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Experimental protocols and preparations to study respiratory long term facilitation

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Abstract

Respiratory long-term facilitation is a form of neuronal plasticity that is induced following exposure to intermittent hypoxia. Long-term facilitation is characterized by a progressive increase in respiratory motor output during normoxic periods that separate hypoxic episodes and by a sustained elevation in respiratory activity for up to 90 min after exposure to intermittent hypoxia. This phenomenon is associated with increases in phrenic, hypoglossal or carotid sinus nerve inspiratory-modulated discharge. The examination of long-term facilitation has been steadily ongoing for approximately 3 decades. During this period of time a variety of animal models (e.g. cats, rats and humans), experimental preparations and intermittent hypoxia protocols have been used to study long-term facilitation. This review is designed to summarize the strengths and weaknesses of the models, preparations and protocols that have been used to study LTF over the past 30 years. The review is divided into two primary sections. Initially, the models and protocols used to study LTF in animals other than humans will be discussed, followed by a section specifically focused on human studies. Each section will begin with a discussion of various factors that must be considered when selecting an experimental preparation and intermittent hypoxia protocol to examine LTF. Model and protocol design recommendations will follow, with the goal of presenting a prevailing model and protocol that will ultimately ensure standardized comparisons across studies.

Keywords

intermittent hypoxia; humans; other animals; ventilation; phrenic nerve

1. Introduction

Respiratory long-term facilitation (LTF) is a form of neuronal plasticity that is induced following exposure to intermittent hypoxia (Mateika & Narwani, 2009), although other stimuli might initiate the phenomenon (see *Selecting a stimulus to initiate LTF*). Long-term facilitation is characterized by a progressive increase in respiratory motor output during normoxic periods that separate hypoxic episodes and by a sustained elevation in respiratory

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activity for up to 90 min after exposure to intermittent hypoxia (Mitchell *et al.*, 2001a). This phenomenon is associated with increases in phrenic, hypoglossal or carotid sinus nerve inspiratory-modulated discharge (Peng *et al.*, 2003;Mahamed & Mitchell, 2007).

The examination of LTF has been steadily ongoing for approximately 3 decades. As a service to the readers of this review a comprehensive summary of the pertinent findings from papers published over this time period has been compiled in an Excel spreadsheet and included with this paper as a supplementary file. This summary is the foundation of our review which is designed to summarize the strengths and weaknesses of the models and protocols that have been used to study LTF. The review is divided into two primary sections. Initially, the models and protocols used to study LTF in animals other than humans will be discussed, followed by a section specifically focused on human studies. Each section will begin with a discussion of various factors that must be considered when selecting an experimental preparation and intermittent hypoxia protocol to examine LTF. A commentary segment that is focused on model and protocol design recommendations will follow, with the goal of presenting a prevailing model and protocol(s) that could be employed to ensure standardized comparisons.

2. Experimental protocols used to initiate long-term facilitation in animals other than humans

A variety of factors (e.g. stimulus, anesthetic, stimulus intensity, episode duration, age and gender of animal) must be considered when selecting a specific model and protocol to investigate LTF in animals. We have attempted to provide historical reasons for employing specific factors so that a chronological time line is followed whenever possible. Moreover, in the discussion of each factor we consider how it impacts on the initiation and manifestation of LTF in a variety of models including urethane anesthetized, paralyzed and ventilated animals, and spontaneously breathing preparations. Likewise, for each factor we have considered its impact on various forms of respiratory plasticity (i.e. LTF of phrenic, hypoglossal or carotid sinus nerve activity).

A urethane-anesthetized, paralyzed, mechanically ventilated and bilaterally vagotomized cat was the model used in the inaugural studies that were designed to determine if long-lasting facilitation of phrenic nerve activity could be initiated (Millhorn *et al.*, 1980a). Animals were mechanically ventilated to tightly control arterial blood gases and tracheal pressure, and paralyzed to prevent background myoelectrical activity and respiratory movement in response to hypoxia. The animals were vagotomized to prevent reductions in the amplitude of phrenic nerve activity and to avoid entrainment of phrenic nerve activity with mechanical ventilation. Since the completion of these initial studies the majority of studies completed have employed a similar model (see supplementary file).

The stimulus protocol utilized to initiate LTF in the inaugural studies included five 2 minute episodes of carotid sinus nerve stimulation interspersed by 5 minute recovery periods with the exception of the last recovery period that was 50 minutes in duration (Millhorn *et al.*, 1980a). The initial studies confirmed that both carotid body and carotid sinus nerve stimulation elicited long-facilitation of phrenic nerve activity while stimulation of other afferent inputs did not (Millhorn *et al.*, 1980a). These studies also reported that anesthesia or lack thereof, decerebration and sectioning of the spinal cord at C7-T1 (Millhorn *et al.*, 1980a) did not prevent the initiation of phrenic nerve LTF, while serotonergic antagonists prevented manifestation of the phenomenon (Millhorn *et al.*, 1980b). Following these inaugural studies, a few additional studies were completed from the mid - eighties to the mid-nineties (Millhorn, 1986;Cao *et al.*, 1992;Hayashi *et al.*, 1993;Fregosi & Mitchell, 1994). In the majority of these studies a similar protocol (stimulation of the carotid sinus

nerve) and experimental model (i.e. paralyzed and vagotomized) were used to establish two primary findings. Phrenic nerve LTF could be elicited in other species (i.e. rats) (Hayashi *et al.*, 1993) and LTF was a general property of other spinal motoneurons, since it was recorded from both intercostal and phrenic nerves (Fregosi & Mitchell, 1994). The studies also established that an intact cerebellum is important in the manifestation of LTF (Hayashi *et al.*, 1993). Moreover, these studies confirmed that carotid sinus nerve stimulation was the principle stimulus to initiate LTF and that serotonin appeared to be required to initiate LTF at the level of a variety of motor neuron pools (i.e. phrenic and intercostal motor neurons) (Fregosi & Mitchell, 1994). Collectively, these early ground breaking studies laid the foundation for an upsurge of experiments that began in the mid-nineties. Subsequent studies focused on factors that could impact on the initiation and manifestation of LTF; including stimulus intensity and duration, type of stimulus, arousal state (wake versus sleep), sex, age, species and motor neuronal pool. The studies also took into account various issues regarding the experimental preparation including the role of vagotomy, anesthesia and artificial ventilation in the initiation and manifestation of LTF. All of these variables must be taken into consideration when considering the ideal model and methods to study LTF.

2.1. Selecting a stimulus to initiate LTF

It was recognized early on that electrical stimulation of the carotid sinus nerve, particularly at a frequency (i.e. 25 Hz) that exceeded the range of discharge frequencies in carotid chemoafferents during hypoxia, was not a natural physiological stimulus. Consequently the use of intermittent hypoxia to elicit LTF emerged (Cao *et al.*, 1992; Hayashi *et al.*, 1993; Bach & Mitchell, 1996) (see supplementary file). It was subsequently established that intermittent and not sustained hypoxia was critical for initiating LTF (Baker & Mitchell, 2000), and that the role of this stimulus in the development of LTF was mediated primarily by inspiratory activation mediated by feedback from the carotid bodies (Bach & Mitchell, 1996). These initial findings were challenged subsequently, because carotid body denervated rats exhibited LTF, albeit at a reduced amplitude, indicating that the carotid body was not essential to elicit LTF (Bavis & Mitchell, 2003; Sibigtroth & Mitchell, 2010). Likewise, intermittent hypoxia was shown to be effective in eliciting LTF even when it resulted in the depression of inspiration in post-natal rats (Tadjalli *et al.*, 2007; Tadjalli *et al.*, 2008). Thus, the intermittent nature of the hypoxic stimulus appears to be a powerful mediator of LTF independent of the carotid bodies and inspiratory activation. Additional studies have also revealed that intermittent hypoxia is likely not the sole stimulus that results in the initiation of LTF, since intermittent vagal nerve stimulation (Zhang *et al.*, 2003), brief episodes of simulated apnea (Mahamed & Mitchell, 2008; Tadjalli *et al.*, 2010), and intermittent application of upper airway negative pressure initiate LTF (Ryan & Nolan, 2009b). Thus, the intermittency of a given stimulus, rather than a specific stimulus (i.e. hypoxia), may be the most important requirement for inducing LTF. However, in order to develop a standardized model we propose that intermittent hypoxia be used as the principle stimulus to elicit LTF whenever possible to allow for historical, as well as, across laboratory comparisons.

2.2. Selecting episode duration and hypoxic intensity

2.2.1. Acute Intermittent Hypoxia—To initiate LTF, anesthetized, paralyzed and vagotomized animals were originally exposed to 3 five minute episodes of hypoxia interspersed with 10 minute recovery periods, with the exception of the end-recovery period that was 60 minutes in duration (Hayashi *et al.*, 1993). Over the past 17 years the use of this protocol has remained popular and has changed little with the only modification being a shortening of the recovery intervals from 10 to 5 minutes (see supplementary file). Nonetheless, altering the pattern or intensity of hypoxia appears to have little impact on the magnitude of LTF in anesthetized, paralyzed and vagotomized animals. Variations in

hypoxic intensity between 28 and 60 mmHg do not significantly alter the magnitude of phrenic nerve LTF (Fuller *et al.*, 2000). Moreover, the magnitude of phrenic or hypoglossal LTF following exposure to 3 – 5 minute episodes of hypoxia, 3 – 25 second apneas or 6 – 25 second apneas were not significantly different (Mahamed & Mitchell, 2008). Thus, altering hypoxic intensity or the duration of exposure to a given stimulus does not appear to impact on the magnitude of LTF in anesthetized, paralyzed and vagotomized animals. However, the duration of recovery periods that intersperse hypoxic episodes are important, since phrenic nerve LTF was observed using short recovery intervals (i.e. 5 min) but was absent using longer periods of recovery (i.e. 30 min) (Bach *et al.*, 1999).

In contrast, there is evidence that the intensity and/or number of hypoxic episodes impacts on the initiation and magnitude of LTF in other experimental models. Studies completed using adult rats that were awake (McGuire *et al.*, 2002), or a neonatal rat heart-brainstem preparation (Tadjalli *et al.*, 2007), showed that the magnitude of ventilatory LTF (vLTF) or phrenic nerve LTF induced by acute intermittent hypoxia was dependent on the severity of the stimulus and frequency of exposure. McGuire and colleagues reported that an optimal range of hypoxia (~ 10 % oxygen) was needed to elicit vLTF, because an equivalent number of mild hypoxic episodes (~12 %) did not elicit LTF and an equal number of severe (~8 %) hypoxic episodes resulted in the elimination of LTF (McGuire *et al.*, 2002). Similarly, Tadjalli *et al.* (Tadjalli *et al.*, 2007) demonstrated that the magnitude of LTF was dependent on hypoxic severity in post-natal rats.

McGuire and colleagues also demonstrated that increasing the number of hypoxic episodes from 10 to 20 to 72 impacted on the duration of expression of vLTF (McGuire *et al.*, 2002), although the magnitude of the response was not influenced. Subsequently, Ryan and Nolan reported that the expression of LTF of chest wall and upper airway muscles was evident in anesthetized and spontaneously breathing rats following exposure to 8 episodes of intermittent hypoxia but was not evident after exposure to 3 episodes (Ryan & Nolan, 2009a). Based on these latter findings it is possible that the absence of LTF that was reported in anesthetized awake rats prior to Ryan and Nolan study may have been the consequence of employing a reduced number of hypoxic episodes (n = 3) (Janssen & Fregosi, 2000).

In summary, the findings to date indicate that altering the intensity and/or number of hypoxic episodes does not dramatically impact on the manifestation and magnitude of LTF in anesthetized, paralyzed and vagotomized animals. In contrast, there is some evidence that these variables do impact on the expression of LTF, in spontaneously breathing preparations. Whether the variable impact of altering the duration and intensity of intermittent hypoxia on the expression of LTF reflects mechanistic differences that are dependent on the type of preparation employed remains to be determined. However, there appears to be enough evidence to suggest that the number of episodes and the intensity of hypoxia should be standardized to make within and between lab comparisons of the magnitude of LTF across experimental preparations.

2.2.2. Chronic Intermittent Hypoxia—A number of studies have been completed to determine whether LTF of ventilation, phrenic nerve/diaphragm, hypoglossal nerve/upper airway muscle and carotid sinus nerve activity is gradually enhanced in response to repeated daily exposure to intermittent hypoxia (i.e. chronic intermittent hypoxia) (see supplementary file). The animal models used to study this phenomenon have included rats (Ling *et al.*, 2001; O'Halloran *et al.*, 2002; McGuire *et al.*, 2003; Peng & Prabhakar, 2003; Zabka *et al.*, 2003; McGuire *et al.*, 2004; McGuire & Ling, 2005), cats (Rey *et al.*, 2004), dogs (Katayama *et al.*, 2007) and mice (Peng *et al.*, 2006). The length of exposure to chronic intermittent hypoxia has ranged from 4 days (Rey *et al.*, 2004) to 5 weeks (O'Halloran *et al.*, 2002), with

the length of exposure in the majority of studies being from 7 to 10 days (Ling *et al.*, 2001; McGuire *et al.*, 2003; Peng & Prabhakar, 2003; Zabka *et al.*, 2003; McGuire *et al.*, 2004; McGuire & Ling, 2005). The protocols typically employed by the studies have exposed animals to intermittent hypoxia 8 to 12 hours per day (Ling *et al.*, 2001; McGuire *et al.*, 2003; Peng & Prabhakar, 2003; Zabka *et al.*, 2003; Rey *et al.*, 2004; McGuire *et al.*, 2004; McGuire & Ling, 2005; Katayama *et al.*, 2007). Many protocols were comprised of hypoxic episodes that were 5 minutes in duration and were interspersed with 5 minutes of normoxia (Ling *et al.*, 2001; McGuire *et al.*, 2003; Zabka *et al.*, 2003; McGuire *et al.*, 2004; McGuire & Ling, 2005). However, a range of shorter and more frequent episodes of reduced oxygen levels have also been employed (i.e. the duration of hypoxic episodes ranged from 15 s – 90 s interspersed with 15 s – 5 min of normoxic recovery periods, and the number of episodes ranged from 9 – 120 episodes per hour) (O'Halloran *et al.*, 2002; Peng & Prabhakar, 2003; Rey *et al.*, 2004; Katayama *et al.*, 2007). Moreover, the intensity of hypoxia has varied across protocols with levels ranging from 11 % (Ling *et al.*, 2001; McGuire *et al.*, 2003; Zabka *et al.*, 2003; Rey *et al.*, 2004; McGuire *et al.*, 2004; McGuire & Ling, 2005) to 5 % (O'Halloran *et al.*, 2002; Peng & Prabhakar, 2003; Katayama *et al.*, 2007) during the induction of hypoxia. Despite these differences, the majority of published studies have reported that increases in ventilation (McGuire *et al.*, 2003; McGuire *et al.*, 2004; McGuire & Ling, 2005), phrenic nerve (Ling *et al.*, 2001; Peng & Prabhakar, 2003), hypoglossal nerve (Zabka *et al.*, 2003), sternohyoid muscle (O'Halloran *et al.*, 2002) and carotid sinus nerve activity (Peng *et al.*, 2004) are 2–4 times greater than baseline in animals chronically exposed to intermittent hypoxia or intermittent asphyxia.

Many divergent models and protocols have been used in studies investigating the impact of chronic intermittent hypoxia on LTF, thereby making it difficult to compare results between laboratories. Consequently, it is unclear whether or not a specific protocol is more effective in enhancing LTF following exposure to chronic intermittent hypoxia. However, it is clear from the published data that a significant enhancement of LTF is typically initiated independent of the episode duration and intensity of hypoxia employed.

2.3. Selecting a level of carbon dioxide

The apneic and/or recruitment threshold are often measured in LTF studies particularly in anesthetized, paralyzed and ventilated animals (see supplementary file). The apneic threshold represents the point at which the activity of a selected nerve (e.g. phrenic, hypoglossal) ceases in response to a gradual decrease in carbon dioxide. The recruitment threshold represents the point at which phrenic nerve activity is initiated in response to a gradual increase in carbon dioxide levels. The carbon dioxide level that demarcates the recruitment threshold in a given animal is predictably higher than levels associated with the apneic threshold (Leevers *et al.*, 1993). The apneic threshold was typically measured in studies completed early on (Fregosi & Mitchell, 1994), while both the apneic and recruitment threshold have been determined in studies published more recently (Mahamed & Mitchell, 2008).

The apneic or recruitment threshold is determined so that carbon dioxide can be elevated and maintained above a selected threshold during and following exposure to intermittent hypoxia. This practice was initiated early on because investigators intuitively recognized that reductions in chemoreceptor feedback, which was caused by hypocapnia induced by exposure to hypoxia, would likely restrain the manifestation of LTF. This suggestion is supported by results obtained from unanesthetized rats during wakefulness, which showed systematically that the full expression of LTF was prevented if accompanied by hypocapnia (Olson, Jr. *et al.*, 2001). Differences in chemoreceptor restraint could also account for findings which showed that vLTF was evident during wakefulness in mice (Terada *et al.*, 2008) but not in rats (Nakamura *et al.*, 2010). Although measures of the partial pressure of

carbon dioxide were not attainable in these studies to confirm this possibility, differences in the magnitude of the hypercapnic ventilatory response between mouse and rat strains suggest that chemoreceptor feedback restraint of LTF may vary among species. Chemoreceptor feedback restraint of LTF might also explain the findings of McGuire and colleagues (McGuire *et al.*, 2002) who showed that exposure to episodes of 8 % hypoxia did not elicit LTF in awake rats. They hypothesized that the absence of LTF was likely due to chemoreceptor feedback restraint since exposure to episodes of 8 % hypoxia was accompanied by hypocapnia (McGuire *et al.*, 2002).

Studies completed to date have highlighted a few issues that must be considered when selecting the level of carbon dioxide that will be maintained throughout an intermittent hypoxia protocol. First, one must decide whether the apneic or recruitment threshold will be used as a point of reference. Typically, resting levels of ventilation and carbon dioxide reside at or above the recruitment threshold. Given this circumstance, the recruitment threshold should be selected to ensure adequate feedback from the chemoreceptors. Second, the degree to which carbon dioxide is maintained above the threshold must be selected. Typically, in most studies carbon dioxide is maintained 2–4 mmHg above the threshold. Determining the threshold and the level of carbon dioxide that will be sustained is not difficult if a given study is designed to solely measure LTF of activity from a single nerve (i.e. phrenic nerve). However, difficulty arises when more than one nerve will be recorded (e.g. phrenic and hypoglossal nerve), because the apneic/recruitment threshold for each nerve are often not identical. For example, the apneic/recruitment threshold is lower for phrenic compared to hypoglossal nerve activity (Mateika & Fregosi, 1997). Thus, if recordings are made from both nerves and the level of carbon dioxide selected is above the threshold for phrenic nerve activity but below the threshold for hypoglossal nerve activity, then it is likely that LTF of hypoglossal nerve activity may be restrained. Conversely, if the level of carbon dioxide selected resides above the apneic/recruitment threshold for the hypoglossal nerve (e.g. 3 mmHg above the hypoglossal recruitment threshold) it is possible that phrenic nerve activity may saturate because the level of carbon dioxide maintained will be well above the threshold for phrenic nerve activity. Consequently, LTF will not be evident even if the phenomenon is initiated. Thus, nerve recordings (i.e. phrenic vs. hypoglossal), threshold measurements (apneic vs. recruitment) and the level of carbon dioxide to be maintained must all be considered in studies designed to examine LTF.

2.4. Selecting a species

Various forms of respiratory plasticity (vLTF, phrenic, hypoglossal and carotid sinus nerve LTF) have been observed in a variety of animals including cats (Millhorn *et al.*, 1980a; Fregosi & Mitchell, 1994; Mateika & Fregosi, 1997), rats (Hayashi *et al.*, 1993; Bach & Mitchell, 1996; Peng & Prabhakar, 2003), rabbits (Sokolowska & Pokorski, 2006), ducks (Mitchell *et al.*, 2001b), dogs (Cao *et al.*, 1992), mice (Peng *et al.*, 2006) and goats (Turner & Mitchell, 1997). However, the animal used in the majority of studies over the past 20 years has been the rat (see supplementary file). Although popular as an experimental model, there is considerable variation in the manifestation of LTF within genetic populations of rats (Fuller *et al.*, 2001b; Mitchell *et al.*, 2001b; Behan *et al.*, 2003). Fuller and colleagues (Fuller *et al.*, 2001b) reported that significant variation in the magnitude of hypoglossal nerve LTF existed between Sprague–Dawley rats obtained from different suppliers (Harlan Sprague Dawley colony 236 vs. Charles River/Sasco colony K62); hypoglossal nerve LTF was only observed in rats obtained from Charles River/Sasco. Fuller and colleagues also reported that the magnitude of phrenic nerve LTF varied between different rat colonies from the same supplier (Harlan Sprague Dawley; colony 236 vs. 205). Differences in phrenic and hypoglossal nerve LTF also exist between inbred rat strains. The magnitude of phrenic nerve LTF following exposure to a similar intermittent hypoxia protocol was greatest in Lewis

rats, followed by Sprague Dawley, Fischer and Brown Norway rats (Nakamura *et al.*, 2010; Baker-Herman *et al.*, 2010). Interestingly, although this relationship holds true for anesthetized, paralyzed and vagotomized rats the relationship does not carry over to awake spontaneously breathing rats; since vLTF has been observed in Sprague Dawley (Olson, Jr. *et al.*, 2001) but not Lewis rats (Nakamura *et al.*, 2010) during wakefulness. In addition, differences between inbred rat strains are specific to a given motor neuron pool. Differences in the magnitude of hypoglossal nerve LTF do not follow the same trend observed for phrenic nerve LTF; hypoglossal nerve LTF was greatest in Fischer 344 followed by Lewis and Brown Norway rats (Baker-Herman *et al.*, 2010). Collectively, published results indicate that careful consideration must be given to the species selected and the genetic population within a given species since this factor has a profound impact on the manifestation and magnitude of LTF.

2.5. Selecting a sex and age

Sex and age also impact on the manifestation of LTF. Decreased levels of testosterone with age and gonadectomy diminish phrenic and hypoglossal LTF in male rats (Zabka *et al.*, 2001a; Zabka *et al.*, 2005), which are restored by testosterone replacement in gonadectomized male rats. In females, LTF is modulated by the estrus cycle (Zabka *et al.*, 2001b). The most significant increase in LTF has been observed in middle aged female rats during diestrus, when 5-HT levels are high (Zabka *et al.*, 2001b). Also, the magnitude of both phrenic and hypoglossal LTF is inversely related to the ratio of progesterone to estrogen (Zabka *et al.*, 2003).

Long term facilitation of genioglossus muscle activity (McKay *et al.*, 2004) and vLTF (Julien *et al.*, 2008) has been observed in anesthetized and awake rats, and in anesthetized mice (Berner *et al.*, 2007), ranging in age from 2 to 10 days old. Once LTF is evident it appears to decline with age particularly in male rats, since vLTF has been shown to be greater in 1 versus 2 month old rats during wakefulness following exposure to acute intermittent hypoxia (McGuire & Ling, 2005). Both hypoglossal and phrenic nerve LTF decrease with age in males, but the decrease is more prominent in hypoglossal versus phrenic nerve activity, leading to a complete absence of hypoglossal LTF in middle aged male rats (13 months old) (Zabka *et al.*, 2001a). In contrast to males, phrenic and hypoglossal LTF is greatest in middle aged female rats when compared to young and geriatric female rats (Zabka *et al.*, 2001b; Zabka *et al.*, 2003). Thus, phrenic and hypoglossal LTF in female rats appears to be strongest at an age when the reproductive system reaches a stable plateau of circulating sex hormones (Zabka *et al.*, 2001b; Zabka *et al.*, 2003).

The impact that pre-treatment with chronic intermittent hypoxia has on the expression of LTF following acute exposure to intermittent hypoxia also appears to be a function of age. Exposure to chronic intermittent hypoxia is known to enhance the response to acute intermittent hypoxia in young to middle aged adult rats; although the response to acute intermittent hypoxia is also enhanced following exposure to chronic intermittent hypoxia in geriatric rats (Zabka *et al.*, 2003). This phenomenon however does not appear to be consistently observed in younger rats. Julian and colleagues reported that vLTF following acute exposure to intermittent hypoxia in 10 day old rats was not evident if rats were exposed to intermittent hypoxia every day for 10 days prior to the acute exposure (Julien *et al.*, 2008). Likewise, Reeves and colleagues (Reeves *et al.*, 2006) revealed that exposure to chronic intermittent hypoxia for the first month of life resulted in a reduction in phrenic LTF in 2 month old rats. Similarly, Pawar and colleagues (Pawar *et al.*, 2008) reported that exposure to chronic intermittent hypoxia did not induce carotid sensory LTF after exposure to acute intermittent hypoxia in neonatal rats (2–6 hours old). However, in contrast to Julien *et al.* (Julien *et al.*, 2008) and Reeves *et al.* (Reeves *et al.*, 2006) findings, exposure to chronic intermittent hypoxia early in life was accompanied by LTF of carotid sensory

activity in 2 month old rats following exposure to acute intermittent hypoxia (Pawar *et al.*, 2008). The reason for the discrepant findings is not known, although it is possible that the impact of chronic intermittent hypoxia on the expression of LTF following acute exposure to intermittent hypoxia may be specific to a given neuronal pool (i.e. phrenic motor neuronal pool vs. carotid body). This cannot be the complete answer however since McGuire and Ling (McGuire & Ling, 2005) showed that when newborn rats were exposed to 7 days of chronic intermittent hypoxia vLTF was enhanced 1 month later. Varying preparations, hypoxic exposure paradigm and recording approaches might account for some of the discrepancies.

2.6. Vagotomized vs. non-vagotomized preparations

As mentioned earlier animals used in studies over the past 30 years were vagotomized (see supplementary file) to prevent the reduction of peak activity of nerves innervating both chest wall and upper airway muscles via vagally mediated lung-volume feedback (Fukuda & Honda, 1982; Kuna, 1986). Moreover, animals were vagotomized because early on it was hypothesized that the magnitude of LTF might be attenuated by a long-lasting inhibitory memory elicited by repeated stimulation of lung stretch receptors (Xi *et al.*, 1993). This possibility was subsequently supported by the findings of Mateika and Fregosi (Mateika & Fregosi, 1997) who established that vagotomy enhanced the magnitude of LTF of upper airway muscle activity in spontaneously breathing cats, particularly at the onset of the end-recovery period. Eleven years later it was also established that activity of the hypoglossal nerve in anesthetized and mechanically ventilated rats increased following vagotomy (Golder & Martinez, 2008). Studies also indicated that the impact of this long-lasting inhibitory memory on the manifestation of LTF was dependent on the motor neuronal population, since LTF of upper airway muscles was diminished while phrenic nerve or other spinal motor neuron activity was similar or increased in vagally intact animals (Mateika & Fregosi, 1997; Golder & Martinez, 2008). Although maintaining integrity of the vagus nerves impacts the magnitude of LTF at the level of the hypoglossal motoneuronal pool, it does not prevent the manifestation of the phenomenon, since studies have shown that LTF of both chest wall and upper airway muscle activity can be initiated and maintained in spontaneously breathing vagally intact rats (Olson, Jr. *et al.*, 2001; Ryan & Nolan, 2009a).

In contrast to findings that have suggested that vagal feedback diminishes the magnitude of LTF, other studies have reported that intermittent activation of the vagus nerve via electrical stimulation elicits LTF of upper airway muscle activity (Zhang *et al.*, 2003). Likewise, brief apneas in spontaneously breathing animals elicited LTF of genioglossus activity in vagally intact but not in vagotomized animals (Tadjalli *et al.*, 2010). This activation was exclusive to upper airway muscle activity since LTF of diaphragmatic activity was not evident (Tadjalli *et al.*, 2010). Lastly, genioglossus LTF was greatest in a small group (n =2) of vagally intact compared to vagotomized rats (Cao & Ling, 2010). It is difficult to reconcile these findings with the more established dogma that activation of an inhibitory memory diminishes LTF. Perhaps the combination of two stimuli (intermittent hypoxia and intermittent vagal nerve stimulation) elicits a response that is much different than the response to activation of vagal stimuli alone. Although additional studies are required to address this question, the primary implication from the published literature is that altering vagal input will have a profound impact on the manifestation of LTF.

2.7. Selecting an anesthetic

Urethane with isoflurane induction has been used overwhelmingly in studies employing vagotomized, paralyzed and mechanically ventilated animals (see supplementary file). Urethane is a popular choice because it has minimal impact on many spinal reflexes, and on the respiratory, cardiovascular and autonomic nervous systems (Cao & Ling, 2010).

Moreover, urethane can be administered via several routes and it typically elicits a long lasting, steady level of surgical anesthesia that can be achieved with no or few additional doses (Cao & Ling, 2010). Over the past 30 years, LTF of phrenic or hypoglossal nerve activity has usually been observed in paralyzed and vagotomized animal preparations using urethane as an anesthetic. In contrast, LTF of upper airway and pump muscle activity was not evident in spontaneously breathing rats anesthetized with urethane (Janssen & Fregosi, 2000). The possibility that urethane might impact on the manifestation of LTF in spontaneously breathing animals was further supported by studies which showed that LTF was initiated in spontaneously breathing preparations using different anesthetic regimes, including a combination of α -chloralose and urethane (Mateika & Fregosi, 1997), subcutaneous ketamine and xylazine (McKay *et al.*, 2004), intraperitoneal equithesin (Dick *et al.*, 2007), and Saffan (alphaxalone/alphadalone) with sodium pentobarbitone induction (Ryan & Nolan, 2009a). More definitively, Cao and Ling showed that LTF of genioglossus muscle activity was not initiated in spontaneously breathing animals anesthetized with urethane but was evident in animals anesthetized with a combination of α -chloralose and urethane, or alphaxalone and urethane (Cao & Ling, 2010). It remains unclear why urethane would be principally responsible for inhibiting LTF of genioglossus muscle activity in spontaneously breathing animals but not elicit a similar response in paralyzed and ventilated animals in which LTF of hypoglossal nerve activity is consistently observed. It is possible that the absence of paralysis in combination with the use of urethane has a primary role in the differential response that has been observed in anesthetized, paralyzed and ventilated animal models vs. spontaneously breathing animals. The role that anesthetic may have in the manifestation of LTF was highlighted further by Carev and colleagues (Carev *et al.*, 2010) who showed that LTF of phrenic nerve activity was absent in propofol anesthetized, paralyzed and vagotomized rats. Although this anesthetic is not typically used in studies examining LTF in rats it does highlight the potential caveats that might be associated with the selection of a given anesthetic regime to examine LTF.

2.8. Selecting a state of arousal

Mitchell and colleagues reported that vLTF was easier to elicit and more robust in anesthetized or decerebrate animals compared to awake animals (Mitchell *et al.*, 2001a). This finding led to the hypothesis that arousal state may play a role in the manifestation of LTF (Mahamed & Mitchell, 2007), wholly or in part because serotonergic neurons in the raphe nucleus discharge at significantly reduced rates during sleep, especially in the deeper stages of non-rapid eye movement sleep (NREM) and rapid eye movement (REM) sleep (Heym *et al.*, 1982; Veasey *et al.*, 1995; Jacobs & Fornal, 1997; Jacobs *et al.*, 2002). Because serotonergic neurons fire at reduced levels during sleep, intermittent hypoxia may stimulate caudal raphe neurons through a greater dynamic range during deep NREM and REM sleep compared to wakefulness, resulting in an increased expression of LTF. Although this was a viable hypothesis, there were other potential explanations for the difference observed between the anesthetized/decerebrate and awake animals. The experimental preparation and design in the anesthetized/decerebrate animals was much different than in awake animals (e.g. vagotomized vs. non-vagotomized; paralyzed vs. non-paralyzed; isocapnia vs. hypocapnia), so that other factors alone or in combination with arousal state could account for the differences observed. Thus, studies which controlled for some or all of these factors were required. This void was filled in part by three investigations completed more recently. However the results have been equivocal with results from only one study supporting the original hypothesis. Terada and colleagues (Terada *et al.*, 2008) reported that vLTF was evident during both wake and sleep in spontaneously breathing mice but the magnitude of the response did not vary between arousal states. In contrast, Nakamura and colleagues (Nakamura *et al.*, 2010) showed that vLTF was easily induced during deep NREM sleep but was absent during wakefulness in spontaneously breathing Lewis male rats (Nakamura *et*

al., 2010). Although supportive of the original hypothesis, Nakamura *et al.* findings results were somewhat surprising because vLTF was absent during wakefulness even though it was previously observed in spontaneously breathing Sprague-Dawley rats (Olson, Jr. *et al.*, 2001). It remains to be determined if this difference is species related. Nonetheless, Nakamura *et al.* findings (Nakamura *et al.*, 2010) were not replicated by Boon and Milsom (Boon & Milsom, 2010) who showed that vLTF was not evident in light or deep stages of anesthesia induced by urethane, in spontaneously breathing Sprague-Dawley rats. The lack of vLTF in Boon and Milsom study (Boon & Milsom, 2010) could have been complicated by the use of urethane, since as outlined above (see *Selecting an anesthetic*) genioglossus muscle LTF was not initiated in urethane anesthetized non-paralyzed animals while it was evident using other anesthetic regimes (Cao & Ling, 2010). Thus, findings to date are equivocal and it remains to be determined definitively in animals other than humans if arousal state impacts on the initiation and magnitude of LTF. To make this determination it is important that in future studies the same species, preparation and anesthetic (or lack thereof) are used to definitively answer whether or not the initiation and magnitude of LTF are linked to arousal state (see *Recommendations* for further discussion).

2.9. Recommendations

As implied by the historical summary, there are numerous variables that impact on the initiation, manifestation and maintenance of LTF. Thus, selecting the ideal model and experimental preparation is a difficult decision, however in our opinion there are two overriding factors that should be considered. First, one must consider whether the results obtained using the selected model and protocol can be compared to findings from other studies completed in the same laboratory or a different laboratory. If the results cannot be compared across laboratories explicit reasons for selecting a specific model and preparation should be carefully considered. Secondly, one should contemplate if the results obtained using the selected model and protocol will translate to humans. Specific reasons for selecting a given model and preparation is necessary, if it is clear that results may not easily translate to humans. Although great strides have been made in unlocking the mysteries of LTF, it remains to be determined whether or not a large majority of the results obtained using the animal models and experimental protocols described above are applicable to humans, healthy or otherwise. In part this may prove to be difficult simply because the nature of the phenomenon may be impacted by the animal studied. However, the ability to translate many of the results obtained from other animals to humans is likely impeded by variability in the experimental models and protocols that were addressed above. To ensure that results obtained from biomedical experiments are translatable to normal human function or a specific human disorder, we recommend that unanesthetized spontaneously breathing animals be used as the model of choice whenever possible to study LTF in future investigations. Utilizing this preparation ensures that issues related to the impact of anesthetic, vagotomy and paralyzes on the initiation of LTF will be eliminated; thus the model will more closely reflect studies completed in intact humans. Moreover, physiological measures and interventions that once were difficult to attain in spontaneously breathing models are now more routinely attained. For example, measures of minute ventilation are now routinely obtained by plethysmography. Likewise, electromyography and encephalography can now be measured from spontaneously breathing rats, allowing investigators to record upper airway muscle activity and differentiate arousal states (e.g. wake vs. sleep), respectively (Morrison *et al.*, 2003). Lastly, various agonists or antagonists can now be applied to spontaneously breathing preparations so that the impact of these agents on the manifestation of LTF can be investigated (Morrison *et al.*, 2003).

In addition to employing an unanesthetized spontaneously breathing model, we suggest that the standard species and animal of choice should be the Sprague - Dawley rat. Although we

recognize that other species or animals may be more appropriate in some experiments we recommend the Sprague-Dawley rat because LTF has been observed most consistently in this species under a variety of experimental conditions and states. Moreover specific colonies in which LTF can be initiated have been clearly identified in Sprague - Dawley rats. We also recommend that a specific stimulus and protocol be uniformly applied whenever possible. Although, intermittent hypoxia may not be the only stimulus that initiates LTF, it still remains the most recognized and utilized. Consequently, we propose the continued use of this stimulus over other potential stimuli. Likewise, we recommend a standard intermittent hypoxia protocol; one that will be robust in initiating LTF to study basic mechanisms or to study its role in the treatment of various motor disorders, respiratory or otherwise, without inducing detrimental side effects. We propose that twelve 2 minute episodes interspersed with 2 minute recovery periods be used to study the acute and chronic impact of intermittent hypoxia on LTF. We suggest that the hypoxic intensity selected result in an oxygen saturation of 85–88 %. We propose this as a standard protocol because it could be useful to study basic mechanisms and could serve as a potential starting dose for the treatment of various respiratory or motor disorders. Likewise, although the timing and conditions may not exactly mimic certain conditions (e.g. sleep apnea), the episode duration will allow for precise control of inspired gas within and across animals, which is more difficult using shorter episode durations. Most importantly, this protocol mimics similar protocols that have successfully initiated LTF in humans (see below for details regarding protocols in humans).

Although we recommend that a spontaneously breathing preparation may be the best model to employ at this point in time, for the reasons stated above, we also recognize that the more traditional model (anesthetized, vagotomized and paralyzed) remains important for the investigation of many aspects of LTF, including the discovery of various neuromodulators and cellular pathways that mediate this phenomena. Given the importance of this model we recommend that it be exactly replicated across laboratories. Likewise, we recommend that the intermittent hypoxia protocol that has been used most often (3 five minute episodes interspersed with 5 minute recovery periods) be a standard protocol that is used along with the traditional model. Given its popularity, a good deal of experimental data has been obtained employing this protocol. Thus, for continued historical comparisons maintenance of this protocol across laboratories would be useful. Likewise, because there is some evidence that episode duration and intensity impact on the magnitude of LTF, using a similar protocol will eliminate these potential influences.

3. Experimental Protocols used to initiate Long-Term Facilitation in Humans

Over the past 15 years efforts to examine LTF of respiratory motor activity (i.e. principally minute ventilation and genioglossus muscle activity) in humans were initiated and have increased substantially. In general, the reasons for completing these studies have been two-fold. First, studies have been completed to answer basic fundamental questions regarding the initiation and magnitude of LTF in humans. Studies have also been designed to examine whether or not respiratory plasticity initiated by exposure to intermittent hypoxia can be used as adjunctive therapy in the treatment of a variety of disorders including sleep apnea and spinal cord injury. Despite the completion of a number of studies the best model and method to elicit LTF in humans has not been fully established. To investigate the basic mechanisms of LTF the model should be effective in initiating LTF of a significant magnitude, while minimizing the time to initiate the phenomenon. Moreover, it should be designed to minimize any deleterious responses associated with exposure to intermittent hypoxia. Additionally, if intermittent hypoxia will ultimately be used as a treatment for

specific disorders then the appropriate “dose” and conditions will be required to treat a specific disorder.

Similar to that outlined for other animals the following paragraphs will provide an historical summary of the types of protocols and conditions that have been used in humans over the past 15 years. *The pertinent findings used for the review are summarized in Table 1, in addition to being compiled in the Excel spreadsheet included with this paper as a supplementary file.* Throughout the summary we will discuss the potential reasons for the use of various protocols and the potential benefits and/or limitations to the protocols that have been employed. Lastly, we will outline what we consider to be the ideal model based on the published literature.

3.1. Models and methodologies used during wakefulness

To our knowledge McAvoy and colleagues (McEvoy *et al.*, 1996) published the first study that was designed to initiate LTF in humans (Table 1). These authors exposed males to ten 2 minute periods of hypoxia (oxygen saturation – 80 %) interspersed with 2 min recovery periods of normoxic breathing, except for the final recovery period which was 10 minutes in duration. During the protocol carbon dioxide levels were maintained at isocapnic baseline values and the participants were awake during exposure. The duration of the hypoxic episodes was selected because it approximated the pattern of sleep apnea and 2 min was a convenient interval over which to make measurements of inspired minute ventilation. Utilizing this protocol, LTF of minute ventilation, diaphragmatic activity or genioglossus muscle activity was not initiated (McEvoy *et al.*, 1996). Taken at face value, the results indicated that LTF could not be initiated in relatively young male participants. In retrospect there is a plethora of explanations for the absence of LTF, since the initiation and/or magnitude of LTF in other animals has been shown to be impacted by a number of factors. These include sex, intensity of hypoxia, duration of hypoxic episodes and end-recovery period, pre-conditioning (prior exposure to intermittent hypoxia) and maintenance of carbon dioxide levels to name a few (Mateika & Narwani, 2009).

3.1.1. Sex and duration of hypoxic episodes—Given these possibilities, this same group followed up their initial investigation with a subsequent study in 2002, during which they exposed both men and women to a similar intermittent hypoxia protocol (Jordan *et al.*, 2002). The results remained the same, LTF of minute ventilation and genioglossus muscle activity was not evident (Jordan *et al.*, 2002) (Table 1). In 2004, we began investigating vLTF in humans during wakefulness in my laboratory (Mateika *et al.*, 2004). We initially attempted to address whether or not the lack of LTF observed previously during wakefulness was in part due to the duration of the hypoxic exposure. Consequently, we exposed healthy men and women (Mateika *et al.*, 2004; Morelli *et al.*, 2004) to 8 episodes of hypoxia that were 4 minutes in duration. Each episode was interspersed with 5 minute normoxic recovery periods, with the final recovery period 15 minutes in duration. Thus, the duration of the episodes used in our protocol was double the length employed in previous studies. The intensity of hypoxia (oxygen saturation – 85 to 88 %) was selected to minimize any potential risk associated with hypoxia while eliciting a physiological response that typically is experienced during mild exercise. Both sexes were studied to ensure that the potential response to our protocol was independent of this factor. Despite our efforts we did not observe LTF of minute ventilation in either healthy males or females (Mateika *et al.*, 2004; Morelli *et al.*, 2004) (Table 1).

3.1.2. Pre-conditioning—Individuals with sleep apnea were also examined following our studies in healthy individuals in the event that preconditioning with nocturnal hypoxemia was required for LTF to be initiated (Khodadadeh *et al.*, 2006). However, LTF of minute

ventilation in individuals with sleep apnea was not observed (Khodadadeh *et al.*, 2006) (Table 1). This latter finding was supported by work completed within the same time period and thereafter (Koehle *et al.*, 2007; Pialoux *et al.*, 2009). These studies repeatedly exposed healthy individuals to intermittent hypoxia for a relatively short period (6 episodes of 12 % hypoxia interspersed by 5 minute recovery periods) each day over 12 consecutive days (Koehle *et al.*, 2007) or for a longer period each day (participants were exposed to 2 minute hypoxic episodes interspersed with 2 minute recovery periods for 6 hours) over a 4 day period (Pialoux *et al.*, 2009). These investigations were designed primarily to study the impact of intermittent hypoxia on the hypoxic ventilatory response and did not specifically address the link between intermittent hypoxia and long-term facilitation. Nevertheless, measures of baseline ventilation were obtained throughout the protocol, which could provide clues as to whether or not pre-conditioning (i.e. repeated daily exposure hypoxia) would result in the initiation and magnification of long-term facilitation. The results from both studies revealed that baseline increases in minute ventilation were not clearly evident (Koehle *et al.*, 2007; Pialoux *et al.*, 2009). Thus, a number of studies which addressed the potential role of sex, protocol design (i.e. duration of hypoxic episodes) and pre-conditioning in the initiation of vLTF were unsuccessful in activating the phenomena (Table 1).

3.1.3. Carbon dioxide—Despite these findings or lack thereof we remained convinced that vLTF could be initiated in humans, given that LTF was observed in a multitude of other animals (Mateika & Narwani, 2009). Based in part on Eldridge's work that examined the role of carbon dioxide in the manifestation of short term potentiation (Eldridge, 1980), we hypothesized that perhaps LTF was initiated but not evident because carbon dioxide levels were at or slightly below baseline levels. Consequently, we examined whether or not LTF would manifest itself if carbon dioxide levels were sustained above baseline levels (Harris *et al.*, 2006). Once carbon dioxide was sustained above baseline, LTF was found to be evident in healthy males and females (Harris *et al.*, 2006; Wadhwa *et al.*, 2008) and in individuals with sleep apnea (Lee *et al.*, 2009) following a one-time exposure to our protocol (Table 1). Likewise, we also found that when carbon dioxide was sustained above baseline levels gradual enhancement of vLTF was clearly evident when participants with sleep apnea were exposed to intermittent hypoxia each day over a 10 day time period (Gerst, III *et al.*, 2010). In our initial study, that examined the role of carbon dioxide in the manifestation of LTF, carbon dioxide was sustained 5 mmHg above baseline (Harris *et al.*, 2006). In studies that followed the increase in carbon dioxide above baseline was not as great (i.e. 4 and 3 mmHg above baseline) (Lee *et al.*, 2009; Gerst, III *et al.*, 2010). Whether or not vLTF is evident when $P_{ET}CO_2$ levels are 1 to 2 mmHg above baseline remains to be determined.

3.1.4. Duration of end-recovery—We also hypothesized that the end-recovery period in our initial studies (Mateika *et al.*, 2004; Morelli *et al.*, 2004; Khodadadeh *et al.*, 2006) and other studies (McEvoy *et al.*, 1996) may not have been long enough to allow for the wind-up of LTF that has been observed in other animals. In these animal models, LTF was not evident during the initial 15 minutes of recovery but slowly increased above baseline after this time period [see Fig. 1 in (Mitchell *et al.*, 2001a)]. We selected a relatively short end-recovery in our earlier investigations because we were concerned that restlessness and the anticipation of completing the protocol could impact minute ventilation during the end-recovery period. The downside to this relatively short end-recovery period was that LTF may have become evident if the recovery period had been extended. We do not believe this to be the case. Although we did not record minute ventilation beyond 15 minutes in our initial study we did collect minute ventilation data for a short segment 70 minutes after exposure to the last hypoxic episode (Mateika *et al.*, 2004). Ventilatory LTF was not evident at this time period. Moreover, Jordan and colleagues (Jordan *et al.*, 2002) did not observed

vLTF or other forms of LTF even though the end-recovery period of their intermittent hypoxia protocol was 25 minutes in duration. More convincingly, Harris *et al.* (Harris *et al.*, 2006) showed that LTF was evident during the initial 15 minutes of end-recovery when carbon dioxide was sustained above baseline levels but was absent when carbon dioxide levels were reduced to baseline levels. Nevertheless, in subsequent studies we increased the length of our recovery periods from 15 to 30 minutes recognizing the potential impact that this factor might have on the magnitude of vLTF (Lee *et al.*, 2009; Gerst, III *et al.*, 2010). Since making this alteration, we have found that participants are capable of tolerating the longer end-recovery period as indicated by little to no change in minute ventilation being evident during control studies of a similar length. In all cases since we implemented the 30 minute recovery period LTF has always been evident from the start of the recovery period. However, we have observed in some participants that minute ventilation gradually increased (Lee *et al.*, 2009) over the 30 minute recovery period while in others minute ventilation did not progressively increase (Lee *et al.*, 2009; Gerst, III *et al.*, 2010).

3.1.5. Carbon dioxide and duration of hypoxic episodes—Having determined that sustaining carbon dioxide levels above baseline appears to be crucial for initiating vLTF in humans, the question remained whether episodes of shorter duration similar to those used in the initial experiments of McAvoy could be used to elicit LTF? This is an important question since minimizing or eliminating the detrimental effects associated with exposure to intermittent hypoxia, while initiating vLTF are ideal attributes to consider in designing an intermittent hypoxia protocol. Thus, most recently we employed a protocol comprised of twelve 2 minute episodes of hypoxia interspersed by 2 minute recovery periods (Syed *et al.*, 2011), which is similar to the number and duration of episodes employed 15 years ago (McEvoy *et al.*, 1996). While the duration of the episodes was altered to match the early studies; we maintained the intensity of hypoxia (oxygen saturation – 88 %), level of carbon dioxide (3 mmHg above baseline) and length of the end-recovery period (30 minutes) employed in previous studies. This protocol resulted in the successful initiation of vLTF during wakefulness (Syed *et al.*, 2011), supporting the notion that carbon dioxide levels are crucial in initiating vLTF. From a methodological point of view this is an important finding, because in addition to initiating vLTF, the reduced exposure to hypoxia minimizes risk. Despite our findings, the optimal number and duration of hypoxic episodes for initiating LTF has not been established. For example, it has not been established whether durations shorter than 2 minutes would effectively initiate LTF during wakefulness. Only one study to our knowledge employed episodes of shorter duration during wakefulness and the protocol did not result in the initiation of vLTF (Diep *et al.*, 2007). However, as in previous studies carbon dioxide was not sustained above baseline values following exposure to intermittent hypoxia (Diep *et al.*, 2007). Thus, a study design to specifically investigate the impact of episode duration and number on the magnitude of vLTF in humans is required.

3.1.6. Intermittent hypoxia as a treatment—Establishing the duration and intensity that is optimal in initiating LTF is not only important for studying the basic mechanisms associated with the initiation of LTF, but it is important if intermittent hypoxia will ultimately be used as a treatment for disorders such as sleep apnea and spinal cord injury; since establishing an appropriate “dose” that will be repeatedly applied to treat a given condition will be required. The role of intermittent hypoxia in the treatment of sleep apnea and spinal cord injury in humans is in its infancy and as a result no full-length papers have been published. We recently completed a study to determine whether repeated daily exposure to intermittent hypoxia impacts on apnea severity (Yokana *et al.*, 2010). Because of space constraints we will not outline the detailed results since they are not directly applicable to the topic. However, from a methodological point of view this investigation will serve as the impetus for additional studies that will attempt to determine the optimal

duration and intensity of intermittent hypoxia and level of sustained carbon dioxide required to promote LTF of genioglossus muscle activity and possibly breathing stability; while minimizing the impact on other forms of respiratory plasticity (i.e. chemoreflex plasticity) that are potentially detrimental to maintaining breathing stability (Mateika & Narwani, 2009).

In addition to these studies, preliminary findings have shown that exposure to 15 episodes of hypoxia (duration – 90 s, recovery periods – 2 minutes, oxygen saturation – 85 %), applied each day for a 5 day period resulted in the improvement of motor function in individuals with spinal cord injury, presumably as a consequence of LTF (Rymer *et al.*, 2009; Trumbower *et al.*, 2010). It remains to be determined if this protocol elicits optimal recovery from spinal cord injury. However, at this point it can be concluded that exposure to a minimal amount of hypoxia promotes motor recovery as a consequence of the initiation of LTF while eliminating deleterious side-effects that have been reported to be associated with long-term exposure to hypoxia.

3.2. Models and methodologies during sleep

Studies examining LTF during sleep were initiated at the same time that studies during wakefulness were being completed. The vast majority of this work has been completed by Badr and colleagues (Babcock & Badr, 1998; Aboubakr *et al.*, 2001; Shkoukani *et al.*, 2002; Babcock *et al.*, 2003; Rowley *et al.*, 2007; Pierchala *et al.*, 2008; Chowdhuri *et al.*, 2008; Chowdhuri *et al.*, 2010) (Table 1). In their initial study participants were exposed to 10 episodes of hypoxia that were 3 minutes in duration (Babcock & Badr, 1998). The episodes were separated by 5 minute recovery periods with a 60 minute recovery period following the last hypoxic episode (Babcock & Badr, 1998). As a whole the group did not display vLTF; however Babcock and colleagues reported that vLTF was evident in flow limited participants and absent in non-flow limited individuals twenty minutes into the recovery period (Babcock & Badr, 1998) (Table 1). Whether or not these findings extended later into recovery was not reported. This led to a hypothesis that vLTF initiated in flow limited individuals was a consequence of reduced upper airway resistance manifested by LTF of upper airway muscle activity (Babcock & Badr, 1998). This hypothesis resulted in a series of studies which produced interesting findings. Ventilatory LTF was often observed in flow limited individuals, although there were some instances in which reductions in upper airway resistance indicative of LTF of upper airway activity was not accompanied by vLTF (Aboubakr *et al.*, 2001; Rowley *et al.*, 2007). In contrast, vLTF was often not evident in healthy individuals, although in some instances vLTF was evident in this population (Babcock *et al.*, 2003) (Table 1). Based in part on findings obtained during wakefulness (Harris *et al.*, 2006), Pierchala and colleagues (Pierchala *et al.*, 2008) suggested that the inability to consistently initiate vLTF in healthy individuals during sleep might in part be a consequence of the level of carbon dioxide that was sustained throughout the protocol. Alternatively, they suggested that the lack of response may have been a consequence of the duration of hypoxic episodes. This hypothesis was supported by findings which showed that a protocol comprised of shorter episodes (~ 1 minute) and recovery periods (~ 1–2 minute) resulted in the initiation of vLTF in healthy participants on two separate occasions (Pierchala *et al.*, 2008; Chowdhuri *et al.*, 2010). Whether or not maintenance of carbon dioxide levels as a consequence of the shortened episodes or another mechanism linked to the reduction in episode duration was primarily responsible for this finding remains to be established. Nevertheless, it has now been recognized that vLTF or genioglossus LTF can be induced during sleep in both flow limited and non-flow limited individuals (Babcock & Badr, 1998; Aboubakr *et al.*, 2001; Pierchala *et al.*, 2008; Chowdhuri *et al.*, 2008; Chowdhuri *et al.*, 2010).

Interestingly, independent of the protocol that was used when vLTF was evident during sleep the magnitude of the response was seemingly less than the magnitude typically observed during wakefulness (Table 1). Thus, we began to investigate whether or not arousal level impacts on the magnitude of vLTF. To date 10 healthy males and females have been exposed to twelve 2 min episodes of hypoxia interspersed with 2 minute recovery periods with carbon dioxide levels sustained 3 mmHg above baseline (Syed *et al.*, 2011). As hypothesized vLTF in healthy males and females were evident during wakefulness and sleep, however the magnitude of the response was reduced during sleep compared to wakefulness (Syed *et al.*, 2011).

Collectively, the available evidence suggests that vLTF can be initiated during sleep. The manifestation of the phenomenon may be less dependent on the length of hypoxic episodes and more dependent on the level of carbon dioxide sustained during the protocol, although this suggestion has not been tested during sleep. The magnitude of the phenomenon is less during sleep compared to wakefulness. Consequently, in designing and implementing an intermittent hypoxia protocol it is important to take into account the impact of arousal state on the magnitude of the response.

3.3. Recommendations

Based on the findings summarized above we have designed a protocol that will allow for the investigation of basic mechanisms associated with the initiation and maintenance of LTF, while minimizing or eliminating potential detrimental effects that may be associated with intermittent hypoxia (Dempsey *et al.*, 2010). The outlined protocol could potentially be an effective dose for treatment of sleep apnea or spinal cord injury; however whether or not it is the most effective dose remains to be determined.

After accommodating to the monitoring equipment, baseline minute ventilation and associated measures (e.g. fractional concentration of end-tidal oxygen, fractional concentration of end-tidal carbon dioxide, heart rate and blood pressure) should be monitored and recorded for a minimum of 10 minutes. At a minimum the final 5 minutes of the baseline period should be averaged. Averaging data across this length of time will eliminate bias associated with selecting and averaging data over shorter time intervals. After establishing baseline values, increase carbon dioxide 3 mmHg above baseline and monitor minute ventilation for an additional 15 minutes, with the final 5 minutes of collected data averaged. It is possible that the level of sustained carbon dioxide required for the manifestation of LTF could be less than 3 mmHg; however experimental data to support this possibility is required. Monitoring of minute ventilation for a minimum of 15 minutes is important since achieving a new steady state often takes 5–7 minutes, so it is likely best to average the final 5 minutes of data. The increase in carbon dioxide should be sustained during and following exposure to the intermittent hypoxia protocol. Thereafter, expose the participant to 12 hypoxic episodes that are 2 minutes in duration interspersed with 2 minute recovery periods, with the exception of the final recovery period that should be a minimum of 30 seconds in duration. The number and duration of hypoxic exposures will ensure that vLTF is initiated while at the same time ensuring that detrimental effects that might be associated with repeated daily exposure to intermittent hypoxia are minimized or more likely given the short duration of exposure eliminated. The length of the end-recovery period should be at least 30 minutes to account for any wind-up in minute ventilation that might occur over this period of time. The intensity of hypoxia should be characterized by oxygen saturation values between 85–88%, thereby ensuring a mild physiological response in humans. Typically, increases in minute ventilation and heart rate are similar to that observed while walking at a quick walking pace. Likewise, although blood pressure increases during the hypoxic exposure it typically returns quickly to normal levels. This intensity of hypoxia will minimize risk in regards to the immediate response to the stimulus as well as any

potential long-term detrimental consequences that arise in response to repeated daily exposure.

Future studies might explore the role of shorter episodes in initiating LTF. Some individuals have suggested utilizing episodes as short as 15 s in duration. However, caution should be taken in employing episodes shorter than 90–120 seconds since the shorter episodes will likely result in hypoxic intensities that are relatively mild and difficult to reproduce episode to episode, or across participants. Moreover, if the goal is to sustain carbon dioxide at a specific value throughout the protocol this will likely be more difficult to control. Lastly, the duration of the experimental study may be lengthened if the desired total duration of exposure to hypoxia (i.e. sum of all the hypoxic episodes) is to be similar to that achieved using episodes of longer duration.

It may be argued that the number and duration of hypoxic episodes that we have recommended does not mimic specific features of human disorders associated with intermittent hypoxia (e.g. sleep apnea). However, our goal in recommending this protocol is not to mimic a given condition, since the ability to replicate a specific disorder that has general applicability is difficult. For example, airway morphology, chemoreflex sensitivity, upper airway muscle activity and arousal threshold are only some of the factors that contribute to initiating sleep apnea (Dempsey *et al.*, 2010). Moreover, the dominance of one factor over another in the promotion of apnea varies across phenotypic groups. Thus, attempts to combine intermittent hypoxia with other factors to replicate sleep apnea are difficult and should not be a necessary requirement. Instead, our protocol is primarily designed to initiate respiratory plasticity in order to examine if activating these phenomena ultimately serves a beneficial purpose in the treatment of a given disorder. In our opinion it is less important that the duration of exposure to hypoxia mimics what occurs naturally, if the selected duration of exposure to hypoxia ultimately proves to be beneficial in treating a specific disorder. It may still be of interest to determine if naturally occurring hypoxemia which is observed in some conditions elicits respiratory plasticity; however the end result is not important if respiratory plasticity can be initiated with a protocol that is not naturally induced by a given condition but is effective in treating a condition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Summary of results from human long – term facilitation studies

Reference	Condition	Sex		Age	State	Hypoxic episode			Recovery		CO ₂	Magnitude of LTF				
		M	F			Intensity	Number	Duration	Between	End		V _E	GG	Dia	P _{crit}	UA
McAvoy et al. (1996)	Healthy	11	-	37	Wake	11	10	2	2	10	isocapnia	N	N	N	-	N
Babcock et al. (1998)	Healthy	4	3	NR	Sleep	8	10	3	-	60	hypocapnia	N	-	-	-	-
Babcock et al. (1998)	IFL	5	2	NR	Sleep	8	10	3	-	60	hypocapnia	Y (1.4)	-	-	-	-
Aboubakr et al. (2001)	OSA	9	2	52	Sleep	8	10	3	5	40	isocapnia	N	-	N	-	Y
Jordan et al. (2002)	Healthy	12	10	25	Wake	9	10	2	2	25	isocapnia	N	N	N	-	N
Shkoukani et al. (2002)	Healthy	5	6	29	Sleep	8	10	3	5	40	hypocapnia	Y (1.2)	-	N	-	Y
Babcock et al. (2003)	Healthy	14	11	30	Sleep	8	10	3	5	40	hypocapnia	Y (1.2)	-	-	-	-
Mateika et al. (2004)	Healthy	7	4	29	Wake	8	8	4	5	15 & 70	hypocapnia	N	-	-	-	-
Morelli et al. (2004)	Healthy	11	11	28	Wake	8	8	4	5	15	hypocapnia	N	-	-	-	-
Morris et al. (2004)	Healthy	7	3	31	Wake	10 & 6	3	1	15	NR	isocapnia	N	-	-	-	-
Harris et al. (2006)	Healthy	7	4	26	Wake	8	8	4	5	15	5 mmHg	Y (1.4)	Y (1.5)	-	-	-
Khodadadeh et al. (2006)	OSA	9	-	38	Wake	8	8	4	5	15	isocapnia	N	-	-	-	-
Diep et al. (2007)	Healthy	7	3	23	Wake	6 O ₂ /5 CO ₂	15	30 s	90 s	60	isocapnia	N	-	-	-	-
Rowley et al. (2007)	OSA	8	3	45	Sleep	8	10	3	5	20	isocapnia	N	N	-	N	Y
Chowdhuri et al. (2008)	Healthy	7	5	26	Sleep	8	10	3	5	40	isocapnia	N	Y (2.1)	N	-	N
Pierchala et al. (2008)	Healthy	7	5	26	Sleep	NR	15	69 s	1-2	20	isocapnia	Y (1.1)	-	-	-	Y
Wadhwa et al. (2008)	Healthy	15	15	24	Wake	8	8	4	5	15	4 mmHg	Y (1.5)	-	-	-	-
Lee et al. (2009)	Healthy	13	-	28	Wake	8	12	4	4	30	4 mmHg	Y (1.4)	-	-	-	-
Lee et al. (2009)	OSA	13	-	28	Wake	8	12	4	4	30	4 mmHg	Y (1.6)	-	-	-	-
Chowdhuri et al. (2010)	Healthy	4	7	26	Sleep	NR	15	1	1-2	10	hypocapnia	Y (1.1)	-	-	-	N
Gerst et al. (2010)	OSA	13	-	27	Wake	8	12	4	4	30	3 mmHg	Y (1.3)	-	-	-	-
Gerst et al. (2010)	Healthy	10	10	25	Wake	8	12	2	2	30	3 mmHg	Y (1.3)	-	-	-	-
Gerst et al. (2010)	Healthy	10	10	25	Sleep	8	12	2	2	30	3 mmHg	Y (1.1)	-	-	-	-

NR – not reported. Hypoxic episode intensity reported as % of inspired oxygen. Unless displayed differently hypoxic episode and recovery duration (between episodes and end-recovery after the last episode) are in minutes. The number in CO₂ (carbon dioxide) column indicates the level at which carbon dioxide was maintained above baseline. Y (Yes) indicates the manifestation of LTF. N (No) indicates the absence of LTF. (-) indicates that the measure was not obtained. Numbers in brackets indicate the magnitude of LTF as a fraction of baseline.