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Daily acute intermittent hypoxia improves breathing function with acute and chronic spinal injury via distinct mechanisms

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ABSTRACT

Daily acute intermittent hypoxia (dAIH) elicits respiratory plasticity, enhancing respiratory motor output and restoring breathing capacity after incomplete cervical spinal injuries (cSCI). We hypothesized that dAIH-induced functional recovery of breathing capacity would occur after both acute (2 weeks) and chronic (8 weeks) cSCI, but through distinct cellular mechanisms. Specifically, we hypothesized that dAIH-induced breathing recovery would occur through serotonin-*independent* mechanisms 2wks post C2 cervical hemisection (C2Hs), versus serotonin-*dependent* mechanisms 8wks post C2Hs. In two independent studies, dAIH or sham (normoxia) was initiated 1 week (Study 1) or 7 weeks (Study 2) post-C2Hs to test our hypothesis. Rats were pre-treated with intra-peritoneal vehicle or methysergide, a broad-spectrum serotonin receptor antagonist, to determine the role of serotonin signaling in dAIH-induced functional recovery. Our data support the hypothesis that dAIH-induced recovery of breathing capacity transitions from a serotonin-*independent* mechanism with acute C2Hs to a serotonin-*dependent* mechanism with chronic C2Hs. An understanding of shifting mechanisms giving rise to dAIH-induced respiratory motor plasticity is vital for clinical translation of dAIH as a therapeutic modality.

1. Introduction

Acute intermittent hypoxia (AIH) has emerged as a safe, non-invasive method for enhancing motor function in humans with chronic, incomplete spinal cord injury [SCI; (Dale et al., 2014; Gonzalez-Rothi et al., 2015; Navarrete-Opazo and Mitchell, 2014a)]. Indeed, individuals with chronic SCI (> 5 years post-injury) who received a single AIH session consisting of ten 60–90 sec exposures to 10.5% O_2 (interspersed with 60 sec of room air at 20.9% O_2) displayed a prolonged increase in maximal ankle torque production and enhanced electromyographic (EMG) activity in ankle plantar flexor muscles that persisted for at least an hour (and up to 4 hours) post-treatment (Trumbower et al., 2012). Subsequently, when AIH was repeated over 5 consecutive days (daily, acute intermittent hypoxia; dAIH) and combined with task-specific rehabilitation (locomotor training), additive functional benefits were observed; groups with combined dAIH and locomotor training ambulated longer and faster than groups receiving either treatment individually (Hayes et al., 2014; Navarrete-Opazo et al., 2016a). Improvements in dynamic balance following dAIH have also recently been reported in patients with chronic SCI (Navarrete-Opazo et al., 2016a). Collectively, these studies support dAIH as a promising therapeutic strategy for improving function following SCI, especially in chronic time periods when the prospect of significant functional return remains bleak. Much work remains to fully understand cellular mechanisms leading to AIH and dAIH-induced motor recovery.

Our working knowledge of mechanisms giving rise to AIH-induced motor enhancement originated with rodent studies of AIH-induced plasticity in respiratory motor control, specifically AIH-induced phrenic long-term facilitation [pLTF; (Bach and Mitchell, 1996; Baker and Mitchell, 2000)]. Moderate AIH (3, 5-min episodes of PaO_2 = 35-45mmHG; 5 min normoxic intervals) elicits prolonged increases in phrenic neural output lasting hours post-AIH. This form of AIH-induced plasticity requires intermittent serotonin release and serotonin receptor activation on or near phrenic motor neurons (Baker-Herman and Mitchell, 2002; Fuller et al., 2001b; Kinkead and Mitchell, 1999). Downstream signaling in the cellular cascade leading to AIH-induced pLTF requires: ERK MAP kinase signaling (Hoffman et al., 2012), new synthesis and release of brain-derived neurotrophic factor [BNDF; (Baker-Herman et al., 2004)], activation of the high-affinity BDNF

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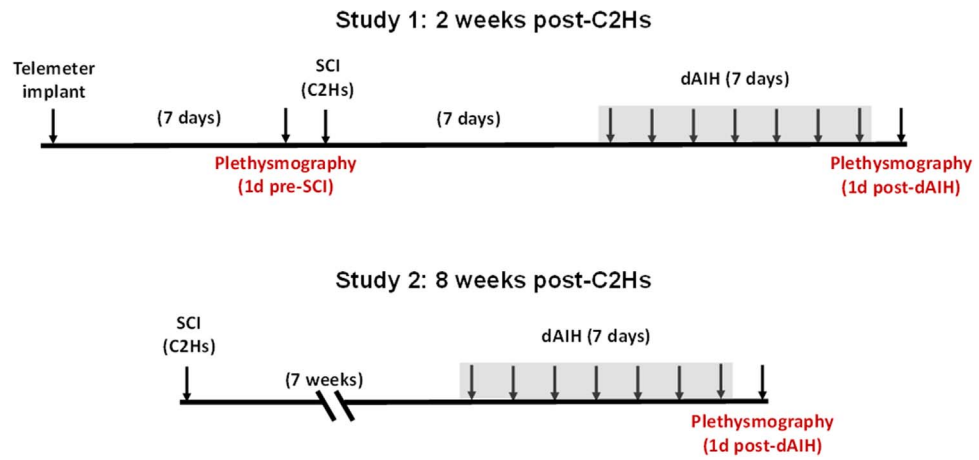

Fig. 1. Study design. Schematic timeline of Study 1 (2wk C2Hs) and Study 2 (8wk C2Hs). Daily, acute intermittent hypoxia was administered for 7 consecutive days beginning one week post-SCI for Study 1 and 7 weeks post-SCI for Study 2. Intraperitoneal injections of methysergide (4 mg x kg-1) or vehicle were given prior to each dAIH session (i.e. daily for 7 consecutive days).

receptor TrkB (Baker-Herman et al., 2004; Dale et al., 2016), and downstream activation of a protein kinase C isoform [PKCθ; (Devinney et al., 2015)]. More severe AIH protocols (sAIH; 3, 5-min exposures of PaO_2 = 25-30mmHG, 5 min normoxic intervals) also elicit pLTF, although through a unique, adenosine-dependent mechanism (Nichols et al., 2012). With sAIH, extracellular adenosine accumulation activates adenosine 2A receptors on or near phrenic motor neurons (Nichols et al., 2012), leading to activation of phosphatidylinositol 3 (PI3)-kinase/Akt (Golder et al., 2008), EPAC (Fields et al., 2015) and mTORC1 (Dougherty et al., 2015), followed by new synthesis of an immature TrkB isoform (Golder et al., 2008). These parallel intracellular pathways to phrenic motor facilitation interact via mutual inhibition (Hoffman et al., 2010), permitting only one pathway to be expressed at a time (depending on severity of dose) with the other acting as an "anchor" to constrain its expression. This mechanistic interplay imparts flexibility in respiratory control and could be critical for maintaining respiratory function following SCI (Devinney et al., 2013).

Cervical SCI (cSCI) at or above the phrenic motor nucleus (C3-C5) may cause respiratory motor neuron death and disrupt descending neural input to respiratory motor neurons (Nicaise et al., 2012). Such injuries reduce the ability to recruit respiratory muscles, particularly during conditions of increased respiratory demand (Alvarez-Argote et al., 2016). cSCI may also disrupt descending neuromodulatory pathways decreasing neuronal excitability and/or the capacity for adaptive plasticity in surviving motor circuits (Dougherty et al., 2016; Golder and Mitchell, 2005; Saruhashi et al., 1996; Zhou and Goshgarian, 1999). With C2 hemisection (C2Hs), an initial decline and subsequent return of serotonergic innervation within the phrenic motor nucleus over 8 weeks post-injury strongly correlates with ipsilateral expression of AIH-induced pLTF (Golder and Mitchell, 2005). When serotonin or serotonin receptor agonists are pharmacologically administered (Zhou and Goshgarian, 2000; Zimmer and Goshgarian, 2006), or serotonergic producing cells are transplanted below C2Hs lesions (Dougherty et al., 2016), enhanced recovery of respiratory motor output is observed and the capacity to express plasticity is restored. Thus, invoking AIH-induced plasticity with cSCI is most effective with chronic injuries after the level of serotonin has had time to recover (Golder and Mitchell, 2005).

Daily AIH (dAIH; 7 consecutive days; 10 5-min episodes per day; 5-min intervals) initiates functional recovery of breathing capacity in rats when initiated as early as 2 weeks post-C2Hs (Lovett-Barr et al., 2012), although the mechanism of this recovery has not been confirmed. In a recent series of studies, Navarette-Opazo and colleagues demonstrated that dAIH initiated one-week post-C2Hs induces functional recovery by a mechanism that requires adenosine 2A (A2A) receptor activation (Navarrete-Opazo et al., 2015), but reverts to an adenosine-constrained mechanism when initiated 8 weeks post-C2Hs (Navarrete-Opazo et al., 2016b), more like the response in uninjured rats (Navarrete-Opazo and Mitchell, 2014b). Thus, different mechanisms appear to contribute to dAIH-induced functional recovery 1–2 versus 7–8 weeks post-cSCI. However, the role of serotonin receptor activation in this functional recovery has never been reported.

We hypothesized that dAIH-induced functional recovery of breathing capacity with acute (1–2 weeks post) C2Hs is driven by serotonin-*independent* mechanisms, since serotonin availability below the injury is limited (Golder and Mitchell, 2005). We also hypothesized that serotonin-dependence of dAIH-induced functional recovery would revert to serotonin-dependent mechanisms with chronic (7–8 weeks) C2Hs due to restoration of serotonergic innervation below the injury (Golder and Mitchell, 2005). To test these hypotheses, we synthesized unpublished data from two independent studies exploring the effects of methysergide pretreatment on dAIH induced functional recovery at 1–2 (Study 1) and 7–8 weeks (Study 2) post-C2Hs. Methysergide is a broad-spectrum serotonin receptor antagonist known to cross the blood brain barrier and to block moderate AIH-induced respiratory motor plasticity (Bach and Mitchell, 1996). The collective data from these studies support a temporal shift in mechanisms underlying dAIH-induced recovery of breathing capacity, transitioning from a serotonin-*independent* mechanism with acute C2Hs, to a serotonin-dependent mechanism with chronic injuries. These findings have important implications for the translation of this promising therapeutic approach.

2. Materials and methods

All experimental procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison, and conformed to policies in the NIH Guide for the Care and Use of Laboratory Animals. Experiments were performed on 3–5 month-old male Lewis (Charles River colony P06; **Study 1**) or Sprague Dawley (Harlan colony 211; **Study 2**) rats. Rats had access to food and water *ad libitum* and were housed in 12hr light-dark cycles in an AAALAC-accredited animal facility. Experimental designs for each study are presented in Fig. 1.

2.1. Surgical preparation (Radio telemetry)

In Study 1, rats were instrumented with an abdominal telemeter for continuous monitoring of real-time body temperature during ventilatory assessments (Nakamura et al., 2010). Rats were anesthetized with isoflurane in 100% O_2. A sterilized temperature telemeter (Mini-Mitter,