Published in final edited form as:

Respir Physiol Neurobiol. 2011 October 15; 179(1): 48–56. doi:10.1016/j.resp.2011.06.022.

Similarities and differences in mechanisms of phrenic and hypoglossal motor facilitation

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Abstract

Intermittent hypoxia-induced long-term facilitation (LTF) is variably expressed in the motor output of several inspiratory nerves, such as the phrenic and hypoglossal. Compared to phrenic LTF (pLTF), less is known about hypoglossal LTF (hLTF), although it is often assumed that cellular mechanisms are the same. While fundamental mechanisms appear to be similar, potentially important differences exist in the modulation of pLTF and hLTF. The primary objectives of this paper are to: 1) review similarities and differences in pLTF and hLTF, pointing out knowledge gaps, and 2) present new data suggesting that reduced respiratory neural activity elicits differential plasticity in phrenic and hypoglossal output (inactivity-induced phrenic and hypoglossal motor facilitation, iPMF and iHMF), suggesting that these motor pool specific differences are not unique to LTF. Differences in fundamental mechanisms or modulation of plasticity among motor pools may confer the capacity to mount a complex ventilatory response to specific challenges, particularly in motor pools with different “jobs” in the control of breathing.

Keywords

long-term facilitation; inactivity-induced phrenic motor facilitation; hypocapnia; inactivity; phrenic motor facilitation; control of breathing; plasticity; reduced respiratory neural activity; intermittent hypoxia; LTF; hypoglossal; phrenic; iPMF; iHMF

1. Introduction

The respiratory control system coordinates the regular, rhythmic contraction of multiple respiratory muscles to achieve one of the most fundamental of mammalian behaviors: breathing. The networks controlling the respiratory musculature are not static, but adapt neural output to meet the challenges of an ever-changing organism. One mechanism whereby the respiratory control system adapts breathing is through plasticity, a change in system performance based on experience (Mitchell and Johnson, 2003). While plasticity may occur at multiple levels in the neural networks underlying breathing (Mitchell and Johnson, 2003), plasticity within or near respiratory motor pools is a fundamental property of respiratory control.

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The most extensively investigated forms of respiratory motor plasticity are those that elicit long-lasting increases in phrenic burst amplitude. Recently, it has become clear that phenotypically similar increases in phrenic burst amplitude can be elicited by multiple stimuli through distinct cellular pathways (Dale-Nagle et al., 2010a,b; Golder et al., 2008; Hoffman and Mitchell, 2011; MacFarlane et al., 2011; Mahamed et al., 2011; Tadjalli et al., 2010; Zhang et al., 2004). Thus, the term “phrenic motor facilitation” (pMF) has been coined to describe a prolonged increase in phrenic burst amplitude regardless of the inducing stimuli or the evoked cellular pathway (Dale-Nagle et al., 2010a). The most frequently studied form of pMF is phrenic long-term facilitation (pLTF) following acute exposure to intermittent hypoxia (AIH). Rapid progress has been made over the last decade in understanding the mechanisms giving rise to pLTF (see Terada and Mitchell, 2011 and Dale-Nagle et al., 2010a,b for review).

AIH-induced LTF is also expressed in the motor output of other respiratory-related nerves, including the hypoglossal (Bach and Mitchell, 1996), glossopharyngeal (Cao et al., 2010) and intercostal (Fregosi and Mitchell, 1994). In comparison to pLTF, far less is known about mechanisms of LTF in other motor pools, although it is generally assumed that cellular mechanisms are the same (Baker-Herman et al., 2010; Cao et al., 2010; Feldman et al., 2003; Mahamed and Mitchell, 2007; Wilkerson and Mitchell, 2009). However, accumulating evidence suggests that, although LTF in different respiratory motor pools is fundamentally similar, there are also interesting and important mechanistic differences that may be appropriate for their respective physiological roles. For example, LTF in upper airway motor activity (e.g., hypoglossal) is expected to affect upper airway resistance and patency in contrast to LTF of respiratory pump muscle activity (e.g., phrenic and intercostal), which would act to increase inspiratory efforts.

One primary purpose of this review is to compare LTF in a motor pool innervating a respiratory pump muscle (phrenic/diaphragm; Figure 1A) with a motor pool innervating muscles contributing to the regulation of upper airway patency (hypoglossal/tongue; Fuller, 2005; Figure 1B), and to point out gaps in current knowledge. We define phrenic and hypoglossal LTF (pLTF and hLTF, respectively) as increased integrated nerve burst amplitude following AIH because available evidence suggests that: 1) burst amplitude LTF arises from mechanisms operating in or near the respiratory motor pool, whereas respiratory frequency LTF likely arises from mechanisms operating in the brainstem networks generating respiratory rhythm or in afferent pathways leading to those rhythm generating centers (Baker-Herman and Mitchell, 2008; Blitz and Ramirez, 2002; Powell et al., 1998); and 2) profound mechanistic differences can exist between AIH-induced increases in burst amplitude and burst amplitude facilitation elicited by other stimuli, such as pulses of serotonin receptor agonists (Hoffman et al., 2011; MacFarlane et al., 2011), adenosine receptor agonists (Golder et al., 2008), vagal feedback (Tadjalli et al., 2010; Zhang et al., 2003, 2004) or reduced respiratory neural activity (Baker-Herman, 2009; Mahamed et al., 2011). As a general term for burst amplitude plasticity, we follow the lead of Dale-Nagle and colleagues (2010a) by using the terms phrenic motor facilitation (pMF) and hypoglossal motor facilitation (hMF). Only when the motor facilitation is caused by AIH *per se* will we refer to it as long-term facilitation or LTF (Dale-Nagle et al., 2010a).

A second purpose of this review is to discuss a recently described form of plasticity elicited by a prolonged neural apnea: inactivity-induced facilitation (Mahamed et al., 2011). We present limited new data suggesting that burst amplitude facilitation induced by reduced respiratory neural activity differs between phrenic and hypoglossal motor outputs, similar to AIH-induced LTF. Thus, the concept that plasticity in the strength of respiratory motor output is differentially and locally regulated at the level of the motor neuron pool (or motor nucleus) may be a common feature of many forms of respiratory plasticity. An

understanding of mechanistic differences in plasticity among respiratory motor outputs that create a breath is critical to understand the physiological significance of respiratory plasticity. Further, a detailed understanding of differences in the capacity for plasticity in different respiratory motor pools may offer unique opportunities as we attempt to target key molecules to selectively harness endogenous or induced plasticity in specific motor pools as a treatment for diverse ventilatory control disorders.

2. Does LTF reflect mechanisms operating within motor neurons?

Since this special issue is focused on respiratory motor neurons, we will briefly comment on evidence that the mechanisms responsible for pLTF and hLTF occur (at least in part) *within* phrenic and hypoglossal motor neurons, respectively. Although LTF of nerve burst amplitude is frequently hypothesized to reflect mechanisms operating within respiratory motor neurons (Baker-Herman et al., 2010; Feldman et al., 2003; Ling, 2008; Mahamed and Mitchell, 2007; Mitchell et al., 2001), there is no definitive evidence to date that supports or refutes this hypothesis. Local (spinal) mechanisms are certainly important for pLTF since inhibition of key molecules in regions of the cervical spinal cord associated with the phrenic motor nucleus selectively block pLTF, but not hLTF (Baker-Herman and Mitchell, 2002; MacFarlane et al., 2009; McGuire et al., 2005; Mahamed and Mitchell, 2008). Although a similar dissociation of pLTF and hLTF by local, targeted inhibition of key cellular pathways in or near the hypoglossal motor nucleus has not yet been demonstrated, local mechanisms are also likely required for hLTF. Indeed, local mechanisms operating at the level of the hypoglossal motor nucleus are required for hypoglossal motor facilitation (hMF) induced by vagal feedback (Tadjalli et al., 2010).

While available data suggest that mechanisms in or near the respiratory motor pool are key for LTF, the cell types (i.e., glia, interneurons, motor neurons, presynaptic terminals of brainstem neurons providing respiratory drive, etc) in which these mechanisms are occurring is unknown. Mechanisms operating within motor neurons are sufficient to induce prolonged increases in respiratory-related burst amplitude (discussed below; Bocchiaro and Feldman, 2004; Neverova et al., 2007), at least for neonatal hypoglossal motor neurons. However, it is not yet known if these same mechanisms are relevant in adult rats or following AIH.

3. What similarities exist between pLTF and hLTF?

To our knowledge, only three features of LTF are currently known to be similar in the phrenic and hypoglossal motor pools: pattern sensitivity (J.E.R. Wilkerson and G.S. Mitchell, personal communication), 5-HT₂ receptor dependence (Fuller et al., 2001b) and the requirement for reactive oxygen species (ROS; MacFarlane and Mitchell, 2008).

3.1 pLTF and hLTF are pattern sensitive

Similar to other forms of neuroplasticity, pLTF and hLTF are exquisitely sensitive to the pattern of stimulation. Specifically, pLTF (Baker and Mitchell, 2000) and hLTF (J.E.R. Wilkerson and G.S. Mitchell, personal communication) are elicited following intermittent, but not sustained hypoxia. LTF induction is independent of the duration of the hypoxic exposure within a limited range since pLTF and hLTF can be elicited by hypoxic episodes ranging from 15 seconds (Peng and Prabhakar, 2003; Dick et al., 2007; Mahamed and Mitchell, 2008) to 5 minutes (Bach and Mitchell, 1996; Hayashi et al., 1993). Peripheral chemoreceptor feedback is proposed to be the relevant LTF stimulus since intermittent electrical stimulation of the carotid sinus nerve (without hypoxia) elicits a serotonin-dependent increase in inspiratory burst amplitude in multiple respiratory motor pools (Mateika and Fregosi, 1997; Fregosi and Mitchell, 1994; Millhorn et al., 1980). However, additional direct effects of hypoxia are indicated since a modest phrenic facilitation is

elicited in carotid chemoreceptor denervated rats (Bavis and Mitchell, 2003; Sigbigtroth and Mitchell, 2011).

3.2 pLTF and hLTF require 5-HT₂ receptor activation

Intermittent hypoxia stimulates medullary raphe serotonergic neurons (Erickson and Millhorn, 1991; Morris et al., 1996; Teppema et al., 1997), which is then proposed to result in the release of serotonin in relevant respiratory motor neuron pools to elicit LTF (Bach and Mitchell, 1996; Baker-Herman et al., 2010; Fregosi and Mitchell, 1994; Kinkead et al., 2001; Fuller et al., 2001a; Cao et al., 2010). Indeed, serotonin receptor activation is sufficient to elicit prolonged increases in inspiratory burst amplitude without intermittent hypoxia since intermittent (but not sustained) application of serotonin or 5-HT receptor agonists on or near the motor pool elicits pMF (MacFarlane and Mitchell, 2009; MacFarlane et al., 2011) and hMF (Bocchiaro and Feldman, 2004). Although multiple serotonin receptor subtypes elicit increased phrenic burst amplitude (MacFarlane et al., 2011; Hoffman et al., 2011), the 5-HT₂ receptor appears to be necessary for the induction of LTF since 5-HT₂ receptor antagonists block LTF expression in all inspiratory motor pools studied to date (Bach and Mitchell, 1996; Cao et al., 2010; Fuller et al., 2001b).

One unanswered question is: where in the respiratory network are the relevant 5-HT₂ receptors? For pLTF, local (i.e., spinal) 5-HT₂ receptor activation is indicated, since blocking serotonin receptors in regions of the spinal cord encompassing the phrenic motor nucleus abolishes pLTF (Baker-Herman and Mitchell, 2002). While it is clear that hLTF is also 5-HT₂ dependent (Fuller et al., 2001b), experiments have not been done testing the location of the relevant 5-HT₂ receptors *in vivo*. 5-HT₂ receptors on hypoglossal motor neurons may be indicated since prolonged increases in hypoglossal AMPA receptor currents are induced by episodic application of a 5-HT₂ receptor agonist onto synaptically isolated hypoglossal motor neurons in an *in vitro* brainstem slice (Bocchiaro and Feldman, 2004). An important caveat is that these experiments were performed in neonatal rat preparations, and the expression of LTF and serotonin receptors change considerably during development (McGuire and Ling, 2005; Reklung et al., 2000; Volgin et al., 2003; Lovett-Barr et al., 2006). Although these *in vitro* experiments make clear that intermittent serotonin receptor activation on hypoglossal motor neurons is sufficient to elicit a long-lasting hMF, there is little evidence as yet that the same mechanism is operative during AIH-induced LTF in adult rats *in vivo*, or if serotonin receptor activation on other, adjacent cell types (astrocytes, microglia or interneurons) contributes to the full response following AIH.

3.3 pLTF and hLTF require ROS formation

Both pLTF and hLTF require ROS formation since systemic application of a superoxide dismutase mimetic blocks LTF in both motor pools (MacFarlane and Mitchell, 2008). Of interest, hLTF and pLTF are differentially sensitive to inhibition of ROS formation since low doses of the compound abolishes hLTF, but only attenuates pLTF (MacFarlane and Mitchell, 2008). Since spinal injections of the superoxide dismutase mimetic blocks pLTF (MacFarlane and Mitchell, 2008), the requirement for ROS formation is in or near the phrenic motor nucleus. Similar experiments have not been done for hLTF; thus, we cannot conclude that the relevant ROS formation is within the hypoglossal motor nucleus at this time since systemic injections do not differentiate between peripheral or brainstem sites of action. It has been hypothesized that ROS formation is a critical aspect of the pattern sensitivity characteristic of LTF (MacFarlane et al., 2008; Wilkerson et al., 2007), and this feature is discussed below.

Beyond these three similarities in the mechanisms underlying pLTF and hLTF, other aspects have simply not been tested in a rigorous and systematic manner.

4. What is currently known about differences in pLTF and hLTF?

Accumulating evidence suggests that there are important differences in several factors that modulate LTF expression. Understanding these differences in pLTF and hLTF is critical to understand the physiological significance of LTF and may offer unique opportunities to selectively harness plasticity in specific motor pools as a treatment for diverse ventilatory control disorders.

4.1 Differential LTF expression among inspiratory motor pools

LTF magnitude varies widely among different inspiratory motor outputs (Baker-Herman and Mitchell, 2011; Cao et al., 2010; Fregosi and Mitchell, 1994; Fuller et al., 2001a). For example, inspiratory intercostal LTF is 7-fold larger than pLTF under the same experimental conditions in anesthetized cats (Fregosi and Mitchell, 1994). In addition, several anesthetized rat strains (Baker-Herman and Mitchell, 2011) and substrains (Fuller et al., 2001a) express robust pLTF, but have attenuated or absent hLTF. Differences in LTF expression between motor pools may involve differential expression of key molecules involved in the cellular cascade leading to LTF (Baker-Herman et al., 2011; Wilkerson and Mitchell, 2009) due to genetic or epigenetic factors. On the other hand, it is premature to rule out distinct cellular pathway(s) giving rise to (or limiting) LTF in different motor pools.

4.2 Differential effects of age and sex on hLTF and pLTF

Age and sex exert complex effects on hLTF, with lesser impact on pLTF (Behan et al., 2002). For example, in young male rats (3–4 months), both pLTF and hLTF are robust (Zabka et al., 2001a, 2005). By contrast, young female rats only express pLTF, with no evidence for hLTF (Zabka et al., 2001b; 2003). As the rats age, pLTF and hLTF are diminished in males, with particularly large effects on hLTF (Nelson et al., 2011; Zabka et al., 2001a, 2005). By contrast, females increase their ability to express pLTF and hLTF as they reach middle age (Zabka et al., 2001b). In old female rats, the capacity for hLTF is lost, whereas pLTF continues to be expressed (Zabka et al., 2003).

Although mechanisms underlying age/sex interactions in modulating hLTF and (to a lesser extent) pLTF are not understood, Behan and colleagues suggest that these differences result from changes in sex hormone levels (Behan et al., 2002; Zabka et al., 2005). To more fully understand the role of sex hormones in LTF, Behan and colleagues gonadectomized young male rats and found that hLTF was greatly reduced, with little (Zabka et al., 2006) or no (Zabka et al., 2005) effect on pLTF. hLTF in gonadectomized male rats was restored by testosterone supplementation, an effect that was dependent on the conversion of testosterone to estrogen by aromatase (Zabka et al., 2006). Circulating testosterone levels decrease in ageing male rats (Smith et al. 1992, Wu & Gore, 2009, Zabka et al., 2006), and serum testosterone levels correlate with hLTF (but not pLTF; Zabka et al., 2005, 2006); thus, decreased testosterone (and central neural estrogen) levels may underlie the age-related decline in hLTF in male rats (Zabka et al., 2005, 2006; Nelson et al., 2011). This hypothesis is consistent with observations that hLTF is restored by testosterone supplementation in old male rats, with little effect on pLTF (Nelson et al., 2011). Collectively, these studies demonstrate that age and sex have greater impact on hLTF *versus* pLTF, suggesting differential modulation *via* sex hormones.

4.3 Differential regulation of LTF due to sensory afferent feedback

Most studies investigating LTF of inspiratory motor output use preparations in which the nerve from which LTF measurements will be made is transected, and efferent motor outflow is measured from the cut central end of the nerve. However, removal of afferent sensory feedback from the respiratory muscles modulates the capacity for burst amplitude plasticity

(Sandhu et al., 2010), at least in the phrenic nerve. For example, pLTF is attenuated in anesthetized, paralyzed and pump-ventilated rats in which the phrenic nerve has been left intact (Sandhu et al., 2010). These data are consistent with reports that prolonged increases in diaphragm EMG activity following AIH or carotid nerve stimulation is absent (Mateika and Fregosi, 1997; Shkoukani et al., 2002; Tadjalli et al., 2010) or requires additional hypoxic episodes to trigger the response (Ryan and Nolan, 2009; Terada and Mitchell, 2011) in spontaneously breathing animals. Interestingly, spontaneously breathing humans or other animals express robust increases in genioglossus EMG activity following AIH or carotid sinus nerve stimulation (Chowdhuri et al., 2008; Harris et al., 2006; Mateika and Fregosi, 1997; McKay et al., 2004; Ryan and Nolan, 2009; but see Janssen and Fregosi, 2000), even in the absence of increases in diaphragm EMG activity, suggesting that hLTF may not be as sensitive as pLTF to sensory afferent feedback from muscle.

Lung mechanoreceptor feedback *via* the vagus nerve also differentially modulates LTF expression. Most studies investigating LTF in anesthetized animals transect the vagus nerve to prevent entrainment of respiratory neural output with the ventilator; however, accumulating evidence suggests that this procedure may have important effects on LTF expression (Golder and Martinez, 2008; Tadjalli et al., 2010; Zhang et al., 2003, 2004). For example, vagally intact, anesthetized and pump-ventilated rats express robust pLTF, but no hLTF (Golder and Martinez, 2008); following vagotomy, pLTF is diminished, but hLTF is robustly expressed (Golder and Martinez, 2008). These data are consistent with findings that carotid sinus nerve stimulation elicits LTF of upper airway EMG activity in vagotomized, but not vagus-intact cats (Mateika and Fregosi, 1997).

Mechanisms whereby lung mechanoreceptor feedback differentially modulates phrenic *versus* hypoglossal LTF are not understood, but may involve recruitment of other forms of respiratory plasticity. For example, reversible disruption of vagal feedback, either through repeated obstructive apneas or vagal cooling, induces a long-lasting increase in inspiratory genioglossus muscle activity, with no change in diaphragm activity (Tadjalli et al., 2010). This vagally-induced facilitation requires α 1-adrenergic (but not 5-HT₂) receptor activation in the hypoglossal motor nucleus. Interactions between distinct mechanisms of respiratory motor facilitation are an important area for future research since this apparent complexity likely confers a rich array of responses to disturbances and/or challenges to respiratory motor control.

4.4 Lack of pattern sensitivity in inspiratory intercostal LTF?

While both hLTF (J.E.R. Wilkerson and G.S. Mitchell, personal communication) and pLTF (Baker and Mitchell, 2000) are pattern sensitive, it is important to not assume that all respiratory motor pools share the same requirement for intermittent stimulation to induce LTF. For example, whereas serotonin-induced increases in phrenic (Lovett-Barr et al., 2006; MacFarlane and Mitchell, 2009) and hypoglossal (Bocchiaro and Feldman, 2004) burst amplitude require intermittent patterns of serotonin receptor activation, a single, sustained serotonin application is sufficient to elicit increased thoracic nerve motor output in the neonatal rat brainstem/spinal cord preparation (Lovett-Barr et al., 2006). While not conclusive, these data support the possibility that LTF is not pattern sensitive in all respiratory motor pools, and point to the need for further investigation.

5. What known mechanisms of pLTF have not yet been tested in hLTF?

Given emerging evidence that the modulation of pLTF and hLTF differ in important respects, it is not appropriate to assume that the “mechanistic backbone” of inspiratory burst amplitude LTF is completely similar in all motor pools. Although we expect key similarities, there will be great interest in defining differences between different motor pools, since these

differences may have critical impact on physiological/functional significance of the plasticity. Thus, we will briefly review areas investigated with respect to pLTF that have not yet been studied in hLTF.

5.1 Mechanisms downstream from 5-HT₂ receptor activation

Although both pLTF and hLTF require 5-HT₂ receptor activation, mechanisms downstream from 5-HT₂ receptor activation have only been investigated in pLTF. 5-HT₂ receptor activation induces, but does not maintain pLTF (Fuller et al., 2001b). Intermittent hypoxia elicits a serotonin-dependent increase in BDNF synthesis within cervical spinal segments encompassing the phrenic motor nucleus (Baker-Herman and Mitchell, 2002; Baker-Herman et al., 2004); blocking this spinal BDNF synthesis abolishes pLTF (Baker-Herman et al., 2004). Although the pathway from 5-HT₂ receptor activation to BDNF synthesis is not yet known, a prominent role for protein kinase C (PKC) has been proposed (Terada and Mitchell, 2011) since 5-HT₂ receptors are positively coupled to PKC (Leysen, 2004) and increased PKC activity increases BDNF synthesis in other systems (Juric et al., 2008). This hypothesis has not yet been rigorously tested.

Activation of the high affinity BDNF receptor, TrkB, is proposed to be a key step in the cascade to LTF (Baker-Herman et al., 2004). TrkB receptors initiate a wide range of signal transduction cascades, including activation of ERK MAP kinases (Kaplan and Miller, 2000), which are necessary for pLTF (MS Hoffman and GS Mitchell, personal communication). Thus, our working model suggests that BDNF elicits pLTF *via* TrkB receptor-induced activation of ERK MAP kinases (Dale-Nagle et al., 2010a,b; Terada et al., 2011), although the link from TrkB to ERK has not yet been directly tested in this system. The final step(s) giving rise to pLTF is (are) unknown, but is hypothesized reflect an increase in synaptic efficacy between glutamatergic respiratory pre-motor neurons and phrenic motor neurons due to increased trafficking of post-synaptic AMPA and/or NMDA receptors (Mitchell et al., 2001; McGuire et al., 2005; Fuller et al., 2000). Such an effect is supported by the experiments of Bocchiario and Feldman (2004) using the neonatal rat brainstem slice preparation in which hMF was associated with a prolonged increase in hypoglossal AMPA receptor currents following intermittent 5-HT₂ receptor activation in the hypoglossal motor nucleus.

5.2 Regulatory constraints on pLTF

Similar to other forms of neuroplasticity, pLTF is subject to important regulatory constraints, but little is known regarding these pathways in LTF in other respiratory motor pools. Of particular interest, protein phosphatases constrain pLTF during sustained hypoxia (Wilkerson et al., 2007, 2008). Indeed, pretreatment with okadaic acid (a serine-threonine protein phosphatase inhibitor) in the cervical spinal cord reveals a serotonin-dependent pLTF following sustained hypoxia (Wilkerson et al., 2008). In contrast to sustained hypoxia, spinal okadaic acid has no effect on intermittent hypoxia-induced pLTF, indicating that this phosphatase constraint is somehow relieved by intermittent (*versus* sustained) hypoxia (Wilkerson et al., 2008). A specific role for reactive oxygen species (ROS) from NADPH oxidases in relieving this regulatory constraint has been demonstrated (Macfarlane and Mitchell, 2008; Macfarlane et al., 2008, 2009). The stimulus regulating NADPH oxidase activity is currently unknown, but has been suggested to arise from AIH-induced, serotonin-dependent PKC activation (MacFarlane et al., 2009).

An additional constraint to pLTF is posed by cross-talk inhibition from metabotropic receptors coupled to Gs proteins (Hoffman et al., 2010; Terada and Mitchell, 2011). For example, spinal adenosine 2A (A2A) receptor activation elicits pMF through an increase in TrkB (not BDNF) synthesis and Akt (not ERK MAP kinase) activation (Golder et al., 2008).

Since extracellular adenosine levels are increased during hypoxia (Winn et al., 1981, Gourine et al., 2002), Hoffman and colleagues hypothesized that A2A receptor activation contributes to pLTF expression (Hoffman et al., 2010). However, in contrary to this hypothesis, Hoffman and colleagues found that A2A receptor inhibition prior to moderate AIH did not diminish pLTF as expected, but actually enhanced it (Hoffman et al., 2010). Thus, A2A receptor activation during AIH partially dampens pLTF through cross-talk inhibition of the 5-HT₂ receptor-BDNF dependent pathway (Terada and Mitchell, 2011). Similar results have been demonstrated for other metabotropic receptors coupled to Gs proteins, such as 5-HT₇ receptors (Hoffman and Mitchell, 2008; Hoffman et al., 2011; Terada and Mitchell, 2011). Precise interactions between A2A (and/or 5-HT₇) and 5-HT₂ receptor pathways may be influenced by the severity of the hypoxic exposure (Terada and Mitchell, 2011) or integrity of sensory inputs (Sigbigtroth and Mitchell, 2011), but this issue has not been completely explored.

5.3 Activation of hLTF via other receptor systems

One potential contributor to hLTF that has not been adequately explored in pLTF is the potential role of α 1-adrenergic receptors. For example, hLTF is abolished following α 1-adrenergic receptor inhibition (Neverova et al., 2007), suggesting a key requirement for norepinephrine in the mechanisms underlying hLTF. Norepinephrine is implicated in other forms of hMF that are independent of AIH (Tadjalli et al., 2010), suggesting that key pathways leading to hLTF may overlap in multiple forms of hypoglossal burst amplitude plasticity. Mechanisms whereby norepinephrine contributes to hLTF are unknown. Alpha1-adrenergic receptors on hypoglossal motor neurons are indicated since prolonged increases in AMPA receptor currents are induced by intermittent application of an α 1-adrenergic receptor agonist to synaptically isolated hypoglossal motor neurons in an *in vitro* brainstem slice preparation (Neverova et al., 2007). While these data indicate that norepinephrine is capable of eliciting hMF *via* increased trafficking of AMPA receptors within hypoglossal motor neurons, there is little evidence that these mechanisms are relevant to hLTF *in vivo* and do not rule out a role for other cell types in the full response. Although local application of an α 1-adrenergic receptor agonist is sufficient to elicit pMF in adult rats (Dale-Nagle et al., 2010a), the role of norepinephrine in AIH-induced pLTF has not been explored.

6. What are the gaps in knowledge regarding mechanisms of LTF?

Despite the rapid progress, there are important gaps in our knowledge of the mechanisms giving rise to pLTF. For example, what cellular pathways convert 5-HT₂ receptor activation into BDNF synthesis? How does BDNF-induced TrkB receptor activation enhance phrenic burst amplitude? How do protein phosphatases constrain pLTF following sustained hypoxia? How does sensory feedback influence pLTF?

There are even more gaps in our knowledge concerning LTF in other respiratory motor pools, where there is little information concerning any aspect of the underlying mechanisms. One key question is: can pLTF be used as a model for LTF in other motor outputs? Do differences in LTF expression reflect differential modulation/regulation of the same cellular mechanistic “backbone” common to all respiratory motor pools? In order to address these questions, it is essential to determine the respective roles of different neurotransmitter/modulator systems (e.g., adenosine, norepinephrine, serotonin, NMDA), local BDNF synthesis and ERK MAP kinase activation in LTF expressed in other (non-phrenic) respiratory motor outputs. It is also important to understand differences in regulatory constraints (e.g., sensory feedback, protein phosphatases, ROS formation, cross talk inhibition) imposed on LTF in different respiratory motor pools.

Aside from these detailed mechanistic questions, major unexplored areas of research with respect to inspiratory burst amplitude LTF include: 1) what cell types contribute to AIH-induced LTF (i.e., is there a role for cells other than motor neurons)? 2) what cellular/synaptic mechanisms create the facilitation (e.g., glutamate receptor insertion, changes in membrane excitability, downregulation of inhibitory inputs, increased presynaptic neurotransmitter release)? 3) what mechanisms give rise to cross-talk inhibition from different pathways leading to inspiratory burst amplitude facilitation and when are these alternate pathways expressed? 4) how do the multiple pathways to inspiratory motor facilitation interact to create and adapt breathing to existing physiological conditions?

7. Inactivity induced phrenic and hypoglossal motor facilitation

It is now clear that multiple stimuli elicit phenotypically similar increases in phrenic and/or hypoglossal burst amplitude through pathways distinct from the “classic” model of inspiratory burst amplitude LTF (Dale-Nagle et al., 2010a; Golder et al., 2008; Hoffman and Mitchell, 2011; MacFarlane et al., 2011; Mahamed et al., 2011; Mitchell and Terada, 2011; Tadjalli et al., 2011; Zhang et al., 2003, 2004). We recently reported that a prolonged (25–30 min) neural apnea in anesthetized, pump-ventilated rats elicits a novel form of spinal plasticity known as inactivity-induced phrenic motor facilitation (iPMF; Mahamed et al., 2011). Specifically, iPMF is a “rebound” increase in phrenic burst amplitude observed following restoration of phrenic activity (figure 1C). iPMF is elicited by inducing a neural apnea by either: 1) lowering arterial CO₂ levels below the apneic threshold (hypocapnia), 2) high frequency ventilation to elicit a vagally mediated reflex inhibition of breathing, or 3) use of a respiratory depressant, such as isoflurane (Mahamed et al., 2011). Since these methods create a neural apnea by different means, yet all three methods elicit similar iPMF, we suggested that iPMF is likely due to reduced respiratory neural activity (*versus* hypocapnia, vagal input or isoflurane *per se*). Here we present data showing the impact of a neural apnea on hypoglossal motor output. These new data demonstrate yet again, that there can be differential expression of respiratory plasticity among respiratory motor pools.

To determine if reduced respiratory neural activity elicits hypoglossal motor facilitation, we created a neural apnea (with modest hypocapnia) in urethane-anesthetized, vagotomized, paralyzed and ventilated Harlan Sprague-Dawley rats (colony 217). Basic procedures have been described elsewhere (Mahamed et al., 2011). Baseline hypoglossal nerve activity was established with PaCO₂ ~47 mmHg. To reduce respiratory neural activity, rats were hyperventilated to lower PaCO₂ below the CO₂ apneic threshold for breathing (PaCO₂ ~37 mmHg) for 30 minutes. Rats were then returned to baseline PaCO₂ conditions, and hypoglossal motor output was monitored for 1 hour. To control for non-specific effects of surgery and recording duration on hypoglossal nerve activity, hypoglossal motor output was monitored for a similar duration in time control rats not exposed to a neural apnea. Blood gases were measured before, during, and 5, 15, 30 and 60 min following restoration of respiratory neural activity to ensure that PaCO₂ was within 1.5 mmHg of baseline values. hypoglossal burst amplitude following restoration of hypoglossal neural activity was expressed as a percentage change from baseline.

A hypocapnia-induced neural apnea elicited only a transient facilitation of hypoglossal motor output once respiratory neural activity was restored, much in contrast to the more prolonged effects observed in the phrenic nerve (Figures 1C, 1D and 2). hypoglossal burst amplitude at 5 and 15 minutes (46 ± 11% and 40 ± 11% baseline, respectively) post-restoration of respiratory neural activity was significantly increased *versus* time control rats at the same time points (−13 ± 7% and −14 ± 8% baseline, respectively, *p* < 0.05), indicating inactivity-induced hypoglossal motor facilitation (iHMF). iHMF was transient since there were no longer significant differences between treated (30 ± 12% and 25 ± 14% baseline, at

30 and 60 min post-neural apnea, respectively) and time control ($-1 \pm 9\%$ and $1 \pm 15\%$ baseline, respectively; $p > 0.05$) rats at 30 and 60 min following restoration of respiratory neural activity. Thus, a prolonged (30 min) neural apnea (without hypoxia) elicits a transient (~ 15 min) iHMF. These data contrast with the phrenic, which exhibits prolonged (> 60 min) facilitation of burst amplitude in similar experiments (iPMF at 60 min: $92 \pm 22\%$ baseline; Mahamed et al., 2011).

Mechanisms giving rise to increased respiratory motor output following a neural apnea are not yet understood, although preliminary investigations from our laboratory suggest that iPMF requires spinal tumor necrosis factor- α (TNF α ; Brody et al., 2010) and atypical PKC activity (Baker-Herman, 2009). We are now actively investigating the basis for differential expression of inactivity induced motor facilitation in phrenic *versus* hypoglossal motor output.

8. What is the physiological significance of differential regulation of phrenic and hypoglossal motor facilitation?

The physiological significance of any form of respiratory plasticity remains to be fully elucidated. However, it is hypothesized that plasticity allows the respiratory control system to compensate for physiological and/or pathophysiological changes that perturb ventilation, including growth, development, aging, pregnancy, ascent to altitude or early onset of lung/neural disease (Mitchell et al., 2001). In recent years it has become clear that respiratory plasticity does not consist of a single mechanism induced by a lone stimulus; indeed, there is a bewildering array of stimuli and cellular pathways capable of eliciting (or limiting) plasticity in the neural network controlling breathing (Baker et al., 2001; Baker-Herman et al., 2004; Bach and Mitchell, 1996; Behan et al., 2002; Blitz and Ramirez, 2002; Dale-Nagle et al., 2011; Dick et al., 2007; Golder et al., 2008; Hoffman and Mitchell, 2011; Janssen and Fregosi, 2000; Kinkead et al., 2001; Kline, 2008; Ling et al., 2001; MacFarlane et al., 2011; Mahamed et al., 2011; Millhorn et al., 1980; Neverova et al., 2007; Peng et al., 2003; Ryan and Nolan, 2009; Sandhu et al., 2010; Tadjalli et al., 2010; Wilkerson et al., 2008; Zhang et al., 2003). This rich array of diverse mechanisms and regulatory processes may impart precision and specificity to ventilatory responses in specific circumstances, thereby allowing the respiratory control system to “fine tune” changes in system performance *via* plasticity in discrete respiratory motor outputs (while limiting plasticity in other motor outputs). For example, the pattern of plasticity necessary to adapt respiratory output to mechanical restrictions of late pregnancy would be different than those associated with overcoming a narrow airway. Understanding similarities and differences in mechanisms of plasticity in different respiratory motor pools may yield novel insights into the physiological role that plasticity plays in the control of breathing.

Summary

Intermittent hypoxia-induced LTF is variably expressed in the motor output of several inspiratory nerves (Fuller et al., 2001a, Baker-Herman et al., 2011), but most frequently studied are pLTF and hLTF. Compared to pLTF, far less is known about mechanisms of hLTF (or mechanisms of LTF in other inspiratory nerves). For the most part, LTF is phenotypically similar in phrenic and hypoglossal motor output, leading to the assumption that similar mechanisms give rise to LTF in these motor pools. However, this assumption has not yet been adequately tested and evidence is accumulating to suggest that while fundamental mechanisms are similar, pLTF and hLTF are differentially modulated. For example: 1) LTF magnitude varies in different inspiratory motor pools (Baker-Herman and Mitchell, 2011; Cao et al., 2010; Fregosi and Mitchell, 1994; Fuller et al., 2001a; Kinkead and Mitchell, 1999; Mateika and Fregosi, 1997; Ryan and Nolan, 2009; Wilkerson and

Mitchell, 2009), with some expressing robust LTF, while others express minimal LTF; 2) aging and sex hormones have a greater impact on hLTF *versus* pLTF (Behan et al., 2002; Nelson et al., 2011; Zabka et al., 2001a,b, 2003a,b, 2005); and 3) sensory afferent feedback differentially influences hLTF and pLTF (Golder and Martinez, 2008; Mateika and Fregosi, 1997; Sandhu et al., 2010). Further, although both pLTF and hLTF require intermittent *versus* sustained hypoxia, there is suggestive evidence that all inspiratory motor pools do not require intermittent stimulation to induce LTF (Lovett-Barr et al., 2006).

Although far less is known about plasticity induced by prolonged neural apnea, early evidence demonstrates that this unique form of respiratory plasticity is also differentially expressed in phrenic and hypoglossal motor output. Specifically, hypocapnia-induced neural apnea elicits a long-lasting (>60 min) iPMF, whereas the same stimulus elicits relatively brief iHMF (~15 min). Mechanisms underlying this differential expression of inactivity-induced plasticity are unknown, but are under active investigation in our laboratory.

Since it is now apparent that multiple, distinct cellular pathways elicit phenotypically similar facilitation (Dale-Nagle et al., 2010a; Golder et al., 2008; Hoffman and Mitchell, 2011; MacFarlane et al., 2011; Mahamed et al., 2011; Tadjalli et al., 2010; Zhang et al., 2004), it is important not to assume that phenotypic similarity demonstrates similar mechanisms. Understanding the mechanisms and/or regulation of plasticity unique to specific motor pools involved in sculpting a breath is key to understanding the full physiological significance of respiratory motor plasticity. Differences in the manifestations of plasticity in respiratory motor pools may confer the capacity to mount a complex ventilatory response to specific physiological or pathophysiological challenges, particularly in motor pools that make substantially different contributions to the overall regulation of ventilation.

Acknowledgments

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

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Figure 1.

Intermittent hypoxia and a prolonged, hypocapnia-induced neural apnea elicit different forms of phrenic and hypoglossal motor plasticity. (A,B) Representative compressed integrated phrenic (A) and hypoglossal (B) neurograms measured before, during and for 60 minutes after 3, 5 min episodes of hypoxia (11% O₂), separated by a 5 min return to baseline conditions. A phenotypically similar LTF is present in both phrenic (pLTF) and hypoglossal (hLTF) motor output, with a progressive increase in burst amplitude from baseline levels that develops slowly over the course of an hour following intermittent hypoxia exposure. (C,D) Representative compressed integrated phrenic (C) and hypoglossal (D) neurograms measured before, during and for 60 minutes after a 30 min neural apnea in pump-ventilated rats. Upon restoration of respiratory neural activity, phrenic burst amplitude is immediately elevated above baseline, and remains above baseline levels for at least one hour, indicating a long-lasting inactivity-induced phrenic motor facilitation (iPMF). By contrast, hypoglossal burst amplitude is transiently elevated above baseline levels upon restoration of respiratory neural activity, indicating that inactivity-induced hypoglossal motor facilitation (iHMF) is only briefly expressed.

Figure 2.

Reduced respiratory neural activity elicits a transient increase in hypoglossal burst amplitude when respiratory neural activity is restored. Average change in hypoglossal burst amplitude from baseline for 60 min following the resumption of respiratory neural activity in rats exposed to a neural apnea (●) or in time control rats (■) exposed to the same surgical preparation, but not receiving reduced respiratory neural activity. In contrast to iPMF (Mahamed et al., 2011), iHMF is expressed only for 15 min following the resumption of respiratory neural activity. * indicates significant increase over time controls ($p < 0.05$).

Table 1

Summary of some of the similarities, differences and knowledge gaps in phrenic and hypoglossal LTF.

	pLTF	hLTF	Reference
Pattern sensitive	Yes	Yes	Baker and Mitchell, 2000; JER Wilkerson and GS Mitchell, personal communication
Serotonin-dependent	Yes	Yes	Bach and Mitchell, 1996; Fuller et al., 2001a
Requires local 5-HT ₂ receptor activation	Yes	Unknown	Baker-Herman and Mitchell, 2002
Requires local BDNF synthesis	Yes	Unknown	Baker-Herman et al., 2004
Requires local NMDA receptor activation	Yes	Unknown	McGuire et al., 2005
Requires local ROS formation from NADPH oxidases	Yes	Unknown	MacFarlane and Mitchell, 2008; MacFarlane et al., 2009
Requires local ERK MAP kinase activation	Yes	Unknown	MS Hoffman and GS Mitchell, personal communication; Nicols and Mitchell, 2009
Regulated by protein phosphatases	Yes	Unknown	Wilkerson et al., 2008
Requires α -1 adrenergic receptor activation	Unknown	Yes	Neverova et al., 2007
Influenced by sex hormones	Modest effects	Yes	Zabka et al., 2001b, 2005, 2006
Influenced by gender	Modest effects	Yes	Zabka et al., 2001a,b, 2003
Influenced by age	Modest effects	Yes	Zabka et al., 2001a,b, 2003, 2005
Influenced by vagotomy	Yes/increased	Yes/decreased	Golder and Mitchell, 2008
Influenced by orexin	Yes	Unknown	Toyama et al., 2009
Influenced by afferent feedback from muscle	Yes	Unknown	Sandhu et al., 2010
Subject to local cross-talk inhibition by alternative cellular pathways	Yes	Unknown	Hoffman et al., 2010