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.

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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 $PaO_2 = 35\text{-}45\text{mmHG}$; 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 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 $PaO_2 = 25\text{-}30\text{mmHG}$, 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 (A$_{2A}$) 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 Figure 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, Sun River, OR) was inserted into the rat's peritoneal cavity under aseptic conditions. At the end of surgery, triple-antibiotic ointment was applied to incisions, and analgesic (buprenorphine) was administered (0.03 mg $\times$ kg^{-1}, s.q.) at 12-h intervals for 48 h post-surgery. Rats were visually monitored and weighed daily, and topical triple-antibiotic ointment was continued twice daily as needed. Rats recovered for 7 days prior to initial pre-SCI ventilatory measures. Rats in Study 2 (8wk post-SCI) did not receive telemeter implants; body temperature was assessed immediately before and after ventilatory measurements as described below.

2.2. Surgical Preparation (C2 hemisection)

For both Studies, spinal hemisections at the second cervical segment (C2Hs) were performed in accordance with previous publications (Dougherty et al., 2012; Keomani et al., 2014; Navarrete-Opazo et al., 2015; Sandhu et al., 2009). Rats were pre-medicated with subcutaneous buprenorphine (0.03 mg/kg), carprofen (Rimadyl, 5 mg/kg) and enrofloxacin (Baytril, 4 mg/kg). Body temperature was maintained between 36.5 and 37.5 °C via heated surgical pad. After tracheal intubation, rats were artificially ventilated (Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA) with 1.5–2.5% isoflurane in 100% O_2. Effective anesthesia was judged by abolition of pedal withdrawal and corneal blink reflexes. Oxygen saturation during surgery was monitored via pulse oximetry (Nonin Medical Inc. Plymouth, MN). After anesthetic induction and pre-operative care, the spinal cord was exposed at C2 *via* dorsal laminectomy. The dura matter was cut and a left C2Hs performed using a micro-scalpel followed by aspiration. The overlying muscles were sutured with polysorb 3.0 and the skin closed with stainless steel wound clips. Sham surgeries were completed for Study 2 and consisted of cervical laminectomy without spinal injury. Following surgery, animals received buprenorphine (0.03 mg $\times$ kg^{-1}, s.q.) and sterile Lactated Ringers solution (5 ml s.q.). Post-surgical care included administration of buprenorphine (0.03 mg $\times$ kg^{-1}, s.q.) during the initial 48h post-injury, delivery of Lactated Ringers solution (5 ml $\times$ day^{-1}, s.q.) and oral Nutri-cal supplements (1–3 ml, Webster Veterinary, MA, USA) as needed until adequate volitional drinking and eating resumed.

2.3. Daily Acute Intermittent Hypoxia (dAIH)

The protocol for dAIH is described in previous reports (Lovett-Barr et al., 2012) and lasted for 7 days in both Study 1 and Study 2. In Study 1, exposures began 7d post-C2Hs (Fig. 1). For Study 2, dAIH was initiated 7wks post-C2Hs. Rats recovered in their home cages without additional interventions until initiation of dAIH (Fig. 1). Each day for 7 consecutive days, rats were weighed and placed in a Plexiglas tube flushed with a mixture of N_2/O_2 (4 L $\times$ min^{-1}) to attain continuous normoxia or intermittent hypoxia (75 s equilibration; 10 - 5 min episodes of 10.5% O_2; 5 min 21% O_2 intervals). All rats were pre-treated with an i.p. injection of either methysergide [4mg/kg; (Bach and Mitchell, 1996; Nichols et al., 2014)] or corresponding vehicle (10%DMSO in sterile saline) five minutes prior to the start of each dAIH session. After dAIH, or the equivalent duration of normoxia (110 min) for non-dAIH groups, rats were returned to their cages until the following day. All groups presented as "SCI" alone received daily exposures to normoxia with i.p. vehicle injections as did all non-injured Control groups (pre-injury group in Study 1 and sham SCI groups in Study 2).

2.4. Barometric Plethysmography

Barometric plethysmography [DSI for Study 1 (Navarrete-Opazo et al., 2015); Buxco for Study 2 (Lovett-Barr et al., 2012)] was used to measure breathing in unanesthetized rats following C2Hs and dAIH. Ventilation was measured 24 hrs after dAIH completion; 2wks post-C2Hs in Study 1 and 8wks post-C2Hs in Study 2. Identical protocols were used in both Study 1 and Study 2 and were described previously (Lovett-Barr et al., 2012). Briefly, following a 30 min acclimation period, baseline ventilation was recorded under normoxic conditions (21% O_2) for 30 min. Rats were subsequently exposed to a combined hypoxic/ hypercapnic gas mixture (7% CO_2 in 10.5% O_2, balance N_2) for 25 mins to assess maximal chemo-reflex stimulated breathing (i.e. MCS). Gas flowed continuously through the chambers at 2L $\times$ min^{-1} to prevent CO_2 buildup and allow control of rapidly changing gas concentrations. Chamber pressure, chamber temperature, humidity and body temperature were used to calculate V$_T$ (ml $\times$ br^{-1}) and V̇$_E$ (ml $\times$ min^{-1}) according to the Drorbaugh and Fenn equation (Drorbaugh and Fenn, 1955). In Study 1, telemetric body temperature was recoded in real time during plethysmography and used for calculations. In Study 2, the rats had not been implanted with telemeters prior to C2Hs. Instead, rectal temperatures were taken immediately prior to entering the plethysmograph chambers, and immediately following the Max response. Adjustments were made to V$_T$ and V̇$_E$ calculations, with the initial temperature used for air breathing, and the latter temperature used to calculate the Max response. There were no differences in initial rectal temperatures (p=0.392) or final temperatures (p=0.848) across groups. Within each study, data were expressed as absolute units (e.g., breaths $\times$ min^{-1} for frequency), relative to body weight (e.g., ml $\times$ min^{-1} $\times$ 100g^{-1}) or relative to values obtained in control rats (%control). "Control" rats were defined as a group of spinally intact rats receiving normoxia (versus dAIH) and intrathecal vehicle injections. For Study 1, plethysmography measures were obtained one day prior to SCI surgeries in all experimental rats and the combined pre-injury group data were used for statistical comparisons (n=22). For Study 2, no pre-injury plethysmography data were collected. Instead, a separate group of rats received sham C2Hs surgeries as described above and these spinally intact rats were used for statistical comparisons. As anticipated, SCI caused a reduction in body weight relative to spinally intact control groups regardless of

treatment; all experimental groups weighed less than control groups in Study 1 (p<0.001) and Study 2 (p<0.03). Data were analyzed in 5 min bins using commercially available statistical software (SigmaPlot, Version 11, Systat Software, Inc.) and are presented as means ± 1 SEM. Statistical tests included One-way ANOVA for body weight and temperature values and 2-way RM ANOVA for ventilatory parameters; Fisher's LSD *post-hoc* test was used to identify statistically significant individual comparisons. Differences were considered significant if p<0.05.

3. Results

3.1. Study 1: 2 weeks post-C2Hs

As reported often in the literature, C2Hs reduced V_T generating capacity with a compensatory increase in respiratory frequency that preserved $\dot{V}_E$ during BL (i.e room air) conditions (n=7; Fig. 2A). Exposure to hypoxia/hypercapnic challenge (i.e. MCS) revealed significant reductions in $\dot{V}_E$ relative to pre-injury control values (p<0.001; Fig. 2A), confirming persistent respiratory impairment. Reductions in $\dot{V}_E$ were characterized by diminished V_T generating capacity during MCS (Fig 2D), while the ability to increase respiratory rate was retained (Fig. 2C). This transition to a rapid, shallow pattern of BL breathing and the reduced capacity to increase V_T during MCS are indicative of disrupted neural input to respiratory motor neurons following C2Hs.

3.2 dAIH restores ventilation 2wks post-C2Hs by a serotonin-independent mechanism

Reductions in $\dot{V}_E$ during MCS were mitigated by dAIH. Indeed, $\dot{V}_E$ returned to pre-injury control levels in groups receiving dAIH (n=8), and was significantly higher than C2Hs rats during MCS at 2wks post-injury (p=0.003; Fig. 2A). Daily pre-treatment with methysergide (n=7) had minimal impact on dAIH-induced $\dot{V}_E$ recovery (Fig. 2A), suggesting a *serotonin-independent* mechanism. Rats receiving methysergide + dAIH produced $\dot{V}_E$ nearly identical to rats receiving dAIH alone during MCS (p=0.727), but significantly higher than rats with C2Hs during BL (p=0.050) or MCS (p=0.002; Fig. 2A). When $\dot{V}_E$ was analyzed as a %pre-injury to determine relative return of respiratory function, dAIH (with and without methysergide) significantly elevated $\dot{V}_E$ during BL and MCS relative to C2Hs alone (Fig. 2B).

Recovery of $\dot{V}_E$ with dAIH (with and without methysergide) 2wks post-C2Hs occurred primarily through increased respiratory frequency and *not* enhanced V_T capacity as previously reported. BL frequency in dAIH rats and dAIH + methysergide rats was elevated to a similar extent as C2Hs rats and was significantly higher than pre-injury values (p<0.001; Fig. 2C). With MCS, dAIH groups demonstrated significantly elevated frequency relative to control rats (p<0.001; Fig. 2C) and the frequency in dAIH + methysergide rats was elevated significantly above C2Hs rats (p=0.031; Fig. 2C). Conversely, dAIH did not significantly impact V_T 2wks post-C2Hs (Fig. 2D). dAIH and dAIH + methysergide groups demonstrated similar V_T to C2Hs during BL and MCS and these remained significantly below pre-injury values (p<0.001; Fig. 2D). When V_T was compared as a %difference from pre-injury, dAIH (with and without methysergide) increased V_T relative to C2Hs rats with BL breathing (Fig. 2E). However, this difference in V_T generation was lost during MCS (Fig. 2E).

3.3 Study 2: 8 weeks post-C2Hs

The functional impact of chronic C2Hs on ventilation was similar to that seen at the 2wk time point. In Study 2, rats 8wks post-C2Hs displayed persistent reductions in V_T generating capacity and a compensatory increase in respiratory frequency that sustained $\dot{V}_E$ during BL conditions (n=11; Fig. 3A). As with acute C2Hs rats, MCS revealed significant reductions in $\dot{V}_E$ versus normoxia treated controls (Sham SCI; n=18; p<0.001; Fig. 3A), confirming persistent respiratory impairment 8 weeks post-C2Hs. Reduced $\dot{V}_E$ was characterized by diminished V_T generating capacity with MCS (Fig. 3D), while frequency remained similar to all other groups (Fig. 3C). The persistence of a rapid, shallow pattern with BL breathing and the inability increase V_T with the respiratory challenge 8wks post C2Hs is consistent with previous studies (Fuller et al., 2008; Navarrete-Opazo et al., 2015).

3.4 dAIH restores ventilation 8wks post-C2Hs

dAIH initiated with chronic C2Hs restored $\dot{V}_E$ capacity, although the strategies employed to enhance $\dot{V}_E$ differed substantially from acute C2Hs in Study 1. Rats receiving dAIH 7wks post-injury (n=14) showed similar $\dot{V}_E$ during BL when compared with control and C2Hs groups (Fig. 3A). But, with MCS, dAIH induced substantial functional recovery such that $\dot{V}_E$ was restored to values equivalent to sham controls without hemisection (p=0.516), and was significantly elevated above C2Hs rats (p=0.012; Fig. 3A). This elevated $\dot{V}_E$ was also apparent when the data were expressed as %control, although differences were only marginally significant (p=0.085; Fig. 3B). Unlike Study 1, dAIH-induced recovery of $\dot{V}_E$ 8wks post-C2Hs resulted from increased V_T capacity and *not* changes in breathing frequency. Breathing frequency was elevated relative to control rats in all SCI groups during BL, but was similar across all groups during MCS (Fig. 3C). Conversely, dAIH rats generated larger V_T with BL and MCS, similar to control rats during BL (p=0.066) and significantly greater than C2Hs rats during MCS (p=0.016; Fig. 3D) (though still impaired versus controls; p<0.001). When expressed as %control, dAIH-induced improvements in V_T capacity during MCS were also observed, though once again they were only marginally significant (p=0.053; Fig. 3E).

3.5 Serotonin-dependent mechanisms underlie dAIH-induced recovery 8wks post-C2Hs

dAIH-induced recovery of $\dot{V}_E$ was abolished by methysergide pretreatment 8wks post-C2Hs (n=13). These data are in stark contrast to the minimal effects of methysergide pre-treatment observed in Study 1, and suggest that recovery of $\dot{V}_E$ with dAIH occurs via serotonin-dependent mechanisms 8wks post-C2Hs. In specific, dAIH + methesergide rats showed $\dot{V}_E$ similar to C2Hs rats during MCS (p=0.919; Fig. 3A), and were significantly reduced compared to controls (p<0.001) and dAIH (p=0.009; Fig. 3A). When expressed as %control, the reduced $\dot{V}_E$ with dAIH + methysergide was again apparent versus dAIH rats, but was only marginally significant (p=0.054; Fig. 3B). The impact of methysergide on $\dot{V}_E$ appeared specific to changes in V_T capacity. Methysergide pretreatment reversed dAIH-induced improvements in V_T generation 8wks post-C2Hs, with lesser impact on respiratory frequency. dAIH + methysergide rats continued to show significantly higher BL frequencies versus controls (p=0.022), but were significantly reduced versus C2Hs rats (p=0.028; Fig. 3C). Differences in breathing frequency were no longer apparent during MCS; dAIH +

methysergide rats had similar breathing frequency as all other groups (Fig. 3C). Deficits in V_T capacity were observed with BL and MCS when rats were treated with methysergide prior to dAIH. V_T in dAIH + methysergide rats was similar to C2Hs rats in each condition and remained significantly below control rats during BL (p=0.028) and MCS (p<0.001; Fig. 3D). In addition, methysergide blunted V_T generation during MCS significantly below rats receiving dAIH alone (p=0.009; Fig. 3D). This was again apparent when V_T was expressed as %control (p=0.035; Fig. 3E). Collectively, these data suggest that dAIH was effective in restoring $\dot{V}_E$ in rats with chronic C2Hs through enhanced V_T generating capacity, an effect mediated through serotonin-dependent mechanisms.

4. Discussion

Taken together, data from these independent studies support the hypothesis that dAIH improves breathing function with acute (2wks post-injury) and chronic (8wks post-injury) C2Hs through distinct cellular mechanisms. Specifically, we confirm that dAIH initiated one week post C2Hs augments $\dot{V}_E$, as previously described (Lovett-Barr et al., 2012; Navarrete-Opazo et al., 2015), and extend these findings to demonstrate that dAIH-induced recovery of $\dot{V}_E$ occurs via a serotonin-*independent* mechanism since methysergide pre-treatment had minimal impact on dAIH-induced recovery; methysergide at this same dose is known to block moderate AIH-induced phrenic long-term facilitation in rats (Bach and Mitchell, 1996). Conversely, when rats were allowed to recover for 7 weeks post-C2Hs, methysergide pre-treatment abolished dAIH-induced recovery of $\dot{V}_E$, suggesting conversion to serotonin-*dependent* mechanisms. This transition from serotonin-independent to serotonin-dependent mechanisms of dAIH-induced functional recovery parallels our previous reports that dAIH induced functional recovery shifts from an adenosine-dependent [acute; (Navarrete-Opazo et al., 2015)] to an adenosine-constrained mechanism [chronic; (Navarrete-Opazo et al., 2016b)]. An understanding of these changing mechanisms will be of importance for future pre-clinical studies and translational applications of dAIH since something as simple as consumption of caffeine (an A2a antagonist) could greatly affect the impact of dAIH therapy depending on the time post-injury.

In study 1, dAIH-induced improvements in $\dot{V}_E$ 2wk post-C2Hs occurred through enhancement of respiratory frequency versus V_T generating capacity in contrast with our previous reports (Lovett-Barr et al., 2012). Indeed, Lovett-Barr et al. (2012) found minimal changes in respiratory frequency and improved V_T during Max respiratory challenge when dAIH was initiated 1wk post-injury. Although the overall impact of dAIH on $\dot{V}_E$ was similar to the current study (significantly enhanced versus C2Hs alone), the observed enhancement in respiratory frequency (and not V_T) was a surprise. There are a few considerations for interpretation of these data. First, the plethysmograph used for Study 1 (Data Science International) was different from those used in previous studies (Buxco) (Doperalski et al., 2008; Dougherty et al., 2016; Fuller et al., 2008; Fuller et al., 2006; Lovett-Barr et al., 2012) in order to facilitate simultaneous radio telemetry measures of body temperature. It is possible that differences in gas flow dynamics and/or internal algorithms applied to calculate respiratory measures between plethysmography systems may introduce subtle variability to V_T and $\dot{V}_E$ data. However, since volume calibrations were completed for each plethysmograph prior to all experiments, it is unlikely that differences in equipment

accounted for the notable differences in respiratory dynamics with dAIH 2wks post-C2Hs observed in Study 1. Also, this same DSI system was used by Navarette-Opazo and colleagues (2015), who reported primary effects of dAIH on V_T. It is more likely that variations in breathing patterns with dAIH represent either genetic variance among colonies and/or strains of experimental rats (Baker-Herman et al., 2010; Golder et al., 2005). Indeed, previous studies of dAIH-induced recovery of breathing function with C2Hs (and Study 2 herein) were completed using Harlan Sprague-Dawley rats (Lovett-Barr et al., 2012); Study 1 was completed using Charles River Lewis rats from colony P06. It is known that hypoxic ventilatory responses (Golder et al., 2005) and expression of respiratory motor plasticity (Baker-Herman et al., 2010) can vary between strains, and even sub-strains (Fuller et al., 2001a) of experimental rats. Genetic factors, such as differences in serotonin or TrkB receptor expression (Baker-Herman et al., 2010) may have contributed to the unique enhancement of respiratory frequency and minimal increase in V_T observed with dAIH in acute C2Hs. Though, unique plasticity in afferent projections to the brainstem rhythm generating circuits (or specifically within rhythm generating circuits) in P06 rats cannot be completely ruled out. Unfortunately, the PO6 colony from Charles River was discontinued prior to the start of Study 2, making direct comparisons between studies untenable.

Despite differences in the respiratory patterns used, net improvements in $\dot{V}_E$ at 2wks post-C2Hs with dAIH were similar to previous reports (Lovett-Barr et al., 2012), strengthening the hypothesis that dAIH improves respiratory motor function in acute SCI. To begin discerning mechanisms of $\dot{V}_E$ recovery and the specific role for serotonin signaling mechanisms 2wks post-C2Hs, rats were pre-treated with methysergide prior to AIH in each session of the dAIH protocol. Methysergide had minimal impact on dAIH-induced $\dot{V}_E$ improvements or on component variables giving rise to $\dot{V}_E$ (frequency and V_T). These data support a non-serotonergic mechanism of dAIH-induced functional recovery 2wks post-C2Hs. C2Hs interrupts descending raphe-spinal projections to phrenic and intercostal motor neurons leading to reduced expression of serotonin-dependent plasticity (Dougherty et al., 2016; Golder and Mitchell, 2005). In addition, C2Hs may induce vascular changes that alter blood flow to spinal tissue below the injury, especially during acute stages of recovery (Holtz et al., 1990). Reduced perfusion of spinal tissue in the vicinity of phrenic motor neurons (just caudal to C2Hs) during dAIH may produce more severe hypoxemia for a given level of FIO_2 compared to spinal-intact control rats. Repeated exposure to severe hypoxia induces respiratory motor plasticity through adenosine-dependent (versus serotonin-dependent) mechanisms (Nichols et al., 2012). Thus, the combined loss of serotonergic innervation to respiratory motor neurons and possible increased hypoxemia and tissue hypoxia during respiratory challenge may have been sufficient to shift dAIH-induced functional recovery towards adenosine-dependent mechanisms. Nevertheless, we cannot rule out overall strain differences in 5-HT function between P06 and SD rats as another plausible explanation, though recent reports of dAIH effects on breathing function 2wks (Navarrete-Opazo et al., 2015) versus 9 wks post- C2Hs (Navarrete-Opazo et al., 2016b) support a shift from an adenosine-dependent to an adenosine-constrained mechanism, consistent with the proposed shift in serotonin function in this same time frame.

In contrast to the serotonin-*independent* mechanism of dAIH-induced recovery with acute C2Hs, dAIH with chronic C2Hs appears to rely on serotonergic signaling mechanisms. In

study 2, we demonstrated that pre-treating rats with methysergide prior to dAIH with more chronic C2Hs eliminates the functional impact of dAIH. Indeed, dAIH treatment alone enhanced V_T generating capacity to normal levels, whereas methysergide pretreatment reduced V_T to levels similar to C2Hs alone. The mechanistic transition to serotonin-dependent mechanisms with chronic C2Hs is likely due to restoration of descending serotonergic innervation to respiratory motor nuclei over time post-injury. Golder et al. (2005) demonstrated a gradual return of serotonergic terminal density ipsilateral to a C2Hs lesion over 8 weeks, with subsequent return of serotonin-dependent phrenic long-term facilitation ipsilateral to injury following moderate AIH (Golder and Mitchell, 2005). In addition, restoring serotonin signaling via pharmacological means (Hsu and Lee, 2015; Zhou and Goshgarian, 2000; Zimmer and Goshgarian, 2006), or through cell transplants designed to restore serotonin near respiratory motor neurons (Dougherty et al., 2016), enhances respiratory motor output with chronic C2Hs, underscoring the importance of serotonergic signaling for functional respiratory motor recovery.

The return of serotonin-dependent mechanisms with chronic C2Hs may be accompanied by restoration of cross-talk inhibition between serotonergic and adenosinergic pathways to phrenic motor plasticity (Devinney et al., 2013; Hoffman et al., 2010; Hoffman and Mitchell, 2013). Indeed, serotonin and adenosine signaling likely occur simultaneously with dAIH, with one pathway emerging as the dominant mechanism of plasticity, and the other acting as a "brake" (Devinney et al., 2013; Navarrete-Opazo and Mitchell, 2014a). With chronic C2Hs, pre-treatment with an adenosine receptor antagonist prior to dAIH should remove the inhibitory "brake" on the dominant serotonin-dependent pathway, thereby enhancing the impact of dAIH (versus abolishing dAIH-induced recovery as seen with acute C2Hs). Indeed, in rats with chronic C2Hs pre-treated with KW-6002, enhanced recovery of V_T generating capacity and diaphragm motor output are observed following dAIH (Navarrete-Opazo et al., 2016b). Thus, our data are consistent with prior studies, and provide compelling support for shifting mechanisms of dAIH-induced functional recovery of breathing capacity.

The appreciation for shifting mechanisms of dAIH-induced recovery of motor function is vital for translational applications of dAIH to humans with SCI. Rodent studies designed to unravel the complex, evolving mechanisms of intermittent-hypoxia induced plasticity are likely to uncover targets for pharmacological manipulation or rehabilitation-specific co-treatments to maximize the impact of dAIH; perhaps even uncovering methods for driving recovery without the necessity for hypoxic exposures. However, of equal importance is our understanding of conditions that may undermine the effectiveness of dAIH as a therapeutic adjunct. For example, even mild inflammatory states can undermine the expression of AIH-induced plasticity (Huxtable et al., 2011; Vinit et al., 2011), and may impact the magnitude of dAIH-induced recovery in individuals with chronic, incomplete SCI. Something as simple as consuming caffeinated beverages (caffeine is an adenosine receptor antagonist) may undermine the benefits of dAIH on motor recovery with acute SCI whereas the same "pre-treatment" may enhance dAIH-induced functional recovery with chronic SCI. Ultimately, the meaningful clinical application of dAIH hinges upon clear understanding of these complex (and changing) mechanisms.

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Highlights

- Daily acute intermittent hypoxia (dAIH) improves breathing function following cervical spinal cord injury (C2 hemisection; C2Hs) in rats.

- dAIH-induced recovery of breathing occurs in both acute (2 weeks) and chronic (8 weeks) C2Hs, but through unique cellular mechanisms.

- Pre-treatment with methysergide, a broad spectrum serotonin receptor antagonist, prior to dAIH has minimal effect on dAIH-induced recovery in acute C2Hs, suggesting a serotonin-independent mechanisms of action.

- However, methysergide reduces dAIH-induced recovery in chronic C2Hs, suggesting a temporal shift to serotonin-dependent mechanisms.

Study 1: 2 weeks post-C2Hs

## Study 2: 8 weeks post-C2Hs
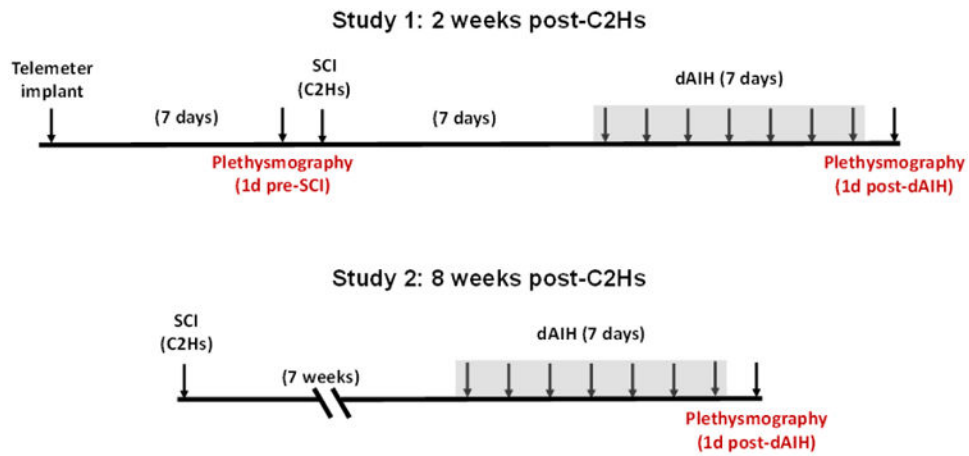
Figure 1. Study design
Schematic timeline of Study 1 (2wk C2Hs) and Study 2 (8wk C2Hs). Daily, acute intermittent hypoxia was administered for 7 consecutive days beginning one week post-SCI for Study 1 and 7 weeks post-SCI for Study 2. Intraperitoneal injections of methysergide (4 mg × kg-1) or vehicle were given prior to each dAIH session (i.e. daily for 7 consecutive days).
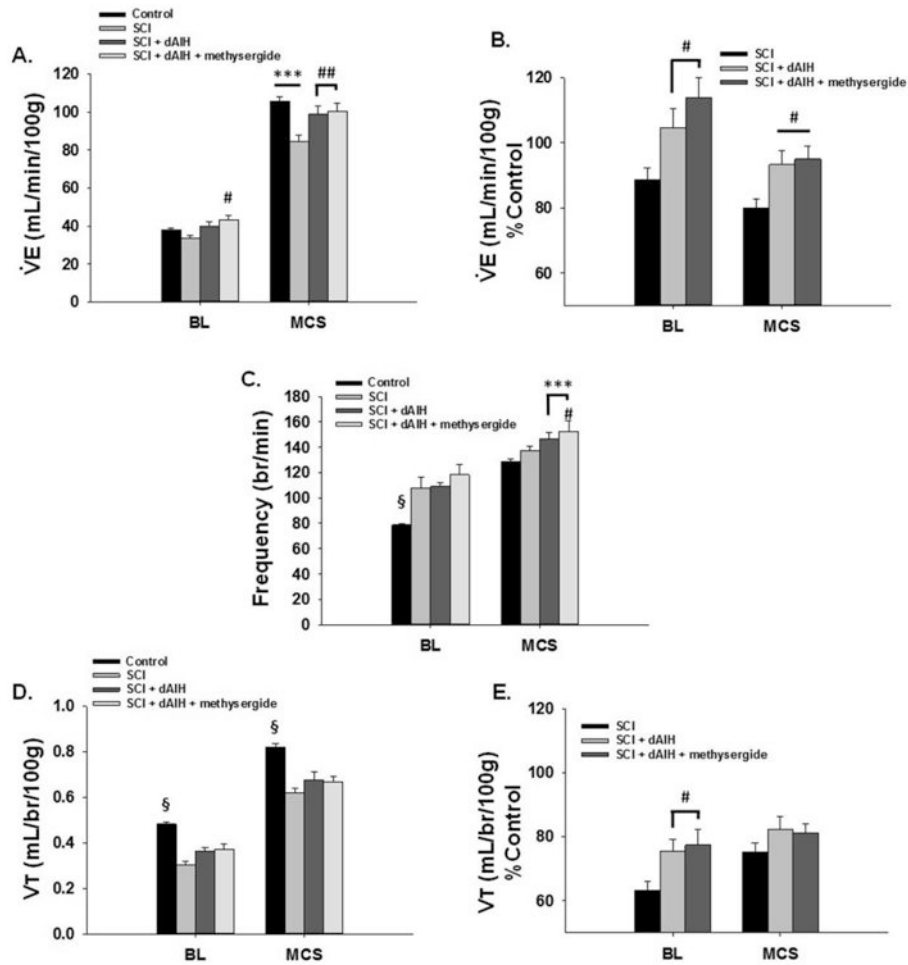
Figure 2. Restoration of ventilation with dAIH 2wks post-C2Hs by serotonin-independent mechanisms

C2Hs caused a reduction in $\dot{V}_E$ that was revealed with maximal chemoreceptor stimulated breathing (MCS; hypoxia/hypercapnia). In (A.), rats with C2Hs alone (n=7) showed a significantly diminished MCS response relative to the control group 7d post injury (the control group consisted of averaged plethysmography data from all spinally-intact experimental rats one day prior to C2Hs; n=22). The C2Hs-induced reductions in $\dot{V}_E$ were brought back to control levels when dAIH was administered beginning 7d post-injury (n=8). Pre-treatment with methysergide, a broad spectrum serotonin receptor antagonist did not affect the MSC (vs Control; n=7) suggesting dAIH-induced recovery is not serotonin-dependent. In (B.) $\dot{V}_E$ data are presented as % Control to further demonstrate enhanced $\dot{V}_E$ with dAIH. (C.) All experimental groups showed increased respiratory frequency with BL when compared to controls. dAIH appeared to enhance frequency during MCS, while C2Hs rats showed similar frequency to controls. (D.) C2Hs reduced V_T generating capacity in all experimental groups in BL and with MCS. dAIH (with and without methysergide) had no significant effect on V_T compared to controls. However, when expressed as % Control (E.) dAIH appeared to enhance V_T capacity in BL. #: $p < 0.05$, ##: $p < 0.01$ compared to SCI; ***:$p < 0.001$ compared to control; §:$p < 0.001$ from all other groups within condition.
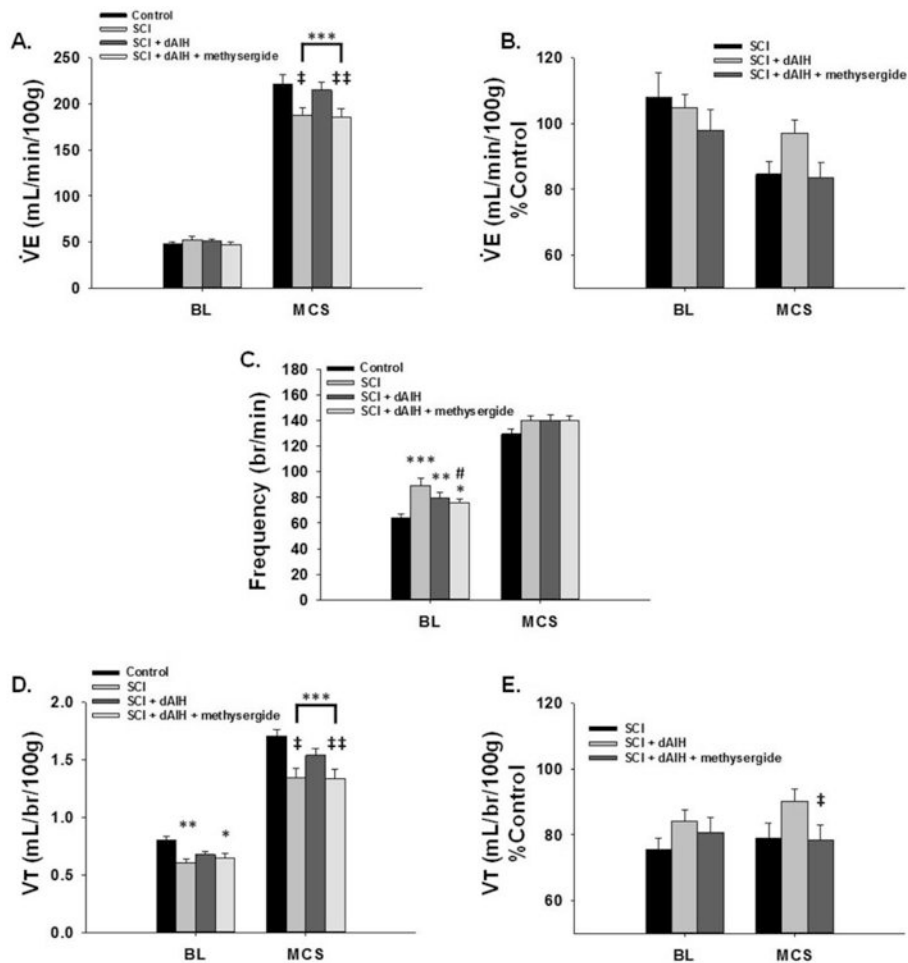
Figure 3. dAIH enhances ventilation 8wks post-C2Hs through a serotonin-dependent mechanism
Persistent reductions in $\dot{V}E$ were seen 8wks post-C2Hs with Maximal chemoreceptor stimulated breathing (MCS). In (A.), BL $\dot{V}E$ was similar among groups, but MCS revealed significantly reduced $\dot{V}E$ in C2Hs rats (n=18). dAIH (n=14) restored $\dot{V}E$ to level equivalent with the control group with MCS while pre-treatment with methysergide (n=13) significantly blunted dAIH-induced recovery of $\dot{V}E$. Changes in $\dot{V}E$ were observable when expressed as %Control, though they did not reach statistical significance (B.). The control group for Study 2 consisted of a unique group of spinally intact rats that received sham SCI surgeries (n=18), daily NX and intrathecal vehicle pre-treatment. Unlike at the 2wk time point, dAIH had minimal effect on respiratory frequency, but significant impact on V_T generation 8wks post-C2Hs. (C.) All C2Hs groups showed significant elevations in frequency with BL breathing, though rats pre-treated with methysergide prior to dAIH displayed reduced frequency relative to C2Hs rats. No differences in frequency were observed between groups during MCS. V_T was reduced in C2Hs rats with BL and MCS relative to controls (D.). dAIH restored V_T generating capacity in each condition to levels similar to controls; significantly above C2Hs rats. Methysergide pre-treatment blunted the dAIH-induced recovery of V_T. Reduced V_T capacity with methysergide was also apparent when V_T was expressed as %Control; dAIH-methysergide rats demonstrated significantly less V_T capacity relative to dAIH treatment alone (E.). #: $p<0.05$ compared to SCI;

*:p<0.05, **:p<0.01, ***:p<0.001 compared to control; ‡:p<0.05, ‡‡: p<0.01 compared to dAIH group.