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# Enhanced recovery of breathing capacity from combined adenosine 2A receptor inhibition and daily acute intermittent hypoxia after chronic cervical spinal injury

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## Abstract

Daily acute intermittent hypoxia (dAIH) improves breathing capacity after C2 spinal hemisection (C2HS) in rats. Since C2HS disrupts spinal serotonergic innervation below the injury, adenosine-dependent mechanisms underlie dAIH-induced functional recovery 2 weeks post-injury. We hypothesized that dAIH-induced functional recovery converts from an adenosine-dependent to a serotonin-dependent, adenosine-constrained mechanism with chronic injury. Eight weeks post-C2HS, rats began dAIH (10, 5-min episodes, 10.5% $O_2$; 5-min intervals; 7 days) followed by AIH 3× per week (3×wAIH) for 8 additional weeks with/without systemic $A_{2A}$ receptor inhibition (KW6002) on each AIH exposure day. Tidal volume ($V_T$) and bilateral diaphragm (Dia) and T2 external intercostal motor activity were assessed in unanesthetized rats breathing air and during maximum chemoreflex stimulation (MCS: 7% $CO_2$, 10.5% $O_2$). Nine weeks post-C2HS, dAIH increased $V_T$ versus time controls ($p < 0.05$), an effect enhanced by KW6002 ($p < 0.05$). dAIH increased bilateral Dia activity ($p < 0.05$), and KW6002 enhanced this effect in contralateral ($p < 0.05$) and ipsilateral Dia activity ($p < 0.001$), but not $T_2$ inspiratory activity. Functional benefits of combined AIH plus systemic $A_{2A}$ receptor inhibition were maintained for 4 weeks. Thus, in rats with chronic injuries: 1) dAIH improves $V_T$ and bilateral diaphragm activity; 2) $V_T$ recovery is enhanced by $A_{2A}$ receptor inhibition; and 3) functional recovery with A2A receptor inhibition and AIH "reminders" last 4 weeks. Combined dAIH and $A_{2A}$ receptor inhibition may be a simple, safe, and effective strategy to accelerate/enhance functional recovery of breathing capacity in patients with respiratory impairment from chronic spinal injury.

### Keywords

Intermittent hypoxia; Spinal cord injury; Chronic; Functional recovery; Breathing; Spinal plasticity; Adenosine receptors; Rehabilitation; Hemisection; Cervical

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## 1. Introduction

Most spinal cord injuries (SCI) are incomplete (Lee et al., 2014), leaving spared synaptic pathways below the site of the injury. Thus, a viable therapeutic strategy to improve motor deficits following SCI is to strengthen these spared synaptic pathways with treatments known to induce spinal motor plasticity (Dale et al., 2014; Vinit et al., 2009). Intermittent hypoxia (IH) elicits spinal, respiratory motor plasticity, strengthening synaptic pathways to respiratory motor neurons (Fuller et al., 2003; Golder and Mitchell, 2005; Lovett-Barr et al., 2012). For example, repetitive IH improves phrenic nerve/diaphragm muscle activity after C2 cervical hemisection (C2HS) (Fuller et al., 2003; Navarrete-Opazo and Mitchell, 2014), at least partially restoring breathing capacity (Fuller et al., 2006; Lovett-Barr et al., 2012; Navarrete-Opazo et al., 2015). To date, studies attempting to harness IH as a therapeutic tool to restore breathing capacity have been performed in rats with sub-acute SCI (<4 weeks).

The rationale to use IH as a means of restoring breathing capacity after SCI was based on studies of phrenic long-term facilitation (pLTF), a model of acute intermittent hypoxia (AIH)-induced phrenic motor facilitation (pMF) (Devinney et al., 2013; Feldman et al., 2003; Mahamed and Mitchell, 2007; Mitchell et al., 2001). In normal rats, AIH-induced pLTF is serotonin-dependent (Bach and Mitchell, 1996; Baker-Herman and Mitchell, 2002), and is constrained by spinal adenosine 2A ($A_{2A}$) receptor activation (Hoffman et al., 2010). Thus, $A_{2A}$ receptor inhibition enhances AIH-induced phrenic and diaphragm LTF in normal rats (Navarrete-Opazo et al., 2014). In contrast, AIH-induced $T_2$ intercostal LTF is greater than that observed in the diaphragm (Navarrete-Opazo et al., 2014), but A2A receptor inhibition has minimal impact on this response.

When one week of daily AIH (dAIH) begins one week post-C2HS, breathing capacity and phrenic nerve activity are increased (Lovett-Barr et al., 2012) Although we originally hypothesized that A2A receptor inhibition would enhance IH-induced functional recovery of breathing capacity following $C_2$HS, it actually blocked the effects of dAIH at this time (Navarrete-Opazo et al., 2014). In retrospect, this observation is consistent with reports that AIH fails to elicit pLTF two weeks post-C2HS due to reduced serotonergic innervation in the phrenic motor nucleus (Golder and Mitchell, 2005). Instead, daily AIH beginning one-week post-C2HS elicits an alternate, adenosine-dependent mechanism of phrenic motor plasticity (Dale-Nagle et al., 2010). In normal rats, this alternate mechanism known as the “S pathway to pMF” can be elicited by spinal A2A receptor agonist administration (Golder et al., 2008) or severe AIH (Nichols et al., 2012).

With time, serotonergic innervation below C2HS recovers progressively in the phrenic motor nucleus (Golder and Mitchell, 2005; Saruhashi et al., 1996; Tai et al., 1997). Thus, 8 weeks post-C2HS, AIH once again elicits pLTF ipsilateral to injury (Golder and Mitchell, 2005). The impact of dAIH on breathing capacity has not been investigated after serotonergic innervation has had time to recover following C2HS.

Here, we tested the hypothesis that daily AIH (10 episodes/day; 7 days) beginning 8 weeks post-C2HS elicits functional recovery of breathing capacity, as well as inspiratory diaphragm and $T_2$ intercostal muscle activation. We further hypothesized that dAIH effects

on breathing capacity and diaphragm, but not T2 intercostal, activity would be enhanced by pretreatment with a systemic A2A receptor antagonist (KW6002). Finally we tested the hypothesis that combined A2A pre-treatment and AIH "reminders" 3 times per week (3×wAIH) extend the benefits of dAIH.

We demonstrate that AIH induces functional recovery of breathing capacity and diaphragm (not intercostal) muscle activity in rats with chronic C2HS (9 weeks post-injury), and that pretreatment with an A2A receptor antagonist (KW6002) augments the impact of AIH on the capacity to increase tidal volume and diaphragm activity. These functional benefits remained until 4 weeks post-dAIH, particularly with KW6002 pretreatment, but could not be sustained by 3×wAIH beyond that time. Thus, mechanisms of dAIH-induced functional recovery 9 weeks post-injury are similar to dAIH-induced phrenic motor plasticity in uninjured rats, but contrast strikingly with rats exposed to dAIH beginning 1 week post-injury. Since combined dAIH and A2A receptor inhibition amplifies and extends the functional benefits of dAIH alone, this study further supports the use of repetitive AIH as a simple, safe and effective therapeutic approach to accelerate recovery of lost breathing capacity in patients with chronic SCI.

## 2. Methods

### 2.1. Animals

Experiments began with 3–4 months old, male Sprague-Dawley rats (310–400 g, colony 211, Harlan, Indianapolis, IN). Rats were individually housed in a controlled environment (12-h light/dark cycle). The Institutional Animal Care and Use Committee at the University of Wisconsin approved all procedures.

### 2.2. Experimental preparation

**2.2.1. Surgical preparation—**Telemetry implantation and C2 cervical hemisection were performed under sterile conditions as described previously (Navarrete-Opazo et al., 2014, 2015). Rats were pre-medicated with subcutaneous buprenorphine (0.03 mg/kg), carprofen (Rimadyl, 5 mg/kg) and enrofloxacin (Baytril, 4 mg/kg); additional injections were made every 12 h for 2 days post-surgery to minimize post-operative pain and infection. Body temperature was maintained between 36.5 and 37.5 °C. 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).

**2.2.2. Telemetry transmitter implantation—**A sterilized telemeter (model 4ET-S1/2; Data Sciences International [DSI], St. Paul, MN) was inserted into the peritoneal cavity. Briefly, both hemi-diaphragms were exposed via midline incision. Electrode leads were implanted into the right and left $T_2$ EIC muscles ~1.0 cm from the sternum. Diaphragm and $T_2$ EIC leads were implanted using a 23- G syringe needle guide and tissue adhesive (Vetbond 1469SB; 3M Animal care product, St. Paul, MN).

**2.2.3. Cervical C2 hemisection—**One week post-telemetry implantation, C2 hemisections (C2HS) were performed as described previously (Dougherty et al., 2012b; Fuller et al., 2009; Vinit et al., 2009). The C2 spinal cord was exposed via dorsal laminectomy, and the duramater cut to enable left C2HS via microscalpel and aspiration. Sham rats underwent cervical laminectomy without spinal injury.

**2.2.4. Whole-body plethysmography—**Unanesthetized rats were placed individually in a 4 L whole body plethysmograph (4 L/min flow; model 600-1211-001; DSI, St. Paul, MN) positioned on a telemetry receiver to enable simultaneous EMG recordings. Tidal volume ($V_T$) and respiratory frequency were analyzed in 1 min bins during baseline, normoxia (20 min) and maximum chemoreceptor stimulation (MCS: 10.5% $O_2$ and 7% $CO_2$, 20 min). Intraperitoneal temperature was monitored with the telemetry system and used to calculate $V_T$ and compensate for changes in body temperature that may introduce calibration errors when ventilation is measured via whole body plethysmography (Stephenson and Gucciardi, 2002).

**2.2.5. EMG telemetry—**Daily AIH exposures occurred in Plexiglas chambers positioned on telemetry receivers (model RPC-2; DSI, St. Paul, MN). Signals from the implanted telemeter were detected by the receivers and sent to a data exchange matrix (model ACQ-7700; DSI, St Paul, MN). Four EMG channels, body temperature and locomotor activity were monitored during the protocol (PONEMAH Physiology Platform; DSI, St. Paul, MN). EMG signals were sampled at 1200 Hz, and analyzed with Neuroscore software (DSI, St. Paul, MN).

**2.2.6. Drug preparation—**KW-6002 (Istradefylline, Sigma-Aldrich) is a selective $A_{2A}$ receptor antagonist with a Ki of 29.6 nM in rats. It's half-life of 110 min and 97% CNS bioavailability after intraperitoneal injections (Yang et al., 2007) make it suitable for use in prolonged in vivo experiments. KW6002 was dissolved in DMSO at 9.3 mg/mL, sonicated and stored at 4 °C in a dark vial.

**2.2.7. Experimental design—**Five days post-telemeter implantation, simultaneous plethysmography and EMG recordings were made during normoxia and MCS to establish pre-treatment values. One day post-injury, the same protocols were repeated. Twenty-seven rats were randomly allocated into five groups: 1) daily AIH (dAIH) + KW6002 (n = 8); 2) dAIH + vehicle (n = 8); 3) daily Nx (dNx) + KW6002 (n = 4); 4) dNx + vehicle (n = 4); and 5) recording controls (n = 3). Group 1 rats received intra-peritoneal KW6002 injections (0.5 mg/kg) immediately before AIH on 7 consecutive days (i.e. dAIH) beginning 8 weeks post-C2HS (i.e. weeks 8–9 post-C2HS), and then before thrice-weekly AIH until 16 weeks post-C2HS. Group 2 received daily AIH, but with vehicle injections (DMSO). Group 3 and 4 rats (drug time controls) were exposed to normoxia only (room air), with $A_{2A}$ antagonist or vehicle injections as appropriate. Recording controls (laminectomy only) received normoxia only, without intraperitoneal injections (see Fig. 1 for experimental design).

**2.2.8. Acute intermittent hypoxia—**Normoxic (21% $O_2$) and hypoxia (10.5% $O_2$) were established in custom-made, cylindrical Plexiglas chambers (12 in. long, 4 in. inner diameter; 1 rat per chamber; rats rested on flat flooring) by mixing $O_2$ and $N_2$ with a

custom-made, computer-controlled mass-flow controller system. Within the chambers, $CO_2$ and $O_2$ levels were continuously monitored (VacuMed Inc, Ventura, CA). Gas flowed through the chambers at 4 L/min, keeping chamber $CO_2$ concentration below 0.2%. At 9:00 a.m., rats received intraperitoneal KW6002 or vehicle injections, followed by AIH (10, 5-min hypoxic episodes, 10.5% $O_2$; 5-min 21% $O_2$). Chamber temperature was kept between 22.5 and 24.5 °C.

**2.2.9. Maximum chemoreceptor stimulation—**Hypoxic (10.5% $O_2$) and hypercapnic conditions (7% $CO_2$) were established in plethysmography chambers by mixing $O_2$, $N_2$ and $CO_2$ via a custom-made, computer-controlled mass flow controller system. After 30 min, baseline measurements were made breathing air (20 min) followed by MCS (20 min). We previously demonstrated that combined hypoxia and hypercapnia elicits a greater breathing response (tidal volume, frequency and diaphragm amplitude) versus continuous hypoxia (Navarrete-Opazo and Mitchell, 2014).

**2.2.10. Tissue processing—**To verify the extent of C2HS, rats were deeply anesthetized, their spinal cords removed and immersion fixed in paraformaldehyde (4%, overnight at 4 °C). Spinal tissues were cryoprotected with sucrose (20–30%), frozen in isopentane (−45 °C) and then stored at −80 °C. Longitudinal spinal sections (C1 to C6, 30 μm thick) were stained with cresyl violet and examined histologically with light microscopy to reconstruct the injury (Vinit et al., 2006) as described by Paxinos and Watson (Paxinos and Watson, 1998). NIH ImageJ software (National Institute of Health; http://rsb.info.nih.gov/jj) was used to measure hemisection area.

### 2.3. Data analyses

EMG signals were analyzed with Neuroscore software. Each signal was filtered (100–624 Hz), rectified, integrated (100 msec) and averaged (bilateral diaphragm/T2 EIC). Values during active locomotor and grooming activity were excluded from analysis. Inspiratory EMG burst amplitude from each muscle was normalized as a percent change from its own pre-injury values.

All variables were compared within and among groups for time (baseline and MCS) and treatment via two-way, repeated measures ANOVA with Fisher's LSD *post hoc* tests (Sigma-Stat version 2.03, Systat Software Inc, San Jose, CA, USA). Differences were considered significant if $p < 0.05$. Values are expressed as means ± 1 SEM.

## 3. Results

### 3.1. Reduced ipsilateral motor activity one-day post-C2HS

To establish baseline pre-injury values, rats were exposed to 20 min of air (normoxia) and 20 min of MCS with EMG and ventilatory measurements five days post-telemeter implantation (Fig. 2). One day post-$C_2$HS, their EMG activity was significantly reduced versus pre-injury values in ipsilateral diaphragm and $T_2$ EIC muscle with no differences among groups ($p > 0.05$, Fig. 3A, B). Unlike EMG recordings in anesthetized rats, EMG telemetry in unanesthetized, freely moving rats may lead to residual activity in ipsilateral diaphragm and

$T_2$ intercostal muscles due to: 1) greater respiratory drive in unanesthetized versus anesthetized rats, or 2) electrical interference from nearby muscles. Similar observations were made in previous telemetry studies assessing respiratory muscle activity after injury (Navarrete-Opazo et al., 2014, 2015).

$C_2$HS reconstruction after experiments demonstrated similar injury areas in all groups (expressed as percent of total cross sectional area; dAIH + vehicle: 49.0 ± 0.8%, dAIH + KW6002: 47.1 ± 0.9%, dNx + vehicle: 49.4 ± 0.9%, dNx + KW6002: 48.7 ± 1.3%, $p > 0.05$).

### 3.2. Stability of recording controls

To determine diaphragm and $T_2$ EIC EMG recording stability, recording controls received sham surgery (laminectomy), but did not receive hypoxia or intraperitoneal injections. Recording controls exhibited stable diaphragm and $T_2$ EIC signals between 9 and 16 weeks post-sham surgery (both $p > 0.05$).

### 3.3. Breathing frequency is not affected by AIH or AIH combined with A2A antagonist

Breathing frequency was increased from pre-injury values (84 ± 2 bpm vs. 106 ± 2 bpm; $p < 0.001$), compensating for reduced $V_T$. However, by 9 weeks post-C2HS, breathing frequency had returned to normal levels (~87 bpm), with no differences among groups ($p > 0.05$). During MCS, breathing frequency was significantly increased versus pre-injury values one day post injury (137 ± 2 bpm vs. 148 ± 2 bpm, $p < 0.05$), and remained elevated throughout the study.

Daily AIH alone or in combination with and A2A receptor inhibition had no effect on respiratory frequency during normoxia or MCS at any time ($p > 0.05$).

### 3.4. $A_{2A}$ inhibition enhances dAIH-increased tidal volume

One day post-C2HS, tidal volume ($V_T$) was significantly reduced versus pre-injury values with no differences among groups (Fig. 4A).

Nine weeks post-C2HS, drug time controls showed normal values of $V_T$ that were not significantly different from recording controls during normoxia ($p > 0.05$; Fig. 4A). However, at this same time, $V_T$ during MCS (10.5% $O_2$, 7% $CO_2$) was significantly reduced versus recording controls ($p < 0.05$, Fig. 4B). Thus, despite substantial spontaneous recovery during normal breathing post-C2HS, ventilatory deficits persist with increased ventilatory demand.

Daily AIH significantly increased $V_T$ versus time controls (dAIH + vehicle: 0.57 ± 0.01 mL/100 g vs. dNx + vehicle: 0.52 ± 0.01, $p < 0.05$; Fig. 4A) and recording controls (0.53 ± 0.02; $p < 0.05$; Fig. 4A) one day post-dAIH (i.e. 9 weeks post-C2HS) during air breathing (normoxia), but not during MCS ($p > 0.05$; Fig. 4B). Thus, the extent of dAIH-induced functional recovery is less robust at 9 versus 2 weeks post-C2HS (Lovett-Barr et al., 2012; Navarrete-Opazo et al., 2015).

Combined dAIH plus A2A receptor inhibition further increased $V_T$ at 9 weeks post-C2HS versus dAIH alone during air breathing (dAIH + KW6002: 0.60 ± 0.08 vs. dAIH + vehicle: 0.57 ± 0.01 mL/100 g, $p < 0.05$; Fig. 4A) and MCS (dAIH + KW6002: 0.95 ± 0.01 vs. dAIH + vehicle: 0.88 ± 0.02 mL/100 g, $p < 0.05$, Fig. 4B). During MCS, $V_T$ was no longer different from recording controls in this group ($p = 0.07$), showing that combined KW6002 and dAIH restored the ability to increase VT to near-normal levels (Fig. 4B).

Three times per week AIH maintained the effects of dAIH on $V_T$ up to 12 weeks post-C2HS (i.e. 3 weeks post-dAIH) with or without the A2A antagonist during air breathing and MCS (Fig. 4A, B).

### 3.5. A2A inhibition enhances dAIH-induced contralateral diaphragm motor recovery

Contralateral (uninjured) diaphragm EMG amplitude significantly increased from pre-injury values one day post-C2HS during air breathing (~130% of pre-injury values, $p > 0.05$), and remained above pre-injury values at all times. Thus, compensatory mechanisms begin shortly after C2HS (Fig. 5A).

Daily AIH increased contralateral diaphragm EMG amplitude 9 weeks post-C2HS versus time controls during air breathing (dAIH + vehicle: 138.3 ± 5.4% vs. dNx + vehicle: 127.0 ± 2.9% pre-injury values; $p < 0.05$; Fig. 5A, C).

A2A receptor inhibition enhanced dAIH-induced contralateral diaphragm activity 9 weeks post-C2HS during air breathing (dAIH + vehicle: 138.3 ± 5.4% vs. dAIH + KW6002: 157.5 ± 5.3% pre-injury values; $p < 0.05$; Fig. 5A, C). Thus, dAIH effects on diaphragm activity are constrained by A2A receptors at this time; by relieving that constraint, the functional impact of dAIH is enhanced.

Three times per week AIH presentations maintained the dAIH effects up to 12 weeks post-C2HS (ie. 3 weeks post-dAIH) with or without A2A antagonist during air breathing (Fig. 5A).

### 3.6. A2A inhibition enhances dAIH-induced ipsilateral diaphragm motor recovery

Ipsilateral (injured) diaphragm activity showed modest spontaneous recovery in control rats 9 weeks post-C2HS (~19% pre-injury values) at 16 weeks post-C2HS (~29% pre-injury values; Fig. 5B), confirming previous reports (Dougherty et al., 2012b).

Daily AIH significantly increased ipsilateral (injured) diaphragm EMG activity versus time controls 9 weeks post-C2HS during normoxia (dAIH + vehicle: 36.1 ± 4.8%, vs. dNx + vehicle: 21.0 ± 1.8% pre-injury values, $p < 0.001$; Fig. 5B, C).

A2A receptor inhibition enhanced dAIH-induced motor recovery in ipsilateral (injured) diaphragm at 9 weeks post-C2HS during normoxia (dAIH + vehicle: 36.1 ± 4.8% vs. dAIH + KW6002: 56.0 ± 7.3% pre-injury values; $p < 0.001$; Fig. 5B, C).

Three times per week AIH maintained dAIH effects up to 12 weeks post-C2HS (i.e. 3 weeks post-dAIH) with or without A2A antagonist during air breathing (Fig. 5B).

### 3.7. A2A receptor inhibition enhanced dAIH-induced MCS response in contralateral diaphragm

EMG motor activity in contralateral diaphragm during MCS was significantly reduced throughout the study (~80% pre-injury vales; Fig. 6A).

Daily AIH increased contralateral diaphragm EMG amplitude 9 weeks post-C2HS versus time controls during (dAIH + vehicle: 108.0 ± 1.9% vs. dNx + KW6002: 92.0 ± 2.8% of pre-injury values, $p < 0.05$; Fig. 6A), demonstrating that dAIH increases motor output of the intact, contralateral diaphragm during increased respiratory drive.

A2A receptor inhibition enhanced dAIH-induced contralateral diaphragm activity 9 and 10 weeks post-C2HS during MCS ($p < 0.05$; Fig. 6A). At later time-points there was no difference between dAIH alone or combined with A2A antagonist (Fig. 6A).

### 3.8. Ipsilateral diaphragm MCS response is not affected by AIH or A2A antagonist

As expected, the MCS response one day post-C2HS was abolished in ipsilateral diaphragm but increased by 9 weeks post-C2HS (~40% pre-injury values), without significant change thereafter (Fig. 6B). Daily AIH alone or combined with A2A antagonists does not increase injured diaphragm activity during MCS (Fig. 6B).

### 3.9. Daily AIH does not affect $T_2$ EIC activity

Contralateral (uninjured) $T_2$ EIC EMG amplitude exhibited a small increase one-day post-C2HS (~113% pre-injury values), but had returned to pre-injury levels by 9 weeks post-C2HS. At this same time, ipsilateral (injured) $T_2$ EIC exhibited complete recovery of its activity after an initial decrease (Fig. 7). Surprisingly, no MCS response was observed in either contralateral or ipsilateral $T_2$ EIC EMG peak activity at any time post-injury (Fig. 7).

Daily AIH alone or combined with A2A antagonist had no significant effects on bilateral $T_2$ EIC EMG activity, a possible indication of a “ceiling effect.”

## 4. Discussion

The essential results of this study were: 1) spontaneous motor recovery is modest in ipsilateral diaphragm, but complete in ipsilateral $T_2$ EIC muscle 9 weeks post-C2HS; 2) dAIH increases tidal volume during normoxia, but not MCS 9 weeks post-C2HS; 3) dAIH increases bilateral diaphragm activity during normoxia, but only in contralateral diaphragm during MCS 9 weeks post-C2HS; 4) $A_{2A}$ inhibition strongly enhances dAIH-induced $V_T$ and contralateral diaphragm recovery during normoxia and MCS; 5) thrice weekly AIH maintains dAIH effects up to 12 weeks post-C2HS; and 6) no differences among groups could be detected beyond 13 weeks post-C2HS (i.e. 3 weeks post-dAIH). Thus, the functional benefits of dAIH in rats 8 weeks post-C2HS are amplified when combined with $A_{2A}$ inhibition and these effects last at least three weeks post- dAIH. However, continued treatment three times per week is not able to sustain these benefits. Combined dAIH and $A_{2A}$ receptor inhibition may be most effective at accelerating versus augmenting long-lasting functional recovery. Although sustained functional benefits may require continued daily AIH, this conclusion requires further testing.

### 4.1. Breathing pattern after C2HS

After C2HS, rats maintain normal blood gases (Goshgarian, 2009; Goshgarian et al., 1986) by breathing with an altered pattern, characterized by increased frequency with decreased $V_T$ (Golder et al., 2001a, 2001b). Changes in breathing pattern after SCI may result from persistent vagal feedback and/or chest wall receptors (Golder et al., 2001b). However, it may also reflect plasticity within the medullary respiratory control network initiated by C2HS and loss of afferent feedback from spinal sensory pathways (Golder et al., 2003).

The extent of spontaneous $V_T$ recovery 9 weeks post-C2HS is similar to other studies of $C_2$HS or lateralized cervical contusion models (Dougherty et al., 2012b; Golder et al., 2011; Lane et al., 2012; Lovett-Barr et al., 2012). Untreated rats show normal $V_T$ during air breathing at this time, coinciding with restoration of normal breathing frequency. On the other hand, breathing frequency remains elevated during MCS (Fuller et al., 2006, 2009), compensating for the persistent reduction in $V_T$.

### 4.2. Spontaneous contralateral versus ipsilateral recovery

Spontaneous VT recovery may occur from recruitment of less affected (contralateral) respiratory muscles (Brichant and De Troyer, 1997; Johnson and Mitchell, 2013; Katagiri et al., 1994; Teitelbaum et al., 1993), removal of inhibitory sensory inputs to phrenic motor neurons (Goshgarian, 1981) or spontaneous plasticity, partially restoring function in the most affected muscles. Since rats with C2HS maintain normal blood gases (Goshgarian et al., 1986), spontaneous compensation does not result from persistent chemoreceptor stimulation. Here, we confirm a small, compensatory increase in contralateral (uninjured) diaphragm (~20% above pre-injury values) and $T_2$ EIC activity (12%) in control rats even one day post-C2HS (Johnson and Mitchell, 2013).

Compensation is maintained throughout the present study in diaphragm, but not $T_2$ EIC activity, coinciding with the complete recovery of left (injured) $T_2$ EIC activity; thus, spontaneous recovery in the ipsilateral (injured) intercostal muscles may remove the mechanism driving compensation in the contralateral (intact) intercostal muscles. The mechanisms driving this complex, muscle specific, and time-dependent shift in activity remains to be explored.

Plasticity in impaired pathways via increased synaptic strength can restore/establish function in spared pathways to ipsilateral phrenic and thoracic motor neurons (Golder et al., 2011; Johnson and Mitchell, 2013; Mitchell and Johnson, 2003; Nantwi et al., 1999). We observed small, variable spontaneous recovery of injured diaphragm activity between 16% to 31% of pre-injury values 4 months post-C2HS, similar to previous reports (Dougherty et al., 2012b). In contrast, injured $T_2$ EIC muscle was completely recovered 9 weeks post-C2HS in control rats, suggesting that accessory inspiratory muscles contribute relatively more to spontaneous recovery of VT during normoxia (Dougherty et al., 2012a; Sherrey and Megirian, 1990). Further studies are needed to determine mechanisms of spontaneous recovery in inspiratory intercostal activity following C2HS. Loss of inhibitory phrenic afferents may excite intercostal motoneurons after SCI since diaphragm paralysis increases inspiratory intercostal muscle activity in dogs (De Troyer, 1998).

### 4.3. Daily AIH induces respiratory functional recovery

Nine weeks post-C2HS, dAIH increases $V_T$ above controls breathing air, reflecting dAIH-effects on ipsilateral and (particularly) contralateral diaphragm activity. Thus, both sides of the diaphragm may contribute to dAIH-induced recovery of VT during room air breathing.

Abundant evidence demonstrates that dAIH improves diaphragm activity and breathing capacity ($V_T$) 2 weeks post-$C_2$HS (Lovett-Barr et al., 2012; Navarrete-Opazo et al., 2015). Here, we demonstrate that dAIH also improves function with chronic SCI (9 weeks-post-$C_2$HS) during normoxia. However, at this time post-injury, dAIH alone had no effect on VT during MCS.

Two weeks post-$C_2$HS, dAIH increases contralateral (intact) diaphragm activity, but not ipsilateral (injured) diaphragm activity (Navarrete-Opazo et al., 2015). In contrast, we show here that at 9 weeks post-C2HS, dAIH improves both contralateral and ipsilateral diaphragm activity, consistent with the time-dependent return of serotonergic innervation in the phrenic motor nucleus following C2HS (Golder and Mitchell, 2005).

### 4.4. Adenosine 2A receptor inhibition enhances dAIH-induced functional recovery

A critical finding of this study is that combined dAIH and $A_{2A}$ receptor inhibition have greater effects on breathing capacity than either treatment alone after chronic cervical SCI. This response contrasts dramatically with the same combinatorial treatment initiated at 2 (versus 8) weeks post-C2HS (Navarrete-Opazo et al., 2015). In that earlier study, A2A receptor inhibition impaired the functional benefits of dAIH, suggesting that the underlying recovery was adenosine-dependent. We now show that the system reverts towards the behavior of uninjured rats with time post-injury (see Fig. 8 for hypothesis schematic). Thus, dAIH-induced functional recovery of breathing capacity undergoes a transition from adenosine-dependent (2 weeks) to a serotonin-dependent, adenosine constrained (9 weeks). It is essential to understand such time-dependent shifts in the mechanisms of dAIH-induced functional recovery, or any other treatment modality. For example, caffeine (a common A2A receptor antagonist) would undermine dAIH induced functional recovery with acute injury, but may enhance outcomes in patients with chronic, incomplete SCI.

The relevance of this finding is that combined KW6002 and dAIH may prevent/minimize respiratory complications (e.g. atelectasis, pneumonia) frequently present with cervical SCI (NSCISC, 2005). Since such pulmonary complications increase ventilatory demand to maintain blood gas homeostasis, greater functional recovery such as that provided by combined dAIH and $A_{2A}$ receptor inhibition may be a simple, safe and effective way to increase/accelerate functional recovery of breathing capacity.

### 4.5. Neither dAIH nor adenosine 2A receptor inhibition affect $T_2$-EIC recovery

Complete spontaneous recovery of the ipsilateral (injured) $T_2$ EIC suggests an important role in spontaneous recovery of breathing capacity after C2HS. However, dAIH (with or without KW6002) had no effect on the ipsilateral or contralateral $T_2$ EIC muscle. We hypothesize that crossed spinal synaptic pathways innervating the $T_2$ EIC motor neurons contribute to spontaneous recovery of ipsilateral $T_2$ EIC activity; these crossed-spinal pathways may be

strengthened maximally by spontaneous plasticity, leaving little room for additional plasticity induced by dAIH (i.e. a “ceiling effect”). A2A receptor inhibition has little effect on $T_2$ intercostal muscle activity in rats with or without C2HS (Navarrete-Opazo et al., 2014, 2015). Thus, it is not surprising that A2A receptor inhibition fails to enable dAIH effects on $T_2$ EIC activity.

### 4.6. 3×wAIH may prolong (but not preserve) functional recovery

We previously we demonstrated that once-per-week AIH reminders were not sufficient to maintain dAIH-induced functional recovery two weeks post-C2HS (Navarrete-Opazo et al., 2015). Here we show that dAIH-induced functional recovery persists at least 3 weeks when followed by 3×wAIH; however, the necessity for 3×wAIH in that persistent recovery was not directly tested here. 3×wAIH does increase expression of relevant molecules for AIH-induced phrenic motor plasticity (Satriotomo et al., 2012). On the other hand, between 13 weeks post-C2HS (3 weeks post-dAIH) to the end of the study, differences among treatment groups were no longer apparent, demonstrating a failure to maintain the advantages conferred by dAIH, with or without A2A receptor inhibition. Failure to maintain any advantage with 3×wAIH may indicate that: 1) 3×wAIH is not sufficient to establish and/or maintain the functional benefits of repetitive AIH therapy; 2) repetitive A2A receptor inhibition may undermine continued benefits due to A2A receptor down-regulation with prolonged drug administration; or 3) the advantages of repetitive AIH may diminish with age.

The rats at the end of this study were ~8 month-old. Serotonin-dependent AIH-induced phrenic motor plasticity diminishes with age-in male rats (Behan et al., 2002; Zabka et al., 2001), largely due to decreasing testosterone/CNS estrogen levels (Behan et al., 2003; Zabka et al., 2005). Similarly, 5-HT2 receptor density in cortex (Hyttel, 1987; Morgan, 1987) and cervical spinal cord (Ko et al., 1997) declines with age. Reduced testosterone/estrogen levels associated with age may diminish the capacity for AIH-induced functional recovery with chronic C2HS.

## 5. Conclusions

In male rats with chronic C2HS, dAIH improves breathing capacity and AIH “reminders” three times per-week may prolong this benefit. Since chronic C2HS enables recovery of serotonergic innervation in the phrenic motor nucleus, dAIH elicits functional recovery by a serotonin-dependent, adenosine-constrained mechanism versus adenosine-dependent functional recovery 2 weeks post-injury. Thus, cellular mechanisms of dAIH induced functional recovery shift with time post injury. This finding has important implications as we develop repetitive acute intermittent hypoxia as a treatment for patients with spinal injury. For example, caffeine should be avoided with acute injury, but may be advantageous in patients with chronic spinal injury.

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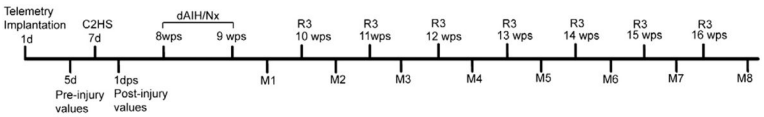


**Fig. 1.**

Timeline describing the study design from day 1 (1d) until 16 weeks post-spinal cord injury (wps). C2HS: cervical hemisection in second segment (C2), dAIH/Nx: daily acute intermittent hypoxia or normoxia for seven days depending on groups (see methods). R3 refers to three times per week presentations of acute intermittent hypoxia or normoxia from week 9–16 post-injury. M1–M8 refers to weekly measurements including simultaneous plethysmography and electromyography of bilateral diaphragm and second external intercostal muscles.

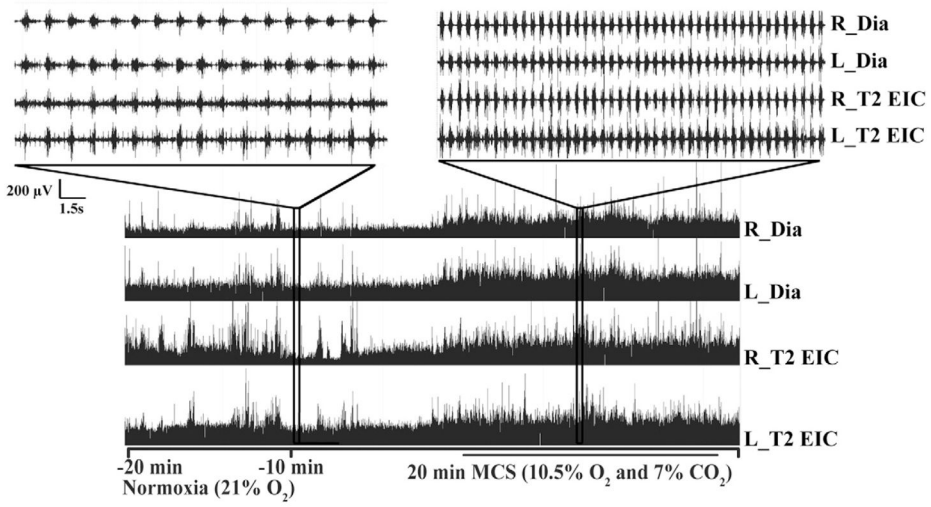


**Fig. 2.**

Representative raw (top) and integrated (bottom) EMG activity of right/left diaphragm (R/L_Dia) and second external intercostal muscle (R/L_$T_2$ EIC) before cervical C2 hemisection during 20 min of room air breathing and during maximum chemoreceptor stimulation (MCS). Note the robust increase EMG amplitude and breathing frequency during MCS in all muscles.

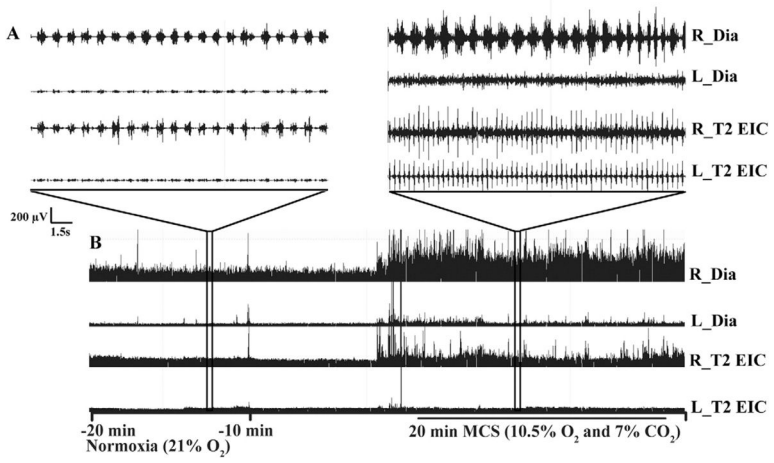


**Fig. 3.**

Representative raw (A, top) and integrated (C, bottom) EMG activity of right (intact)/left (injured) diaphragm (R/L_Dia) and second external intercostal muscle (R/L_$T_2$ EIC) one day post-C2HS during 20 min of room air breathing and maximum chemoreceptor stimulation (MCS). Note: 1) significantly reduced EMG activity in left diaphragm and T2 EIC during room air breathing and MCS, consistent with left C2HS; 2) reduced MCS response in right $T_2$ EIC muscle; 3) increased MCS response in right diaphragm.

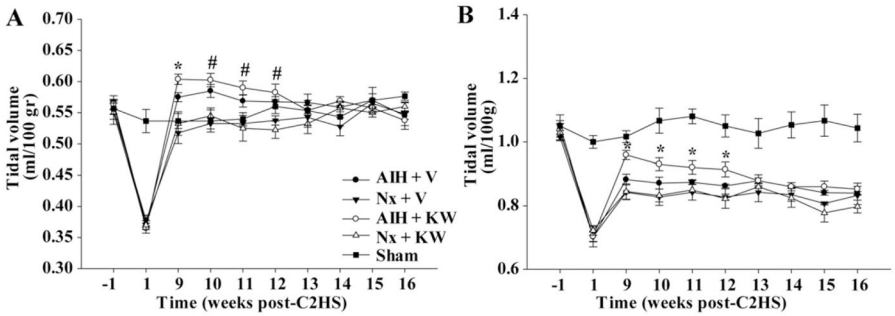


**Fig. 4.**

Absolute values of tidal volume ($V_T$; mL per 100 gram body mass) during room air breathing (normoxia, Nx; A) and maximum chemoreceptor stimulation (MCS; B) one week before spinal injury and then, 1 to 16 weeks post C2HS. Note: 1) time control rats (Nx + V, Nx + KW6002; no AIH) show normal tidal volumes at 9 weeks post-C2HS during normoxia (A); 2) dAIH plus vehicle-treated rats show significantly increased $V_T$ versus drug and time controls (sham rats) for up to 11 weeks post-C2HS during room air breathing; this effect was significantly enhanced by $A_{2A}$ receptor inhibition with KW6002 at 9 weeks post-C2HS, and this effect was seen through 12 weeks post-injury (A); 3) during MCS (B), KW6002 significantly enhances $V_T$ versus dAIH plus vehicle-treated rats for up to 12 weeks post-C2HS (3 weeks post-dAIH); 4) from 13 up to 16 weeks post-C2HS there were no differences among groups. dAIH: daily acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002, Nx (normoxia, control rats). Values are means ± SEM. *significantly different from AIH + V and controls, # significantly different from controls; $p < 0.05$.

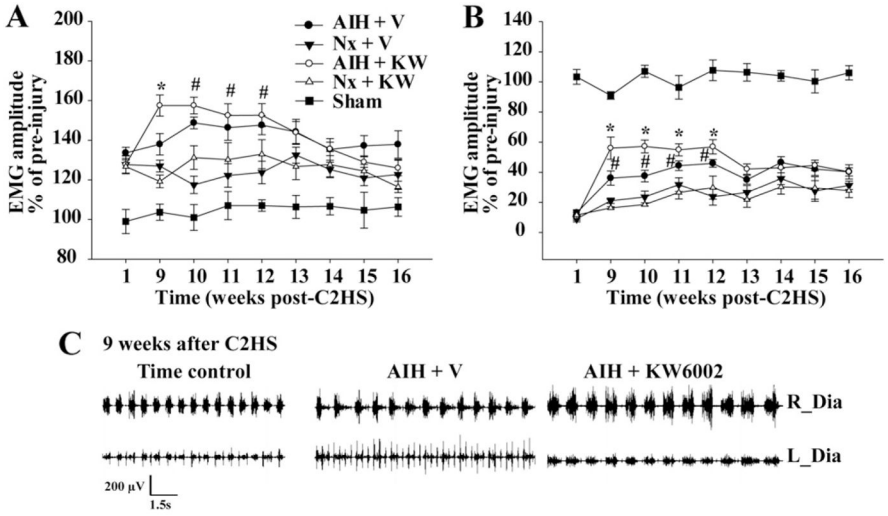


**Fig. 5.**

Changes in contralateral (uninjured, A) and ipsilateral (injured, B) diaphragm muscle activity (peak integrated amplitude) during room air breathing (normoxia) expressed as a percent change from pre-injury values from 1 to 16 weeks post-C2HS. Note: 1) contralateral diaphragm shows increased activity in all groups throughout the study; 2) there was a limited spontaneous recovery in ipsilateral diaphragm; 3) dAIH significantly enhances contralateral amplitude (A) up to 12 weeks post-C2HS, an effect significantly enhanced by $A_{2A}$ receptor inhibition (KW6002) at 9 weeks post-C2HS and beyond; (4) dAIH significantly enhances ipsilateral diaphragm activity (B) up to 12 weeks post-C2HS, an effect significantly enhanced by KW6002 for up to 12 weeks post-C2HS; 5) from week 13 to the end of the study, there was no significant difference among groups (see Discussion). (C) Representative raw EMG activity of right/left diaphragm (R/L_Dia) 9 weeks post-C2HS during room air breathing (normoxia). Notice increased EMG amplitude of bilateral diaphragm activity in dAIH plus KW6002-treated rats versus dAIH plus vehicle (DMSO) and time control rats. dAIH: daily acute intermittent hypoxia, Nx: normoxia, V: vehicle (DMSO), KW: KW6002. Values are mean ± SEM. * significantly different from AIH + V and controls, # significantly different from controls (Nx + V, Nx + KW6002); $p < 0.05$.

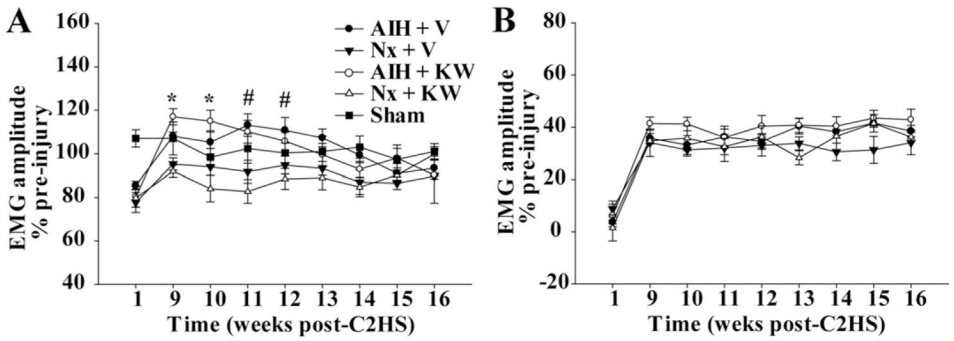


**Fig. 6.**

Changes in contralateral (uninjured, A) and ipsilateral (injured, B) diaphragm muscle amplitude during maximum chemoreceptor stimulation (MCS) expressed as percent change of pre-injury values between 1 and 16 weeks post-C2HS. Note: 1) AIH and $A_{2A}$ receptor inhibition (KW6002) significantly increased EMG peak integrated amplitude in contralateral diaphragm at 9 and 10 weeks post-C2HS (A); 2) both dAIH alone or in combination with KW6002 significantly increased contralateral EMG amplitude at 11 and 12 weeks post-C2HS; 3) Only a small MCS response is observed in the ipsilateral diaphragm (B) throughout the study with no differences among groups. dAIH: daily acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002. Values are mean ± SEM. *AIH + V significantly different from time controls rats (Nx + V, Nx + KW6002), # AIH + KW6002 significantly different from controls; p < 0, 05.

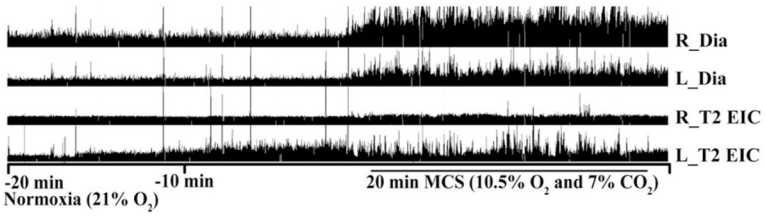


**Fig. 7.**

Representative integrated EMG activity of right/left diaphragm (R/L_Dia) and second external intercostal muscle (R/L_$T_2$ EIC) at 16 weeks post-C2HS) during maximum chemoreceptor stimulation (MCS). Note: 1) small MCS response in left diaphragm; 2) robust MCS response in right diaphragm; and 3) abolished MCS response in bilateral $T_2$ EIC activity.

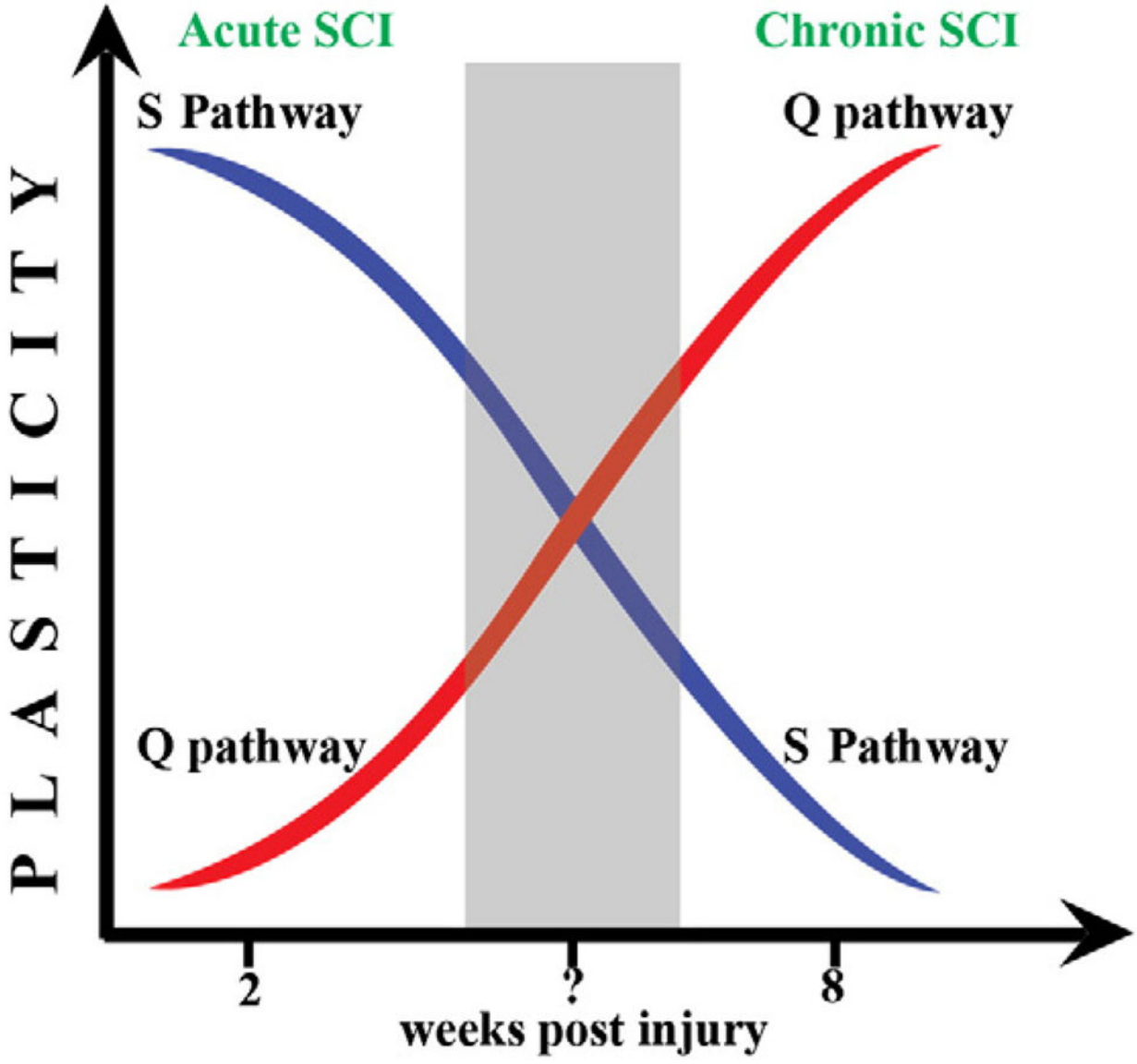


**Fig. 8.**

Schematic illustration of hypothetical mechanisms of acute intermittent hypoxia (AIH)-induced plasticity after SCI. During early SCI (<8 weeks post-injury), serotonin terminals innervating phrenic motor nuclei have been disrupted and, thus, AIH induces respiratory functional recovery via an adenosine-dependent mechanism (S pathway, blue line). With more chronic injury, serotonin terminal density is at least partially restored (8 weeks post-injury), and AIH once again induces plasticity and functional recovery via a serotonin-dependent mechanism (Q pathway, red line). The precise time the serotonin-dependent mechanism shifts to an adenosine-dependent is not known (grey zone). Collectively, our data paint the emerging picture that mechanisms of AIH induced functional recovery shift in complex ways with time following spinal injury; further, the possibility exists that AIH may

not have detectable functional benefits at certain times post-injury due shifts in the balance of these mechanisms.