratory plasticity are inactivity-induced phrenic motor facilitation (iPMF; Mahamed et al., 2011) and hypoxia-induced phrenic long-term facilitation (pLTF; Bach and Mitchell, 1996). Whereas iPMF is elicited by intermittent reductions in respiratory neural activity (i.e. neural apneas), pLTF develops following several episodes of moderate hypoxia (35-45 mmHg PaO_2; Fuller et al., 2000); both are expressed as a persistent enhancement in phrenic motor output. While clinically significant neural apneas rarely occur without concurrent hypoxia, the impact of hypoxia on inactivity-induced iPMF has never been studied.

Recent developments in our understanding of iPMF and pLTF signaling processes have determined that these two forms of plasticity operate through independent signaling pathways

(Strey et al., 2013; Fields and Mitchell, 2015). Specifically, signaling mechanisms giving rise to iPMF and pLTF exhibit converging, yet contrasting, roles for spinal NMDA receptor (NMDAR) activation. Whereas spinal NMDAR activation is necessary for both induction and maintenance of pLTF (McGuire et al., 2005; 2008; Golder, 2008), inhibition of spinal NMDARs is necessary for (at least some forms of) iPMF (Streeter et al., 2014). Thus, we propose that a contrasting drive to enhance and reduce NMDAR activity mechanistically undermines the capacity for either experience to elicit plasticity during concurrent reductions in respiratory neural activity with hypoxia, potentially explaining absent compensatory respiratory behaviors in patients with periodic breathing disorders characterized by neural apneas with hypoxia (i.e. sleep apnea).

Here, we demonstrate that while reduced respiratory neural activity and hypoxia are independently sufficient for respiratory plasticity (pLTF and iPMF, respectively), a concurrent reduction in neural activity with moderate (but not mild) hypoxia undermines expression of compensatory respiratory plasticity. Further, inhibition of hypoxia enhanced spinal NMDARs removes signaling constraints to enable plasticity. Alternatively, we show that hypoxia enhanced NMDARs can be bypassed through application of retinoic acid, thus rescuing several stabilizing behaviors for improved breathing control. This is the first study to provide a possible mechanistic explanation for why patients with sleep apnea lack compensatory respiratory behaviors to stabilize breathing, rendering them susceptible to recurrent apneas/hypoxia and secondary long-term complications associated with unstable breathing control. Recognition of hypoxia enhanced NMDARs as a key constraint to retinoic acid dependent inactivity-induced plasticity is an important step in identifying novel targets for pharmacologically treating CSA, OSA and other periodic breathing disorders.

Methods

Animals. Experiments were performed on 2.5-3.5month old male Sprague-Dawley rats from Harlan Laboratories (HSD; colony 217). Rats were housed 2 per cage with 12hr light/dark

cycles and food/water *ad libitum*. The University of Wisconsin, Madison animal care and use committee approved all experimental protocols.

Surgical Preparation. Isoflurane anesthesia was induced in a closed container and continued on a nose cone of 2.5% isoflurane with 30/70 O_2/N_2 balance. Tail vein catheter was placed for fluid infusion (10/5/1 lactated rings solution/hetastarch/8% sodium bicarbonate solution) in order to maintain blood pressure and pH homeostasis throughout experimental protocol. The trachea was cannulated, and mechanical ventilation was begun (Model 683, Harvard Apparatus, Holliston Massachusetts; ~70 breaths/min, 2.5-3ml; 30/70 O_2/N_2 balance) with CO_2 added to maintain an end-tidal CO_2 ($ETCO_2$) near ~45mmHg (assessed through an expired airflow capnograph; Respironics) and avoid unintended neural apnea during surgery or anesthesia conversion. The vagus nerve was isolated and cut bilaterally at the cervical level to prevent entrainment of spontaneous respiratory frequency to the ventilator. The right femoral artery was isolated and catheterized to monitor arterial blood pressure and draw blood samples (0.3ml per sample) for pH and blood-gas analysis (ABL800; Radiometer, Copenhagen, Denmark). The left hypoglossal (XII) and left phrenic nerves were dissected by dorsal approach, cut distally, de-sheathed, and covered with a moist cotton balls to prevent drying. The nerves were placed on bipolar silver electrodes prior to recording neural output. To place the intrathecal catheter, a lamenectomy was performed at cervical vertebrae 2 (C2) with a small hole cut in the dura. Rats were then converted to urethane anesthesia (0.180g/100g rat) as isoflurane was gradually withdrawn over a period of ~15min. Body temperature was monitored by rectal thermometer and maintained near 37.5°C (± 1°C) with a custom heated surgery table. Just prior to experimental protocol, a silicone catheter (2 French, Access Technologies) connected to a 50µl syringe (Hamilton) was inserted into the intrathecal space and advanced caudally (~5mm) to rest above spinal segment C4. In addition, pancuronium bromide was infused through the tail vein (3mg/kg, i.v.) to induce neuromuscular paralysis.

Experimental Protocols. Blood pressure and phrenic/XII nerve amplitude responses to paw pad-pinch were tested to confirm adequate depth of urethane anesthesia. At least one hour following termination of isoflurane anesthesia, the apneic threshold (measured by $ETCO_2$) was identified by slowly reducing inspired CO_2 until phrenic/XII bursting ceased for at least 20sec. Inspired CO_2 was then slowly increased until nerve activity returned (i.e. recruitment threshold; measured by $ETCO_2$) and raised an additional 2-3mmHg above RT for "baseline" nerve recordings. Baseline nerve activity was recorded for 15min with 2 arterial blood samples drawn 5min apart to obtain baseline $PaCO_2$, PaO_2, and pH measurements (temperature corrected). For the remainder of the protocol baseline parameters were maintained within ±1.5mmHg for $PaCO_2$ (of eupneic baseline), ±10mmHg for PaO_2 (baseline approx. 100mmHg PaO_2), ±1.5 for pH, and ±1.0°C for temperature. Blood gases were taken 15, 30, and 60min after the final neural/ventilator apnea challenges with necessary adjustments to gas flow and pH being made after each blood test. At the end of each protocol rats were exposed to a maximum ventilatory challenge consisting of hypercapnia (90mmHg < $ETCO_2$ < 100mmHg) with anoxia to ensure observed results were not due to deterioration of the preparation or a lack of dynamic range in respiratory motor output. Rats who did not experience at least a 100% increase in nerve activity during the maximum ventilatory challenge were removed from data analysis.

Following baseline recordings, rats were exposed to one of five protocols: **1)** 5 neural apnea episodes (~1.25min each, separated by 5min of baseline blood gases; n = 8), **2)** 5, 25sec ventilator apnea episodes (i.e. hypoxia challenges), in which mechanical ventilation was stopped while neural activity remained intact (each episode separated by 5min of baseline blood gases; n = 7), **3)** 5, 6sec ventilator apnea episodes (each episode separated by 5min of baseline blood gases; n = 8) with intact neural activity, **4)** 5, 25sec ventilator apnea episodes during concurrent neural apnea (each episode separates by 5min of baseline blood gases; n = 7), and **5)** 5, 6sec ventilator apnea episodes during concurrent neural apnea (each episode

separated by 5min of baseline blood gases; n = 8). In order to assess natural drifts in nerve activity, a "time control" group received the same surgical procedure without neural apnea or ventilator apnea experiences (n = 7).

Isolated neural apneas (i.e. neural inactivity)

In a similar manner to the apneic threshold, neural apneas were induced by lowering inspired CO_2 until rhythmic phrenic and XII activity ceased. Neural apnea was confirmed for 10sec before inspired CO_2 was returned to baseline levels, though nerve activity did not return until ~ 1min after $ETCO_2$ reached baseline levels. This delay in spontaneous respiratory activity is consistent with previously published work from our lab and others (Baertsch and Baker-Herman 2013; 2015). 5 episodes of neural apnea were given with each episode separated by 5min of baseline inspired CO_2.

Isolated ventilator apneas (i.e. hypoxia)

Rats experiencing ventilator apneas were ventilated as described above (with baseline parameters recorded) before the mechanical ventilator was turned off for either 6sec or 25sec, depending on the experimental group. This was repeated 5 times with 5min separating each ventilator apneic episode. Reactive increases in respiratory effort (phrenic and XII) were observed during 6sec and 25sec ventilator apnea episodes. A consistent decrease in arterial O_2 (measured by pulse oximeter; 8600V, Nonin Medical Inc., Plymouth, MN, USA) confirmed a consistent desaturation (**Supplementary Figure 1**). Following cessation of hypoxic event, ventilator was turned back on at pre-challenge baseline settings. Each animal naturally corrected their breathing activity and arterial blood gases between hypoxic episodes without any adjustments being made to the ventilator or inspired gases.

Neural apneas with concurrent ventilator apneas

Rats were mechanically ventilated while baseline neural activity was recorded for at least 15min. As described above, inspired CO_2 was decreased to induce a neural apnea. Following a 10sec period of absent phrenic firing activity, the ventilator was turned off for either 6sec or 25sec. The acute hypoxic episode resulted in a natural rescue from neural apnea (approx. 4sec after turning the ventilator off), though the ventilator is kept off for the full 6sec or 25sec duration to maintain a consistent drop in arterial oxygen (confirmed with pulse oximetry). This experience was repeated 5 times, with 5min separating each episode. Blood gases were not tested/adjusted until test bloods were taken 15, 30 and 60min after the final challenge.

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

Solutions were dissolved in DMSO and stored at -20°C for up to 1 week. Prior to injecting, stock solutions were diluted with artificial CSF (aCSF; 120 NaCl, 3 KCl, 2 CaCl, 2 MgCl, 23 $NaHCO_3$, 10 glucose bubbled with 95% O_2/5% CO_2) to a concentration that was less than 1-10 of stock. Solutions were delivered intrathecally near cervical region C3-C5 over a 2 minute period. Vehicle treated control rats received 10-12 µl of a 20% DMSO in aCSF solution (vehicle). Separate groups of time control rats were also treated with each solution or vehicle. There was no relative or absolute drift in nerve activity within time controls for drug injected or vehicle injected rats.

Immunohistochemistry (IHC). Following inactivity, hypoxia or time control studies, rats were euthanized and perfused transcardially with 1mL/g chilled 0.01M PBS followed by 1mL/g chilled

4% paraformaldehyde (PFA) at a pH of 7.4. The spinal cords were harvested and placed into 4% PFA for 8 hours at 4^{O}C. The tissues were then transferred to 20% sucrose followed by 30% sucrose until sinking. 40μm transverse slices were cut using a microtome (SM2000R Leica, Buffalo Grove, IL) from C3-C5 and placed into antifreeze solution (30% glycerol; 30% ethylene glycol; 40% 0.1M PBS). For each group, 6 slices from 3 rats (18 total slices; 36 total images, per group) were selected for staining (slices were at least 200μm apart). Slices were washed with 0.05M tris-buffered saline with 0.1% Triton-X (TBS-Tx) and blocked with 5.0% bovine serum albumin (BSA) for 1hour. The tissues were stained with rabbit anti-phospho-NR2b-NMDAR (Santa Cruz Biotech), goat anti-CtB (Millipore), mouse anti-ALDH (Santa Cruz Biotech), and mouse anti-retinoic acid receptor alpha (Santa Cruz Biotech). 4°C for 16h. Slices were washed with TBS-Tx and subsequently stained with conjugated donkey anti-goat Alexa Fluor 633 (Invitrogen), conjugated donkey anti-rabbit Alexa Fluor 488 (Invitrogen), conjugated donkey anti-mouse Alexa Fluor 595 (Invitrogen) for 2hr at room temperature. Tissue slices were washed a final time with TBS and mounted with coverslip and an anti-fade solution (Invitrogen). Slices were imaged on an epifluorescence confocal microscope at 40x using Nikon EZ-C1 software. The images were localized onto the phrenic nuclei cluster bilaterally via focusing on the ventral region of cervical spinal cords. CtB was used as a retrograde tracer of phrenic motor neurons to confirm location within respective slices as outlined in previous studies (Mantilla et al., 2009).

Data Analysis Neurophysiology. Phrenic and XII burst activity was amplified (x10k), band-pass filtered (0.3-10kHz; AM Systems), integrated (time constant 50ms), and rectified. The resulting signal was digitized and analyzed with PowerLab (AD Instruments; Lab Chart 7.0 software). 60-breath bins were taken immediately prior to blood samples at baseline, 15, 30, and 60min post final neural apnea or hypoxic experience. Nerve burst amplitude was expressed as a percent change from baseline. Statistical differences between groups and individual time points were determined using a two-way repeated measures ANOVA design and Bonferroni post-hoc test.

Groups were considered significantly different if p-values fell < 0.05. Data is shown as means ± SE. All p values are given relative to baseline amplitude and time matched control group nerve amplitude.

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

Results

Concurrent hypoxia undermines inactivity-induced respiratory plasticity

To determine the impact of concurrent reductions in respiratory neural activity and hypoxia on plasticity rats were exposed to reduced respiratory neural activity with and without hypoxia (**Figure 2B**). In accordance with previous studies, 5 brief episodes of neural apnea elicited a long-lasting increase in phrenic inspiratory burst amplitude 54.3 ± 7.8% (i.e. iPMF; $p < 0.001$; **Figure 2Ai**). In a similar fashion, 5 episodes of hypoxia (25sec ventilator apnea) increased phrenic motor amplitude 59.9 ± 4.1% (i.e. pLTF; $p < 0.001$; **Figure 2Aii**). Conversely, 5 episodes of neural apnea with concurrent hypoxia did not elicit a significant change in phrenic motor output (7.7 ± 6.1%; $p = 1.000$; **Figure 2Aiii**).

To determine if hypoxia-induced constraint of iPMF was a dependent on the magnitude of hypoxia or was rather an artifact of the reduced neural apnea duration, we performed a limited “mild” hypoxic experience with a 6sec ventilator apnea (vs above mentioned 25sec

ventilator apnea) that was sufficient to reduce the neural apnea duration without undermining plasticity expression. First we demonstrate that 6sec of hypoxia results in 5 individual episodes of 56mmHg PaO_2, which when given in isolation were not sufficient to elicit a pLTF (-0.9 ± 2.8%; p = 1.000; **Figure 2Ci**). This is consistent with previous reports demonstrating moderate hypoxia (35-45mmHg) is necessary to initiate the signaling processes essential for hypoxia-induced respiratory adaptions (Fuller et a., 2000; **Supplemental Table 2**). While neural apnea with concurrent mild hypoxia (6sec; 56mmHg PaO_2) vs moderate hypoxia (25sec; 38mmHg PaO_2) had a similar effect of reducing neural apnea duration (13-14sec; **Supplemental Table 2**) concurrent mild hypoxia did not disrupt expression of inactivity-induced iPMF (66.6 ± 9.4%; p < 0.001; **Figure 2Cii**). Collectively, these data suggest that signaling mechanisms giving rise to hypoxia-induced plasticity impair iPMF.

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

New synthesis of retinoic acid is necessary for several well-studied models of plasticity induced by activity deprivation (Aoto et al., 2008; Wang et al., 2011), including iPMF (Baetsch and Baker, in review). Within the hippocampus, retinoic acid receptor alpha (RARα) mediates the necessary signaling cascades for plasticity expression (Aoto et al., 2008), but little is known regarding the receptor RA activates in the respiratory control system. Using IHC we confirmed that retinaldehyde dehydrogenase (RALDH; synthesizes active retinoic acid from retinol) and RARα are both present within CtB labeled spinal respiratory motor neurons (**Figure 3A**). Thus, spinal respiratory motor pools have the capacity to both synthesize and respond to local retinoic acid.

To date, there has been no evidence supporting a role for retinoic acid within hypoxia-induced pLTF. To confirm a mechanistic distinction between plasticity induced by activity deprivation (iPMF) and hypoxia (pLTF), DEAB (10μL x 1.0mM), a RALDH inhibitor, was injected

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

Hypoxia enhances activity of spinal NMDARs to constrain inactivity-induced iPMF

Spinal NMDARs are necessary for pLTF (McGuire et al., 2005; 2008; Golder 2010). In contrast, spinal NMDARs constrain retinoic acid dependent plasticity induced by activity deprivation within the hippocampus (Aoto et al., 2008) and spinal cord (Streeter and Baker-Herman 2014). Using cervical (C3-C6) spinal injections of APV (10μL x 100μM), a selective NMDAR inhibitor, we confirmed these respective roles for spinal NMDAR activation in pLTF and iPMF. Consistent with previous work, pretreatment with APV abolished pLTF (2.1 ± 3.1%; p = 1.000; **Figure 4Aiv**). Conversely, intrathecal APV prior to neural apnea did not impact iPMF expression (73 ± 7.6%; p < 0.001 relative to baseline; p = 0.440 relative to inactivity control; **Figure 4Aii**). Thus, spinal NMDARs are necessary for hypoxia-induced pLTF, but spinal NMDARs do not to contribute to, or constrain, iPMF induced by activity deprivation (**Figure 4B**).

While basal activity of spinal NMDARs do not constrain iPMF, hypoxia experiences are known to enhance NMDARs within the hippocampus (Takagi et al., 2003) and spinal cord (McGuire et al., 2005; 2008). We sought to determine if hypoxia-enhanced NMDARs could therefore conditionally constrain plasticity during concurrent neural apnea with hypoxia. Pretreatment with cervical spinal injections of APV prior to neural apnea with hypoxia revealed a persistent enhancement in phrenic motor output (60.9 ± 8.0%; p < 0.001; **Figure 4Cii and 4D**), demonstrating hypoxia-enhanced NMDARs constrain inactivity-induced respiratory plasticity.

The exact mechanism enabling hypoxia's enhancement of NMDARs for pLTF expression has not been determined but NR2B-NMDAR subunits are known to increase their phosphorylation status in response to hypoxia (Takagi et al., 2003). In addition, phosphorylation of NR2B-NMDAR subunits increases calcium influx through NMDARs (Takasu et al., 2002; Paul and Connor; 2010), to enable some forms of plasticity (Xu et al., 2006); suggesting a potential mechanism for hypoxia-induced, NMDAR dependent, pLTF. To assess if these same changes may be occurring within the respiratory control system we stained cervical spinal tissue to identify phosphorylation changes in NR2B-NMDARs. NeuN staining was used to selectively quantify NR2b changes occurring within neuronal cells. We demonstrate that NR2b phosphorylation was significantly increased within NeuN positive cells following intermittent hypoxia ($p < 0.001$; **Figure 5A**). Conversely, activity deprivation (with or without hypoxia) significantly decreased NR2B phosphorylation within NeuN positive cells ($p < 0.001$; **Figure 5A**), but there was no significant difference between these two groups ($p = 0.213$). In addition, there was no difference in the NeuN expression amongst the individual groups (data not shown).

Further, to confirm that these signaling processes were contributing to the observed physiological changes, we used Co 101244 (10μL x 1.0mM) and eliprodil 10μL x 100μM), two NR2B selective NMDAR inhibitors (Bath et al., 1996; Zhou et al., 1999; Gill et al., 2002), to inhibit NR2B containing NMDARs. Pretreatment of either CO101244 or eliprodil prior to neural apnea with concurrent hypoxia rescued respiratory plasticity expression (61.6 ± 5.01% $p < 0.001$ and 63.6 ± 11.2% $p < 0.001$; **Figure 5B**).

Exogenous retinoic acid rescues respiratory plasticity for improved breathing stability

Calcium inhibits RALDH to constrain plasticity expression induced by activity deprivation in the hippocampus (Wang et al., 2011). Since NR2B phosphorylation increases calcium influx

through NMDA receptors (Viviani et al., 2003; Strack and Colbran, 1998), and hypoxia-enhanced NR2B-NMDA receptors constrain iPMF (**Figure 5**), we hypothesized that hypoxia may be undermining inactivity-induced iPMF by inhibiting calcium sensitive RALDH. Therefore, we hypothesized that exogenous retinoic acid would circumvent RALDH inhibition to rescue plasticity during concurrent inactivity with hypoxia.

Cervical spinal injections of trans-retinoic acid (10μL x 50μM) within control rats (without neural apnea or hypoxia) gave rise to enhanced phrenic inspiratory burst amplitude (41.6 ± 5.9%; $p < 0.001$; **Figure 6Aii and Figure 6B**), without a significant change in XII motor output (20.9 ± 9.0%; $p = 1.000$; **Figure 6C**) apneic threshold (-0.2 ± 0.4; $p = 1.000$; **Figure 6D**), or respiratory frequency (**Supplemental Table 1**).

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

Discussion

Continuous, rhythmic firing of the neural network controlling breathing is ensured through intrinsic plasticity mechanisms that detect perturbations and respond through compensatory respiratory behaviors (i.e. respiratory plasticity: Strey et al., 2013; Braegelmann et al., 2017). Despite the existence of these safeguards, periodic breathing disorders exist. Previous investigations into respiratory plasticity have significantly advanced our understanding of the cellular events enabling breathing stabilization, but unfortunately, most of these models have not been able to mimic the pervasive pattern of respiratory neural activity deprivation with hypoxia that characterizes sleep apnea and other periodic breathing disorders. This gap in understanding of the cellular mechanisms underlying unstable breathing patterns (vs respiratory plasticity) has hindered development of targeted pharmacological therapies for periodic breathing disorders.

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

Hypoxia-enhanced spinal NMDARs constrain inactivity-induced iPMF

While enhanced NMDARs are necessary for hypoxia-induced pLTF, NMDARs constrain

some forms of hippocampal (Aoto et al., 2008) and spinal (Streeter and Baker-Herman, 2014) plasticity induced by neuronal activity deprivation. We thus hypothesized that contending roles for NMDARs undermines respiratory plasticity expression during concurrent reductions in respiratory neural activity with hypoxia. Within the present study we demonstrate that hypoxia-enhanced, but not basally active, spinal NMDARs constrain iPMF (**Figure 4**). While this is in contrast to other forms of activity deprivation-induced plasticity in which basal NMDAR activity constrains plasticity expression (Aoto et al., 2008; Steeter and Baker-Herman, 2014), slight differences in induction paradigms may account for the differences in NMDAR sensitivity. For example, within this study we induced iPMF through several bouts of intermittent neural apneas while Streeter and Baker-Herman utilized a single prolonged (60min) neural apnea, similar to the single prolonged (24hr) episode of neural activity deprivation used by Aoto et al., 2008. These different patterns can result in a profound difference in signaling mechanisms, consistent with previous work demonstrating pattern sensitivity for distinct signaling cascades within respiratory (Baertsch and Baker-Herman, 2013; Devinney et al., 2016), as well as non-respiratory plasticity (Sutton and Carew, 2000; Muller and Carew, 1998).

Mechanisms rendering iPMF following intermittent reductions in respiratory neual activity resistant to basal NMDAR activity are not well understood but may involve desensitization of post-synaptic membranes to NMDA activation. Though intermittent neural apnea is not intermittent stimulation per se, the periods separating individual inactivity episodes mimic intermittent stimulation and could lead to similar molecular adaptions. Through negative feedback, intermittent stimulation of NMDARs promotes their removal from the membrane (Joshi et al. 2007; Thiagarajan et al. 2002), decreasing NMDAR mediated calcium influx, effectively mimicking pharmacological inhibition with AP-V. If correct, this hypothesis would suggest that while intermittent activity deprivation may down regulate NMDAR expression to inhibit hypoxia-induced pLTF, hypoxia enhanced activation of spinal NMDARs overcomes this by facilitating remaining NMDARs to suppress necessary retinoic acid synthesis for iPMF

induction (**Figure 6D**). This would theoretically result in bidirectional cross-talk inhibition of iPMF and pLTF pathways during concurrent neural apnea with hypoxia. Further work is still needed to test this hypothesis and to explain the inhibition of pLTF by reduced respiratory neural activity with concurrent hypoxia.

Exogenous application of trans-retinoic acid rescues compensatory respiratory behaviors

Here we demonstrate that iPMF and pLTF can be mechanistically distinguished by their requirement for retinoic acid synthesis, with iPMF requiring new retinoic acid synthesis and pLTF being retinoic acid synthesis independent (**Figure 3**). Further, we have demonstrated that hypoxia-enhanced spinal NMDARs constrain iPMF, likely by inhibiting calcium sensitive RALDH (Wang et al., 2011) within the cervical spinal cord. While NMDAR inhibition rescues respiratory plasticity expression (**Figure 4**), previous failed attempts to treat OSA and CSA with NMDAR antagonists were limited by the psychotropic side effects that are characteristic of NMDAR modulation (Hedner et al., 1996; Torvaldsson et al., 2005). These investigations hypothesized that excess glutamate release in response to hypoxia was leading to neuronal excitotoxicity and secondary breathing instability. While NMDAR inhibition was effective in improving breathing control (Hedner et al., 1996), researchers were not able to confirm a correlation between hypoxia and glutamate levels (Torvaldsson et al., 2005). Conversely, our data suggest that hypoxia-enhanced NMDARs (vs glutamate release) interrupt intrinsic stabilizing mechanisms (vs excitotoxicity) for breathing control. We thus utilized exogenous retinoic acid to bypass NMDAR constraints (without associated psychotropic side effects) and enable compensatory respiratory behaviors during neural apnea with hypoxia.

To our surprise, spinal retinoic acid enabled phrenic motor plasticity, XII motor plasticity, and an apneic threshold reduction (**Figure 6**), without modulating respiratory frequency (**Supplemental Figure**). The absence of respiratory frequency changes suggest intrathecal retinoic acid injections remained local to the spinal cord and did not diffuse to the brainstem

where retinoic acid stimulation of the PBC promotes hyperpneic breathing activity (Guimarães et al., 2007). Further, our IHC results demonstrate that spinal respiratory motor pools have the capacity to synthesize and respond to local retinoic acid synthesis (**Figure 2**) independent of PBC input, providing further evidence that these respiratory behaviors are occurring local to the spinal cord.

While the proposal of a local (spinal) mechanism for stabilizing respiratory motor output contends with the traditional PBC centered dogma of respiratory motor control, others studies have demonstrated that transmission failures from a normally firing PBC can mimic irregular respiratory motor activity. For example, some forms of intermittent hypoxia impair transmission fidelity from the PBC to hypoglossal motor pool, without directly modulating firing rhythm within the PBC (Garcia et al., 2016). Consistent with our data, mechanisms that enhance firing fidelity through plasticity mechanisms local to respiratory motor pools may be one mechanism to improve network connectivity and stabilize breathing control.

Still, the question remains, how can spinal application of retinoic acid affect XII motor output when the XII motor nuclei is located within the brainstem? While an anatomical connection between spinal motor nuclei and brainstem nuclei has not been definitively identified, there is evidence suggesting the existence of a spinal pattern integrator enabling communication between individual respiratory motor pools independent of the PBC (Butler et al., 2014; Rice et al., 2011). Spinal afferent fibers are a likely conduit for communication between individual motor pools as they directly sense changes in motor activity and have the capacity to communicate with brainstem nuclei to modulate breathing activity (Yazawa et al., 2014; Potts et al., 2005). Further work is still needed to understand how spinal networks regulate non-spinal respiratory motor systems, but these observations provide some evidence as to its existence.

Clinical implications for CSA, OSA and other periodic breathing disorders

Previous studies investigating periodic breathing disorders have focused on aberrant PBC activity within the brainstem. We take an alternative perspective and seek to explain the absence of compensatory respiratory behaviors during periodic breathing events. Specifically, we demonstrate that respiratory plasticity that is initiated to correct/prevent reduced respiratory motor output is conditionally constrained during concurrent hypoxia. These experimental observations are of central significance as they begin to explain several important clinical observations that have thus far been poorly understood.

First, patients with mixed/complex sleep apnea receiving CPAP or tracheostomy will continue to have apneas initially upon treatment; however, in many of these patients, central events will spontaneously self-resolve through mechanisms that are not understood (Arzt et al., 2009, Deacon and Catcheside, 2015; Salloum et al., 2010). Secondly, many patients with primary CSA experience a reduction in central events following treatment with inspired oxygen (Chowdhuri et al., 2012). Finally, periodic breathing is common in both full-term and premature infants whom experience transient episodes of mild apnea associated hypoxic events. While many infants receive supplemental oxygen, most infants with mild hypoxic events naturally correct their breathing pattern within 6 months of life, without intervention (Kelly et al., 1985). Collectively, these data are consistent with the hypothesis that (moderate; 35-45mmHg) hypoxia constrains compensatory mechanisms that correct breathing instability, and when hypoxia is resolved, these endogenous mechanisms emerge to restore respiratory stability.

In summary, within sleep apnea, and other periodic breathing disorders, neural apneas lead to acute hypoxic events. Recent clinical observations suggest, and our present study explains, that secondary hypoxia may operate in a feed forward manner to undermine compensatory mechanisms thus propagating subclinical apneic events into periodic breathing disorders. By addressing hypoxemia with CPAP or oxygen therapy, intrinsic compensatory mechanisms will naturally develop to stabilize breathing control. Alternatively, retinoic acid treatment may rescue compensatory signaling mechanism to stabilize breathing control when

hypoxemia cannot be corrected; i.e patients with chronic cardiopulmonary disease. Further work is still needed to confirm the efficacy of retinoic acid treatment in patients with periodic breathing patterns.

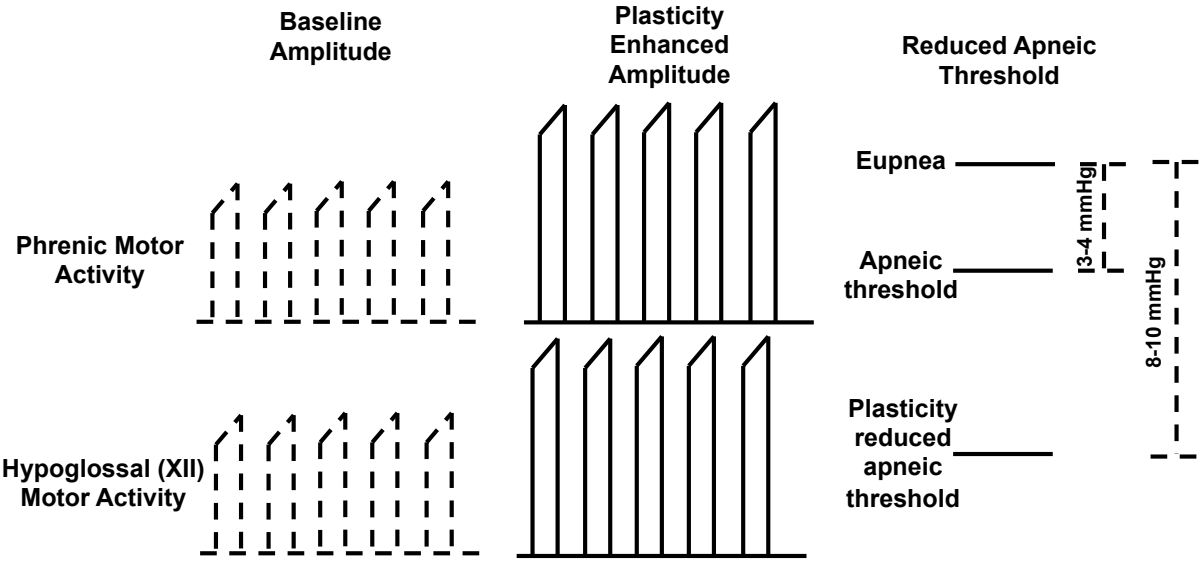

Figure 1: Compensatory respiratory behaviors. Expression of long lasting respiratory plasticity presents in several ways. Three well studied examples are **A)** phrenic motor facilitation for enhanced diaphragm muscle control, **B)** XII motor facilitation for improved upper airway patency, and **C)**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

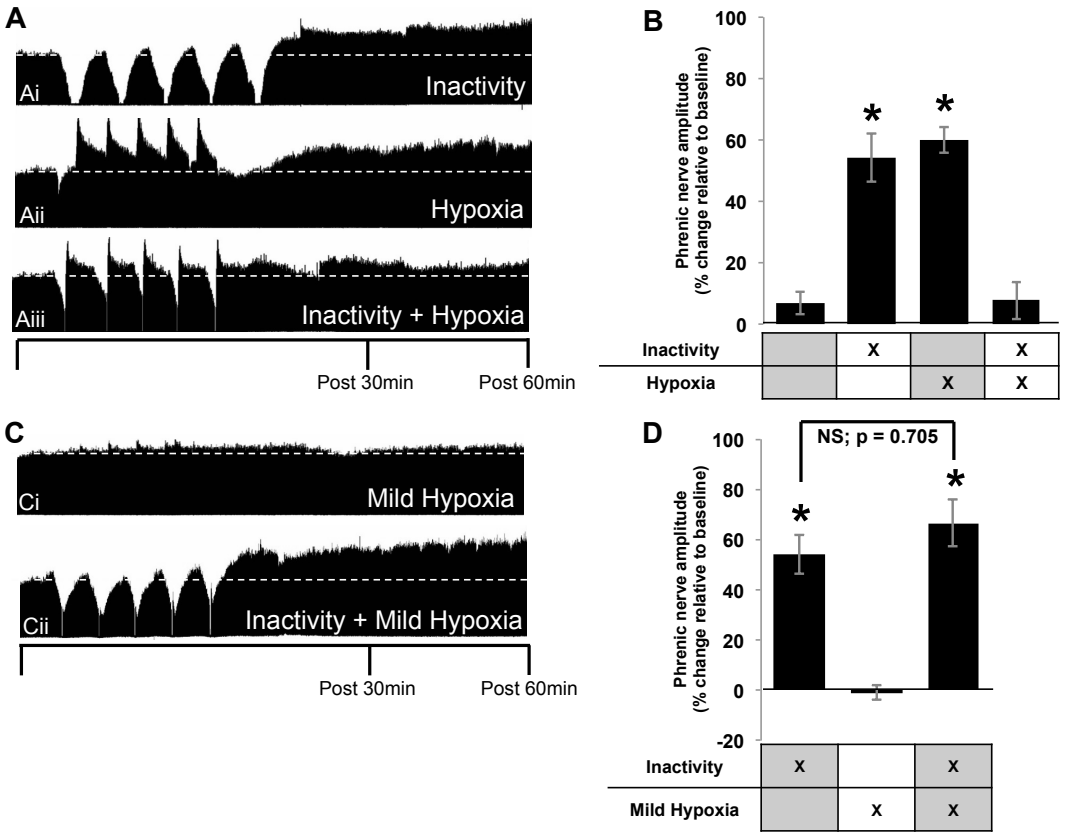

Figure 2: **Concurrent hypoxia undermines inactivity-induced respiratory plasticity.** **A)** Representative phrenic neurogram traces: **i)** neural apnea elicited iPMF, **ii)** hypoxia episodes elicited long-lasting pLTF, **iii)** neural apnea with concurrent hypoxia does not elicit long-lasting plasticity. **B)** Average data. Neural apnea gave rise to a long-lasting enhancement in phrenic motor output (60min: 54.3 ± 7.8%; n = 8; p < 0.001). Hypoxia gave rise to a long-lasting enhancement in phrenic motor output (60min: 59.9 ± 4.1%; n = 7; p < 0.001). Neural apnea with concurrent hypoxia did not significantly affect phrenic motor output (60min: 7.7 ± 6.1%; n = 7; p = 1.000). Combined neural apnea with hypoxia was significantly different from the response when neural apnea and hypoxia were presented in isolation (p < 0.001). **C)** Representative phrenic neurogram traces: **i)** mild hypoxia does not elicit pLTF, **ii)** i neural apnea with concurrent mild hypoxia elicits iPMF. **D)** Average data. Mild hypoxia neither elicits pLTF (60min: -0.9 ± 2.8%; n = 8; p = 1.000) or obstructs expression of inactivity-induced iPMF (60min: 66.6 ± 9.4%; n = 8; p < 0.001). There was no difference in phrenic motor amplitude changes between the neural apnea and neural apnea with mild hypoxia groups (p = 0.705). Data represent mean values ± 1 SEM. Significant differences from baseline and control are denoted with (*). NS denotes non-significant difference p > 0.05.

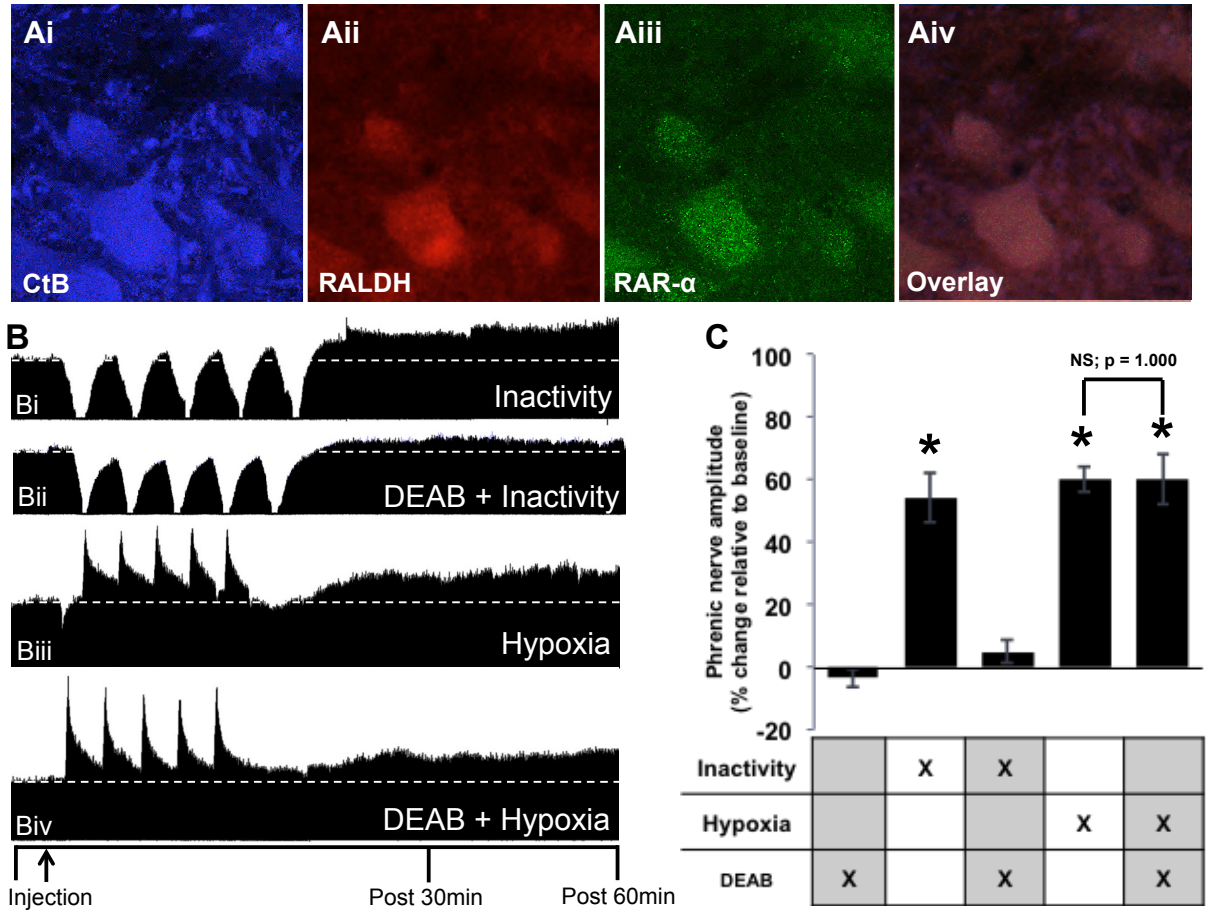

Figure 3: **New retinoic acid synthesis is necessary for inactivity-induced, but not hypoxia-induced, plasticity. A)** Representative immunofluorescence images (100x magnification) identifying RALDH and RARα within CtB labeled cervical spinal phrenic motor neurons. Ai) CtB labeled (blue) phrenic respiratory neurons within the cervical spinal cord. Aii) RALDH (red) within cervical spinal cord tissue. Aiii) RARα (green) within cervical spinal cord tissue. Aiv) Overlay of CtB, RALDH, and RARα within cervical spinal cord tissue. **B)** Representative phrenic neurogram traces: **i)** neural apnea elicits iPMF, **ii)** pretreatment with DEAB, a RALDH inhibitor, prevents iPMF, **iii)** hypoxia elicits pLTF, and **iv)** DEAB pretreatment does not effect hypoxia-induced pLTF. **C)** Summary of DEAB treated groups. DEAB pretreatment abolished iPMF (5.0 ± 3.7%; n = 6; p = 1.000). DEAB pretreated neural apnea group was not statistically different from vehicle treated neural apnea control group ($p < 0.001$). Conversely, DEAB pretreatment did not effect hypoxia induced pLTF (60.1 ± 7.9%; n = 7; $p < 0.001$). Comparing DEAB pretreated hypoxia with the hypoxia control group there was no statistical difference (p = 1.000). Data represent mean values ± 1 SEM. Significant differences from baseline and control are denoted with (*). NS denotes non-significant differences $p > 0.05$.

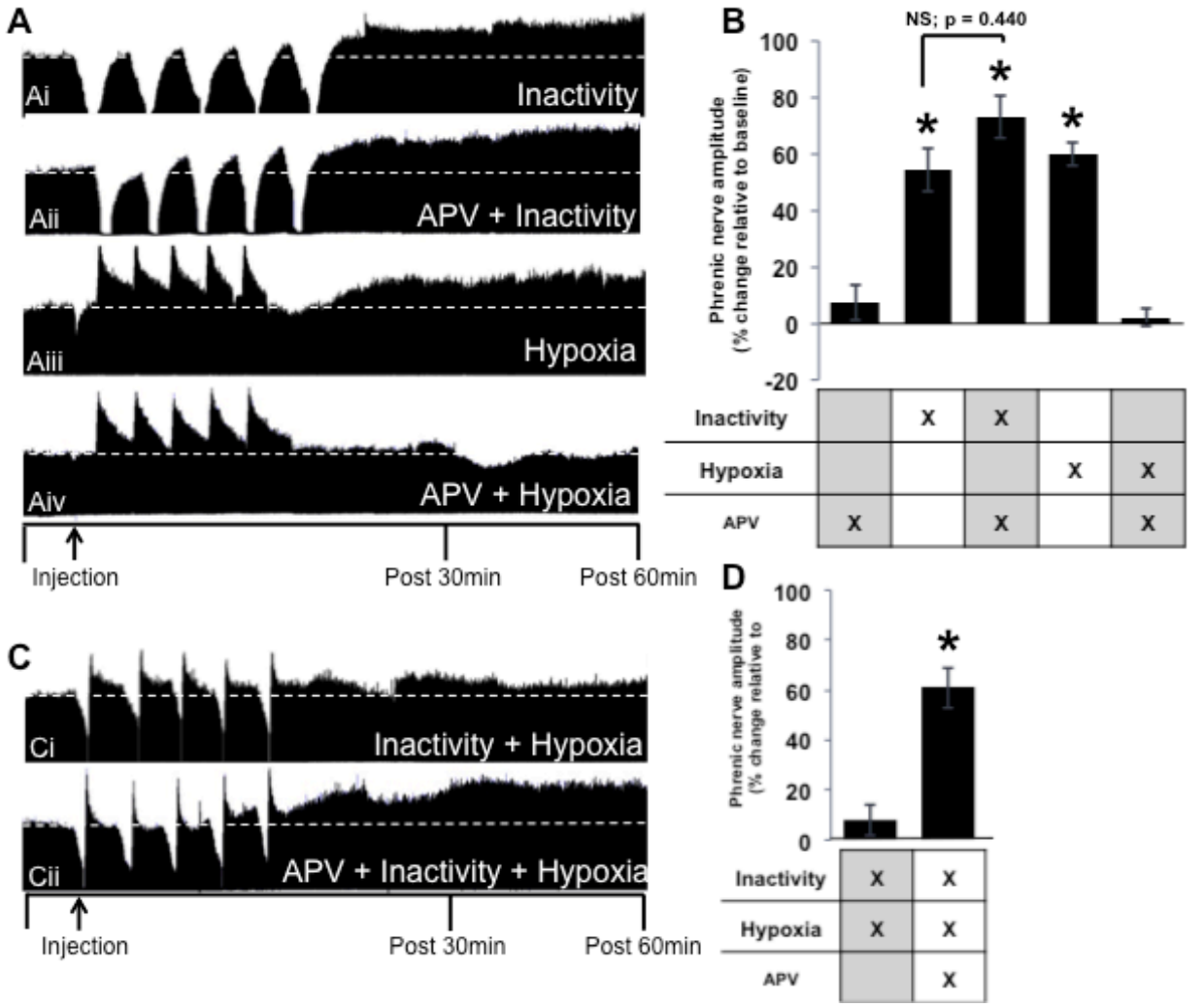

Figure 4: **Hypoxia-enhanced NMDA receptors constrain inactivity-induced iPMF. A)** Representative neurogram traces: **i)** neural apnea elicits iPMF expression, **ii)** Pretreatment with APV, NMDA receptor inhibitor, does not effect iPMF, **iii)** hypoxia elicits pLTF, and **iv)** pretreatment with APV abolishes pLTF. **B)** Summary of APV treated groups. Pretreatment with APV does not effect iPMF (73 ± 7.6%; n = 8; $p < 0.001$). Comparing APV pretreated neural apnea group with vehicle treated neural apnea control group, there was no significant difference ($p = 0.440$). APV pretreated hypoxia animals did not express an enhancement in phrenic motor output (2.1 ± 3.1%; n = 8; $p = 1.000$) Comparing APV pretreated hypoxia group with vehicle treated hypoxia control group, APV pretreatment group was significantly different from hypoxia control ($p = 0.001$). **C)** Representative neurogram traces: **i)** neural apnea with hypoxia abolishes plasticity expression, and **ii)** APV pretreatment rescues plasticity during neural apnea with hypoxia. **D)** Summary of APV treated groups. Pretreatment with APV rescued plasticity expression in animals experiencing neural apnea with hypoxia (60.9 ± 8.0%; $p < 0.001$) Data represent mean values ± 1 SEM. Significant differences from baseline and control are denoted with (*). NS denotes non-significant differences $p > 0.05$.

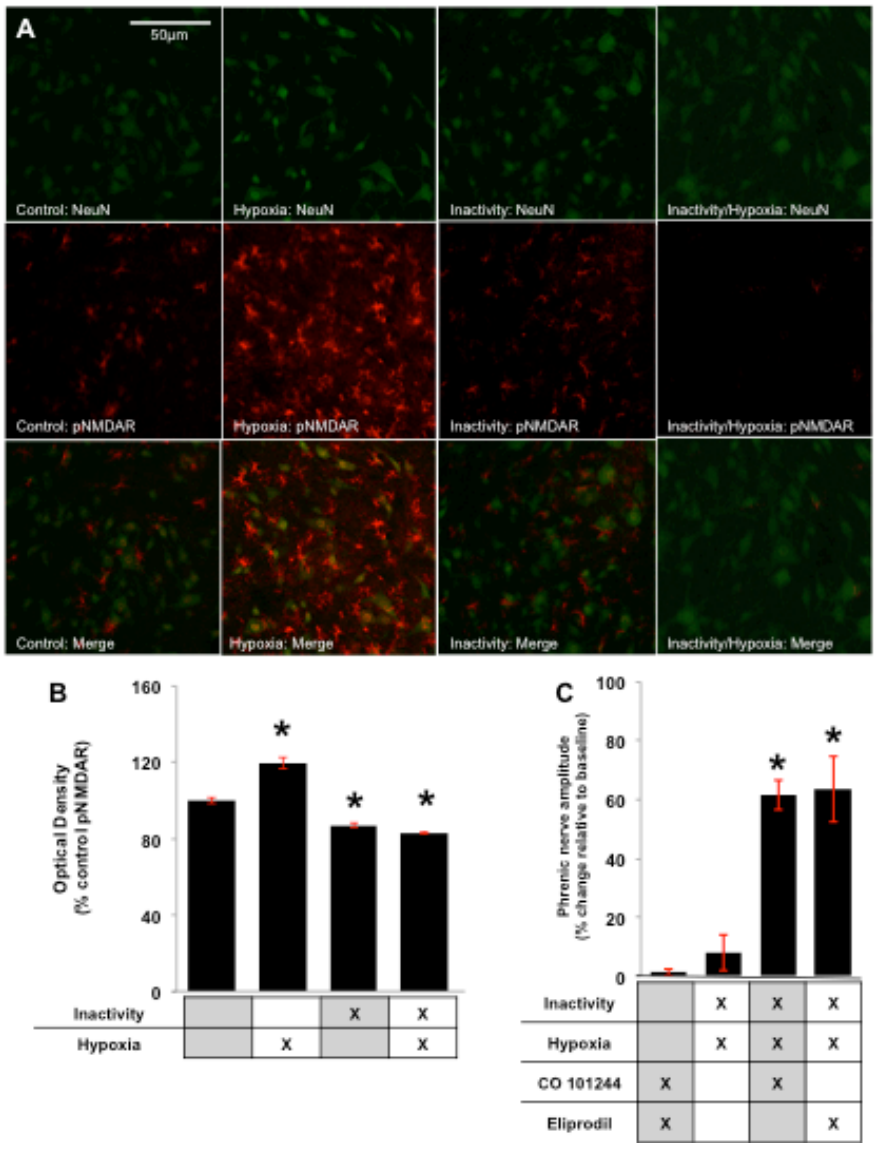

Figure 5: **NR2B modulated NMDA receptors constrain inactivity-induced iPMF. A)** Hypoxia enhanced NR2B phosphorylation of cervical spinal NMDA receptors. Ai) control Neuon, Aii) control pNMDA, Aiii) control NeuN – pNMDA overlay. Aiv) hypoxia NeuN, Av) hypoxia pNMDA, Avi) hypoxia NeuN – pNMDA overaly. Avii) inactivity NeuN, Aviii) inactivity pNMDA, Aix) inactivity NeuN – pNMDA overaly. Ax) inactivity/hypoxia NeuN, Axi) inactivity/hypoxia pNMDA, Axii) inactivity/hypoxia NeuN – pNMDA overlay. **B)** Summary of NR2B data. Pretreatment with selective NR2B inhibitors Co 101244 (61.6 ± 5.01%; n = 5; $p < 0.001$) and eliprodil (63.6 ± 11.2%; n = 5; $p < 0.001$) revealed enhanced phrenic motor output following inactivity with hypoxia. When compared to inactivity with hypoxia control groups, both Co 101244 and eliprodil pretreated groups were significantly different ($p < 0.001$). There was no significant difference between CO 101244 and eliprodil pretreated inactivity with hypoxia groups ($p = 1.000$). Data represent mean values ± 1 SEM. Significant differences from baseline and control are denoted with (*).

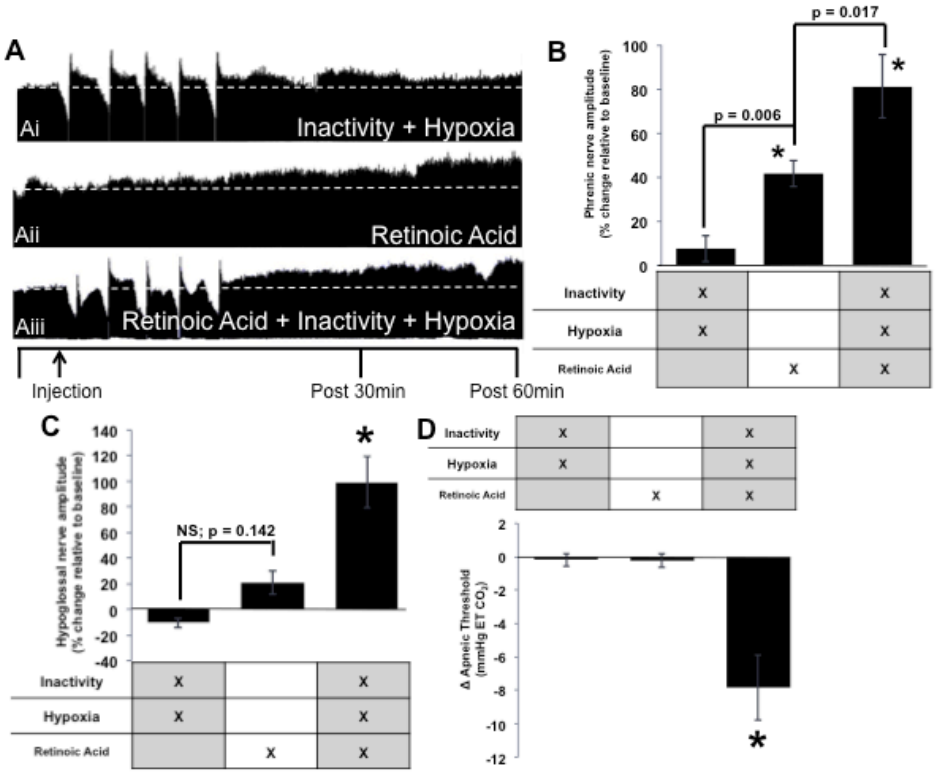

Figure 6: Exogenous retinoic acid reveals phrenic plasticity following reduced respiratory neural activity with concurrent hypoxia. A) Representative phrenic neurogram traces: **i)** neural apnea with hypoxia does not elicit a long-lasting change in phrenic motor output, **ii)** exogenous retinoic acid elicits a progressive enhancement in phrenic motor output, and **iii)** exogenous retinoic acid pretreatment prior to neural apnea with hypoxia revealed enhanced phrenic motor output. **B)** Summary of phrenic retinoic acid data. Retinoic acid enhances phrenic motor output within controls (41.6 ± 5.9%; n = 7; $p < 0.001$) and following neural apnea with hypoxia (81.3 ± 14.4%; n = 6; $p < 0.001$). Phrenic motor output was significantly higher in retinoic acid with neural apnea and hypoxia group relative to retinoic acid controls ($p < 0.001$). **C)** Summary of XII retinoic acid data. Retinoic acid alone does not modulate XII motor output (20.9 ± 9.9%; n = 6; $p = 0.142$), but does enhance XII motor output following neural apnea with hypoxia (99.0 ± 22.0%; n = 6; $p < 0.001$). Summary of apneic threshold retinoic acid data. Retinoic acid alone does not modulate apneic threshold (-0.2 ± 0.4; n = 7; $p = 1.000$), but does reduce apneic threshold following neural apnea with hypoxia (-7.8 ± 1.9mmHg $PaCO_2$; n = 6; $p = 0.007$). Data represent mean values ± 1 SEM. Significant differences from baseline and control are denoted with (*). NS denotes non-significant differences $p > 0.05$.

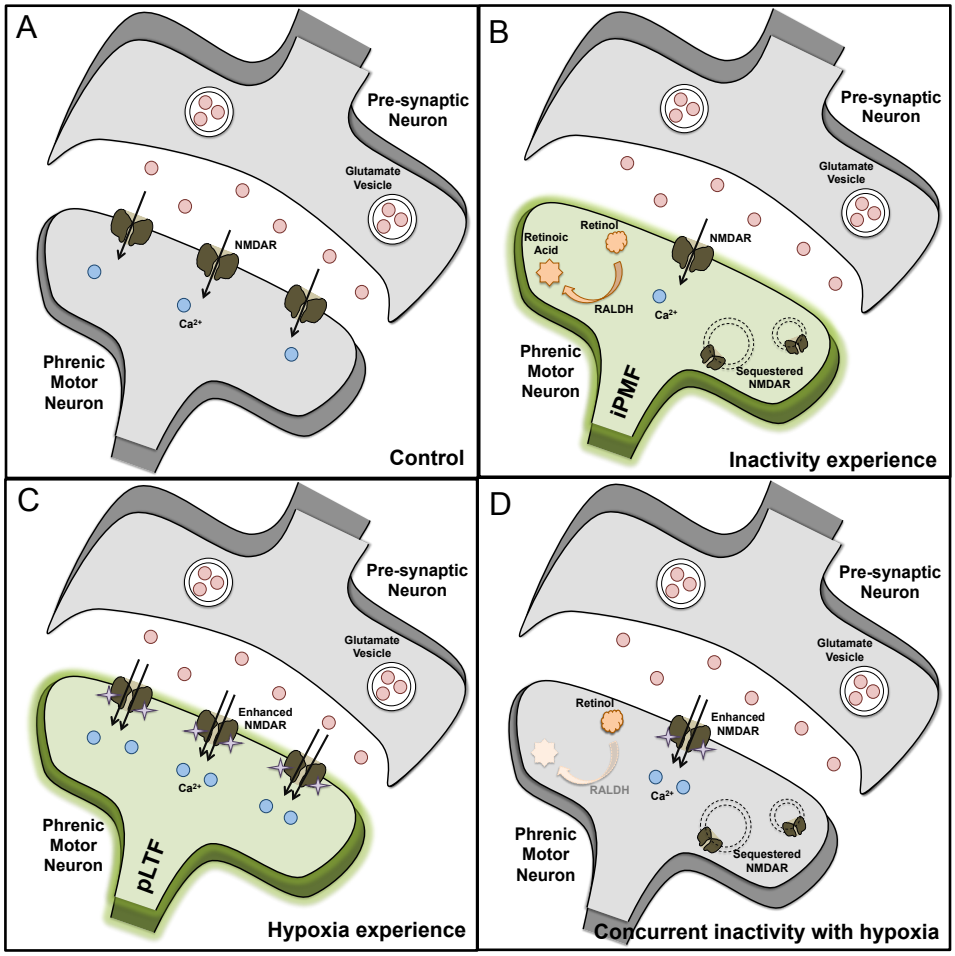

Figure 6: **Proposed signaling mechanism for sleep apnea. A)** Basal phrenic motor activity is dependent on presynaptic glutamate release, driving cation influx through glutamate sensitive channels (NMDA receptors; NMDAR) on phrenic motor neurons. **B)** Following reduced respiratory neural activity, transient suppression of calcium ion influx through decreases NMDAR dependent currents, enabling retinaldehyde dehydrogenase (RALDH) activity to synthesize new retinoic acid from retinol. Through activation of phrenic RARα, plasticity enabling signaling processes are initiated. **C)** Hypoxia-induced phosphorylation of NR2B subunits potentiates NMDA currents to enable pLTF. **D)** Concurrent reductions in respiratory neural activity with hypoxia promotes contending drives to enhance (hypoxia-induced) and blunt (inactivity-induced) NMDAR activity, resulting in a net obstruction of respiratory plasticity.

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

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

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

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

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

Bibliography:

Abbott S.M., and Videnovic A. (2016). Chronic sleep disturbance and neural injury: links to neurodegenerative disease. Nat Sci Sleep 25, 55-61.

Amann M., Blain G.M., Proctor L.T., Sebranek J.J., Pegelow D.F., Dempsey J.A. (2010). Group III and IV muscle afferents contribute to ventilatory and cardiovascular response to rhythmic exercise in humans. J Appl Physiol *109*, 966-976.

Aoto J., Nam C.L., Poon M.M., Ting, P., and Chen L. (2008). Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. Neuron *60*, 308-320.

Bach, K.B., and Mitchell, G.S. (1996). Hypoxia-induced long-term facilitation of respiratory activity is serotonin dependent. Respir Physiol 104, 251-260.

Baertsch N.A., and Baker-Herman T.L. (2013). Inactivity-induced phrenic and hypoglossal motor facilitation are differentially expressed following intermittent vs. sustained neural apnea. J Apply Physiol *114*, 1388-1395.

Baertsch N.A., and Baker-Herman T.L. (2013). Inactivity-induced phrenic and hypoglossal motor facilitation are differentially expressed following intermittent vs. sustained neural apnea. J Appl Physiol *114*, 1388-1395.

Baertsch N.A., and Baker-Herman T.L. (2015). Intermittent reductions in respiratory neural activity elicit spinal TNF-α-independent, atypical PKC-dependent inactivity-induced phrenic motor facilitation. Am J Physiol Regul Integr Comp Physiol *308*, R700-R707.

Baker-Herman T.L., and Mitchell G.S. (2002). Phrenic long-term facilitation requires spinal serotonin receptor activation and protein synthesis. J Neurosci *22*, 6239-6246.

Broytman O., Baertsch N.A., and Baker-Herman, T.L. (2013). Spinal TNFa is necessary for inactivity phrenic motor facilitation. J. Physiol *591*, 5585-98.

Butler J.E., Hudson A.L., and Gandevia S.C. (2014). The neural control of human inspiratory muscles. Prog Brain Res *209*, 295-308.

Chen L., Lau A.G., and Sarti F. (2014). Synaptic retinoic acid signaling and homeostatic synaptic plasticity. Neuropharm *78*, 3-12.

Chowdhuri S., Quan S.F., Almeida F., Ayappa I., Batool-Anwar S., Budhiraja R., Cruse P.E., Drager L.F., Griss B., Marshall N., Patel S.R., Patil S., Knight S.L., Rowley J.A., and Slyman A. (2016). An Official American Thoracic Society Research Statement: Impact of Mild Obstructive Sleep Apnea in Adults. Am J Respir Crit Care Med *193*, e37-54.

Dale, EA, Mabrouk F., and Mitchell G.S. (2014). Unexpected benefits of intermittent hypoxia: enhanced respiratory and nonrespiratory motor function. Physiology (Bethesda) *29*, 39-48.

Devinney M.J., Huxtable A.G., Nichols N.L., and Mitchell G.S. (2013). Hypoxia-induced phrenic long-term facilitation: emergent properties. Ann. N Y Acad. Sci. *1279*, 143-153.

Fields D.P., and Mitchell G.S. (2015). Spinal metaplasticity in respiratory motor control. Front Neural Circuits *11*, 2.

Fuller D.D., Bach K.B., Baker T.L., Kinkead R., and Mitchell G.S. (2000). Long-term facilitation of phrenic motor output. Respir Physiol *121*, 135-146.

Garcia A.J., Zanella S., Dashevskiy T., Khan S.A., Khuu M.A., Prabhakar N.R., and Ramirez J.M. (2016). Chronic Intermittent Hypoxia Alters Local Respiratory Circuit Function at the Level of the preBötzinger Complex. Front Neurosci 10, 4.

Golder F.J. (2008). Spinal NMDA receptor activation is necessary for de novo, but not the maintenance of, A2a receptor-mediated phrenic motor facilitation. J Appl Physiol *107*, 217-223.

Guimarães L., Domínguez-del-Toro E., Chatonnet F., Wrobel L., Pujades C., Monteiro L.S., and Champagnat J. (2007). Exposure to retinoic acid at the onset of hindbrain segmentation induces episodic breathing in mice. Eur J Neurosci *25*, 3526-3536.

Heinzer R., Vat S., Marques-Vidal P., Marti-Soler H., Andries D., Tobback N., Mooser V., Preisig M., Malhotra A., Waeber G., Vollenweider P., Tafti M., Haba-Rubio J. (2015). Prevalence of sleep-disordered breathing in the general population: the HypnoLaus study. The Lancet Respir Med *3*, 310-318.

Hoffman M., and Schulman D.A. (2012). The appearance of central sleep apnea after treatment of obstructive sleep apnea. Chest *142*, 517–522.

Joshi, I., Yang, Y.M., and Wang, L.Y. (2007). Coincident activation of metabotropic glutamate receptors and NMDA receptors (NMDARs) downregulates perisynaptic / extrasynaptic NMDARs and enhances high-fidelity neurotransmission at the developing calyx of Held synapse. J Neurosci *27*, 9989-99.

Leung R.S. (2009). Sleep-disordered breathing: autonomic mechanisms and arrhythmias. Prog Cardiovasc Dis *51*, 324-338.

Mahamed S., and Mitchell G.S. (2008). Simulated apneas induce serotonin-dependent respiratory long-term facilitation in rats. J Physiol *586*, 2171-2181.

Mahamed S., Strey K.A., Mitchell G.S., and Baker-Herman T.L. (2011). Reduced respiratory neural activity elicits phrenic motor facilitation. Respir Physiol Neurobiol 175, 303-309.

McGuire M., Liu C., Cao Y., and Ling L. (2008). Formation and maintenance of ventilatory long-term facilitation require NMDA but not non-NMDA receptors in awake rats. J Appl Physiol *105*, 942-950.

McGuire M., Zhang Y., White D.P. and Ling L. (2005). Phrenic long-term facilitation requires NMDA receptors in the phrenic motonucleus in rats. J Physiol *567*, 599-611.

McKay L.C., and Feldman J.L. (2008). Unilateral ablation of pre-Botzinger complex disrupts breathing during sleep but not wakefulness. Am J Respir Crit Care Med *178*, 89-95.

Mehra R., and Redline S. (2014). Arrhythmia risk associated with sleep disordered breathing in chronic heart failure. Curr Heart Fail Rep *11*, 88-97.

Rice A., Fuglevand A.J., Laine C.M., and Fregosi R.F. (2011). Synchronization of presynaptic input to motor units of tongue, inspiratory intercostal, and diaphragm muscles. J Neurophysiol *105*, 2330-2336.

Streeter K.A., and Baker-Herman T.L. (2014). Spinal NMDA receptor activation constrains inactivity-induced phrenic motor facilitation in Charles River Sprague-Dawley rats. J Appl Physiol *117*, 682-693.

Strey K.A., Baertsch N.A., and Baker-Herman T.L. (2013). Inactivity-induced respiratory plasticity: protecting the drive to breathe in disorders that reduce respiratory neural activity. Respir Phyiol Neurobiol *189*, 384-394.

Strey K.A., Nichols N.L., Baertsch N.A., Broytman O., and Baker-Herman T.L. (2012). Spinal atypical protein kinase C activity is necessary to stabilize inactivity-induced phrenic motor facilitation. J Neurosci *32*, 16510-20.

Thiagarajan, T.C., Piedras-Renteria, E.S., and Tsien, R.W., (2002). Alpha and beta CaMKII: inverse regulation by neuronal activity and opposing effects on synaptic strength. Neuron *36*, 1103-14.

Torvaldsson S., Grote L., Peker Y., Basun H., and Hedner J. (2005). A randomized placebo-controlled trial of an NMDA receptor antagonist in sleep-disordered breathing. J Sleep Res *14*, 149-155.

Valic M., Pecotic R., Pavlinac I., Valic Z., Stipica I., and Dogas Z. (2016). Intermittent hypercapnia-induced phrenic long-term depression is revealed after serotonin receptor blockade with methysergide in anaesthetized rats. Exp Phyiol *101*, 319-331.

Wang H.L., Zhang Z., Hintze M., and Chen L. (2011). Decrease in calcium concentration triggers neuronal retinoic acid synthesis during homeostatic synaptic plasticity. J Neurosci *31*, 17764-17771.

Westhoff M., Arzt M., and Litterst P. (2012). Prevalence and treatment of central sleep apnoea emerging after initiation of continuous positive airway pressure in patients with obstructive sleep apnoea without evidence of heart failure. Sleep Breath *16*, 71-78.

Wheatley J.R., Mezzanotte W.S., Tangel D.J., and White D.P. (1993). Influence of sleep on genioglossus muscle activation by negative pressure in normal men. Am Rev Respir Dis *148*, 597-605.

Wilkinson V., Malhotra A., Nicholas C.L., Worsnop C., Jordan A.S., Butler J.E., Saboisky J.P., Gandevia S.C., White D.P., and Trinder J. (2008). Discharge patterns of human genioglossus motor units during sleep onset. Sleep *31*, 525-533.

Xu F., Plummer M.R., Len G.W., Nakazawa T., Yamamoto T., Black I.B., and Wu K. (2006). Brain-derived neurotrophic factor rapidly increases NMDA receptor channel activity through Fyn-mediated phosphorylation. Brain Res *1121*, 22-34.

Young T., Palta, M., Dempsey, J., Skatrud, J., Weber, S., and Badr, S. (1993). The occurrence of sleep-disordered breathing among middle-aged adults. N Eng J Med *328*, 1230-1235.

Discussion

A hallmark feature of the neural system controlling breathing is its capacity to express long-lasting plasticity; a persistent change in system performance following a previous experience (Mitchell and Johnson, 2003). Plasticity has been identified within peripheral chemoreceptors, rhythm generating nuclei with the brainstem, spinal motor neuron pools, as well as neuromuscular junctions (Fields and Mitchell, 2015; Strey et al., 2013; Dale et al., 2010). With the existence of both anatomical and molecular redundancy within the neural system controlling breathing, the potential for contending signaling pathways to obstruct plasticity expression is readily evident (reviewed in Fields and Mitchell, 2015). Advancing our understanding of how, when, and under what conditions contending signaling pathways regulate plasticity is an important goal as we continue to translate our basic science discoveries into novel therapeutic strategies for treating incurable neurological conditions.

Significance of cross-talk interactions between spinal serotonin receptors

Although 5-HT2 and 5-HT7 -dependent pMF are phenotypically similar, they operate through distinct signaling pathways referred respectively as the Q and S pathways to pMF (Dale-Nagle et al., 2010); these pathways are named for the G proteins most often coupled to their initiating receptors (i.e., Gq for serotonin type 2 and Gs for serotonin type 7 receptors). The Q pathway to pMF consists of: spinal Gq-linked G protein coupled receptors activation (Fuller et al., 2001; Baker-Herman and Mitchell, 2002; MacFarlane et al., 2011), protein kinase C (PKC) activation (Devinney et al., 2015) new synthesis of BDNF (Baker-Herman and Mitchell, 2002; Baker-Herman et al., 2004), TrkB receptors (Baker-Herman et al., 2004; Dale et al., unpublished), and downstream signaling via ERK MAP kinases (Wilkerson and Mitchell, 2009; Hoffman et al., 2012). In contrast, the S pathway to pMF involves: spinal Gs-linked G protein coupled receptors (Hoffman and Mitchell, 2011; Nichols et al., 2012), adenylyl cyclase activation with synthesis of

cyclic AMP, new protein synthesis of an immature TrkB isoform (vs. BDNF; Golder et al., 2008; Hoffman and Mitchell, 2011) and downstream signaling via Akt (vs. ERK; Golder et al., 2008; Hoffman et al., 2012).

The Q and S pathways interact in interesting and complex ways that we have defined as "cross-talk inhibition" (Dale-Nagle et al., 2010; Hoffman et al., 2010; Hoffman and Mitchell, 2013). Our working hypothesis is that manipulations of these cross-talk interactions during intermittent hypoxia training may underlie at least some forms of spinal respiratory metaplasticity. During moderate AIH-induced pLTF, serotonin release activates abundant, high affinity 5-HT2 receptors; driving the dominant Q pathway to pLTF. Although off-target activation of Gs protein linked 5-HT7 receptors is insufficient to trigger the S pathway at these levels of hypoxia (moderate AIH-induced pLTF is exclusively Q pathway dependent plasticity), sub-threshold activation of Gs-linked receptors constrains the Q pathway and reduces pLTF magnitude. Inhibition of spinal 5-HT7 receptors (Hoffman and Mitchell, 2013) during moderate AIH eliminates this cross-talk constraint thereby enhancing moderate AIH-induced pLTF. We suspect that all Gs-linked G protein coupled receptors have a capacity to constrain the Q pathway to pMF since cross-talk inhibition is mediated by protein kinase A (PKA); a prominent effector of downstream Gs protein/cAMP signaling. Whereas spinal PKA inhibition relieves cross-talk inhibition and enhances moderate AIH induced pLTF (to a similar extent as 5-HT7 inhibition; Fields and Mitchell, unpublished results), PKA activation suppresses moderate AIH-induced pLTF (Hoffman and Mitchell, 2013). Since Gs-linked G protein coupled receptors induce S pathway dependent pMF through a mechanistically distinct pathway that requires EPAC, and not PKA activity (Fields et al., 2015), there exists the possibility of selectively activating S pathway plasticity (EPAC dependent) without enabling cross-talk (PKA dependent); thus enabling enhanced pLTF induced plasticity through concurrent contributions from S and Q pathway for pMF. This hypothesis is well supported by unpublished data demonstrating while concurrent PKA activation constrains 5-HT2A induced pMF through cross-talk inhibition,

concurrent EPAC activation additively enhances 5-HT2A induced pMF (Fields and Mitchell, unpublished)

Although considerable effort has been devoted to investigations of detailed cellular mechanisms giving rise to AIH-induced pLTF (Devinney et al., 2013; Dale et al., 2014), relatively little attention has been given to the equally important concept of respiratory metaplasticity; adaptive changes in plasticity expression (i.e. plastic plasticity; Abraham and Bear, 1996). We still lack a fundamental understanding of when, where and how respiratory metaplasticity occurs. Even though metaplasticity confers a remarkable potential to amplify (and harness) existing mechanisms of plasticity, metaplasticity also offers promise to reveal new forms of plasticity not present in normal animals. Examples of the latter include sensory LTF after CIH preconditioning (Peng and Prabhakar, 2003, 2004), and dAIH-revealed, AIH-induced hypoglossal LTF in Brown Norway rats; a strain that does not normally exhibit hypoglossal LTF (Wilkerson and Mitchell, 2009). While hypoglossal LTF and pLTF are phenotypically similar (i.e., enhanced nerve activity following prior experience), the threshold for eliciting each differs based on triggering stimulus and strain/species (reviewed in Golder et al., 2005), strongly suggesting an adaptive change in plasticity capacity/threshold following dAIH training.

With our significant progress in understanding cellular mechanisms of AIH-induced pLTF, it is an advantageous model to study metaplasticity in respiratory motor control. Such studies are warranted from a basic science perspective, but also because rAIH is rapidly moving towards clinical application as a treatment for respiratory insufficiency in disorders that compromise breathing capacity (Lovett-Barr et al., 2012; Nichols et al., 2013) as well as motor deficits in non-respiratory motor systems (Lovett-Barr et al., 2012; Hayes et al., 2014). Principles elucidated by studies of plasticity and metaplasticity in respiratory motor control may be an essential guide for understanding the plasticity, metaplasticity and functional recovery of a diverse range of clinical disorders that compromise movement (Lovett-Barr et al., 2012; Dale et al., 2014; Hayes et al., 2014).

Significance of understanding hypoxia's constraint of iPMF

A common complication of chronic cardiorespiratory disease (CRD) is development of abnormal breathing patterns; including Cheyne-Stokes breathing while awake, and central sleep apnea during sleep. If left untreated, these neural impairments in the drive to breathe can lead to hypoxemia. We hypothesized that respiratory plasticity (i.e. iPMF) normally protects from neural apneas by reactively enhancing respiratory control, thus preventing acute hypoxic episodes. However, patients with CRD exhibit chronic compensated hypoxemia that progresses to acute moderate hypoxia before onset of iPMF mechanisms. Through concurrent initiation of hypoxia-induced and inactivity-induced respiratory plasticity, cross-talk interactions constrain expression of respiratory plasticity; potentially explaining absence of compensatory respiratory behaviors in patients with CRD. Of clinical importance, the relationship between CRD disease and abnormal breathing is cyclic. While CRD is a primary cause of sleep apnea, sleep apnea is an independent risk factor for cardiac dysrhythmia; a precipitating event for myocardial infarctions in patients with CRD (Leung, 2009). Thus, patients with pre-existing CRD are rendered susceptible to neural apneas, but are also at an increased risk of complications from apnea-associated hypoxemia. Therefore, therapeutic advancements for stabilizing breathing control may be an important technique for limiting secondary complications for patients with CRD.

While current treatment options for sleep apnea (i.e. CPAP) prevent dangerous drops in arterial oxygen while asleep, fewer options are available for patients with central sleep apnea. By understanding how concurrent hypoxia undermines the capacity for inactivity-induced iPMF, novel drug targets may be identified for improving breathing control and reducing myocardial infarctions in patients with pre-existing CRD (Leung, 2009). Although the manipulation of NMDARs for therapeutic advantage has lead to poor outcomes in patients with sleep apnea, an understanding of how NMDARs undermine compensatory behaviors has lead to the

identification of retinoic acid as an important stabilizer of respiratory control. Further research is warranted to understand how retinoic acid may benefit other respiratory control disorders.

Future directions

This work presented within this thesis has elucidated some of the unique interpathway interactions within the respiratory control network that dictate the capacity for adaptive change following previous experience; i.e. respiratory motor plasticity. While we have advanced our understanding of serotonin-induced plasticity (chapters II and III), it still remains to be determined if these interpathway regulatory sites also dictate expression of plasticity elicited from other ligands such as dopamine, catecholamines or erythropoietin. All of these ligands operate through GPCR to enhance spinal respiratory motor plasticity, but do they converge on similar downstream signaling cascades as serotonin 5-HT2 and 5-HT7 receptors. A better understanding of inter-molecular, inter-pathway interactions will advance our understanding of how respiratory control network adapt to natural experience that may operate through multiple ligands and thus be dependent on unique interactions that we have not yet studied.

In addition, we have shown that protective respiratory behaviors can be revealed during concurrent inactivity with hypoxia challenges by either blocking spinal NR2B-modified NMDA receptors, or by providing exogenous retinoic acid. Both of these techniques are designed to rescue inactivity-induced plasticity. Yet, our data suggests that constraints are bi-directional with inactivity-induced plasticity also constraining hypoxia-induced plasticity. Potential sites for constraining hypoxia-induced plasticity are speculated to involve the internalization of NMDA membrane receptors (----), but this has not been definitively shown. Future work looking into membrane protein shifting may provide a better understanding of this phenomenon in addition to providing alternative treatment strategies for central and obstructive sleep apnea.

Bibliography

Abraham, W. C., and Bear, M. F. (1996). Metaplasticity: the plasticity of synaptic plasticity. Trends Neurosci. 19, 126–130.

Baker-Herman, T. L., and Mitchell, G. S. (2002). Phrenic long-term facilitation requires spinal serotonin receptor activation and protein synthesis. J. Neurosci. 22, 6239–6246.

Baker-Herman, T. L., Fuller, D. D., Bavis, R. W., Zabka, A. G., Golder, F. J., Doperalski, N. J., et al. (2004). BDNF is necessary and sufficient for spinal respiratory plasticity following intermittent hypoxia. Nat. Neurosci. 7, 48–55.

Dale-Nagle, E. A., Hoffman, M. S., MacFarlane, P. M., Satriotomo, I., Lovett-Barr, M. R., Vinit, S., et al. (2010). Spinal plasticity following intermittent hypoxia: implications for spinal injury. Ann. N Y Acad. Sci. 1198, 252–259.

Dale, EA, Mabrouk F., and Mitchell G.S. (2014). Unexpected benefits of intermittent hypoxia: enhanced respiratory and nonrespiratory motor function. Physiology (Bethesda) *29*, 39-48.

Devinney M.J., Fields D.P., Huxtable A.G., Peterson T.J., Dale E.A., and Mitchell G.S. (2015). Phrenic long-term facilitation requires PKCθ activity within phrenic motor neurons. J Neurosci 35, 8107-8117.

Devinney M.J., Huxtable A.G., Nichols N.L., and Mitchell G.S. (2013). Hypoxia-induced phrenic long-term facilitation: emergent properties. Ann. N Y Acad. Sci. *1279*, 143-153.

Fuller, D. D., Zabka, A. G., Baker, T. L., and Mitchell, G. S. (2001). Phrenic long-term facilitation requires 5-HT receptor activation during but not following episodic hypoxia. J. Appl. Physiol. (1985) 90, 2001–2006.

Golder, F. J., and Mitchell, G. S. (2005). Spinal synaptic enhancement with acute intermittent hypoxia improves respiratory function after chronic cervical spinal cord injury. J. Neurosci. 25, 2925–2932.

Golder, F. J., Ranganathan, L., Satriotomo, I., Hoffman, M., Lovett-Barr, M. R., Watters, J. J., et al. (2008). Spinal adenosine A2a receptor activation elicits long-lasting phrenic motor facilitation. J. Neurosci. 28, 2033–2042.

Hayes, H. B., Jayaraman, A., Herrmann, M., Mitchell, G. S., Rymer, W. Z., and Trumbower, R. D. (2014). Daily intermittent hypoxia enhances walking after chronic spinal cord injury: a randomized trial. Neurology 82, 104–113.

Hoffman, M. S., and Mitchell, G. S. (2011). Spinal 5-HT7 receptor activation induces long-lasting phrenic motor facilitation. J. Physiol. 589, 1397–1407.

Hoffman, M. S., and Mitchell, G. S. (2013). Spinal 5-HT7 receptors and protein kinase A constrain intermittent hypoxia-induced phrenic long-term facilitation. Neuroscience 250, 632–643.

Hoffman, M. S., Golder, F. J., Mahamed, S., and Mitchell, G. S. (2010). Spinal adenosine A2(A) receptor inhibition enhances phrenic long term facilitation following acute intermittent hypoxia. J. Physiol. 588, 255–266.

Leung R.S. (2009). Sleep-disordered breathing: autonomic mechanisms and arrhythmias. Prog Cardiovasc Dis *51*, 324-338.

Lovett-Barr, M. R., Satriotomo, I., Muir, G. D., Wilkerson, J. E., Hoffman, M. S., Vinit, S., et al. (2012). Repetitive intermittent hypoxia induces respiratory and somatic motor recovery after chronic cervical spinal injury. J. Neurosci. 32, 3591–3600.

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

Nichols, N. L., Van Dyke, J., Nashold, L., Satriotomo, I., Suzuki, M., and Mitchell, G. S. (2013). Ventilatory control in ALS. Respir. Physiol. Neurobiol. 189, 429–437.

Peng, Y. J., and Prabhakar, N. R. (2003). Reactive oxygen species in the plasticity of respiratory behavior elicited by chronic intermittent hypoxia. J. Appl. Physiol. (1985) 94, 2342–2349.

Peng, Y. J., and Prabhakar, N. R. (2004). Effect of two paradigms of chronic intermittent hypoxia on carotid body sensory activity. J. Appl. Physiol. (1985) 96, 1236–1242.

Strey K.A., Baertsch N.A., and Baker-Herman T.L. (2013). Inactivity-induced respiratory plasticity: protecting the drive to breathe in disorders that reduce respiratory neural activity. Respir Phyiol Neurobiol *189*, 384-394.

Wilkerson, J. E., and Mitchell, G. S. (2009). Daily intermittent hypoxia augments spinal BDNF levels, ERK phosphorylation and respiratory long-term facilitation. Exp. Neurol. 217, 116–123.