INVITED MINI-REVIEW

Intermittent Hypoxia and Neurorehabilitation

Gonzalez-Rothi EJ¹, Lee KZ², Dale EA³, Reier PJ⁴, Mitchell GS¹, Fuller DD¹*

Author contributions: EGR drafted the manuscript, and GSM and DDF revised and edited the text. KZL collected the data shown in Fig. 4. PJR and EAD contributed to original ideas presented herein, as well as manuscript revisions.

Addresses:
¹Department of Physical Therapy
College of Public Health and Health Professions
University of Florida
P.O. Box 100154, 100 S. Newell Drive
Gainesville, FL 32610, USA

²Department of Biological Sciences
College of Science
National Sun Yat-sen University
No. 70 Lien-Hai Rd.
Kaohsiung City, 804, Taiwan

³Department of Integrative Biology and Physiology
Terasaki Life Sciences Building
University of California – Los Angeles
610 Charlse E Young Drive, South
Los Angeles, CA 90095, USA

⁴Department of Neuroscience
College of Medicine
University of Florida
P.O. Box 100154, 100 S. Newell Drive
Gainesville, FL 32610, USA

*, corresponding author
David D. Fuller
Department of Physical Therapy
University of Florida
P.O. Box 100154, 100 S. Newell Drive
Gainesville, FL 32610, USA
Email: ddf@phhp.ufl.edu

RUNNING HEAD: Intermittent Hypoxia and Neurorehabilitation
Intermittent Hypoxia and Neurorehabilitation

Abstract

In recent years, it has become clear that brief, repeated presentations of hypoxia (i.e., acute intermittent hypoxia, AIH) can boost the efficacy of more traditional therapeutic strategies in certain cases of neurologic dysfunction. This hypothesis derives from a series of studies in animal models and human subjects performed over the past 35 years. In 1980, Milhorn and colleagues showed that electrical stimulation of carotid chemosensitive neurons produced a persistent, serotonin-dependent increase in phrenic motor output that outlasts the stimulus for more than 90 minutes (i.e., a “respiratory memory”). AIH elicits similar phrenic “long term facilitation” (LTF) by a mechanism that requires cervical spinal serotonin receptor activation and de novo protein synthesis. From 2003-present, a series of studies demonstrated that AIH can induce neuroplasticity in the injured spinal cord, causing functional recovery of breathing capacity after cervical spinal injury. Subsequently, it was demonstrated that repeated AIH (rAIH) can induce recovery of limb function, and the functional benefits of rAIH are greatest when paired with task-specific training. Since uncontrolled and/or prolonged intermittent hypoxia can elicit pathophysiology, a challenge of intermittent hypoxia research is to ensure that therapeutic protocols are well below the threshold for pathogenesis. This is possible since many low dose rAIH protocols have induced functional benefits without evidence of pathology. We propose that carefully controlled rAIH is a safe and non-invasive modality that can be paired with other neurorehabilitative strategies including traditional activity-based physical therapy or cell-based therapies such as intraspinal transplantation of neural progenitors.

Key Words

Intermittent Hypoxia, Neurehabilitation, Spinal Cord Injury, Cellular transplantation
Intermittent Hypoxia and Neurorehabilitation

Introduction

The fundamental theme of this review article is that brief, repeated presentations of hypoxia (i.e., acute intermittent hypoxia, AIH) are a safe and (relatively) easily applied therapeutic modality that triggers beneficial neuroplasticity. Although we will focus on potential applications of AIH to neurorehabilitation after spinal cord injury (SCI), the fundamental principles of AIH-induced neuroplasticity are likely to apply to other movement disorders (65, 77). Here, we review evidence that repetitive AIH provides direct benefits to respiratory and non-respiratory motor function, and discuss the potential use of AIH in conjunction with other treatment modalities, including electrical stimulation of the spinal cord and cell-based therapies (i.e., cell transplantation). Like all therapeutic agents, the dose-response curve must be carefully considered. A guiding principle of this article is that appropriate “doses” of AIH evoke beneficial plasticity while minimizing negative consequences associated with more severe or prolonged protocols of intermittent hypoxia (73). Indeed, chronic intermittent hypoxia can lead to a range of pathophysiological outcomes. Thus, one challenge in IH research is to ensure that the AIH paradigm is well below the threshold for pathogenesis.

Brief history of AIH and respiratory motor plasticity. Initial studies of AIH and neuroplasticity focused exclusively on the respiratory system. A timeline which highlights key advances over the past 35 years is shown in Fig. 1. The seminal work from Millhorn and colleagues showed that electrical stimulation of carotid chemoafferent neurons (from the primary hypoxia-sensitive chemoreceptors, the carotid bodies) produced a persistent increase in phrenic motor output in anesthetized cats (64). This study elaborated the concept of long-lasting “memories in breathing,” a topic formally reviewed in 1986 in the Handbook of Physiology (20). Subsequently, Hayashi and colleagues showed that similar neuroplasticity could be evoked by replacing electrical stimulation of the carotid sinus nerve with AIH (37). In this paper, the term “long term facilitation” (LTF) was introduced to describe the persistent increase of phrenic nerve
burst amplitude that occurred after exposure to brief periods of intermittent hypoxia. Shortly thereafter, Fregosi and Mitchell (1994) used electrical stimulation of the carotid sinus nerve in cats to show that LTF also occurs in inspiratory intercostal nerve activity (26). A few years later, Bach and Mitchell showed that AlH-induced phrenic LTF in rats required activation of serotonin receptors, and was expressed in upper airway motor outputs, particularly in hypoglossal nerve activity (4). Other notable advances in the AlH/LTF field include: 1) establishing that, whereas acute intermittent exposures to hypoxia (i.e. AlH) evokes LTF, exposure to a comparable duration of continuous hypoxia does not (7); 2) a series of papers (using in vivo and in vitro preparations) localizing the relevant cellular mechanisms at or near the specific motoneuron pool exhibiting LTF (5, 6); 3) confirming that ventilatory LTF can be elicited in unanesthetized animals of multiple species (66)); and 4) the demonstration that ventilatory LTF in unanesthetized animals is greater during sleep versus wakefulness (71, 92). Since the initial descriptions of respiratory LTF in animal models, subsequent work has shown that LTF of ventilation can be evoked in humans during sleep (3, 78) and wakefulness (36, 62), although it may require mild background hypercapnia (61).

Work in animal models has generated considerable information concerning cellular/molecular mechanisms of AlH-induced LTF (10, 66). These studies have identified multiple, distinct cellular cascades capable of giving rise to phrenic motor plasticity under different conditions (10). Most forms of AlH-induced LTF require serotonin receptor activation on or near respiratory motoneurons (6, 10, 29). Cellular mechanisms of phrenic motor facilitation (pMF) initiated by 5-HT2 (type A or B) receptor activation have been described as the Q pathway to pMF since these are Gq coupled metabotropic receptors. Necessary downstream signaling includes activation of protein kinase C, de novo synthesis of brain derived neurotrophic factor (BDNF) (5), activation of the high affinity BDNF receptor tropomyosin-related kinase B (TrkB), and ERK MAP kinase signaling (43). The Q pathway also requires superoxide formation via
NADPH oxidase (58, 59). The reactive oxygen species apparently inhibit okadaic-acid sensitive serine/threonine phosphatases that normally constrain expression of phrenic LTF (60, 100, 102).

A distinct pathway to pMF, referred to as the S pathway, is initiated by Gs protein-coupled metabotropic adenosine (A<sub>2A</sub>) (33) and/or 5-HT<sub>7</sub> receptors (41). The S pathway involves necessary downstream signaling via cyclic AMP, de novo synthesis of an immature TrkB isoform) (33), and Akt signaling. Importantly, the Q and S pathways do not summate to produce enhanced LTF; rather, their complex interactions are characterized by mutual “cross-talk inhibition” (14, 40, 76). For example, moderate protocols of AIH stimulate serotonin release and activation of high-affinity 5-HT<sub>2</sub> receptors which enables the Q pathway to dominate. In contrast, more severe AIH protocols result in activation of A<sub>2A</sub> and 5-HT<sub>7</sub> receptors, which blocks the Q pathway and thus enables the S pathway to drive pMF. Although the mechanisms of cross talk inhibition from the Q to S pathway are unknown, protein kinase A mediates cross-talk inhibition from the S to the Q pathway (42). A summary of essential elements in the S and Q pathways to pMF is provided in Fig. 2.

**Metaplasticity and intermittent hypoxia.** With repeated exposure to AIH (rAIH) it is essential to know if the effects accumulate, habituate, or remain unchanged. To be useful therapeutically, it is critical that repeated AIH presentations provide cumulative and continued impact in terms of both magnitude and duration of its beneficial effects. Cumulative effects can be considered “metaplasticity,” since the plasticity (ie. LTF of phrenic motor output) itself exhibits plasticity (24, 67). Pre-clinical work in rat models has established that repeated intermittent hypoxia elicits phrenic LTF metaplasticity. For example, Ling et al. (2001) first reported that pre-treatment with chronic intermittent hypoxia (i.e., IH exposures 8-12 hours per day for one week) enhances AIH-induced phrenic LTF (56). However, chronic intermittent hypoxia is too severe for clinical application; subsequent work focused on more modest rAIH protocols (73). Wilkerson et al.
Intermittent Hypoxia and Neurorehabilitation

(2009) set the stage for future therapeutic applications of rAIH by showing that a paradigm of 10, 5-min episodes of 11% O₂ for 7 consecutive days evokes LTF metaplasticity without evidence for systemic hypertension (101). This rAIH paradigm also increased BDNF expression in the vicinity of phrenic motoneurons and enhanced the capacity for LTF (101).

**Intermittent hypoxia and spinal cord injury.** Since intermittent hypoxia elicits both respiratory motor plasticity and metaplasticity, several investigations have been performed to examine the therapeutic potential of AIH or rAIH to treat neurological conditions associated with impaired respiratory motor output (11, 12, 97). Spinal cord injury (SCI) represents one condition that can benefit from increased spinal synaptic transmission to respiratory motoneurons and/or associated neural networks (11, 65).

Axonal connections between brain and spinal cord are at least partially interrupted by SCI, and the loss of motor, sensory and autonomic function below the injury has devastating consequences. More than half of SCI occur in the cervical region, impairing motor function and sensory feedback from the limbs. The majority of spinal injuries are incomplete, and some degree of spontaneous functional recovery does occur in the weeks to months after injury. Such spontaneous recovery is often attributed to plasticity in uninjured spinal pathways or other areas of the central nervous system. Unfortunately, spontaneous recovery of motor and/or sensory function is modest at best, and seldom enables complete restoration of function. Thus a major focus of contemporary clinical and pre-clinical SCI research is aimed at enhancing CNS plasticity to enhance functional recovery. Although numerous therapeutic strategies have been investigated to enhance motor recovery after SCI, few have yielded robust outcomes when replicated or translated to clinical populations. There is a clear need for new therapeutic strategies to promote plasticity and restore motor function. Of equal importance, these strategies must be “translatable” to the clinic.
Intermittent Hypoxia and Neurorehabilitation

Intermittent Hypoxia and spinal cord injury: preclinical respiratory studies. The first study to explore the therapeutic potential of intermittent hypoxia for mitigating respiratory impairment after SCI was published in 2003 (31). In this study, rats were exposed to either chronic intermittent hypoxia (5 min episodes of 11% O₂, 5 min intervals, 12 hours per night, 7 days) or a similar protocol of normoxia beginning one week following C2 spinal hemisection (C2Hx). Increased spontaneous inspiratory phrenic burst amplitude was observed ipsilateral to C2Hx in hypoxia treated rats. Furthermore, electrical stimulation of the contralateral spinal cord above the injury demonstrated increased strength of crossed-spinal synaptic pathways to phrenic motor neurons following hypoxia conditioning. The authors were careful to point out potential shortcomings that may limit or constrain the use of chronic IH as a therapeutic tool; specifically, the potential for pathophysiological comorbidities made it unsuitable as a treatment.

Subsequently, Golder et al. (2005) demonstrated that even a single presentation of AIH was sufficient to enhance respiratory motor output (i.e. phrenic LTF) below a C2Hx but only following chronic injury (8-, but not 2-weeks post-injury) (32). Furthermore, the time-dependence of AIH effects on phrenic LTF following C2Hx was strongly correlated with the initial loss and subsequent partial recovery of serotonergic innervation in the phrenic motor nucleus.

A subsequent study focused on daily AIH as a therapeutic approach (10, 5-minute hypoxic episodes (11% O₂), 5-minute normoxic intervals, 7 consecutive days beginning 1 week post-C2Hx injury)(57). Tested one day after the final AIH exposure (2-weeks post-injury), daily AIH treated rats demonstrated: 1) substantial recovery of breathing capacity (unanæsthetized, spontaneously breathing); 2) increased spontaneous phrenic motor output ipsilateral to injury; 3) strengthened crossed-spinal synaptic pathways to phrenic motor neurons; and 4) increased BDNF, TrkB and phospho-TrkB expression in and around phrenic motor neurons (57). These functional benefits were not associated with evidence of CNS pathology, such as hippocampal cell death and/or reactive gliosis. More recent studies investigating respiratory muscle EMG...
Intermittent Hypoxia and Neurorehabilitation

activity (72, 74) confirm this finding, and indicate that both AIH and daily AIH act primarily on respiratory pathways not directly impacted by SCI; specifically, increased activity occurred predominantly in contralateral (intact) diaphragm and intercostal muscle EMG activity 7 days post-injury. In a study by Navarrete-Opazo and colleagues (2015) rats were exposed to daily AIH (10, 5-minute hypoxic episodes (11% O₂), 5-minute normoxic intervals, 7 consecutive days beginning 1 week post-C2Hx injury) followed by weekly presentations of AIH for an additional 8 weeks. The functional respiratory benefits of daily AIH persisted at least one week after completion of daily AIH exposures, and were shown to be mediated by an adenosine (not serotonin) dependent mechanism (74). Interestingly, the subsequent weekly “reminder” presentations of AIH were not sufficient to maintain these effects, raising the possibility that more frequent rAIH is necessary to prolong the observed effects. Collectively, these studies suggest that rAIH will be an effective therapeutic for restoring breathing capacity in patients with SCI. Further, these studies represent an important step in the clinical translation of rAIH to clinical populations since the daily AIH protocol confers functional benefits without attendant pathology (57, 87).

Intermittent Hypoxia and spinal cord injury – non-respiratory motor systems. The response to hypoxia is not restricted to respiratory neurons/networks. There is abundant literature suggesting that hypoxia activates raphe serotonergic neurons, and that serotonin is subsequently released in diverse regions of the brainstem and spinal cord (49, 66). For example: 1) carotid chemoafferent neuron activation (68) increases caudal raphe neuron activity, 2) hypoxia increases c-fos labeling in caudal raphe serotonergic neurons, consistent with increased activity (21, 22), and 3) hypoxia releases serotonin in the brainstem of anesthetized cats (83) and the spinal cord of anesthetized rats (49). Since raphe-spinal serotonergic neurons project throughout the spinal cord, including non-respiratory regions, and most motoneurons express serotonin receptors (39), it is not unlikely that the same AIH-
Intermittent Hypoxia and Neurorehabilitation

induced, serotonin-dependent mechanisms of spinal motor plasticity are manifest in non-respiratory motor systems. In fact, there is accumulating evidence that AIH-induced neuroplasticity is not limited to the neural system controlling breathing, and can also occur in other motor systems (Fig. 3).

Lovett-Barr and colleagues (2012) were the first to systematically investigate the impact of rAIH in non-respiratory motor systems after SCI (57). One month after cervical SCI in rats, rAIH for seven consecutive days improved skilled forelimb function in a horizontal ladder-walking task. These functional benefits lasted more than one three weeks post-treatment, and were not associated with any evidence of CNS comorbidities characteristic of chronic IH protocols (hippocampal cell death/reactive gliosis). Improvements in motor function coincided with increased expression of BDNF and TrkB receptors in non-respiratory motor nuclei, consistent with the hypothesis that similar molecular mechanisms underlie the improvements in respiratory and non-respiratory motor function following daily AIH. In a follow up study, Prosser-Loose et al. (2015) demonstrated that pairing AIH (11% O₂; 10, 5-min episodes per day x 7 days) with task specific training was necessary for observed functional benefits (81). Rats exposed to daily AIH (7 days) beginning 4 weeks post-SCI exhibited the most robust functional recovery when AIH was paired with ladder walking practice versus treadmill training or AIH alone. These functional improvements were retained up to 8 weeks post-treatment, and were not found in non-trained motor behaviors, such as reach to grasp, grip strength and paw use preference. These results confirm the effectiveness of repeated AIH as a therapeutic approach to restore motor function, but demonstrate that rehabilitation is optimal with combined AIH + task-specific training, at least in non-respiratory motor systems.

Translation to clinical populations. The first report that AIH could be safely implemented in a clinical SCI population was published in 2012 (96). Trumbower and colleagues showed that AIH could modulate somatic motor function in humans by demonstrating that a single AIH
Intermittent Hypoxia and Neurorehabilitation

presentation increases ankle plantar flexor strength in 13 humans with chronic (~15 years post-injury) incomplete SCI. Specifically, changes in maximal voluntary isometric ankle plantar flexion torque and plantar flexor electromyogram activity were assessed before and after AIH (15, 1 min hypoxic exposures – inspired O\textsubscript{2}=0.09, SaO\textsubscript{2} ~ 80%; 1 minute intervals). Using a double blinded, cross-over design, results were compared between AIH and sham normoxia (e.g. room air – inspired O\textsubscript{2}=0.21). Immediately following AIH, plantar flexion torque increased 82% above baseline, and increased strength continued for more than 90 minutes post-AIH. Improved muscle strength remained elevated for more than four hours in several of the study participants, suggesting the potential for long-lasting effects. Increased ankle plantar flexor EMG activity correlated with increased torque. No differences in plantar flexion strength or electromyogram activity were observed in sham experiments.

A recent study by Tester \textit{et al.} was the first to assess rAIH effects on respiratory function in a clinical population (93). Humans with chronic cervical or thoracic SCI were exposed to 8, 2-minute intervals of 8% inspired O\textsubscript{2} for 10 consecutive days. Significant and sustained increases in minute ventilation (\textit{i.e.} ventilatory LTF) were observed on each day of AIH exposure. However, LTF magnitude was constant across the 10 day period; cumulative responses were not detected. This study provided proof-of-principle that AIH could be used to enhance breathing function in individuals with chronic SCI, laying a foundation for future studies concerning appropriate intermittent hypoxia protocols for humans.

Collectively, initial AIH studies in humans with chronic SCI are promising; no adverse events have been reported in the initial trials (48, 93, 96).

**Combinatorial therapies.** AIH may serve as a “stand alone” therapy, or may be more effective when paired with other rehabilitation (task specific) approaches (Fig. 3). For example, the finding that AIH increases ankle strength may be important clinically, as reduced ankle strength is a major factor affecting mobility after SCI (96). Plantar flexor muscle activation contributes
Intermittent Hypoxia and Neurorehabilitation

significantly to the energetics of normal walking; thus, pairing AIH with traditional plantar flexor
strength training might further enhance recovery. AIH may also have utility in the context of
newer (still experimental) interventions, such as cell transplantation (53). In the next section, we
review three substantially different approaches to spinal cord rehabilitation that have a common
thread: each has potential to benefit if paired appropriate AIH “priming.” Specific examples
include: 1) locomotor training (i.e., traditional “activity based” physical therapy), 2) electrical
stimulation of the spinal cord, and 3) cell transplantation (e.g. stem/progenitor cell therapy).

Paired AIH and task-specific rehabilitation/training. Hayes and colleagues tested the
hypotheses that: 1) daily AIH (5 days) augments locomotor function (e.g. walking speed and
endurance) in patients with chronic, incomplete SCI, and 2) combined daily AIH with walking
practice enhances the functional benefits of AIH alone (38). In their study, nineteen individuals
with incomplete SCI received AIH (15 x 90 sec hypoxic episodes of 0.09 inspired O2; 60 sec
normoxic intervals), or daily sham normoxia exposures. AIH was given alone, or combined with
30 minutes of overground walking practice 1 hour later. Daily AIH improved both walking speed
(10 meter walk test) and endurance (6 minute walk test). Combined daily AIH and walking
practice resulted in greater improvements in walking endurance than daily AIH treatment alone.
These benefits persisted up to one week post-training, but by 2 weeks, walking endurance was
not different from daily AIH alone. Participants were monitored for negative side effects during
AIH exposures, including assessment of headaches, pain, lightheadedness, dizziness, altered
vision, respiratory distress, cyanosis, spasticity, autonomic dysreflexia, cardiopulmonary/circulatory instability or changes in motor or sensory function. The rAIH
paradigm was well tolerated by all study participants, and no adverse events were observed or
reported during or following the study.

One of the most intriguing findings of the Hayes study relates to the magnitude of the
effects given the limited treatment duration. Contemporary therapies for individuals with SCI
Interruption Hypoxia and Neurorehabilitation typically consist of several weeks-months of intensive training, however clinically meaningful improvements are often limited. Indeed, when compared with a recent study that examined the effects of 60 sessions (12 weeks) of four different locomotor therapies, the effect sizes for walking speed and endurance following AIH and AIH + walking were more than twice those achieved by traditional locomotor therapies. Furthermore, AIH and AIH + walking resulted in walking speeds and walking endurance that was comparable to or greater than those seen in training studies of much longer durations. Indeed, more than 30% of subjects that received AIH + walking practice achieved a clinically meaningful change in walking speed and more than 70% achieved a clinically meaningful change in walking endurance (38).

Spinal Cord Electrical Stimulation and AIH. Tonic, spinal cord stimulation has recently garnered a great deal of attention as a powerful tool to restore lost function following SCI. Although the underlying mechanisms are not yet clear, applying electrical currents to the injured spinal cord can induce profound functional benefits. For example, electrical stimulation of the ventral surface of the thoracic spinal cord triggers rhythmic diaphragm contractions after complete transection of the high cervical spinal cord in dogs and rats (16-18, 50). Further, epidural stimulation of the lumbar spinal cord in humans with SCI restores voluntary activation of distal muscles, even in patients with severe SCI (2, 35). In animal models, the efficacy of spinal cord stimulation is enhanced by pharmacologic interventions that activate monoamine receptors. For example, serotonin receptor agonists produce rhythmic stepping when coupled with spinal stimulation in rats (69, 70). Since rAIH enhances spinal synaptic efficacy (27, 57), and increases spinal serotonin terminal density and receptor expression (87), AIH may be a modality that can increase the efficacy of electrical spinal stimulation paradigms.

Cellular therapies. The goals of tissue transplantation after SCI include filling and/or “bridging” cyst cavities, replacing lost cells (e.g. motoneurons, interneurons, oligodendrocytes), and creating a favorable environment for growth (82). Fundamental to the therapeutic success of cell
transplantation is the optimization of cell yield, generation of neuronal phenotypes, and enhancement of graft survival and graft-host connectivity post-transplantation. Very little effort has been made to date directed at enhancing host-graft functional integration with preconditioning and/or training paradigms. Studies using “enriched environments” (e.g., larger cage, toys, running wheels, etc.) in association with cell transplants have demonstrated improved functional outcomes of cell transplantation following SCI (25). Furthermore, these studies suggest that therapies altering the “trophic environment” may enhance host-graft integration.

Our group and others have explored the use of fetal spinal cord (FSC) transplants to reconstruct disrupted spinal circuitries (i.e., “gray matter repair”) and/or to create novel neuronal relays (reviewed in (82)). FSC grafts represent transplants of lineage-restricted precursors (i.e., cells with a pre-determined fate e.g., interneurons), and residual stem cell populations (9, 54, 55, 94, 95). FSC grafts typically fill spinal lesion cavities by one-month post-transplantation (95), become anatomically integrated with host-spinal interneurons (52), and are associated with improved locomotion after SCI (reviewed in (82)). We recently found that neurons within mature FSC transplants in the C2 spinal cord respond to hypoxia with increased bursting (52). Since FSC graft neurons respond to hypoxia (52), and host neurons in the immediate vicinity of a cervical graft (e.g., phrenic motoneurons, interneurons) also robustly increase discharge during hypoxia (86), we reasoned that rAIH may be a stimulus that could enhance the modest synaptic connectivity that appears to develop between the host respiratory circuitry and FSC graft neurons following cervical transplantation (52, 99). In a preliminary study, we therefore explored the impact of rAIH on neuronal discharge in mature FSC grafts in the cervical spinal cord (53). Using previously published methods (52), adult male rats with C2Hx received FSC transplants (embryonic day 14 tissue(82)) at the time of injury. Beginning 1 week or 6 weeks after the FSC transplant procedure, a paradigm of rAIH was initiated (10, 5-minute episodes of 10% inspired
Intermittent Hypoxia and Neurorehabilitation

O₂, 3 days per week). At 10 weeks post-injury, electrical activity of graft neurons and the ipsilateral phrenic nerve were recorded in anesthetized, vagotomized and ventilated rats using published methods (52). Synchronized bursting of FSC graft neuron activity (i.e., “population activity”) was observed in 7/12 (58%) of transplanted animals that received hypoxia training. We have never observed this response in control (i.e., non rAIH exposed) FSC grafts (52). Moreover, we found two very clear examples of FSC grafts which displayed respiratory-related discharge patterns during baseline or hypoxia (see Fig. 4). During these recordings, we repeatedly removed the recording electrode from the visibly discernable graft tissue (see examples in (52, 99)), and then reinserted the electrode to confirm the observation. In prior experiments, we have never observed respiratory-related neuronal discharge in control (non-hypoxia treated) FSC grafts in the cervical spinal cord (52). Further work is obviously needed to validate these initial observations, and to determine the functional significance of respiratory-related discharge in graft neurons. Nevertheless, these data suggest that rAIH exposure may provide a tool to enhance the “functional integration” of neuronal progenitor transplants with host respiratory neurons.

Another potential use of AIH in the context of cellular transplantation is to “pre-condition” cells prior to transplantation. For instance, culture of neural progenitor cells in a low O₂ environment, increases cell proliferation and yield (90), makes cells less susceptible to programmed cell death (90), and elaborates neuronal differentiation (8). Moreover, in vivo AIH exposure prior to harvest of subventricular zone-derived (SVZ) neural progenitor cells results in an increase their subsequent in vitro expansion (84). In that study, rAIH increased Pax6 expression (a transcription factor involved in neuronal fate determination) in the harvested SVZ cells, and was associated with increased neuronal differentiation. Thus in vivo rAIH can enhance the viability of subsequent in vitro SVZ-derived cell cultures, and may provide a means to “prime” neural progenitor cells prior to transplantation into the injured CNS.
Optimization of rAIH protocols. To realize the potential of rAIH for clinical use, it is necessary to determine optimal protocols including the number, frequency and severity of hypoxic episodes in patients (97). It will be of fundamental importance to minimize or eliminate potentially adverse consequences of rAIH such as hypertension, autonomic dysreflexia, neuroinflammation, reactive gliosis and hippocampal cell death (34, 79, 80, 85, 105). Whereas severe intermittent hypoxia (as in obstructive sleep apnea) causes severe morbidity, low-moderate doses of rAIH do not appear to induce pathology (73). It is not yet possible to definitively state the appropriate (or “best practice”) rAIH paradigm, but some clear guidelines are emerging (see (73). Paradigms using inspired O₂ in the range of approximately 9-16% and with relatively low numbers of exposures (e.g. 3-15 episodes) have predominantly beneficial effects whereas more severe protocols of inspired O₂ (e.g.<9%) and more episodes per day (>50), produce dose-dependent pathology. It is possible that variations in the number exposures or the duration of exposures or treatments may yield even greater therapeutic benefit in humans. Future studies are warranted to maximize rAIH-induced functional gains and sustainability without invoking maladaptive plasticity and adverse consequences.

Additional considerations regarding AIH as a therapeutic modality. Multiple factors may undermine (or enhance) AIH-induced plasticity and, therefore, influence the therapeutic efficacy of rAIH. We will briefly comment on a few variables that merit particular consideration.

Inflammation. Systemic inflammation persists for months and even years after SCI, and can have considerable impact on the degree of motor recovery (1, 88). The impact of inflammation on neurons and networks that regulate breathing has only recently been appreciated (15, 98). Initial explorations of this topic indicate that even low-grade systemic inflammation dramatically impairs the ability to express AIH-induced respiratory plasticity (46, 47). Thus, AIH-induced LTF of phrenic motor output is abolished by the modest inflammation caused by low-dose systemic lipopolysaccharide (46, 98) or a “high dose” of intermittent hypoxia (e.g., repeated IH exposures
Intermittent Hypoxia and Neurorehabilitation

throughout a 24 hr period (45). In both cases, phrenic LTF can be restored by a non-steroidal
anti-inflammatory drug (ketoprophen), which is thought to act either via inhibition of COX$_{1/2}$
enzymatic activity or via inhibition of the pro-inflammatory transcription factor NFκB (47).

In addition to spontaneous, ongoing systemic inflammation associated with SCI, individuals
with SCI are highly susceptible to acute inflammation associated with, for example, bladder
infections or skin lesions. Unfortunately, at this point little is known concerning how ongoing or
acute systemic or neuro-inflammation influence spontaneous functional recovery of breathing
capacity following SCI, or how it impacts the therapeutic efficacy of rAIH. It may be that anti-
inflammatory treatments increase the efficacy of rAIH therapy.

Sleep disordered breathing. SCI is associated with a considerable increase in the prevalence of
sleep disordered breathing, including both central and obstructive sleep apnea (OSA) (23). This
may be an important consideration since AIH-induced ventilatory LTF is enhanced in
neurologically intact individuals with OSA (30, 51, 91). One theory is that IH associated with
chronic OSA triggers metaplasticity in phrenic LTF (56) and ventilatory LTF (63). One prior
study with a small sample size failed to detect any difference in the ventilatory LTF following AIH
in individuals with SCI, with or without co-morbid OSA (93). From a different perspective, a
single night of central or obstructive OSA may trigger neuroinflammation that is qualitatively
different from that experience after chronic IH or OSA, and this short-term effect serves to
undermine AIH-induced phrenic LTF(45) (Huxtable, A.G. et al, Neuroscience 2015). The topic
of sleep disordered breathing in individuals with SCI is complex (28) and we suggest that the
past history of hypoxic exposures is an important variable to consider in the context of rAIH
therapy and SCI.

Drug therapies. Concomitant use of certain drugs may alter the impact of rAIH on spinal
neuroplasticity. Drugs that reduce spasticity, such as baclofen, activate inhibitory
neurotransmitter receptors on spinal neurons (13), potentially reducing the excitability of spinal
motoneurons and undermining the therapeutic efficacy of rAIH. In contrast, pharmacological inactivation of A2A receptors (40, 75) or alterations in serotonin receptor activity may enhance or undermine the therapeutic efficacy of rAIH in restoring motor function. For example, caffeine is an A2A receptor antagonist, and may be highly beneficial in amplifying the impact of rAIH therapy in individuals with chronic SCI.

Many individuals with chronic SCI take antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) or monoamine oxidase inhibitors. These types of drugs elicit complex effects on serotonergic function, increasing serotonin availability in the extraceullar space, yet down-regulating key serotonin receptor subtypes (eg. 5-HT2A) with chronic administration. Because serotonin elevations are sustained and key receptor subtypes decrease their expression with chronic SSRI administration, their impact on rAIH induced functional recovery is uncertain.

Gender. Consideration of sex-related differences in response to rAIH is important, as sex hormones greatly influence the magnitude of the AIH induced phrenic and hypoglossal LTF. Specifically, LTF is absent in male rats after gonadectomy but restored with testosterone replacement (104). Furthermore, the phase of the estrous cycle and levels of progesterone and estrogen in females influences the magnitude of LTF and ventilatory responses (103). After SCI, low testosterone is common in males (19), and altered menstrual cycles are common in females (89). Both of these effects may influence the extent to which individuals respond to rAIH therapy. The study by Tester and colleagues investigating daily AIH in subjects with chronic SCI provides suggestive evidence that sex/sex hormones may indeed be an important consideration for the therapeutic efficacy of rAIH. In their study, repeated AIH appeared to enhance ventilatory LTF in males, and to actually reduce this response in females (93). However heterogeneity of injury and the limited sample size (N=8) in this study precluded the authors from making firm
Intermittent Hypoxia and Neurorehabilitation

conclusions concerning the impact of sex differences on the magnitude of LTF; further studies in
a larger population of individuals with SCI are warranted.

Level, Severity and Chronicity of Injury. The neurological level of SCI is always an important
consideration for neurorehabilitation strategies. In regards to breathing, cervical vs. thoracic
spinal cord injuries are likely to have differential effects on inspiratory (diaphragm/intercostal)
versus expiratory (intercostal/abdominal) motor function. In the study by Tester et al. (93), two
individuals with thoracic SCI showed no AIH-induced improvements in ventilatory outcomes,
whereas the majority of cervically injured participants showed improvements in forced vital
capacity (FVC) and forced expiratory volume in 1 second (FEV₁). Accordingly, rAIH may be
more beneficial to individuals with cervical versus thoracic injuries since they have greater
deficits in inspiratory function. The severity of the SCI is also an important consideration, as
severe disruption of descending neural pathways may limit the potential substrate for IH-
induced plasticity. Lastly, the chronicity of injury is known to influence the extent of
neuroplasticity and therapy-induced functional gains in persons with SCI (44). The fact that
improvements in both respiratory and non-respiratory motor function were observed in all three
human trials of AIH is promising, as these trials were conducted in individuals with chronic
injuries, suggesting that this therapy has the potential to elicit functional improvements long after
the onset of injury.

Interactions between the molecular pathways that trigger respiratory plasticity. There is “cross-
talk inhibition” between pathways to long-lasting phrenic motor facilitation, and these
interactions may influence the degree of functional recovery induced by rAIH (14). For example,
during severe AIH, greater adenosine formation/accumulation increases A₂A receptor activation,
resulting in dominant S pathway activation (76) with subsequent Q pathway suppression.
Conversely, moderate AIH stimulates serotonin release with coincident adenosine
accumulation, leading to predominant Q pathway activation (6, 29). The interactions between
pathways enable either pathway to predominate depending on condition; however, it is also possible for them to reach a balance where they offset one another, effectively cancelling the functional benefits of AIH. Accordingly, one possibility to consider is that at certain times post-SCI, or with certain rAIH protocols, these inhibitory interactions could compete, creating an impasse where neither the S nor the Q pathways will be expressed as motor facilitation. In this circumstance, the potential benefits of rAIH therapy would be undermined.

**Conclusion.** Recent trends in SCI rehabilitation highlight the benefits of utilizing combinatorial approaches to amplify small improvements of single interventions. Combining traditional rehabilitation approaches with therapies designed promote neuroplasticity or regeneration (e.g., cellular transplantation, gene therapy, etc.) have had some success in animal models, but translation to humans has been challenging. rAIH is a very attractive approach in this regard since it can noninvasively induce spinal plasticity, and has had remarkable functional impact in animal models of SCI and in a limited number of human studies. Initial observations suggest that AIH can be effectively paired with traditional rehabilitation paradigms (e.g., locomotor training) and with more experimental approaches such as cell transplantation (Fig. 4).

One of the most important barriers to overcome is to change the attitudes of clinicians and patients regarding the potential therapeutic benefits of breathing low levels of O2. Recent trials in humans with SCI will help in this regard, as they demonstrate that rAIH can be safely administered to humans with chronic, incomplete SCI without negative consequences. To translate rAIH to clinical and/or at home use as a therapeutic tool, design of suitable devices and FDA approval is essential. Salient features to consider in the design of the device include safety, cost (initial purchase as well as the cost of daily use/maintenance), size, personnel requirements for operation, and ease of set-up and use.
Acknowledgements. This research was supported by grants from the National Institutes of Health HD-052682 (DDF), HL-69064 (GSM), HL-080209 (GSM), and NS-054025 (PJR), the Department of Defense (GSM), and the Brain and Spinal Cord Research Trust of Florida (PJR and DDF).
Figure Legends

Figure 1. Timeline depicting some of the important milestones in the study of intermittent hypoxia and respiratory neuroplasticity. Specific emphasis is placed on the initial seminal work that established the concept of “respiratory memories”, and then on publications that have directly lead to the use of intermittent hypoxia as a potential therapeutic modality in humans with SCI. This timeline represents a summary, and many important contributions were not included.

Figure 2. Working model of the cellular pathways contributing to long-lasting phrenic motor facilitation triggered by intermittent hypoxia. The highly simplified drawing depicts “respiratory drive” as a single axon terminal originating from the brainstem. Membrane proteins and intracellular pathways are depicted on a phrenic motoneuron. The “Q” pathway (left side of the figure) is elicited by intermittent activation of Gq-coupled metabotropic receptors (e.g., 5-HT₂ or α₁), followed by activation of PKC, new synthesis of BDNF, activation of TrkB, and activation of ERK MAP kinases (pERK). The “S” pathway (right side of the figure) is elicited by Gs-coupled metabotropic receptors (e.g., 5-HT₇ and A₂A), followed by activation of cAMP, new synthesis of an immature TrkB isoform, and downstream signaling via Akt phosphorylation/activation (pAkt). The specific mechanisms by which pERK (“Q” pathway) and pAkt (“S” pathway) elicit persistent increases in phrenic motor output are not known but are likely to involve changes in motoneuron excitability and/or synaptic strength.
Figure 3. Intermittent hypoxia can act throughout the spinal neuraxis following SCI. Initial work on AlH focused on the respiratory system, but recent work (see Fig. 1) demonstrates that upper and lower extremity motor function is also enhanced after daily AlH. This conceptual diagram depicts strengthening of synaptic connections in the mid-cervical (phrenic-diaphragm motor system), low cervical (forelimb / upper extremities) and lumbo-sacral spinal cord (leg / ankle function). We propose that common mechanisms contribute to plasticity in these respiratory and nonrespiratory motor systems after AlH therapy (e.g., triggered by serotonin receptor activation; see Fig. 2 for a more comprehensive summary). Collectively, evidence from both human and animal models indicates that AlH is safe and easy to administer, induces robust spinal neuroplasticity, and may be an effective therapeutic approach to enhance motor function in persons with chronic SCI.

Figure 4. Anecdotal data suggests that rAlH can “train” a spinal cord transplant following cervical SCI. In this experiment, rat embryonic day 14 FSC tissue was transplanted into acute C2 hemileision (C2Hx) cavity in adult rats. Rats were then exposed to 10, 5 min hypoxic episodes (10% O₂, balance N₂) for 10 week, beginning 1 week post-C2Hx. After 10 weeks, electrical activity of graft tissue and the ipsilateral phrenic nerve were recorded in anesthetized, vagotomized and ventilated rats during baseline (FIO₂ = 0.5-0.6) and hypoxia (FIO₂ = 0.13-0.15). The graft tissue was visualized using a dorsal surgical approach, and a 0.4-0.8 MΩ electrode (carbostar-3, Kation Scientific) was inserted directly into the graft. Panel A shows examples of graft and phrenic nerve activity (both “raw” and “integrated”, ∫) during baseline conditions and hypoxic challenge. The graft recording shows considerable tonic activity, but a clear respiratory-related discharge can be appreciated. Panel B shows cross-correlation analysis of the graft and phrenic bursting depicted in Panel A. The correlogram shows a clear central peak which is consistent with the hypothesis that the graft and host...
phrenic motor neuron pool shared a common synaptic input. Lastly, Panel C shows waveform averages (several minutes of data) of graft and phrenic signals. The averages were generated using phrenic burst onset as a trigger (arrows). Graft neurons exhibit inspiratory activity during baseline, and hypoxia triggered pre-inspiratory activity in the graft recording. Note also that tonic activity increased during hypoxia in the graft, but decreased in the host phrenic activity.
Citations


Intermittent Hypoxia and Neurorehabilitation


Intermittent Hypoxia and Neurorehabilitation


Intermittent Hypoxia and Neurorehabilitation


<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Key Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Millhorn et al.</td>
<td>1980</td>
<td>Carotid sinus nerve stimulation elicits a lasting respiratory “memory”</td>
</tr>
<tr>
<td>Hayashi et al.</td>
<td>1993</td>
<td>Hypoxia elicits a similar response; Coined the term “long term facilitation” (LTF)</td>
</tr>
<tr>
<td>Bach et al.</td>
<td>1996</td>
<td>Hypoxia-induced LTF is serotonin dependent</td>
</tr>
<tr>
<td>Baker et al.</td>
<td>2000</td>
<td>Hypoxia-induced LTF is pattern sensitive</td>
</tr>
<tr>
<td>Fuller et al.</td>
<td>2003</td>
<td>Chronic intermittent hypoxia (CIH) enhances respiratory motor output after cervical SCI in rats</td>
</tr>
<tr>
<td>Baker-Herman et al.</td>
<td>2004</td>
<td>Acute intermittent hypoxia (AIH)-induced LTF requires new BDNF synthesis</td>
</tr>
<tr>
<td>Golder et al.</td>
<td>2005</td>
<td>AIH enhances respiratory motor output after cervical SCI in rats</td>
</tr>
<tr>
<td>Wilkerson et al.</td>
<td>2009</td>
<td>Daily AIH elicits respiratory metaplastcity</td>
</tr>
<tr>
<td>Lovett-Barr et al.</td>
<td>2012</td>
<td>Daily AIH improves motor function and upregulates protein expression in respiratory and non-respiratory motor neurons after cervical SCI</td>
</tr>
<tr>
<td>Trumbower et al.</td>
<td>2012</td>
<td>AIH enhances voluntary ankle strength in humans with SCI</td>
</tr>
<tr>
<td>Tester et al.</td>
<td>2014</td>
<td>Daily AIH enhances ventilatory LTF in humans with SCI</td>
</tr>
<tr>
<td>Hayes et al.</td>
<td>2014</td>
<td>Daily AIH paired with walking training enhances locomotor outcomes in humans with SCI</td>
</tr>
</tbody>
</table>