Intermittent Hypoxia: a Potential Therapy for Spinal Cord Injuries

By

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I dedicate this thesis to my husband, Joe, who patiently accompanied me during my long hours of writing and study. Without his support and infinite love I could have not focused in my career as I did.

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ABSTRACT

Traumatic cervical spinal cord injury (SCI) interrupts descending synaptic pathways from brainstem pre-motor neurons to spinal respiratory motor neurons, thereby paralyzing respiratory muscles and compromising breathing. In recent years, considerable effort has been devoted towards an understanding of the capacity for respiratory motor plasticity following acute intermittent hypoxia (AIH). The fundamental hypothesis guiding this thesis is that AIH-induced respiratory motor plasticity can be "harnessed" to restore breathing capacity after incomplete cervical spinal injury. Further, I postulate that moderate AIH combined with adenosine A_{2A} receptor inhibition enhances that functional recovery of breathing capacity and respiratory muscle activity. In this thesis, radiotelemetry of respiratory muscle electromyogram activity and plethysmography were used in normal (intact) and C_2 cervical hemisected (C_2HS) unanesthetized rats to test these hypotheses. First, I demonstrated that moderate AIH elicits long-term facilitation of diaphragm (DiaLTF) and external inspiratory intercostal (EIC) muscle activity ($T_{2,4,5}$ EIC LTF); thus AIH-induces plasticity in multiple inspiratory muscles. In aim 2, I demonstrated that systemic adenosine A_{2A} receptor inhibition enhances DiaLTF, but not T_2 EIC LTF, suggesting differences in their cellular mechanisms. Potential therapeutic effects of AIH-induced respiratory plasticity (and enhanced plasticity) after acute and chronic C_2HS were tested in aims 3 and 4. Shortly after cervical injury, when serotoninergic innervation of the phrenic motor nucleus is greatly reduced (<4 weeks post-injury), repetitive AIH (rAIH) elicits functional recovery of breathing capacity by an adenosine-dependent (serotonin-independent) mechanism. In contrast, with chronic spinal injury (>8 weeks post-injury), rAIH-induces functional recovery via a serotonin-dependent, adenosine-constrained mechanism. Systemic A_{2A} receptor antagonist (Istradefylline) administration impairs rAIH-induced functional recovery 2 weeks post-C_2HS, but enhances rAIH-induced functional recovery with chronic injury (> 8 weeks). Repetitive AIH (rAIH) represents a simple, safe and effective protocol eliciting respiratory functional recovery after cervical spinal cord injury. Furthermore, adenosine A_{2A} antagonist administration may amplify rAIH-induced functional recovery in humans with chronic, incomplete spinal injuries, where the potential for further functional gain is limited.

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INTRODUCTION

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Abstract

Intermittent hypoxia (IH) evokes respiratory, cardiovascular and metabolic adaptations. These adaptations are largely associated with detrimental effects when the intermittent hypoxic stimulus is severe/chronic. However, IH is also associated with beneficial effects, such as motor plasticity and increased growth factor expression in the central nervous system when the stimulus is modest/acute. In this chapter, I focus on the factors leading to detrimental versus beneficial IH effects. Specifically, this chapter is concerned with relevant characteristics of IH that lead (differentially) to safe, protective and/or therapeutic effects versus pathology in multiple physiological systems. Extensive literature concerning IH is surveyed, and the impact assessed on the nervous, cardiovascular and immune systems, as well as metabolic regulation. To understand the effects of IH, it is imperative to define a number of critical characteristics of the IH protocol under investigation, including the severity of hypoxia within each hypoxic episode (often expressed as the inspired oxygen fraction), the duration of each hypoxic episode, the number of cycles per day (i.e. frequency of episodes), the total time of exposures and, finally, the pattern of presentation across time (within day versus consecutive days versus alternating days). Key features of the IH protocol are highly associated with the functional outcome. Namely, moderate hypoxic episodes (inspired $O_2 \geq 9\%$) and a low number of cycles per day ($\leq$15 cycles/day) are associated with beneficial effects of IH. In contrast, severe hypoxia within episodes (inspired $O_2 < 9\%$) and more frequent episodes per day (40-2400 cycles) are more likely to elicit deleterious effects. Accumulating evidence suggests that "low dose" IH (modest hypoxia, few episodes) may be a simple, safe and effective treatment with considerable therapeutic potential in multiple clinical disorders.

Introduction

Intermittent hypoxia (IH) has been a topic of considerable research in the past few decades. However, a full understanding of IH and its biological effects remains elusive; some reports claim that IH elicits pathology, whereas others focus on its beneficial effects. To some extent, this apparent discrepancy can be explained by the wide range of experimental procedures/protocols described as "intermittent hypoxia" among investigators. The essential feature of IH is repeated episodes of low oxygen (hypoxia), interspersed with periods of normal oxygen (normoxia). However, this definition does not begin to capture the range of protocols reported in the literature. Indeed, there is no real consensus regarding key variables defining the biological impact of IH. The fundamental goal of this brief review is to assess relevant characteristics that lead to beneficial/compensatory versus mal-adaptive/pathological outcomes.

The specific protocols/paradigms of IH reported in the literature are often associated with the specific perspective of the investigators (i.e. their field of study). Reported IH protocols vary greatly in terms of: 1) the severity of hypoxia (i.e. the inspired oxygen fraction), 2) the duration of hypoxia, 3) the number of hypoxia/reoxygenation cycles per day, 4) the overall duration of exposure, and 5) regulation of other relevant variables, such as the prevailing level of arterial carbon dioxide. The severity of hypoxia within episodes ranges broadly from 2% to 16% inspired oxygen. The duration of hypoxic episodes can be as little as 15-30 seconds, or up to 12 hours. Studies use IH protocols that vary from 3 to 2400 cycles of hypoxia/reoxygenation per day. The overall IH protocol duration ranges from less than an hour to exposures lasting between 2 to 90 days. Most chronic IH protocols involve hypoxic episodes on consecutive days, although some emphasize hypoxia/reoxygenation episodes on alternating days (for example every other day or 3 times per week). These experimental variables must be carefully considered before we can understand the biological impact of IH, and the distinctions between protocols leading to beneficial versus detrimental IH effects (Dale et al., 2013).

Since each laboratory applies its own unique IH paradigm, discrepancies in terminology make generalizations difficult, and obstruct efforts to understand systemic IH effects. On the other hand, it is hazardous to standardize protocols since such standardization will obscure the range of IH prevalent in

life, and may obscure appreciation of the wide impact of IH on physiology (Mitchell and Terada, 2011). To maximize the progress towards therapeutic applications of IH, detailed understanding of different animal models and mechanisms underlying each particular disorder will be useful to define optimal IH protocols for each clinical condition.

This review does not attempt to discuss in great detail the different cellular mechanisms underlying therapeutic benefits of IH. Rather, the purpose of this introductory chapter is to examine key features of experimental IH protocols used to simulate or treat different clinical conditions, with a special focus on *in vivo* animal models and human studies. Finally, common features of IH paradigms eliciting beneficial versus deleterious effects is proposed in a range of clinical conditions.

Inflammatory/immune responses to IH

Individuals with Obstructive Sleep Apnea (OSA) have increased levels of systemic inflammation versus controls (Vgontzas et al., 1997, Lavie, 2003, Ryan et al., 2006), and markers of systemic inflammation correlate with cardiovascular disease in both OSA and non-OSA cohorts (Lavie, 2003, Minoguchi et al., 2005). It is hypothesized that CIH activates nuclear factor kappa B (NFκB)-mediated inflammatory pathways (Ryan et al., 2006), leading to increased expression of the inflammatory mediators tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and c-reactive protein (CRP) (Lavie, 2003, Saito et al., 2003, Kofler et al., 2005). CIH-induced inflammation may lead to endothelial dysfunction and injury, contributing to atherosclerosis associated with OSA (Libby, 2002). From this perspective, IH is deleterious as a result of its pro-inflammatory effects.

In contrast to these studies of CIH (intending to simulate OSA), other studies using less robust IH protocols found no evidence for increased inflammation after IH in animal models (Tam et al., 2007). For instance, IH consisting of 6 minutes of hypercapnic hypoxia (8% O_2, 7% CO_2), alternating with 6 minutes of air, for 90 minutes does not increase blood levels of TNF-α or CRP in male piglets (Tam et al., 2007). Moreover, exposure to a single daily isocapnic hypoxia exposure (oxyhemoglobin saturation: 80% ~ 48 mmHg PaO_2, 1 h/day) for 10 consecutive days shows no increase in markers of inflammation in healthy

young men (Querido et al., 2012), suggesting that modest protocols of IH can be potentially used as a therapy without an increase in inflammation. Further, some found no evidence for systemic inflammation in OSA patients (Guilleminault et al., 2004, Phillips et al., 2007, Kohler et al., 2009) and no differences in circulating CRP levels were reported (Guilleminault et al., 2004).

Inflammation elicited by chronic IH, like in OSA, may relate to multiple factors in addition to IH per se, such as obesity or nocturnal arousal. For example, after 12 weeks of CIH (30 sec 5% O_2, alternated with 30 sec normoxia, 12 h/day), increased hepatic TNF-α gene expression only in mice fed with a high cholesterol diet (Savransky et al., 2007). In OSA patients, CRP levels are significantly correlated with the body mass index (BMI), esophageal pressures, hip/waist ratio and neck circumference (Guilleminault et al., 2004). Inflammation may be also related to the frequent arousals experienced by patients with OSA (Meier-Ewert et al., 2004). Yet, mechanisms underlying inflammation in OSA patients requires further investigation.

Interestingly, at few studies suggest that more moderate IH protocols may actually enhance the innate immune system while having an overall anti-inflammatory effect. In healthy subjects, exposure to 4, 5-min episodes of 10% O_2 interspersed with 5-min room-air intervals, for 14 days augments phagocytic and bactericidal activities of neutrophils, while suppressing pro-inflammatory mediators such as TNF-α and IL-4 by more than 90% (Serebrovskaya et al., 2011). These responses, which persisted at least 7 days post-IH, may augment the body's immune defenses without eliciting inflammation.

Taken together, these studies provide at least some empirical support for the idea that IH can have both detrimental and beneficial effects on the immune/inflammatory system, suggesting that select IH protocols may have eventual clinical application in immunologically compromised patients. However, the potential of IH in immunotherapy has not yet been fully explored. Further research is necessary to identify the optimum IH "dose" (e.g., severity of hypoxia, frequency of cycles) that enhances the innate immune system without coincident inflammation.

Metabolic responses to IH

According to World Health Organization criteria, metabolic syndrome consists of obesity with associated diabetes mellitus, impaired glucose tolerance and fasting glucose levels, or insulin resistance (WHO, 1999). Obesity is a chronic, multifactorial disease resulting from the interaction between genotype, environment and physical activity patterns (Marti et al., 2008). A significant percentage of the obese population suffers from obstructive sleep apnea (OSA) (McCallister et al., 2009). It has been speculated that prolonged CIH simulating OSA might contribute to metabolic syndrome in individuals with OSA. In animal models, typical CIH protocols (40 sec 6% O_2, 40 sec normoxia, 8 h/day, 35 days) elicit profound effects on metabolic hormones, increase sympathetic activation (Zoccal et al., 2007), systemic inflammation (Arnardottir et al., 2009) and levels of the appetite stimulant neuropeptide Y (Tasali and Van Cauter, 2002), as well as elicit pancreatic beta-cell injury (Drager et al., 2010). Collectively, these changes increase food intake, obesity, hypertension and insulin resistance (Rasche et al., 2010).

In contrast, more moderate IH protocols have beneficial effects on metabolism, including body weight, cholesterol and blood sugar levels, and insulin sensitivity. For example, IH exposure with/without physical training (IH-T) consisting of physical activity (20 min strength–resistance exercises and 30 min high-intensity aerobic exercises) under hypoxic conditions (10-12% O_2, 3 times per week, 3-6 weeks) has been proposed as an efficient way to lose weight and increase aerobic capacity (Urdampilleta et al., 2012) without detrimental effects associated with prolonged CIH exposure. This method induces physiological adaptations and enhancement of athletic performance (Wilber, 2007) not observed in normoxic conditions (Roels et al., 2007).

Mechanisms of moderate IH-induced bodyweight loss may include increased serotonin and leptin levels. Acute hypoxia, in both humans and rats, increases blood serotonin levels (Gonzales, 1980). Food intake, protein intake, carbohydrate selection, and body weight are all at least partially regulated by serotonin, a molecule that produces anorexia in rats (Gonzales, 1980). Moderate IH can also reduce bodyweight by increasing blood leptin concentrations and enhancing liver leptin expression (Ling et al., 2008). Leptin is a peptide hormone secreted primarily by white adipose tissue, acting on the hypothalamic

metabolic control center to reduce energy intake, increase energy consumption and reduce body fat composition (Morton et al., 2006). Differentiated human PAZ6 adipocytes cultured for 48 hr in 6% oxygen increase leptin mRNA expression, leptin promoter activity and leptin secretion by 2-3 fold (Grosfeld et al., 2002). Interestingly, hypoxia (8% O_2) for 3h does not significantly alter *in vivo* leptin gene expression in rat adipose tissue; however, increased leptin mRNA expression was observed in liver, kidney and lung tissue (Meissner et al., 2005). Leptin is involved in body weight regulation and plays key roles in inflammation, immunity, tissue repair and angiogenesis; thus, leptin up-regulation during hypoxia in peripheral organs may also play an important role in tissue repair (Fantuzzi and Faggioni, 2000).

Moderate hypoxia (14.6% inspired O_2) reduces blood glucose and cholesterol levels (Chiu et al., 2004, Ling et al., 2008), and increases insulin sensitivity in type 2 diabetic subjects (Mackenzie et al., 2011). Hypoxia also increases mitochondrial enzymatic activity, glycolysis and fatty acid oxidation, but reduces cholesterol synthesis (Takahashi et al., 1996, Ling et al., 2008). Prolonged moderate intermittent hypoxia (12 hr, 14% O_2, 7days/week, 4 weeks), with or without training, improves glucose tolerance and increases glucose transporter GLUT-4 levels in rats (Chiu et al., 2004). Hypoxia stimulates glucose disposal, independent of contractile activity in rodents (Cartee et al., 1991, Chiu et al., 2004), isolated human muscle tissue (Azevedo et al., 1995), and type 2 diabetic patients (Mackenzie et al., 2011). This latter work also demonstrated that the effects of exercise (60 min at 90% of lactate threshold) on glucose disposal and insulin sensitivity are enhanced by moderate hypoxia (Mackenzie et al., 2011), suggesting that insulin signaling and insulin-dependent glucose transport are up-regulated following post-hypoxic exercise (Chiu et al., 2004).

Although the therapeutic IH effects on metabolic diseases has not been fully explored, accumulating evidence suggests possible value of moderate intermittent hypoxia combined with exercise to prevent or correct metabolic defects associated with obesity, insulin resistance and type 2 diabetes.

Bone mineral density

Intermittent hypoxia is reported to have positive effects on the physiological remodeling of bone tissue (Litovka, 2008, Guner et al., 2013). Rats exposed to IH (10 min, 13% O_2 interspersed with 10 min normoxia, 4 h/day, 28 days) increased alkaline phosphatase activity in bone tissue (Litovka, 2008), suggesting high osteoblast activity and new bone formation. Moreover, rats exposed to hypobaric hypoxia (430 mmHg ~ 34 mmHg PaO_2; 5 h/day, 5 days/week, 5 weeks) show greater bone mineral density versus control rats (Guner et al., 2013). This effect may result from high levels of nitric oxide (NO) observed in IH-exposed rats since greater bone mineral density was not observed in rats treated with an NO synthase inhibitor. High NO levels inhibit bone resorption by inhibiting osteoclast formation and resorptive function of mature osteoclasts (Ralston et al., 1995).

Thus, moderate IH protocols may restrain osteoclastic activity and/or stimulate osteoblastic activity, although the mechanisms of such effects remain unknown. Further studies are needed to assess the therapeutic potential of IH in osteopenia and osteoporosis.

Learning and Memory

OSA causes neurocognitive and behavioral deficits (Decary et al., 2000). Similarly CIH protocols that simulate aspects of OSA cause multiple cognitive deficits in rodents. For instance, 14 days of CIH (90 sec episodes of 10% inspired O_2/normoxia, 12 hr/day) during the habitual sleep times of adult male rats reduced REM sleep and impaired a hippocampus-dependent learning task (Gozal et al., 2001). Both rats and mice display cognitive deficits consistent with impaired hippocampal and/or prefrontal cortex function after CIH (Tagaito et al., 2001, Douglas et al., 2007, Row et al., 2007, Perry et al., 2008).

CIH also triggers hypersomnolence, a typical clinical complaint among OSA patients. CIH during the sleep cycle for 8 consecutive weeks hinders maintenance of wakefulness in mice, suggesting that CIH per se an underlying contributor to the excessive daytime sleepiness observed in OSA patients (Veasey et al., 2004). In humans, a randomized crossover design demonstrated that IH (1 min of 6% inspired O_2 interspersed with 1 min normoxia, 6h/day) negatively impacts spatial working memory in healthy young

adults (Champod et al., 2013). Further, high cycle frequency versus the overall duration of CIH exposure has a greater impact on learning and memory.

CIH causes increased neuronal apoptosis, and cytoarchitectural disorganization in brain regions involved in learning and memory, such as the hippocampal CA1 sub-field and the fronto-parietal cortex (Gozal et al., 2001). Increased apoptotic activity peaks after 48 hr of CIH, and then slowly decreases thereafter, albeit remaining above control levels of apoptosis (Gozal et al., 2001). CIH reduces the density of N-Methyl-d-Aspartate (NMDA) receptors and the excitability of hippocampal CA1 neurons (Pichiule et al., 1996), and, thus, diminishes the ability of hippocampal neurons to sustain hippocampal synaptic plasticity, such as long-term potentiation, the NMDA-dependent correlate of memory formation (Payne et al., 2004). In contrast, more moderate IH protocols do not elicit similar central nervous system (CNS) pathology. For example, rats exposed to 10, 5-min episodes of 10.5% inspired O_2 (5 min normoxic intervals) either daily for 7 days, or 3 times per week for 10 weeks, show no signs of hippocampal apoptosis, astrogliosis, or systemic hypertension (Wilkerson and Mitchell, 2009, Lovett-Barr et al., 2012, Satriotomo et al., 2012).

The deleterious effect of CIH may be more pronounced during development. Early life CIH is associated with anomalous brain development (Ment et al., 1998, Scheepens et al., 2003), and clinical conditions such as schizophrenia (Dalman et al., 2001, Rehn et al., 2004), cerebral palsy and mental retardation (Lai and Yang, 2011). However, moderate hypoxia in early life may also increase brain development, leading to increased learning capacity (Zhang et al., 2005, Shao et al., 2006, Lu et al., 2009, Martin et al., 2010). Increased learning capacity has been studied in animal models, expressed as increased development of conditioned reflexes. Neonatal IH exposure in mice (10.8% O_2, 4 hr/day from birth to 4 weeks of age) enhances performance in Morris water maze and 8-arm radial maze tasks (Zhang et al., 2005). A similar protocol with a milder hypoxic severity (16 % O_2) enhanced spatial learning and memory in developing mice (Lu et al., 2009). This increased learning capacity is associated with increased brain DNA concentrations, increased neurogenesis (Martin et al., 2010) and increased expression of proteins involved in synaptic plasticity (Lu et al., 2009).

The discrepancy among studies seems to be related to differences in the severity of the hypoxic stimuli, as well as the frequency of hypoxic episodes. Studies used to mimic aspects of OSA are not expected to have therapeutic benefits because of attendant CNS pathology. In contrast, moderate IH protocols may enhance learning capacity in developing rodents. More moderate repetitive IH protocols (3 times/week, 10 weeks) appear to be safe, offering the possibility that this paradigm may be beneficial in multiple clinical conditions.

Brain ischemia

Preconditioning IH protocols are neuroprotective for subsequent ischemic injury (Dirnagl et al., 2009, Stowe et al., 2011). For example, IH preconditioning (8% inspired O_2, 4 hr/day, 2 weeks) reduces the size of infarction, inflammation, and increased blood–brain barrier permeability after 60-min of transient middle cerebral artery occlusion (MCAO) in mice (Stowe et al., 2011). Changes in gene expression differ markedly between harmful ischemia and ischemic preconditioning. Preconditioning seems to attenuate the response to ischemia, increasing the expression of genes involved in the suppression of metabolic pathways, immune responses, ion-channel activity and blood coagulation (Dirnagl et al., 2009).

Some have suggested that IH has therapeutic potential for chronic cerebral ischemia. Brain ischemia is characterized by reduced brain-derived neurotrophic factor (BDNF), diminished synapse formation and impairments in learning and memory in rodents (Lee et al., 2004, Enright et al., 2007, Tsai et al., 2011). Seven days post-MCAO in rats, moderate IH (12% O_2, 4 hr/day, 7 days beginning 7 days post MCAO) rescues ischemia-induced spatial learning and memory impairment by inducing hippocampal neurogenesis, synaptogenesis and BDNF expression (Tsai et al., 2013). Moderate IH also reduces infarct size without increasing mortality rate versus normoxic controls. In contrast, the same IH protocol administered 1-2 days post-ischemia increases mortality rate (Tsai et al., 2008), suggesting that IH may have therapeutic potential for chronic, not acute brain ischemia.

Although moderate IH may reduce complications associated with chronic brain ischemia in rats, studies are required to support IH as a potential therapy for cerebral ischemia in humans. If verified as

safe and effective, repeated IH exposures may confer long-term brain protection in subpopulations of individuals at identified risk for stroke.

Depression

Many depressed patients show either partial or no response to antidepressants (Fava, 2001). Thus, effective methods of prophylaxis and treatment for human depression are crucial. Preconditioning with mild IH has a preventive/therapeutic effect in rodent models of depression (Rybnikova et al., 2007, Rybnikova et al., 2008). IH preconditioning (10% O_2, 2 hr/day, 3 days) has an antidepressant effect in the learned helplessness model of depression, returning behavioral and hormonal parameters to control values. This effect was not significantly different from rats receiving standard antidepressant drugs (Rybnikova et al., 2007, Rybnikova et al., 2008). IH also exerts therapeutic benefits with ongoing depression in rodent models and in humans. Given the strong link between hippocampal neurogenesis and antidepressant activity (Santarelli et al., 2003, Airan et al., 2007), and observations that IH promotes neurogenesis *in vitro* (Jin et al., 2002) and enhances neuroprogenitor cell proliferation *in vivo* (Zhu et al., 2005), IH may oppose depression by increasing new neuron formation.

Moderate IH (84 mmHg PaO_2, 4hr/day, 14 days) produces antidepressant-like effects in multiple animal models screening for antidepressant activity, including the forced swimming test, chronic mild stress paradigm, and novelty-suppressed feeding test (Zhu et al., 2010). The latter study showed enhanced cell proliferation and BDNF expression in the hippocampus, an effect that requires BDNF–TrkB signaling. Thus, neurogenic and antidepressant-like effects of IH may involve BDNF.

Although evidence demonstrating therapeutic benefits from IH in depression in humans is not yet conclusive, accumulating evidence supports the concept that moderate IH protocols may have protective and therapeutic benefits in depression. For example, 5 min of 10% inspired O_2 interspersed with 5 min normoxic intervals for 120 min/day reduced symptoms of depression in 71% of human patients after 4 weeks of treatment (Basovich, 2010). Additional studies are critical to understand the therapeutic potential of IH in cognitive and mental disorders.

Cardiovascular system

The clinical use of intermittent hypoxic training (IHT) was recognized by Russian physicians as a therapeutic modality to prime patients for the stress of disease processes. The rationale was based on the cross-protective value of adaptations to one stress providing resistance to another stress (Meerson et al., 1994). Thereafter, IHT became globally recognized by the sports medicine community as a useful strategy to enhance aerobic exercise performance in athletes (Fulco et al., 2000). For example, IH (2.5 min of 10.5% O_2, 1.5 min intervals, 4hr), in combination with low-intensity exercise, improves blood oxygen transport capacity and aerobic endurance, and induces altitude acclimation (Knaupp et al., 1992, Rodriguez et al., 1999, Casas et al., 2000, Koistinen et al., 2000). In this case, "living high" and "training low" promote hematological adaptations that improve aerobic performance without eliciting the adverse effects characteristic of CIH.

Despite potential beneficial effects of IHT, association of hypertension and heart disease with OSA fostered considerable interest in links between OSA and cardiovascular disease. Accordingly, severe and prolonged protocols of IH that more closely simulate OSA were developed. Such CIH protocols significantly increase blood pressure (Fletcher et al., 1992, Brooks et al., 1997, Lesske et al., 1997, Tahawi et al., 2001), increase right ventricular heart mass (McGuire and Bradford, 1999), and are associated with pulmonary vascular remodeling and hypertension (Nattie et al., 1978). Nonetheless, more moderate IH protocols elicit beneficial cardiovascular effects in animal models and humans, suggesting a dose-response relationship between aspects of the IH protocol and its physiological impact.

Arterial Hypertension

During hypoxic episodes, chemoreceptor-mediated sympathetic activity increases heart rate, cardiac output, peripheral resistance and systemic arterial pressure. However, different protocols of prolonged intermittent hypoxia produce divergent effects on systemic arterial blood pressure post-IH. The hypertensive effects of severe IH (mimicking OSA) versus depressor effects of more modest IH exemplify this divergence. OSA imposes a series of brief, intense hypoxic episodes leading to persistent,

maladaptive chemoreflex-mediated activation of the sympathetic nervous system, culminating in hypertension (Lavie et al., 2000, Pepperell et al., 2002, Prabhakar et al., 2005). Conversely, accumulating evidence in animal models and humans demonstrates that moderate IH conditioning is safe and effective as a means of prevention and treatment for systemic hypertension (Serebrovskaya et al., 2008, Shatilo et al., 2008).

CIH in humans and rodents elevate blood pressure, and this effect outlasts the period of hypoxic exposure (White et al., 1995). Studies in rodents demonstrate that the dose of IH protocol has a great impact on the magnitude of change in systemic blood pressure, suggesting a (crude) dose-response relationship. Severe protocols of chronic intermittent hypoxia (CIH: 60-120 episodes/hr., 2-5% inspired oxygen, 14-35 consecutive days) increase mean arterial pressure (MAP) by 9-16 mmHg (Fletcher et al., 1992, Brooks et al., 1997, Lesske et al., 1997, Tahawi et al., 2001). Moderate CIH protocols (15-20 episodes/hr., 6-10% inspired oxygen, 14-70 consecutive days) still increase MAP by 12 mmHg (Marcus et al., 1977); however, yet milder protocols (10 episodes/hr., 10% inspired oxygen, 7 consecutive days) increase MAP less than 2 mmHg in female rats (Hinojosa-Laborde and Mifflin, 2005). The greatest increases in blood pressure were observed in studies where hypocapnia was prevented via inspired carbon dioxide supplementation. To mimic the episodic asphyxiations imposed by OSA, McGuire and Bradford used combined hypoxia (6-8%) and hypercapnia (12-14%) for 15 sec, interspersed with 15s normoxia/normocapnia 8h/day, 5d/week for 5 weeks. This protocol increased diurnal mean systemic and pulmonary arterial pressures by 17 mmHg (McGuire and Bradford, 2001). In young, healthy humans, CIH (13% inspired oxygen, 30 episodes/hr, 9h/day, 14 consecutive days) increases the short-term hypoxic ventilatory responses, blood hemoglobin concentration and daytime blood pressure (Tamisier et al., 2011).

Chronic intermittent, but not sustained hypoxia (Fletcher et al., 1992), persistently activates the sympathetic nervous system (Smith et al., 1996, Sica et al., 2000, Fletcher, 2001, Leuenberger et al., 2005, Prabhakar et al., 2005) and the renin-angiotensin system (Fletcher et al., 1999, Foster et al., 2010), culminating in increased blood pressure during apneic events and post-IH wakefulness. Denervation of

carotid body chemoreceptors prevents development of systemic hypertension with CIH in rats, demonstrating that either chemoreceptor or CNS chemoreflex plasticity underlies this response (Fletcher et al., 1992).

The therapeutic potential of IH to treat hypertension has been studied under hypobaric and normobaric conditions. Treatment sessions (30 min to 2–3 h/d for 10–30 days) at simulated altitudes of 1500–3500 m (13-17% inspired oxygen) caused a significant decrease in arterial pressure in 60% of hypertensive patients (Rafibekova et al., 1987). However, hypobaric hypoxic protocols are poorly tolerated in humans (e.g. acute mountain sickness) (Johnson et al., 2001). Some unwanted side-effects of hypobaric hypoxia include headache, pain in the chest associated with an insufficient supply of blood to the heart, palpitations and dizziness (Farinelli et al., 1994). A more practical means of producing IH is normobaric hypoxia by increasing the inspired oxygen fraction. Similar to hypobaric hypoxia, normobaric IH normalizes blood pressure in hypertensive patients (Serebrovskaya et al., 2008, Shatilo et al., 2008). For example, a moderate IH program (10, 5 min cycles/day, 10–14% inspired O_2, 5 min normoxic intervals) administered to 56 patients with stages I–II hypertension reduced both systolic and diastolic BP, heart rate and peripheral resistance (Mukharliamov et al., 2006). Furthermore, IH proved useful and safe in elderly patients, leading to reduction in clinical symptoms of angina, normalization of lipid metabolism, optimization of oxygen consumption and normalization of microcirculation and increased exercise tolerance (Korkushko et al., 2010).

The anti-hypertensive effects of moderate IH may arise from increased endothelial NO production (Cohen et al., 1999, Giles, 2006, Manukhina et al., 2011), provoking vasodilation and opening reserve capillaries (i.e. decreased peripheral resistance), reduced sympathetic nervous activity (Melin et al., 2003, Pshennikova et al., 2005), prevention of calcium overload of vascular smooth muscle (Cohen et al., 1999), improved water and salt metabolism (Behm et al., 1984), increased antioxidant enzyme activity (Asha Devi et al., 2005), and increased synthesis of angiogenic growth factors VEGF and FGF (Wang et al., 2007). Finally, moderate IH augments parasympathetic activity, mimicking acclimatization to altitude, in which parasympathetic activity is enhanced (Reeves, 1993, Hughson et al., 1994).

Although CIH elicits persistent hypertension similar to OSA (Foster et al., 2007), moderate IH protocols reduce blood pressure in hypertensive animals and humans (Serebrovskaya et al., 2008). A major reason for this divergence is that the cardiovascular response to hypoxia directly depends on the "dose" of hypoxic stimuli. Protocols inducing systemic hypertension generally employed brief (6-30sec intervals), severe (3-9% O_2) and prolonged (6-12 h/day) hypoxic exposures (Fletcher et al., 1992, Tahawi et al., 2001, Phillips et al., 2004). In contrast, moderate IH regimens, often involving longer hypoxic episodes (45 sec to several hours), less severe hypoxia (10–12% O_2) and shorter durations of exposure per day (1-2 hr/day) do not increase systemic blood pressure, and actually reduce blood pressure in spontaneously hypertensive rats (Serebrovskaya et al., 2008). Whereas "high dose" CIH ignites a crescendo of factors that activate the sympathetic nervous system (Chen et al., 2005, Leuenberger et al., 2005, Prabhakar et al., 2005), increase oxidative stress (Lavie, 2003, Troncoso Brindeiro et al., 2007), systemic inflammation (Arter et al., 2004, Ryan et al., 2005, Budhiraja et al., 2007, Selmi et al., 2007), and cause persistent systemic hypertension, moderate "low dose" protocols of IH minimally activate and/or even dampens these same factors. Such divergence in the impact of "IH" on physiological functions likely accounts for apparent discrepancies in the literature, and may suggest means of harnessing "low dose" IH for therapeutic benefit without invoking the comorbidities of CIH and OSA. Thus, IH represents a promising therapeutic modality to prevent and treat hypertension. However, details of the IH protocol must be adjusted to optimize benefits and minimize pathogenesis in each patient. The intensity, frequency and duration of intermittent hypoxia cycles are key determinants of its physiological impact. The absence of adverse side-effects often encountered with common anti-hypertensive drug therapies makes IH an interesting alternative therapeutic approach.

Myocardial infarction

Myocardial infarction is the major cause of cardiovascular morbidity and mortality, despite advances in drug therapy and interventional procedures (Rosamond et al., 2008). The heart has the capacity to adapt to stress, meaning that brief episodes of ischemia enhance myocardial tolerance to subsequent ischemic

incidents (Murry et al., 1986). For example, brief ischemia makes the heart more resistant to subsequent ischemic insults (Murry et al., 1986, Tomai et al., 1999). Myocardial ischemic tolerance can also be induced by IH preconditioning, which exerts cardio-protective effects. For instance, 24 hr after IH pretreatment (40 s of 10% O_2, 20 s normoxic intervals, 30 min) rats exhibit reduced myocardial infarct size after global ischemia-reperfusion (Beguin et al., 2005). In humans, Burtscher and colleagues reported that moderate IH (5 min, 10–14% O_2, 3 min normoxic intervals, 15 times/day, 3 weeks) increased peak oxygen consumption in elderly men (50-70 years old) with and without coronary artery disease (Burtscher et al., 2004). Moreover, during sub-maximal exercise (cycling at 1 watt/kg) heart rate, systolic blood pressure, blood lactate concentration, and perceived exertion were diminished after IH (Burtscher et al., 2004). Myocardial protection correlates with the ability of moderate IH (2 min of 10% O_2, 2 min normoxia, 30 min) to increase myocardial vascularity, coronary blood flow, cardiomyoglobin and increase antioxidant enzyme expression (Zhuang and Zhou, 1999).

Ischemic tolerance of the heart can also be induced by long-term intermittent hypobaric hypoxia (Asemu et al., 1999, Neckar et al., 2005, Xie et al., 2005). This form of cardio-protection persists longer than normobaric ischemic preconditioning (Bolli, 2000, Zhang et al., 2000, Cai et al., 2003, Neckar et al., 2004), and is associated with less adverse side-effects, such as right ventricular hypertrophy versus chronic sustained hypoxia (Irlbeck et al., 1997, Asemu et al., 1999, Pei et al., 2003, Xie et al., 2004). For example, rats exposed to intermittent hypobaric hypoxia (7000 m, 8 h/day, 35 days) exhibit significantly reduced infarction size and antiarrhythmic protection after 30-min coronary artery occlusion. Cardiac protection by hypobaric IH has been associated with several mechanisms, including preserved Ca2+ homeostasis (Chen et al., 2006), calcium/calmodulin-dependent protein kinase II activity regulation (Yu et al., 2009), reduced myocardial apoptosis (Dong et al., 2003), and induced opening of mitochondrial ATP-Sensitive Potassium (mitoKATP) channels (Neckar et al., 2002, Kolar et al., 2005).

Intermittent hypobaric hypoxia is not only protective, but also therapeutic in patients with acute myocardial infarction (Neckar et al., 2002, Ding et al., 2004, Chen et al., 2006, Guo et al., 2009, Wang et al., 2011). For instance, Wang et al. observed that intermittent hypobaric hypoxia (404 mmHg, PaO_2 ~84

mmHg, 6h/day, 14 days) improves post-ischemic recovery of myocardial contractile function by elevating reactive oxygen species production during early reperfusion in rats (Wang et al., 2011). Seven days after left anterior descending coronary artery ligation, rats exposed to a similar protocols showed significantly reduced left ventricular dilation and improved cardiac performance (Xu et al., 2011). This effect was accompanied by attenuated infarct size, increased coronary blood flow, capillary density and VEGF expression (Xu et al., 2011), and activation of genes increasing myocardial cell survival (Park et al., 2007).

Some doubts exist as to whether IH is a really a safe technique in post-myocardial infarction patients. IH stimulates erythropoiesis (Brugniaux et al., 2011, Martinez-Bello et al., 2011) and increased erythropoietin (EPO) concentrations enhance hematocrit, blood viscosity and platelet count (Brugniaux et al., 2011). Elevated hematocrit increases the risk of ischemic stroke and myocardial infarction (Lippi et al., 2007, Lippi and Franchini, 2010). However, several studies, have failed to demonstrate significant alterations in the erythropoietic response after different IH protocols (Truijens et al., 2003, Julian et al., 2004). For example, Neya and colleagues showed that IH at 3000 m (14% oxygen equivalent) was insufficient to enhance erythropoiesis (Neya et al., 2007), whereas exposure to greater simulated altitude (5000–6300 m; ~7-8% oxygen equivalent), increased several hematological parameters (Esteva et al., 2010, Vani et al., 2010), demonstrating a dose-response relationship between the severity of hypoxia and the level of erythropoiesis.

Despite the abundant literature supporting cardio-protective and therapeutic effects of IH in myocardial infarction, its clinical translation is still controversial, mainly due to the lack of suitable ischemic/reperfusion (I/R) models to adequately simulate what happens in human patients (Hausenloy et al., 2010). IH appears to be a promising therapeutic strategy for coronary heart disease due to its simplicity and long duration of action, with few adverse effects (Chen et al., 2006, Przyklenk and Whittaker, 2011, Xu et al., 2011). However, further studies are needed to determine the most effective dose of IH and the level of barometric pressure eliciting the optimum therapeutic response.

Intermittent hypoxia induced-respiratory plasticity

In humans and other animals, acute hypoxic exposure increases carotid chemoreceptor activity, stimulating increased in pulmonary ventilation. These are the classical negative feedback effects of hypoxia in respiratory control. We now know that this response varies considerably in different time domains, and that factors such as the pattern of hypoxia (intermittent versus sustained), the duration of exposure (minutes to days), and the severity of the hypoxic stimulus (Powell et al., 1998) explain the great scope of effects described in the literature. Also, poikilocapnic hypoxia (allowing $PaCO_2$ to fall during hyperventilation) masks the ventilatory response to hypoxia, and must be considered in any study of ventilation during or after IH (Bisgard and Neubauer, 1995).

Different IH protocols elicit different mechanisms of plasticity in the CNS (Turner and Mitchell, 1997, Baker and Mitchell, 2000, Mitchell et al., 2001a, Mitchell and Johnson, 2003). For example, moderate acute intermittent hypoxia elicits a long-lasting (hours), serotonin-dependent increase in respiratory motor output known as respiratory long-term facilitation (LTF) (Bach and Mitchell, 1996). LTF appears to be a unique property of intermittent hypoxia, since continuous hypoxia of the same cumulative duration does not elicit similar LTF (Baker and Mitchell, 2000). Exposure to brief IH (3-10 consecutive hypoxic episodes) elicits sustained elevations in phrenic nerve activity, hypoglossal nerve activity and ventilation in a variety of animal species (Cao et al., 1992, Hayashi et al., 1993, Fregosi and Mitchell, 1994, Bach and Mitchell, 1996, Mateika and Fregosi, 1997, Turner and Mitchell, 1997, Mitchell et al., 2001b, McKay et al., 2004).

Intermittent carotid sinus stimulation is sufficient to induce LTF (Hayashi et al., 1993, Fregosi and Mitchell, 1994). Thus, LTF is an expression of central neural rather than peripheral neuroplasticity. Indeed, carotid chemoafferent neuron activation is not essential for IH-induced phrenic LTF in anaesthetized rats (Bavis and Mitchell, 2003) since (functional) carotid denervation attenuates, but does not abolish AIH-induced pLTF. In humans, initial studies indicated that IH-induced LTF may not exist during wakefulness (Jordan et al., 2002, Mateika et al., 2004, Morelli et al., 2004). However, further studies revealed ventilatory and genioglossus LTF in sleeping humans (Pierchala et al., 2008, Chowdhuri

et al., 2010) or awake humans with modest elevations in baseline carbon dioxide (Harris et al., 2006, Wadhwa et al., 2008).

In longer time domains, or with more severe hypoxic episodes, additional forms of plasticity are elicited by IH (Mitchell and Johnson, 2003). For example, CIH (5 min episodes, 10–12% O_2, 8–12 h/night, 7 days) elicits serotonin-dependent enhancement of: 1) baseline phrenic nerve activity, 2) the short-term hypoxic phrenic response, and 3) phrenic LTF induced by acute intermittent hypoxia (Ling et al., 1999, Ling et al., 2001). CIH-enhanced LTF represents a form of metaplasticity (i.e., the ability of prior experience to alter subsequent plasticity) (Abraham and Bear, 1996, Mitchell and Johnson, 2003). CIH-induced metaplasticity represents a potential therapeutic advantage in restoring breathing capacity with clinical disorders that cause respiratory insufficiency (eg. spinal injury, ALS) (Mitchell, 2007). Unfortunately, CIH also elicits considerable morbidity, including hypertension, hippocampal apoptosis and cognitive deficits, among others (Lesske et al., 1997, Yan et al., 2009). More subtle protocols of repetitive acute intermittent hypoxia (rAIH) have been developed to elicit pLTF metaplasticity without the detrimental effects elicited by CIH. For example, rAIH (e.g. 10, 5 min episodes, 10.5% O_2, 5 min intervals, 3 times per week for 10 weeks; or daily for 7 days) increases the expression of key molecules involved in AIH-induced phrenic LTF without any signs of hippocampal apoptosis, astrogliosis or systemic hypertension (Wilkerson and Mitchell, 2009, Lovett-Barr et al., 2012, Satriotomo et al., 2012). Further, we demonstrated that rAIH elicits phrenic LTF metaplasticity, enhancing AIH-induced phrenic LTF when AIH is presented daily for 7 days (Wilkerson and Mitchell, 2009) or three times per week for 4 weeks (MacFarlane et al., 2010, Vinit et al., 2010). Thus, fewer hypoxic episodes per day (even with longer exposure durations) elicit pLTF metaplasticity without attendant pathology. Such protocols have considerable therapeutic potential following, for example, cervical spinal injury (Lovett-Barr et al., 2012, Tester et al., 2013).

Activation of different molecular pathways within phrenic motor nuclei underlies the differential physiological effects of different IH protocols. A hallmark of phrenic LTF is its sensitivity to the pattern of hypoxia; in specific, with moderate hypoxia, intermittent but not sustained hypoxia elicits the

underlying mechanism (Baker-Herman and Mitchell, 2008). One key difference between intermittent and sustained hypoxia appears to be the level of ROS-dependent inhibition of okadaic acid sensitive, serine threonine protein phosphatases (Wilkerson et al., 2007, MacFarlane and Mitchell, 2008, Wilkerson et al., 2008). When ROS are scavenged (MacFarlane and Mitchell, 2008) or their production is blocked (MacFarlane et al., 2009), the phrenic LTF is blocked following moderate AIH. However, by inhibiting spinal protein phosphatases with okadaic acid after reducing ROS formation, phrenic LTF is restored (MacFarlane and Mitchell, 2008). On the other hand, with moderate sustained hypoxia, serotonin-dependent phrenic LTF is revealed following okadaic acid, suggesting that less ROS-dependent inhibition of the relevant phosphatase(s) occurs with sustained hypoxia (Wilkerson et al., 2008). In agreement with this hypothesis, spinal okadaic acid has no effect on phrenic LTF following moderate AIH, suggesting that the relevant phosphatases have already been inhibited by some process unique to intermittent, but not sustained hypoxia (Wilkerson et al., 2007).

The severity of hypoxic stimuli within episodes is a determinant of the specific cellular mechanisms giving rise to phrenic LTF (Nichols et al., 2012). For example, moderate AIH (3, 5-min hypoxic episodes; PaO_2 35–45 mmHg; 5-min intervals) elicits phrenic LTF by a mechanism that requires spinal serotonin type 2 (5-HT2) receptor activation (Bach and Mitchell, 1996, Fuller et al., 2001, Baker-Herman and Mitchell, 2002), new synthesis of brain-derived neurotrophic factor (BDNF) (Baker-Herman et al., 2004) and activation of its high-affinity receptor tyrosine kinase (TrkB) (Baker-Herman et al., 2004, Dale et al., 2013) followed by ERK MAP kinase signaling (Hoffman et al., 2012)(Fig.3). In contrast, a severe protocol of AIH (less than 30 mmHg O2) elicit pLTF by a distinct serotonin-independent cellular mechanism that requires spinal adenosine 2A receptor activation (Nichols et al., 2012). Spinal adenosine 2A (Golder et al., 2008) and 5-HT7 receptor activation (Hoffman and Mitchell, 2011) elicit phrenic motor facilitation by a mechanism that requires new synthesis of an immature TrkB isoform (not BDNF) and downstream signaling via phosphatidylinositol (PI) 3-kinase/protein kinase B (Akt) (not ERK) (Golder et al., 2008). In longer time domains (days) vascular endothelial growth factor (VEGF) or erythropoietin (EPO)-induced phrenic motor facilitation might play a role in longer time domains of IH, such as

during/after CIH or even chronic sustained hypoxia (Dale et al., 2013). Spinal VEGF or EPO receptor activation triggers phrenic motor facilitation via mechanisms that require both ERK and Akt activation (Dale-Nagle et al., 2011, Dale et al., 2012).

Another contributing factor to the pattern-sensitivity of phrenic LTF appears to be balanced cross-talk inhibition between the competing cellular cascades to phrenic motor facilitation described above (Devinney et al., 2013). With moderate AIH, phrenic LTF occurs predominantly via the serotonin/BDNF/ERK dependent Q pathway to phrenic motor facilitation (Dale-Nagle et al., 2010). However, sub-threshold activation of the adenosine/TrkB/Akt dependent S pathway restrains pLTF in this condition via PKA-dependent cross talk inhibition (Hoffman et al., 2010, Hoffman and Mitchell, 2013). At some level of hypoxemia, extracellular ATP/adenosine builds up enough to convert the system from serotonin-dependent (Q) to adenosine-dependent (S) facilitation (Nichols et al., 2012). With moderate, sustained hypoxia, there appears to be sufficient balance between the serotonin- (Q) and adenosine-dependent (S) pathway activation that they cancel each other via cross-talk inhibition (Devinney et al., 2013). The strongest evidence for this is that spinal pretreatment with an adenosine 2A receptor antagonist reveals serotonin-dependent phrenic LTF following moderate sustained hypoxia (Devinney et al., 2013). Thus, there appear to be a number of factors contributing to the hallmark pattern-sensitivity of phrenic LTF. The essential point from the perspective of this review is that these distinctions in the pattern and severity of hypoxia protocols are essential if we are to control the specific physiological outcomes for therapeutic benefit.

Collective evidence strongly suggests that moderate repetitive exposures to acute intermittent hypoxia may be harnessed as a therapeutic approach to restore lost respiratory motor output in clinical disorders that lead to respiratory insufficiency such as amyotrophic lateral sclerosis (Nichols et al., 2013), spinal cord injury (Lovett-Barr et al., 2012) or OSA (Mitchell, 2007), or that compromise breathing due to mechanical constraints such as chronic obstructive pulmonary disease.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a degenerative motor neuron disease, with associated death of respiratory motor neurons (Zinman and Cudkowicz, 2011). Patients with ALS invariably develop respiratory muscle weakness, and the most common cause of death is ventilatory failure (Lechtzin et al., 2002). Transgenic rats over-expressing a mutated form of superoxide dismutase ($SOD1^{G93A}$) have been studied extensively as an animal model of familial ALS. $SOD1^{G93A}$ mutants exhibit progressive motor neuron death, and faithfully mimic many important aspects of familial ALS in humans, including compromise of phrenic motor output (Llado et al., 2006, Nashold et al., 2006). Interestingly, although more than 60-80% of all phrenic motor neurons die at disease end-stage, phrenic motor output only decreases between 40-50% (Nichols et al., 2013), reflecting the onset of ventilatory failure. Nevertheless, despite major losses of intercostal motor neurons, the ability to increase tidal volume during maximal chemoreceptor stimulation is fully preserved at the same disease end-stage (Dale et al., 2006). Thus there is considerable intrinsic capacity to compensate for major losses of key respiratory motor neurons, preserving the capacity to generate tidal volume until late in disease progression. The mechanisms underlying such spontaneous compensation are not yet known, but may reflect forms of plasticity similar to pLTF since surviving phrenic motor neurons at disease end-stage express high levels of BDNF and TrkB protein (Satriotomo et al., 2006). Nevertheless, respiratory motor neuron death eventually overcomes the capacity for spontaneous compensation, leading to overt ventilatory failure (Tankersley et al., 2007).

We recently tested the hypothesis that IH-induced respiratory motor plasticity strengthens synaptic inputs to surviving motor neurons, thereby enhancing respiratory motor output and slowing progression to ventilatory failure in ALS. Indeed, at disease end stage, a single presentation of AIH (3, 5 min episodes, PaO_2 35-45 mmHg, 5 min intervals) fully restores the capacity to increase phrenic motor output in anesthetized rats at disease end stage (Nichols et al., 2013). Further studies are needed to understand the potential of repetitive AIH to preserve ventilatory capacity further into disease progression, hopefully improving the quality of life for patients with this devastating disease.

Spinal Cord Injury

Respiratory complications are the leading causes of morbidity and mortality in patients with spinal cord injury (SCI), especially among cervical and higher-thoracic injuries (NSCISC, 2005). There are few therapeutic options available after the acute, post-injury period, when mechanisms of spontaneous recovery are exhausted and additional functional improvement is unlikely (McDonald et al., 2002). Recent work in rodent models has demonstrated that repetitive AIH is a possible therapeutic approach to restore breathing capacity after cervical spinal hemisections (Lovett-Barr et al., 2012).

Cervical spinal hemisection at C2 (C2HS) causes persistent deficits in the capacity to increase phrenic motor output (Golder et al., 2003) and tidal volume in rats (Fuller et al., 2006, Lovett-Barr et al., 2012). In rats with C2HS, even a single presentation of AIH strengthens spinal synaptic pathways to phrenic motor neurons below the hemisection by activating serotonin-dependent neuroplasticity (Golder and Mitchell, 2005, Fuller et al., 2006). However, the capacity of AIH to induce crossed-spinal synaptic pathways to phrenic motor neurons below the injury is highly dependent on time post-injury. For example, following C2HS, AIH (3 episodes 5 min 11% O_2 interspersed with 5 min normoxia) induces ipsilateral phrenic long-term facilitation (>60 min post-AIH) at 8 weeks post-injury, but not at 2 weeks post-injury in Sprague–Dawley and Lewis rats (Golder and Mitchell, 2005). This increasing ability with time post-injury correlates with spontaneous restoration of serotonergic input to the phrenic motor nucleus below the injury (Golder and Mitchell, 2005). Thus, IH may be more effective at restoring respiratory function in patients with chronic (versus acute) spinal injury, once descending serotonergic innervation has had sufficient time to recover below the injury. Further, in rats 2 weeks post-C2HS, repeated AIH (10, 5 min episodes per day, 10.5% O_2, 5 min intervals, 7 consecutive days beginning 7 days post-C2HS): 1) increases the strength of crossed-spinal synaptic inputs to phrenic motor neurons; and 2) at least partially restores the capacity to increase tidal volume during (Lovett-Barr et al., 2012). This functional recovery is accompanied by an increased expression of key proteins necessary for AIH-induced spinal plasticity in the phrenic motor nucleus (Lovett-Barr et al., 2012). There was no evidence

for hippocampal cell death or reactive gliosis in this latter study. Detailed mechanisms of rAIH-induced functional recovery remain to be explored.

A more aggressive CIH protocol (72, 5 min episodes per day, 10.5% O_2, 5 min intervals, 7 consecutive nights, beginning 7 days post-injury) also induces functional recovery of phrenic motor output and strengthens crossed spinal synaptic inputs to phrenic motor neurons below the injury (Fuller et al., 2003). However, as noted previously, CIH is expected to also elicit morbidity such as systemic hypertension, CNS inflammation and neuronal death. Since the less severe repetitive AIH elicits similar functional recovery without apparent morbidity (Satriotomo et al., 2012), such IH protocols have greater clinical potential.

IH and limb function after spinal injury

Repetitive AIH has also been shown to elicit sustained functional recovery of forelimb function in rats with C2HS (Lovett-Barr et al., 2012). This functional improvement is accompanied by increased BDNF and TrkB levels within cervical (C_7) motor nuclei innervating the forelimb (Lovett-Barr et al., 2012). Although the detailed mechanisms of this functional recovery have not been verified, we have suggested that the same serotonin-dependent mechanisms facilitate motor output in respiratory and non-respiratory motor nuclei (Dale et al., 2013).

The use of IH to improve limb function in humans with incomplete, chronic SCI has shown promising results. A single presentation of IH (15, 1-minute episodes of 9% O_2 alternating with 1-minute of 21% O_2) in incomplete, chronic (>1 year) spinal cord injury patients (American Spinal Cord Injury Association Impairment Scale C or D) increases the ability to voluntarily generate plantar flexion 4 hr post-hypoxia (Trumbower et al., 2012). In a randomized, double-blind, placebo-controlled, crossover design study, the impact of repetitive AIH (15 episodes per day, 90 sec 9% O_2, 60 sec normoxic interval, 5 consecutive days) combined with walking training was studied in 19 chronic, incomplete SCI patients (AIS D) (Hayes et al., 2013). Daily AIH alone increased walking speed 18% three days after treatment (10 m walk test), whereas dAIH combined with walking training improved both walking speed and

distance (37%) after 5 days and 1 week post-dAIH (Hayes et al., 2013). Importantly, no changes in motor or cognitive function were observed after dAIH, suggesting that his moderate IH "dose" is safe in humans.

Sleep Apnea

Considerable progress has been made over the last several decades in our understanding of the pathophysiology of both central and obstructive sleep apnea. Central sleep apnea is generally the product of an unstable ventilatory control system with high hypercapnic responsiveness generally being the cause (Yumino and Bradley, 2008). On the other hand, obstructive sleep apnea (OSA) typically occurs in individuals whose narrow upper airways and the reduction in upper airway muscle activity during sleep causes airway collapse (Stradling and Davies, 2004). Although problems with maintenance of upper airway patency result primarily in obstructive sleep apnea, ventilatory control instability can lead to either central or obstructive apnea depending on the collapsibility of the individual airway.

Intermittent Hypoxia elicits multiple forms of respiratory plasticity, including: 1) ventilatory long term facilitation (McGuire et al., 2003, Gerst et al., 2011), 2) upper airway facilitation (Shkoukani et al., 2002, Chowdhuri et al., 2008, Ryan and Nolan, 2009), and 3) augmentation of the short-term hypoxic ventilatory response (Powell et al.., 1998).

The therapeutic potential of IH in sleep apnea is uncertain since IH has the potential for both stabilizing and destabilizing influences on breathing in anesthetized rats (Mahamed and Mitchell, 2008) and humans (Gerst et al., 2011). For instance, moderate protocols of IH (3 min FiO_2 8% alternating with 5 min of normoxia, 10 times) decreased upper airway resistance (Aboubakr et al., 2001, Shkoukani et al., 2002) in patients with OSA. Same protocol does not alter the critical closing pressure of the upper airway in patients with OSA (Rowley et al., 2007), which suggest that changes in upper airway resistance and caliber can be dissociated from changes in upper airway collapsibility. Repeated daily exposure to intermittent hypoxia (5 min 11–12% O_2 interspersed with 5 min normoxia, 12 h/night, 7days) enhances the magnitude of the hypoxic ventilatory response (Ling et al., 1999, Rey et al., 2004) which can elicit

both beneficial effect in UA patency and detrimental effects in breathing stability. Increases in the hypoxic ventilatory response (HVR) will increase UA dilating muscle activity, decreasing UA resistance (Badr et al., 1994). However, an enhanced HVR may increase the respiratory control system loop gain, which will cause destabilization of UA and respiration. Exaggerated HVR will produce an undershoot of arterial CO2 (Chowdhuri et al., 2010), leading subsequently to hypoventilation and concomitant reduction of UA dilator muscle activity, thereby increasing the likelihood of another collapse. Thus, a high HVR is thought by many to destabilize breathing in OSA. One the other hand, IH-induced ventilatory LTF might serve to promote breathing stability by ensuring that breathing is sustained despite fluctuating levels of carbon dioxide (Mahamed and Mitchell, 2007). In patients with moderate OSA, AIH-induced HVR is greater in the morning whereas vLTF is greater in the evening (Gerst et al., 2011). If exaggerated HVR is detrimental and LTF is beneficial, therapeutic applications of IH during the evening may elicit a greater therapeutic potential.

It is worth mentioning that until recently, both healthy and OSA subjects studied were poikilocapnic and the levels of CO2 were not controlled. Recent evidence shows that the magnitude of IH-induced ventilatory LTF is greater in individuals with sleep apnea in the presence of sustained hypercapnia (Lee et al., 2009, Syed et al., 2013). These findings may indicate that detrimental effects of IH may predominate under conditions in which carbon dioxide levels are not maintained. Recently, Mateika and colleagues propose that administration of moderate intermittent hypoxia and sustained hypercapnia with continuous positive airway pressure may have therapeutic effects in sleep apnea patients (Mateika and Syed, 2013). The application of sustained hypercapnia would ensure the manifestation of long-term facilitation of upper airway muscle activity (increasing the patency) as well as ventilatory LTF. Moreover, the number of apneas and persistent arousal would be eliminated with the application of sustained hypercapnia and positive air pressure. Although promising, additional work is required to determine the optimal intensity, duration and frequency of intermittent hypoxia that will initiate the greatest magnitude of ventilatory and upper airway muscle long-term facilitation without the destabilizing effects of IH in patients with sleep apnea.

Chronic Obstructive Pulmonary Disease (COPD)

COPD is defined as a chronic irreversible pulmonary disease, and includes emphysema, chronic bronchitis, and asthma (Rabe et al., 2007). Intermittent hypoxic training (IHT) may be useful as a therapy for patients suffering from COPD. For many years, Ukrainian and Russian researchers have been using IHT for treatment of diseases such as bronchial asthma and COPD, reporting a significant improvement in the clinical symptoms of COPD without unwanted side effects (Serebrovskaya et al., 2003).

Randomized, double-blind and controlled clinical trials have shown that mild intermittent normobaric IH (3-5 min of hypoxia, 12-15% O_2, 3-5 min normoxia intervals, 5-9 times/day for 15 days) elicit beneficial effects in COPD patients such as increased exercise time, baroreflex sensitivity, hypercapnic ventilatory response (HVR), total hemoglobin mass, forced expiratory volume in the first second (FEV1) and forced expiratory vital capacity (FVC) (Burtscher et al., 2009, Haider et al., 2009). However, longer hypoxic intervals in healthy subjects show no significant differences between IH-groups and controls. For example, normobaric hypoxia (3-10 hr of continuous hypoxia 12-15% O_2 for 7-20 consecutive days) show no difference in the hypoxic ventilatory response or ventilatory capacity among groups (Townsend et al., 2005, Katayama et al., 2009). Similarly, hypobaric intermittent hypoxia (4000, 5500 m, 3h/day, 5 days/week, 4 weeks) shows no difference among groups in terms of the hypoxic ventilatory response and cardiovascular changes (Gore et al., 2006, Fu et al., 2007). Evidence suggests that IH protocols using short hypoxic intervals (3-5 min) are more effective to increase ventilatory capacity than prolonged exposures to hypoxia (3-10 hr). Despite the scarce literature studying the effect of moderate IH in COPD, those studies provide promising results and raise hope that IH could be used in the future as a complementary therapy with few adverse side effects.

Conclusions

Intermittent Hypoxia has been a subject of considerable investigation from the viewpoint of its beneficial and adverse effects. Recent studies reveal that the effects of intermittent hypoxia on the body can greatly vary, depending on the dose of the hypoxic stimulus, including: 1) the severity of hypoxemia, 2) the duration of hypoxia, 3 the number of cycles/day, and 4) the total protocol duration. Accumulating evidence suggests that low-dose IH has considerable therapeutic potential to treat multiple clinical conditions (fig.1).

The problem with high-dose IH is the associated morbidity. For example, high-dose IH protocols (e.g. CIH: 2-8% $O_{2,}$ 40-2400 cycles/day) elicit detrimental effects in multiple systems, including systemic hypertension (Fletcher et al., 1992, Brooks et al., 1997, Lesske et al., 1997, Tahawi et al., 2001), hypercholesterolemia (Savransky et al., 2007), obesity, insulin resistance (Rasche et al., 2010), increased sympathetic activation (Zoccal et al., 2007), pulmonary hypertension (McGuire and Bradford, 2001), cognitive deficits (Gozal et al., 2001, Douglas et al., 2007, Champod et al., 2013), and inflammation (Arnardottir et al., 2009) (fig.2).

In contrast, low dose IH protocols, such as repetitive AIH ($\geq$ 9% $O_{2,}$ < 15 cycles/day) reduce arterial hypertension (Serebrovskaya et al., 2008), strengthen innate immune responses, reduce inflammation (Serebrovskaya et al., 2011), reduce body weight, increase aerobic capacity (Urdampilleta et al., 2012), improve glucose tolerance (Chiu et al., 2004), increase bone mineral density (Guner et al., 2013), enhance spatial learning and memory (Zhang et al., 2005, Lu et al., 2009), rescue ischemia-induced memory impairment (Tsai et al., 2011, Tsai et al., 2013), reduce symptoms of depression (Basovich, 2010), improve post-ischemic recovery of myocardial contractile function (Wang et al., 2011), increase respiratory capacity in COPD (Haider et al., 2009), increase respiratory and non-respiratory somatic motor recovery following spinal injuries in rats and humans (Vinit et al., 2009, Lovett-Barr et al., 2012, Trumbower et al., 2012, Hayes et al., 2013). Repetitive low-dose IH has these benefits without detectable adverse consequences such as hypertension (Wilkerson and Mitchell, 2009), neuronal cell loss and/or

reactive gliosis (Lovett-Barr et al., 2012, Satriotomo et al., 2012) or and systemic inflammation (Tam et al., 2007, Querido et al., 2012) (fig.2).

Detrimental effects induced by high-dose IH often relate to increased oxidative stress and systemic inflammation. Repetitive hypoxia/re-oxygenation is in some respects like repeated ischemia-reperfusion, and increases reactive oxygen species formation (Lavie, 2005). Increased ROS production will activate NF-κB and, hence, expression of NF-κB target genes such as pro-inflammatory cytokines (e.g. TNF-α, IL-6 and ICAM- 1) (Lavie, 2005). These inflammatory molecules lead to cellular damage, endothelial dysfunction and development of several morbidities. In contrast, modest IH protocols do not appear to cause inflammation in humans (Serebrovskaya et al., 2011), and may in fact strengthen the innate immune system while suppressing the production of pro-inflammatory mediators (Serebrovskaya et al., 2011). Thus, there are abundant reasons to suggest that low-dose IH will be simple, safe and effective in the treatment of multiple clinical disorders. To optimize IH as a therapeutic approach to clinical disorders, a balance must be achieved between the highest IH dose possible (i.e. maximize benefits) that is not associated with adverse consequences (i.e. minimize deleterious effects). It is also important to understand conditions that may undermine the therapeutic efficacy of IH. For example, systemic inflammation (commonly present in SCI patients) undermines hippocampal and spinal respiratory and non-respiratory motor plasticity (Hook et al., 2008, Vinit et al., 2011, Huxtable et al., 2013). The potential applications of IH in health and in various pathological states are numerous, since it represents a simple and safe, non-pharmacological method for enhancing physiological functions and rehabilitation in patients with diverse chronic diseases.

Figures

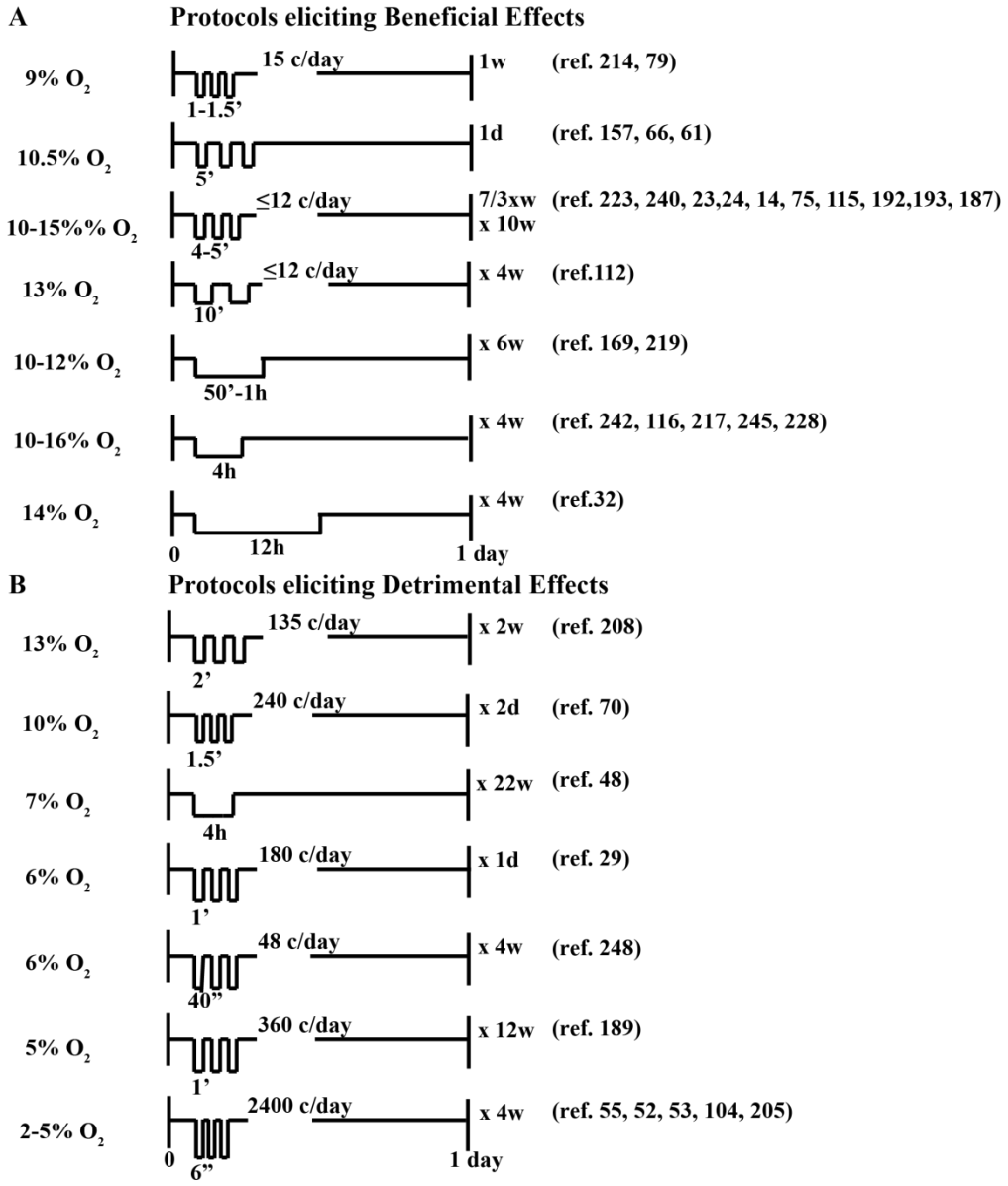

Figure 1. Representative protocols of intermittent hypoxia (IH) eliciting beneficial (A) and detrimental (B) effects in different clinical conditions. Each protocol depicts the dose of intermittent hypoxia (IH) including the severity of hypoxia (inspired oxygen, O_2), the duration of the hypoxic episode (from 6 seconds to 12 hours), the number of cycles per day (c/day) and the total time of exposure. Note that severe protocols (less than 9% O_2) and/or a high number cycles per day (>40 cycles/day) elicit

detrimental effects. In contrast, moderate protocols of IH using ≥9% O_2, and up to 15 cycles per day elicit therapeutic effects. The total time of exposure is not different between beneficial and detrimental protocols of IH.

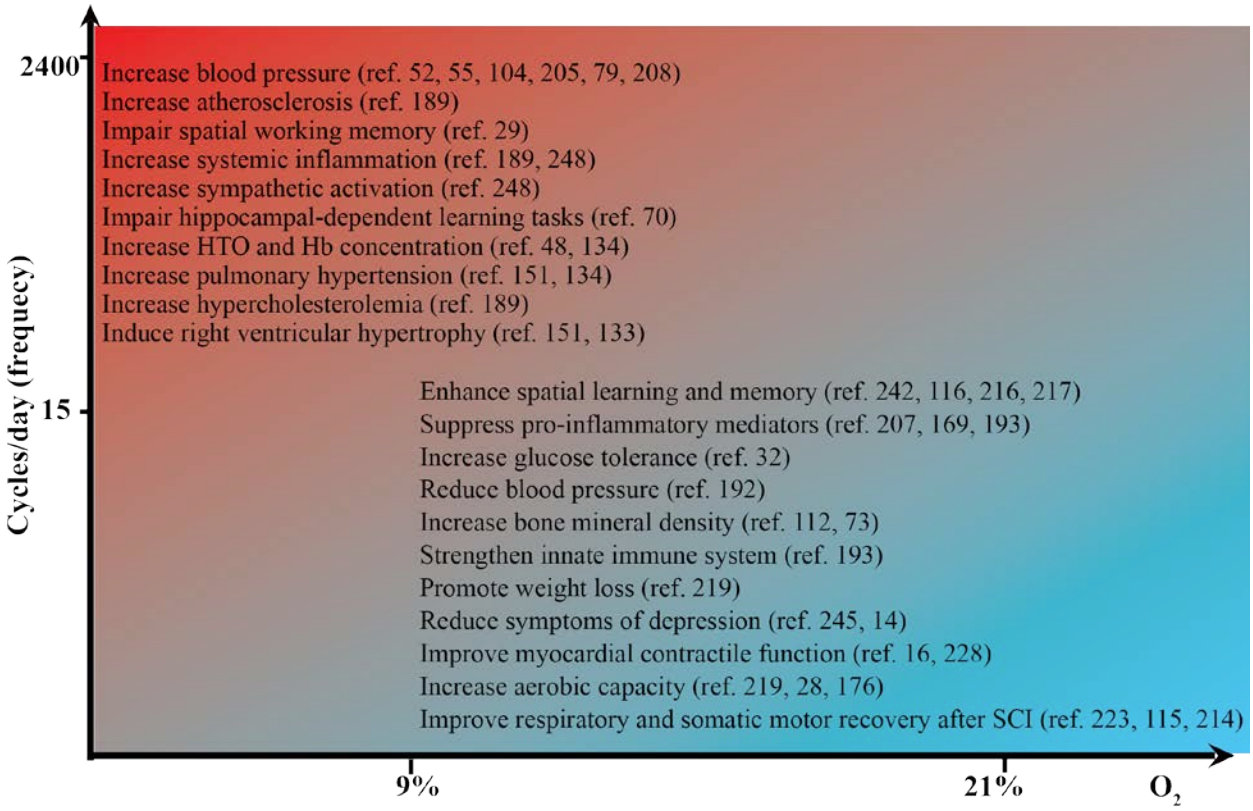

Figure 2. Schematic graph illustrating therapeutic (blue) and pathological (red) effects of intermittent hypoxia (IH). Protocols using severe hypoxia (less than 9% inspired oxygen, O2) and more than 15 cycles per day elicit pathological effects. Moderate protocols of IH using more than 9% O2 and up to 15 cycles per day elicit therapeutic effects. Evidence demonstrates that moderate protocols of IH are safe and have widely distributed beneficial effects among several clinical conditions.

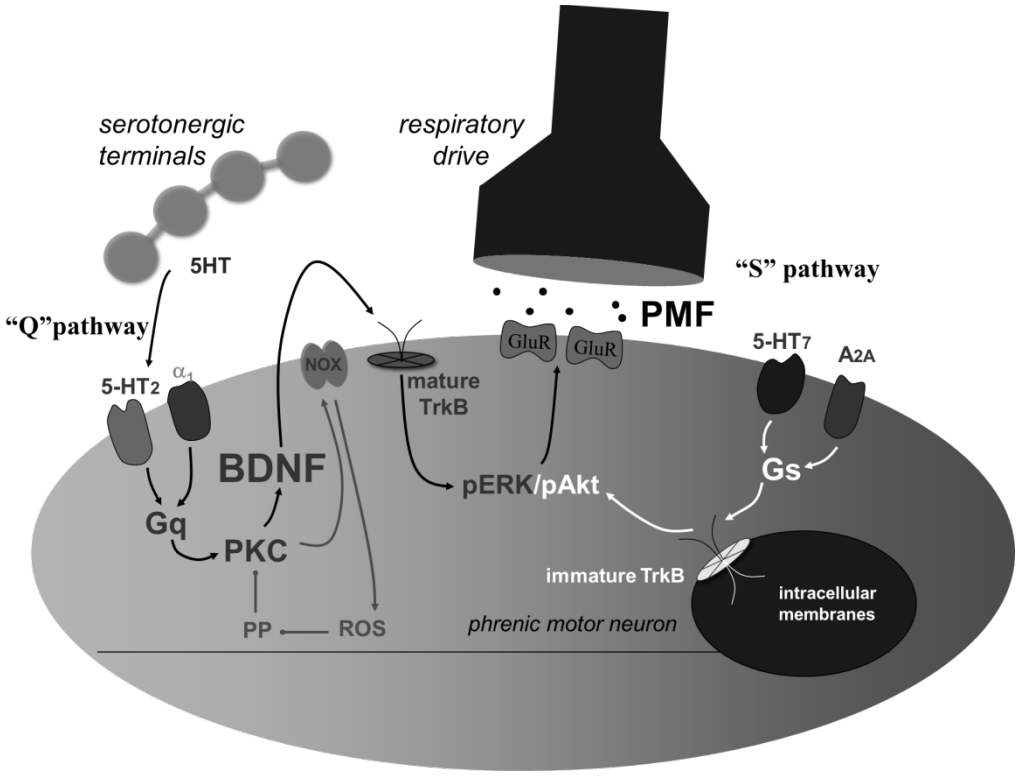

Figure 3. Cellular mechanisms of Phrenic Motor Facilitation (PMF). Intermittent hypoxia stimulates serotonin (5-HT) release from raphe-spinal terminals near phrenic motoneurons. Serotonin receptor and Gq-protein activation increases PKC activity, which in turn increases BDNF synthesis within phrenic motoneurons. BDNF is secreted extracellularly and binds to mature, fully glycosylated TrkB receptors expressed on the outer cell membrane. BDNF phosphorylates mature TrkB receptors, which in turn strengthen excitatory glutamatergic synapses onto phrenic motoneurons via activated MAP kinases (pERK1/2). As the same cascade of event can be elicited by activating serotonin type 2A, 2B as well as adrenergic alpha 1 receptors, this was called the "Q" pathway. A2a receptor agonists activate Gs-protein and increase synthesis and phosphorylation of intracellular, hypoglycosylated, immature TrkB protein without the need for BDNF (transactivation). Phosphorylated intracellular TrkB strengthens excitatory glutamatergic synapses onto phrenic motoneurons via activated PI3K and protein kinase B (pAkt). Since this mechanism can be elicited by activation of adenosine 2A as well as serotonin type 7 receptors we call it the "S" pathway.

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Chapter II

Summary of Thesis Aims

In the United States, there are more than 250,000 individuals living with a chronic spinal cord injury (SCI) (NSCISC, 2005). Currently, scientists are pressured on many fronts to develop a "cure" for paralysis. Although the scientific understanding of central nervous system (CNS) regeneration has advanced greatly in the past twenty years, there are still many unknowns with regard to inducing successful regeneration, especially with chronic SCI. A more realistic approach based on currently available knowledge to improve the quality of life for a large proportion of the paralyzed population may be to develop treatments that elicit partial functional recovery based on improved use of spared neural pathways. In this scenario, the need for alternative approaches to alleviate respiratory deficits and thus improve the lives of ventilator-dependent patients is a critical area of investigation in spinal injury research. **The fundamental hypothesis guiding this thesis is that the inherent capacity for respiratory motor plasticity can be harnessed to (at least partially) offset functional deficits in breathing capacity caused by cervical spinal injuries.** Specifically, I postulate that moderate acute intermittent hypoxia, particularly with concurrent adenosine A_{2A} receptor inhibition; can enhance ventilatory function and motor activity in respiratory muscles below an incomplete cervical injury. Concurrent administration of A_{2A} receptor antagonists is expected to alleviate inhibitory interactions between the S and the Q pathways to phrenic motor facilitation, thus enabling greater Q-dependent plasticity with the same protocol of repetitive AIH (Hoffman et al., 2010).

In recent years, the rat have become an important animal model to study the impact of traumatic spinal cord injuries on respiratory function, at least in part because the morphological, biochemical and functional changes that occur after SCI share some similarities to those seen in humans (Onifer et al., 2007, Lane et al., 2008). However, most studies have emphasized function of the phrenic motor system, and very few studies have been performed characterizing contributions of the accessory respiratory muscles to date, including the intercostal and abdominal muscles. This is particularly true in spontaneously breathing, unanesthetized rats. Thus, Chapter 3 of this thesis aims to characterize external intercostal (EIC) as well as abdominal muscle contributions during quiet breathing (eupnea), sustained hypoxia (10.5% O_2), and maximum chemoreceptor stimulation (10.5% O_2 and 7% CO_2) in

unanesthetized rats using an advanced radiotelemetry system for electromyography (EMG). Additionally, IH-induced plasticity in these accessory respiratory muscles was investigated using a frequently studied protocol of moderate intermittent hypoxia (AIH: 10 episodes of 5 min 10.5% O2 interspersed with 5 min normoxia) (Mitchell and Terada, 2011).

Plasticity is an important property of neural systems, including the neural system controlling breathing (Mitchell and Johnson, 2003). AIH-induced plasticity has functional consequences, expressed as a phrenic long-term facilitation (pLTF), diaphragm long-term facilitation (diaLTF) and ventilatory long-term facilitation (vLTF). Furthermore, since adenosine A_{2A} receptor inhibition enhances AIH-induced pLTF in anesthetized, normal rats, chapter 4 of this thesis aims to investigate the impact of adenosine A_{2A} receptor inhibition on AIH-induced LTF in diaphragm and second EIC muscle activity in unanesthetized normal and C2 cervical hemisected (C2HS) rats. C2HS interrupts bulbospinal drive to all respiratory muscles distal to the lesion (e.g., ipsilateral diaphragm; intercostals, abdominals), allowing studies of functional recovery in both ipsilateral (injured) and contralateral (uninjured) respiratory muscles.

Daily acute intermittent hypoxia (i.e. dAIH, 10 episodes per day, 7 d) strengthens synaptic pathways to phrenic motorneurons and, hence, increases respiratory recovery one week after cervical spinal cord injury (Lovett-Barr MR et al., 2007, Lovett-Barr et al., 2012). While ventilatory measurements reveal functional recovery of breathing capacity, such measures reveal little about the mechanism of recovery (e.g., plasticity in injured side versus compensatory plasticity in uninjured muscle activities). Therefore, chapter 5 aims to investigate the relative contributions of dAIH-dependent respiratory motor plasticity in T2 external intercostal (T2 EIC) and diaphragm muscles to improved ventilatory capacity 1 to 2 weeks following C2 cervical hemisection in rats. Simultaneous electromyography (EMG) of bilateral diaphragm and T2 EIC muscles in unanesthetized rats offers a more complete picture about the respective contributions of different respiratory muscles to functional recovery of breathing capacity after cervical spinal injuries. Additionally, the effect of dAIH on automatic grooming behavior was assessed in forelimbs.

Our lab has evidence suggesting that cellular mechanisms giving rise to dAIH-induced functional recovery are completely different in early (<8 weeks) versus chronic (>8 weeks) SCI (Golder and Mitchell, 2005). It is hypothesized that dAIH elicits plasticity via an adenosine-dependent (serotonin-independent) mechanism with early injury (7 days), but serotonin-dependent (adenosine-constrained) mechanism with chronic cervical spinal injuries (8 weeks). Therefore, chapter 5 of this thesis aims to test the hypothesis that systemic adenosine A_{2A} inhibition impairs dAIH-induced functional recovery with acute spinal injury (1-2 weeks post-C2HS), but actually enhances dAIH-induced functional recovery with chronic injuries (8-16 weeks post- C2HS; chapter 6).

Repetitive acute intermittent hypoxia (rAIH) consisting of AIH three times per week (3×wAIH) for 10 weeks increases the expression of key molecules involved in AIH-induced pLTF (Satriotomo et al., 2012). An additional aim in Chapter 6 was to extend the functional benefits of dAIH by using a less-frequent, but prolonged rAIH protocol to extend the functional benefits. Since 3×wAIH upregulates pro-plasticity molecules in the phrenic motor nucleus without evidence for CNS pathology (Satriotomo et al., 2012), it may be a useful therapeutic approach to extend the functional benefits of dAIH in chronically injured rats. **Currently, there are no approved therapies for chronic thoracic or cervical SCI; therefore, our approach represents a promising new strategy to enhance function in patients with chronic SCI, where the potential for further functional gains is limited.**

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Chapter III

Recruitment and plasticity in accessory respiratory muscles in unanesthezised rats

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ABSTRACT

Although rats have become a frequent model for studies of respiratory control, the relative contributions and expression of respiratory plasticity in accessory respiratory muscles are not well known. These muscles may be critical to enable adequate responses with physiological (e.g. exercise, hypoxia) and pathophysiological challenges (e.g. cervical spinal injury, ALS). Here, we characterized external intercostal (T2, T4, T5, T6, T7, T8, T9 EIC) and abdominal muscle (external oblique and rectus abdominus) electromyogram (EMG) activities in unanesthezised, spontaneously breathing rats with a radiotelemetry system. EIC and abdominal EMG activity were measured during normoxia (21% O_2), and before, and after acute intermittent hypoxia (AIH: 10, 5-min episodes of hypoxia, 10.5%, interspersed with 5-min episodes of normoxia). Additionally, diaphragm and T2-T5 EIC muscle activity was assessed with simultaneous plethysmography under maximum chemoreceptor stimulation (MCS: 10.5% O_2 and 7% CO_2) and sustained hypoxia (SH: 10.5% O_2). During normoxia, T2 EIC shows only inspiratory activity. T4, T5, T6 and T7 EIC muscles show a progressively decreasing rostro-caudal inspiratory activity, superimposed to tonic activity. T8, T9 EIC as well as abdominal muscles show only tonic activity without apparent expiratory activity. MCS elicits a greater increase in EMG amplitude and tidal volume than SH and controls (0.94 ± 0.10 vs. 0.68 ± 0.05 ml/100g and 0.48 ± 0.03 ml/100g, respectively; $p < 0.001$). AIH significantly increases EMG amplitude 0-60 min post-AIH in T2 EIC, which doubles the increase in EMG amplitude in diaphragm (41.49 ± 1.33% vs. 19.05 ± 1.97% above baseline, respectively; $p < 0.001$), representing diaphragm and T2 EIC long term facilitation (LTF). T4-T5 EIC shows either an increase, decrease or no change in EMG amplitude after AIH versus baseline activity; this response was greatly affected by rat posture during the AIH protocol. We conclude that 1) diaphragm and rostral T2-T5 EIC muscles are major contributors to tidal volume during normoxia, SH and MCS; 2) MCS elicit a greater ventilatory and motor activity in diaphragm and rostral T2-T5 EIC muscles, compared to SH; 3) AIH induces consistent diaphragm and T2 EIC LTF.

INTRODUCTION

The spatial and temporal distribution of inspiratory and expiratory activity among intercostal muscles has been extensively investigated in cats, dogs and humans (Taylor, 1960, De Troyer et al., 1985, De Troyer and Wilson, 2002, Wilson and De Troyer, 2004). The pattern of respiratory muscle activation in these species can be described by two general principles: 1) the activity within each muscle layer follows the distribution of mechanical advantage in that layer; and 2) inspiratory activation is restricted to the external layer and the parasternal muscle, whereas expiratory activation is restricted to the internal layer (De Troyer et al., 2005). However, rats appear to diverge from these principles. In rats, some areas of a single intercostal layer can be activated in both inspiration and expiration (Megirian et al., 1987). Moreover, cephalic ribcage muscles (scalenus medius, parasternal and external and internal intercostal muscles) always exhibit inspiratory activity, regardless of the state of consciousness (Megirian et al., 1987). On the other hand, those muscles of the mid-thoracic region, and less so those of the caudal region, may be inspiratory or expiratory (Megirian et al., 1987). It is worth noting that the latter study used a connecting cable to detect EMG signals, which may increase animal stress. It is generally acknowledged that the quality of physiological measurements collected from conscious unstressed animals is superior, since they are collected under conditions that best represent the normal state of the animal and are least influenced by chemical, stress-related and psychological factors. The FDA suggests that this condition is most predictive of results expected in humans (FDA, 2001). Thus, the use of radiotelemetry systems to monitor EMG activity is a valuable tool in unrestrained, unanesthezised animals, potentially allowing more reliable clinical translation.

The effect of hypoxia and hypercapnia in diaphragm and external intercostal muscles has been studied previously in unanesthetized rats. During REM sleep, the mean diaphragm EMG activity is greater during SH (10% O_2) compared to hypercapnia (5% O_2) and MCS (10% O_2 and 4% CO_2) (11.3, 9.3 and 8.3 arbitrary units, respectively) (Megirian et al., 1980). Furthermore, in the same study, respiratory frequency was greater during SH than MCS and hypercapnia alone in all sleep states (Megirian et al., 1980), In the case of external intercostal muscles, gas concentrations have minimal effect on EMG

activity, with the exception of the 2nd and 5th external intercostal muscles, which exhibit a robust increase in EMG activity during 5% CO_2 exposure (Megirian et al., 1987). Although greatly advancing our knowledge, these older studies fail to describe some important experimental conditions, and did not investigate other factors such as the EMG activity during sleep stages different than REM and also the response following repetitive exposure to hypoxia (i.e. respiratory plasticity). There are no studies specifically showing the effect of sustained hypoxia and maximum chemoreceptor stimulation on simultaneous ventilatory measurements and EMG activity for either diaphragm or rostral external intercostal muscle activity using radiotelemetry in unanesthetized rats.

Moderate acute intermittent hypoxia (AIH) elicits plasticity in the phrenic motor system (Feldman et al., 2003, Mitchell and Johnson, 2003, Mahamed and Mitchell, 2007). AIH-induced plasticity has functional consequences, expressed as a persistent increase in phrenic motor output (phrenic long-term facilitation, pLTF); increased diaphragm activity (diaphragm long-term facilitation, diaLTF) and increased ventilation (ventilatory long-term facilitation, vLTF) in spontaneously breathing rats (Terada and Mitchell, 2011), and can increase capacity to generate respiratory and somatic motor recovery after cervical spinal cord injury (Golder and Mitchell, 2005, Lovett-Barr et al., 2012). Although it appears to be greater capacity for LTF in inspiratory intercostal versus phrenic activity in cats (Fregosi and Mitchell, 1994), there are no reports concerning the impact of AIH on LTF in accessory respiratory muscles in unanesthetized rats.

The main purpose of the present investigation is to study the recruitment and plasticity of external intercostal and abdominal muscles using radiotelemetry to assess EMG activity in unanesthetized, spontaneously breathing rats. We tested three specific hypotheses: 1) EIC muscles (T2, T4, T5 EIC) show inspiratory activity whereas caudal T6, T7, T8, T9 EIC and abdominal muscles show expiratory activity during normoxia; 2) maximum chemoreceptor stimulation (7% CO_2 and 10.5% O_2) elicits greater EMG activity and tidal volume versus sustained hypoxia; and 3) AIH elicits LTF in rostral external intercostal muscles (T2, T4, T5) with no effect in caudal segments (T6-T9 EIC) and abdominal muscles.

METHODS

Animals

All experiments were performed on 3-4 month old, male Sprague-Dawley rats (320-360 g, colony 211, Harlan, Indianapolis, IN). Animals were individually housed in a controlled environment (12-h light/dark cycle). The Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin approved all experimental procedures in this study.

Experimental preparation

Surgical preparation. Sterile surgery was performed under anesthesia induced with isoflurane in 100% O_2. The rats were treated with buprenorphine (0.03 mg/kg), carprofen (Rimadyl, 5 mg/kg) and enrofloxacin (Baytril, 4 mg/kg) subcutaneously to minimize potential post-operative pain and infection. Body temperature was maintained at 36.5-37.5°C using a rectal probe and external heating pad. A cannula was inserted into the trachea and the animals were artificially ventilated (tidal volume, 2.0-2.5 ml; Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA) with 1.5-2.5% isoflurane in 100% O_2 during surgery. Effective anesthesia was judged by abolition of pedal withdrawal and corneal blink reflexes. Oxygen saturation was monitored by pulse oximetry (model 8600; Nonin Medical Inc. Plymouth, MN) during the surgical procedures. With the rat supine, the ventral surface of the abdominal muscle was exposed and a sterilized telemetry transmitter body (model 4ET-S1/2; Data Sciences International [DSI], St. Paul, MN) was inserted into the peritoneal cavity. The transmitter allows simultaneous and continuous monitoring of electrical biopotentials, body temperature and general locomotor activity. In the present study the four biopotential channels were used to record EMG of diaphragm and external intercostal muscles (EIC) in different set of experiments. Due to the limited number of channels, 3 set of different experiments were necessary to characterize external intercostal and abdominal muscles. Right diaphragm (R-Dia) muscle was used in all set of experiments as considered gold standard for inspiratory activity. In a first set of experiment the targeted muscles were R-Dia, T2, T4, T5 EIC muscles. In a second time the targets were R-Dia, T6, T7 EIC and external oblique muscles.

Finally, R-Dia, T8, T9 EIC and rectus abdominus muscles were explored. Diaphragm EMG leads were placed on the basis of previous reports (Terada and Mitchell, 2011). Briefly, the right costal part of the diaphragm was exposed and two leads were implanted on the right side of the mid-costal diaphragm using a 23-G syringe needle guide and tissue adhesive (Vetbond 1469SB; 3M Animal care product, St. Paul, MN). Next, the second, fourth, fifth and sixth EIC muscles were exposed 1.0 cm from the sternum and identified by counting intercostal spaces from the first external intercostal muscle. The seventh, eighth and ninth thoracic external intercostal muscles were exposed at the mid axillary line. The left rectus abdominus muscle was identified by exposing the muscle immediately left to the alba line. The left external oblique muscle was identified by exposing the muscle 3 cm apart from the alba line and visualizing the oblique orientation of the muscles fibers. All bi-potential lead pairs (bipolar) targeting external intercostal and abdominal muscles were tunneled subcutaneously from the body of the transmitter in the right ventral abdomen to different intercostal spaces on the right hemithorax and to the left ventral area for the abdominal muscles. As used in the diaphragm, all the leads in EIC and abdominal muscles were implanted using a 23-G syringe needle guide and tissue adhesive to keep the leads on place. At the end of surgery buprenorphine, carprofen and enrofloxacin at the same dose rats received prior to the surgery were administered at 12 h intervals for 48 h post-surgery. Rats were visually monitored and weighed daily. Experiments were not initiated until rats resumed normal weight gain.

Telemetry signal assessment. For the AIH protocol rats were placed in custom-made Plexiglas chambers (see below) positioned on radiotelemetry receivers (model RPC-2; DSI, St. Paul, MN). Signals from the implanted radio-transmitter were detected by the receivers and sent to a data exchange matrix (model ACQ-7700; DSI, St Paul, MN). Four channels of EMG, body temperature and general locomotor activity in unanesthetized freely moving rats were monitored during the experimental protocol on a laboratory computer (data acquisition system: PONEMAH Physiology Platform; DSI, St. Paul, MN). EMG signals were sampled at 1200 Hz, and then filtered (100-624 Hz). An analysis of all parameters was performed with Neuroscore software (DSI, St. Paul, MN) as described below.

Whole-body plethysmography. Rats were placed individually in a 4 L DSI Plethysmography chamber (model 600-1211-001). Pressurized air continuously flowed through the chamber at 4L/min, allowing control of inspired gas composition. The chamber was positioned on a receiver (see above) to measure EMG and ventilation simultaneously during MCS/SH protocols. Compensated whole body plethysmography was used, allowing corrections for chamber temperature and humidity. The compensation was provided by a temperature sensor (model P/N 60-1210-001) and a humidity sensor (model P/N 600-1211-001) connected to the universal signal conditioner. The system used a Buxco transducer (model TRD5700) and a gas analyzer CWE (model Gemini). Plethysmography data were analyzed in 1 min intervals during baseline conditions (20 minutes), MCS (10.5% O_2 and 7% CO_2) and SH (10.5% O_2).

Acute intermittent hypoxia protocol. Normoxic (21% O_2) and hypoxic (10.5% O_2) conditions were established in chambers (Plexiglas cylinder, 12 x 4 inches id; 1 rat per chamber) by mixing O_2 and N_2 gas via a custom-made computer-controlled system to obtain the desired inspired oxygen concentrations. Within the chambers, CO_2 and O_2 levels were continuously monitored (O_2 Analyzer, model 17518; CO_2 Analyzer, model 17515; VacuMed Inc, Ventura, CA). Gas flowed through the chamber at a rate of 4 L/min, keeping CO_2 concentration in the chamber less than 0.5% at all times. 95% of the change in O_2 levels within the chamber (i.e., intermittent hypoxia) was achieved in 25 ± 5 sec. The rats were poikilocapnic; thus, arterial CO_2 levels were not controlled in the present study. Once normal body mass increases resumed following surgery (7 days), the rats were acclimated to the chambers for 2 hours at least 1 day before the experiment. At 8:00 am, on the experimental day, the rats were placed in the chamber above the telemetry signal receivers. At 10:00 am, after the 2-h acclimation for each rat, baseline recording were made during normoxia for 1 hour. At 11:00 am, after baseline recordings, half of the rats were administered AIH (10, 5-min hypoxic episodes with 5-min normoxic intervals, for a total of 95 min, see fig.5); the other half were administered continuous normoxia as a time control (TC). Finally, recordings were made during post-treatment normoxia until 1:35 pm in both TC and AIH treated rats. For

the first set of experiments targeting R-Dia, T2, T4, T5 EIC muscles, 6 rats were used as time controls (TC n=6), and 8 rats were exposed to AIH (AIH n=8). For the second round of experiments targeting R-Dia, T6, T7 EIC and external oblique muscles 4 rats were used as time controls (TC=4) and 6 rats as AIH group (AIH=6). In the third group targeting R-Dia, T8, T9 EIC and rectus abdominus muscles, 4 rats were used as time control (TC=4) and 6 rats were used as AIH group (AIH=6). Rats had *ad libitum* access to food and water throughout experiments. Chamber temperature was 22.5-24.5^{o}C. Chamber O_2 and CO_2 concentrations (inflow and outflow) gases were continuously monitored during the experiment.

Sustained hypoxia and maximum chemoreceptor stimulation protocol. Normoxic (21% O_2) hypoxic (10.5% O_2) and hypercapnic conditions (7% CO_2) were established in plethysmography chambers (see above) by mixing O_2, N_2 and CO_2 via a custom-made, computer-controlled system of mass flow controllers to obtain the desired inspired gas concentrations. After 30 min acclimation, baseline during normoxia was recorded for 20 minutes, followed by 20 minute recordings during maximum chemoreceptor stimulation (MCS) (10.5% oxygen with 7% CO_2 for 20 min, see fig. 2A). Time control (TC) rats were exposed to 20 minutes of normoxia to match the MCS protocol. One week after MCS, the same rats were exposed to SH (20 min normoxia followed by 20 min hypoxia, 10.5%, see fig. 2B). Unlike MCS, rats were poikilocapnic in the SH protocol; thus, arterial P_{CO2} levels decreased during SH. Gas flow through the chamber was 4L/min to enable control of inspired gas composition and prevent CO_2 accumulation within the chamber (CO_2 < 0.5%). For both MCS and SH, targeted muscles were the R-Dia, as well as the T2, T4 and T5 EIC muscles; 4 rats were exposed to TC, and 6 rats underwent SH and MCS. EMG assessment and plethysmography were performed simultaneously in each experiment, enabling correlations between respiratory muscle activity and tidal volume. All volumes were corrected for chamber and body temperature (via telemetry), an important correction since body temperature changes appreciably during hypoxic conditions in rats. Many previous studies examining ventilatory LTF do not account for changes in body temperature within an experimental protocol.

Data analyses

EMG analysis was performed with Neuroscore software. EMG signals were filtered (100-624 Hz), rectified, integrated (100 msec.) and quantified in arbitrary units. Mean integrated amplitude, respiratory frequency and minute activity (amplitude x frequency) were averaged in all rats. For AIH experiments, peak amplitude and burst frequency were averaged before and after AIH (60 min). For the MCS/SH experiments, values were averaged before and during MCS/SH. Values during active rat movement or during sniffing behavior were excluded from analysis. Data obtained during the pre-treatment period represent baseline values. All values during and after treatment are expressed as a percent change from this baseline. Ventilation was analyzed at the same times as EMG activity, but was expressed as absolutes values.

Respiratory variables (diaphragm/external/abdominal amplitude, respiratory frequency, tidal volume, minute activity) were compared for time (pre and post AIH, or pre and during MCS or SH) and treatment (TC vs. AIH and TC vs. MCS/SH) using a 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 statistically significant if $p < 0.05$. All values are expressed as means ± SEM.

RESULTS

External intercostal and abdominal muscle activity during baseline

Right diaphragm EMG activity was used as an indicator of inspiratory activity to characterize respiratory activity of intercostal (T2, T4, T5, T6, T7, T8, T9) and abdominal muscles (external oblique and rectus abdominus). EMG activity during baseline normoxia conditions differed among thoracic external intercostal muscles (Fig. 1). The second thoracic external intercostal muscle (T2 EIC) showed prominent inspiratory activity in all rats studied, with minimal tonic (non-respiratory) activity. In contrast, T4 and T5 EIC exhibited both inspiratory and tonic activity. Whereas T6 and T7 EIC showed minimal inspiratory activity, but often exhibited tonic activity, T8 and T9 showed only tonic activity, with no appreciable inspiratory or expiratory activity. Both the external oblique and rectus abdominus showed tonic activity exclusively, with no clear evidence for respiratory modulated activity.

Sustained hypoxia and maximum chemoreceptor stimulation

To investigate the ability to recruit EIC and abdominal muscle respiratory activity, SH and MCS were used to drive breathing and respiratory muscle EMG activity (only diaphragm and T2, T4 and T5 EIC muscles). Rats were exposed to 20 min normoxia (baseline), followed by 20 min of 10.5% O_2 and 7% CO_2. MCS increased respiratory EMG activity in Dia, T2, T4 and T5 EIC muscle (102 ± 2%, 98 ± 3%, 72 ± 3% and 53 ± 2% respectively, $p < 0.001$; fig. 2A, 3). This decreasing pattern from diaphragm through T5 EIC coincides with the decreasing inspiratory activity from diaphragm through T5 EIC during normoxia. A similar, decreasing pattern was found when rats were exposed to sustained hypoxia (10.5% O_2), although this is not as powerful drive to increased breathing. SH increased EMG amplitude in the Dia, T2, and T4 EIC muscles (56 ± 2%, 55± 2%, 20 ± 3 %, $p < 0.001$; fig. 2B, 3). However, the T5 EIC muscle showed no significant effect during SH relative to baseline or TC rats (5 ± 1% vs. 5 ± 2% respectively, $p=0.563$; fig.2B).

Ventilation during sustained hypoxia and maximum chemoreceptor stimulation

MCS elicits the greatest increase in tidal volume versus SH and TC (0.94 ± 0.1 vs 0.68 ± 0.05 and 0.48 ± 0.03 ml/100g, respectively; $p < 0.001$; fig. 4). Respiratory frequency during MCS was also increased more than in SH or TC (133 ± 1 vs. 118 ± 2 and 75 ± 3 breaths/min, respectively; $p < 0.001$; fig.4). Accordingly, MCS elicits greater minute ventilation (tidal volume x respiratory frequency) than SH (126 ± 5 vs. 80 ± 2 ml/100g/min respectively, $p<0.001$; fig.5).

Effect of acute intermittent hypoxia

A sustained increase in Dia EMG amplitude was observed 0-60 min post AIH (19 ± 2% baseline, $p < 0.001$; fig. 6A, 7A), confirming reports of diaphragm long term facilitation (Dia LTF) (Terada and Mitchell, 2011). External T2 intercostal EMG amplitude also exhibited LTF, but it's magnitude was double that of Dia LTF (41 ± 1% baseline, i.e T2 EIC LTF, $p < 0.001$, see fig. 6A, 7A). Respiratory frequency (14 ± 1% above baseline; $p = 0.004$; fig. 6B) and minute activity (diaphragm, 40 ± 2% baseline; T2 EIC, 63 ± 2% baseline; both $p < 0.001$; fig. 7C) were also increased 0-60 min post-AIH (i.e., frequency and minute activity LTF). On the other hand, normoxia (e.g., TC) did not affect post-treatment EMG amplitude, frequency or minute activity in either diaphragm or T2 EIC muscles (all $p > 0.05$; fig.6D). T4 and T5 EIC muscles exhibited considerable variability in their response following AIH. In T4 EIC, 3 rats increased EMG amplitude 0-60 min post AIH (fig.6A), but 3 did not (fig.6C). T5 EICs showed increased (n = 1; fig.6A), no change (n = 3; fig. 6C) or decreased EMG amplitude (n = 2; fig.6B) 0-60 min post AIH. The presence or absence of T4/T5 LTF, or overt inhibition (long term inhibition, LTI) was not completely predictable, but appeared related to the rat posture when AIH was administered; a curled up position favored LTF (fig.6E), whereas an extended position favored LTI (fig.6F). Semi-curled postures exhibited no change in T4/T5 EIC activity versus baseline (fig.6G). This observation suggest that the rotational function of the ribcage influences respiratory behavior in mid-thoracic EIC muscles. In T6, T7, T8 and T9 EIC muscles, as well as rectus abdominus, neither increased nor decreased respiratory related activity was observed 0-60 minutes post-AIH (figs. 8A, 8B). Interestingly, the external oblique

muscles exhibited inhibition during the AIH protocol (fig. 9), but this effect did not persisit 0-60 min post-AIH.

DISCUSSION

Here, we report four major findings: 1) diaphragm and the second external intercostal muscles show exclusively inspiratory activity in all conditions studied, whereas the third, fourth and fifth EIC exhibit progressively less (rostro-caudal) inspiratory activity and progressively more tonic (non-respiratory) activity; 2) combined hypoxia and hypercapnia is a more powerful stimulus than hypoxia alone in all variables studied; 3) moderate AIH elicits consistent LTF in the diaphragm and second external intercostal muscle; 4) AIH can elicit LTF, LTI or no change post-AIH in mid-thoracic external intercostal muscles, an effect that appears related by rat posture during the study.

Respiratory (phasic) activity in normoxia

Phasic respiratory activity in accessory muscles depends on the particular ribcage muscle under study, the animal's posture and the extent of chemoreflex activation. Thus, it is difficult to categorize the respiratory function of individual external intercostal muscles. Nevertheless, consistent with previous studies in rats and dogs (Megirian et al., 1987, DiMarco et al., 1992), we show that the second EIC muscle always exhibits prominent inspiratory activity during normoxia. The decreasing rostro-caudal gradient of inspiratory activity from T4 through T7 EIC muscles is consistent with electrophysiological studies showing inspiratory discharges in the external intercostal nerves in rats, with stronger activity rostrally than caudally (de Almeida et al., 2010). Similar results were found in dogs where the inspiratory activity of the EIC muscles decreases rapidly from the second to sixth interspace, and actually reverses to an expiratory pattern by the 8th to 10th interspaces (De Troyer, 2005). Mid-thoracic (T6, T7) EIC muscles exhibit high variability in their pattern of discharge, with inspiratory activity superimposed on tonic (non-respiratory) activity, consistent with previous studies in rats (Megirian et al., 1987, de Almeida et al., 2010). However, we do not confirm expiratory activity in mid-costal or caudal EIC muscles. Other studies

in dogs reported variable presence of expiratory and inspiratory activity in mid-costal and caudal EIC muscles. Intercostal nerve recordings in anesthetized rats show discharges for inspiration and expiration in both external and internal caudal intercostal nerves (de Almeida et al., 2010). Furthermore, intracellular recordings confirmed that individual thoracic motorneurons can be excited in both phases of respiration (de Almeida and Kirkwood, 2010). A reasonable explanation for variability of inspiratory/expiratory activity in mid-thoracic and caudal EIC muscles could be the convergence of bulbo-spinal synaptic pathways onto these motor neurons, and cross-talk between the external and internal intercostal muscles. The external intercostals muscles in the caudal interspaces are particularly thin, so the expiratory activity recorded in some studies could simply be the result of contaminations. Legrand and De Troyer (Legrand and De Troyer, 1999) examined with selective denervation procedures the pattern of activity of the caudal canine external intercostal muscles. In agreement with previous observations (Le Bars and Duron, 1984, Carrier, 1996), phasic expiratory discharges were recorded from the external intercostal muscles in the ventrolateral portion of the caudal interspaces. However, this expiratory activity remained unchanged after section of the external intercostal nerve, and it disappeared only after section of the internal intercostal nerve in the same interspace and abolition of expiratory activity in the underlying internal intercostal muscle; that is, the expiratory discharges recorded in the external intercostal muscles in the caudal segments of the rib cage were due to impulses traveling along the internal, rather than the external, intercostal nerves. Moreover, the dorso-ventral gradient of expiratory activity in caudal external intercostals may explain the lack of expiratory activity observed in this study. In dogs (De Troyer et al., 1999) and cats (Greer and Martin, 1990), the external intercostals in the caudal interspaces have larger masses dorsally (vs. ventrally); therefore, an already reduced expiratory activity may be masked by tonic activity in ventral spaces in our ventrally implanted rats.

Details concerning central respiratory drive in spinal thoracic motoneurons are still unresolved. It has been proposed that intercostal activity is driven by a network of spinal interneurons and that direct monosynaptic inputs from brainstem respiratory neurons are rare (Merrill and Lipski, 1987, Saywell et al., 2011). Thus, high frequency stimulation of the T2 to T5 spinal cord in cervical hemisected anaesthetized

dogs activates inspiratory external intercostal motor neurons in a remarkably physiological manner (DiMarco and Kowalski, 2011); thus, breathing and locomotion can be executed by intrinsic networks of interneurons in the vertebrate spinal cord. Interestingly, the vast majority of individual thoracic interneurons produce relatively weak effects on EIC activity (Kirkwood et al., 1988, Kirkwood et al., 1993), suggesting that few, key interneurons drive thoracic motor neurons (McLaughlin, 1972, Saywell et al., 2011). The impact of these spinal interneurons and their tendency to elicit inspiratory, expiratory or combined activity at different levels of the thoracic spinal cord remains to be explored.

Maximum chemoreceptor stimulation versus hypoxia

Maximum chemoreceptor stimulation (10.5% hypoxia and 7% hypercapnia) is a stronger stimulus to accessory respiratory muscle activity than sustained hypoxia, at least in the rostral external intercostal muscles. These results contradict previous studies in rats showing that during REM sleep hypoxia is a more potent stimulus than hypoxia and hypercapnia combined (Megirian et al., 1980). We did not analyze EMG activity in different sleep states which may account for the differences observed. In contrast, studies done in unanesthetized dogs (Cherniack et al., 1973), cats (Fitzgerald, 1973, Gautier, 1976) and humans (Somers et al., 1989) show that hypercapnia alone has a stronger effect in minute ventilation than hypoxia, and that combined hypoxia and hypercapnia has a synergistic effect on minute ventilation (Somers et al., 1989), consistent with our findings in the present study.

Effect of acute intermittent hypoxia in external intercostal and abdominal muscles

We confirm that AIH elicits a long lasting increase in diaphragm peak amplitude above baseline, i.e. long term facilitation (DiaLTF) (Terada and Mitchell, 2011). The cellular mechanisms of pLTF have been explored extensively (Baker-Herman and Mitchell, 2002, Baker-Herman et al., 2004, MacFarlane and Mitchell, 2009). Here, we report for the first time that AIH elicits long lasting increase in peak EMG amplitude above baseline in the second external intercostal (T2 EIC) muscle (i.e. T2 EIC LTF) in unanesthetized, spontaneously breathing and poikilocapnic rats. Interestingly, the increase in peak EMG

amplitude in T2 EIC is double Dia LTF, suggesting a relatively greater contribution of inspiratory accessory muscles to ventilation after intermittent hypoxia.

Variability in the effect of AIH on mid-thoracic (fourth and fifth) EIC may relate to several factors. First, more caudal thoracic segments may be more involved in postural adjustments as rats navigate their world. Respiratory behavior may be influenced by posture. Studies in cats (Duron and Marlot, 1980, Dick et al., 1984) and humans (Goldman et al., 1985, Whitelaw et al., 1992, Rimmer et al., 1995) demonstrates that rotation of head and trunk affect the degree of respiratory activity in middle and lower thoracic intercostal muscles. Second, there may be differences among sleep states that were not accounted for in this study. LTF is bigger during NREM versus quiet wakefulness and REM sleep (Terada and Mitchell, 2011), and it is possible that this effect is greater in some respiratory muscles than in others. Failure to account for sleep state is a limitation of this study (a logistics issue due to limited biopotential leads per transmitter). As the inspiratory activity in mid-thoracic segments exhibits a clear rostrocaudal gradient, the presence of LTF may depend on the degree of inspiratory activity at the time of exposure, which is affected by both posture and sleep stage. When cats adopt a curled position, inspiratory intercostal muscle activity is greater on the concave-upward side versus the convex downward side (Dick et al., 1984). In rats, the inspiratory activity of second and fifth external intercostal muscles is greater when the rat is in curled-up versus in an extended position (Megirian et al., 1987). Indeed, our rats were free to adopt different postures during experimental conditions. In general, we observed that a curled position during AIH increased EMG inspiratory activity and was accompanied by AIH-induced LTF in the T4 and T5 EIC muscles. In contrast, an extended position favored long term inhibition in the T5 EIC muscle, and rats in a semi-curled posture exhibited no change (i.e. no LTF) after AIH. The basis for this strong correlation between LTF and baseline inspiratory activity in the mid-EIC muscles is not yet clear.

AIH-induced LTF may be elicited routinely in exclusively inspiratory muscles, such the diaphragm and T2 EIC muscle. The idea of LTF being a general property of inspiratory motor nerve activity was first brought up by Fregosi and Mitchell (Fregosi and Mitchell, 1994). Repeated carotid sinus nerve (CSN) stimulation evokes a serotonin-dependent LTF of both phrenic and inspiratory internal intercostal (IIC)

(para-sternal) nerve activity in anesthetized cats. They also showed that CSN stimulation-evoked LTF of inspiratory intercostal exceeds that of phrenic nerve activity (Fregosi and Mitchell, 1994), consistent with the greater T2 EIC LTF versus DiaLTF shown here.

The four abdominal muscles with significant respiratory function in quadrupeds and humans are rectus abdominis, external oblique, internal oblique and transversus abdominis. Under normoxic conditions the muscles explored in this study show almost exclusively tonic activity, although weak expiratory activity may have been obscured by postural changes. Our results are consistent with experiments in unanesthetized dogs, where expiratory EMG activity was rarely seen in the external oblique, and was absent in the rectus abdominis (De Troyer et al., 1989, Yasuma et al., 1993). As in the EIC muscles, posture affects the expiratory activity of abdominal muscles. When expiratory activity is present in external oblique muscle of dogs, it was reduced considerably when lying in a prone position (versus standing) (De Troyer et al., 1989). Similar postural issues may help explain the absence of phasic abdominal muscle activity in the rats of this study, since they were always in a prone position.

We report that EMG activity in external oblique was actually inhibited during hypoxic episodes (i.e. during AIH), consistent with previous studies. Peak EMG amplitude in external oblique is greater during hypercapnia, and clearly inhibited during hypoxia (Yasuma et al., 1993) in dogs. Electrophysiological studies confirm that isocapnic hypoxia inhibits iliohypogastric nerve activity in cats (Fregosi et al., 1987).

One purpose of this study was to characterize external intercostal and abdominal muscle activity using a minimally invasive radiotelemetry system; although most findings were consistent with earlier studies, at least some differences from prior accounts may have arisen from the use of telemetry versus traditional wire-implantation techniques used in the earlier studies. Radiotelemetry is expected to cause less animal stress relative to externalized wires, which restrain movement and leave an open (possibly inflamed) exit site. Telemetry represents the most physiologically relevant and humane method for monitoring of physiological parameters in conscious, freely moving laboratory animals (Kramer and Kinter, 2003, Mogensen, 2011). Some have claimed that radiotelemetry minimizes the number of animals required for a given study by up to 60–70% (Van Acker et al., 1996). Radiotelemetry can reduce research

costs by increasing the number of variables monitored from a single animal, eliminating the need for separate studies to collect the same data. Further, radiotelemetry use permits virtually continuous data collection for weeks or longer. This latter feature is critical for studies in chronic disease models, such as spinal cord injury. Most importantly, since radiotelemetry permits investigation of drug effects in freely moving animals, it establishes animal models that are more physiologically relevant, giving advantages to subsequent drug studies and, possibly, more reliable clinical translation.

ACKNOWLEDGEMENT

A Navarrete-Opazo was supported by Fulbright scholarship. This work was supported by NIH grants HL89209 and HL69064. We thank Jiro Terada for dedicated mentoring of A Navarrete-Opazo in the early stages of this study.

FIGURES

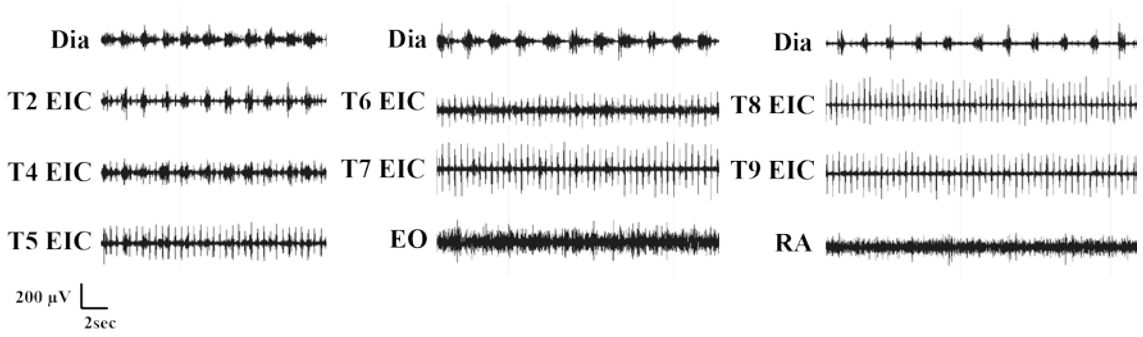

Figure 1. Representative traces of diaphragm (Dia) and external intercostal (EIC) segments T2, T4, T5, T6, T7, T8, T9 as well as abdominal raw EMG activity (RA: rectus abdominus, EO: external oblique) during normoxia in unanesthetized rats. Note: 1) T2 EIC muscle shows inspiratory activity phase-locked with diaphragm; 2) rostro-caudal decrease of inspiratory activity from T2 through T7 EIC muscles; 3) rostro-caudal increase of tonic activity from T2 trough T9; and 4) tonic activity in abdominal muscles.

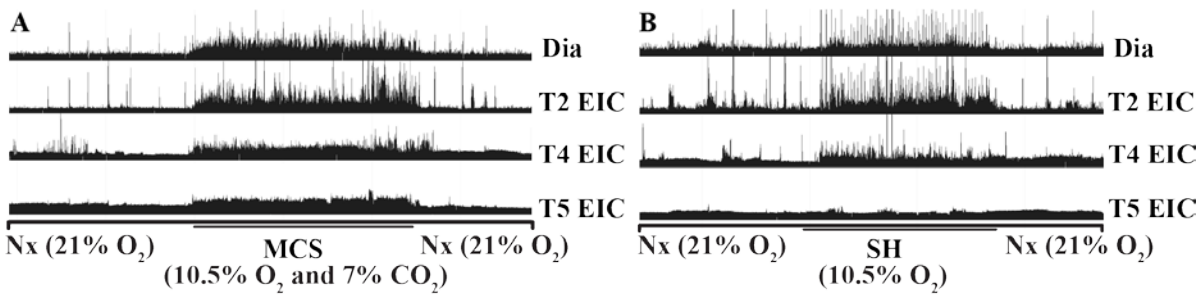

Figure 2. Representative traces of integrated diaphragm and second, fourth and fifth EIC muscle activity in unanesthetized rats during maximum chemoreceptor stimulation (MCS; A) and sustained hypoxia (SH; B) protocols. Note: 1) decreasing rostro-caudal EMG peak amplitude from diaphragm through T5 EIC; 2) MCS elicits a greater EMG activity than SH; 3) T5 EIC does not show increased amplitude during SH. Dia, diaphragm; T2 EIC, second external intercostal muscle; T4 EIC, fourth external intercostal muscle; T5 EIC, fifth external intercostal muscle; MCS, maximum chemoreceptor stimulation; SH, sustained hypoxia; Nx, normoxia.

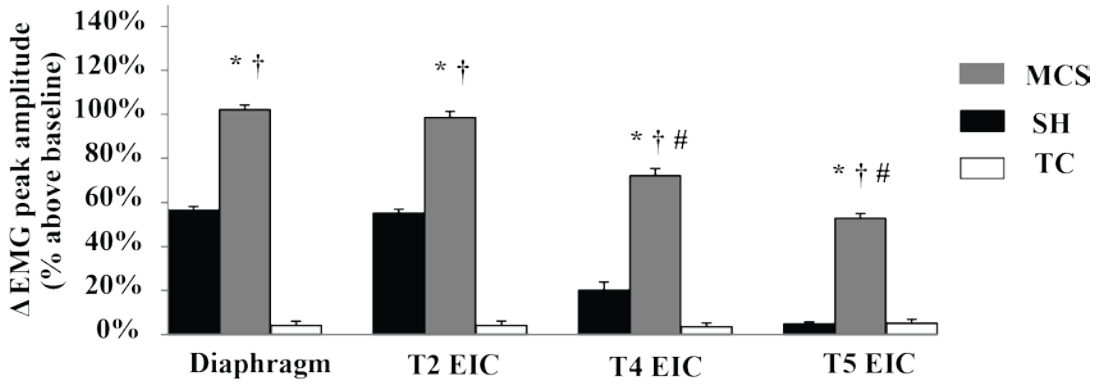

Figure 3. Changes in diaphragm, second, fourth and fifth external intercostal (T2, T4, T5 EIC) EMG peak amplitude expressed as percent change from baseline during maximum chemoreceptor stimulation (MCS) and sustained hypoxia (SH) protocols. Note the rostro-caudal decreasing EMG amplitude from T2 through T5 EIC in both protocols. Values are means ± SEM. * significantly different from SH and TC, † significantly different from baseline. # significantly different from diaphragm and T2 EIC; $p < 0.001$.

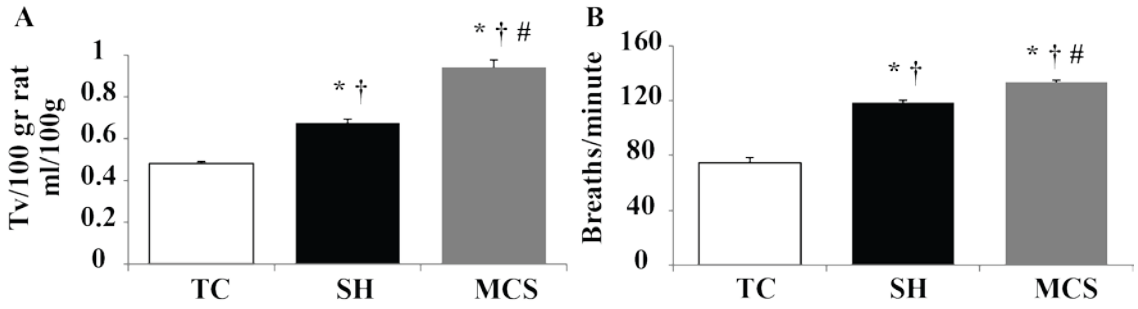

Figure 4. Absolute values of tidal volume (TV, A) per 100 gr rat and respiratory frequency (breaths per minute, B) during maximum chemoreceptor stimulation (MCS), sustained hypoxia (SH) and time control (TC) groups. Note that MCS is a more powerful stimulus for TV and frequency than SH. Values are means ± SEM. *significantly different from TC, † significantly different from baseline, # significantly different from SH; $p < 0.001$.

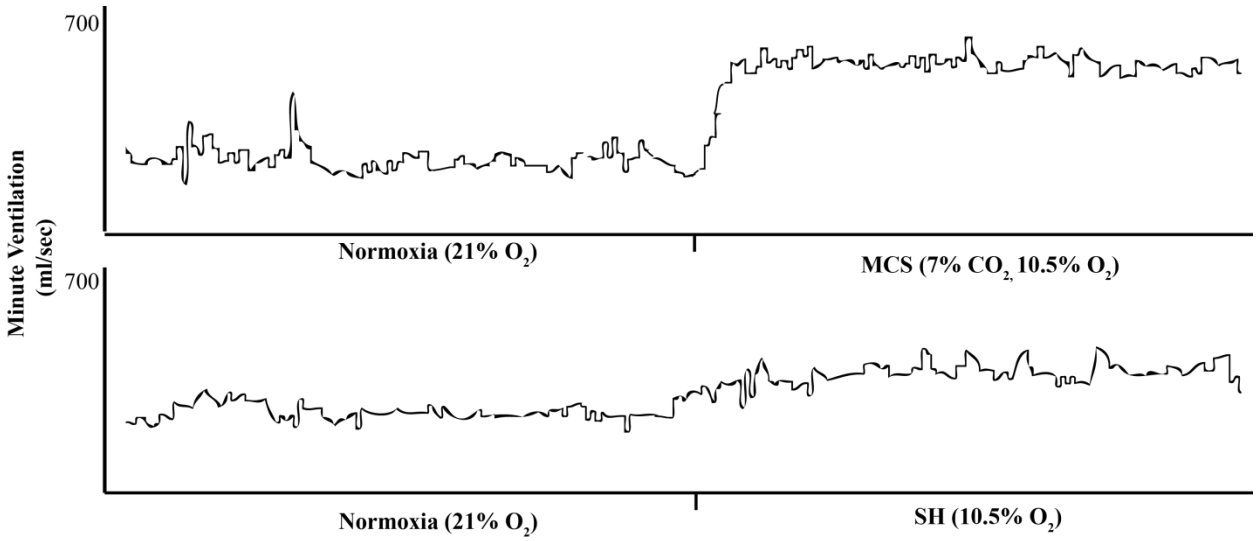

Figure 5. Representative integrated minute ventilation (tidal volume x respiratory frequency, ml/sec) during maximum chemoreceptor stimulation (MCS, top trace) and sustained hypoxia (SH, lower trace). Note the greater minute ventilation elicited by MCS compared to SH.

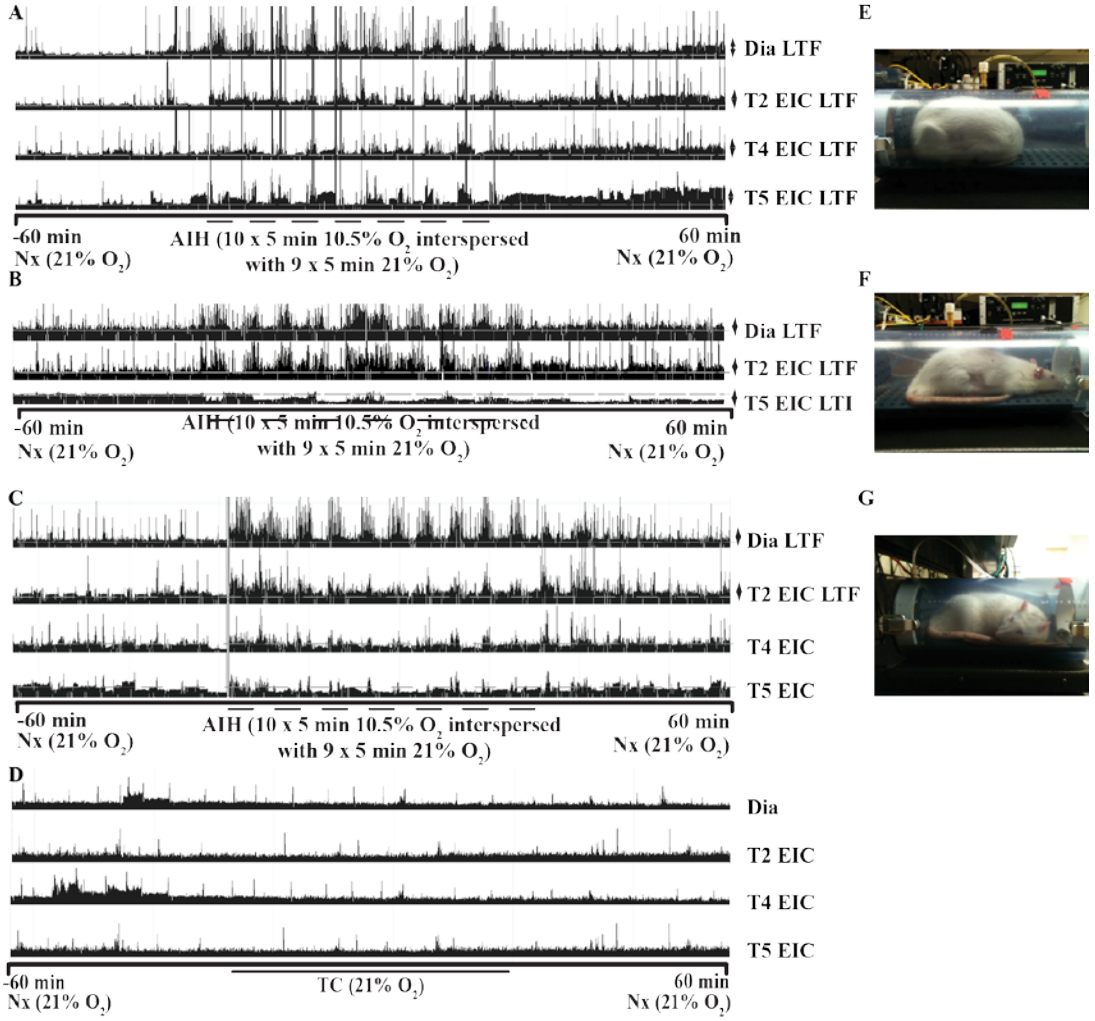

Figure 6. Representative traces of diaphragm (Dia), T2, T4, T5 EIC muscle activity in unanesthetized rats. Integrated Dia and EIC EMG activity before, during and after acute intermittent hypoxia (AIH: trace A, B, C) and normoxia (i.e., time control, TC: trace D) are presented; E) curled-up posture; F) extended posture; G) semi-curled posture. Note: 1) long-lasting increase in diaphragm, T2, T4, T5 EIC amplitude above baseline (*dotted white line*) correspond to diaphragm and T2, T4, T5 EIC long term facilitation (LTF) (*arrows on right*) following AIH, observed when rats adopt a curled-up position (fig. E); 2) long-lasting decrease in T5 EIC amplitude below baseline corresponds to T5 EIC long term inhibition (LTI),

observed when rats adopt an extended position (fig. F); 3) T4 and T5 EIC muscles show no LTF or LTI in trace C, observed with rats in semi-curled position (fig. G). No diaphragm or EIC LTF was observed in TC rats (fig. D).

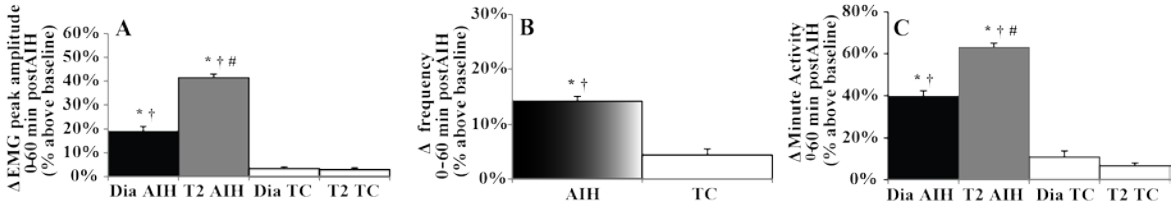

Figure 7. Changes in diaphragm and T2 peak amplitude expressed as percent change from baseline after acute intermittent hypoxia (AIH) and continuous normoxia (time controls, TC) in unanesthetized rats. Significant increase above baseline was observed in diaphragm and T2 EIC EMG amplitude (A), respiratory frequency (B) and calculated values of minute diaphragm and T2 EIC activity (amplitude x frequency) (C) 0-60 min post AIH but not in TC treated (i.e., normoxia) rats, indicative of robust diaphragm and T2 EIC long term facilitation (diaLTF and T2 EIC LTF respectively). Values are means ± SEM. *significantly different from TC, † significantly different from baseline, # significantly different from diaphragm; $p < 0.001$.

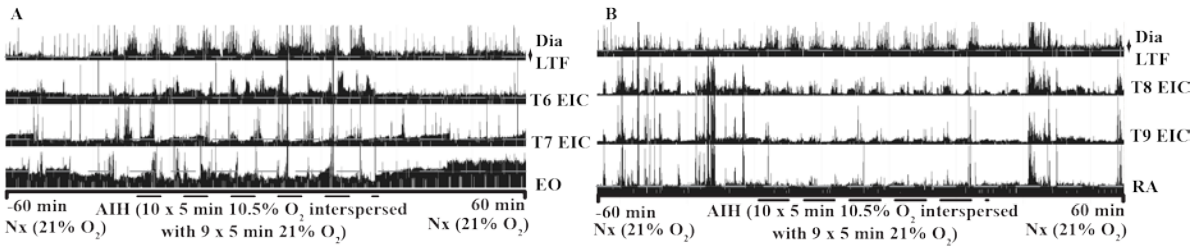

Figure 8. Representative traces of diaphragm (Dia), T6, T7, T8, T9 EIC and abdominal external oblique (EO) and rectus abdominus (RA) muscle activity in unanesthetized rats. Integrated EMG activity before, during and after acute intermittent hypoxia (AIH) are presented for Dia, T6 EIC, T7 EIC and EO muscle (trace A) and Dia, T8, T9 EIC and RA muscle (trace B). Note: (1) long-lasting increase in diaphragm amplitude above baseline (*dotted white line*) corresponds to Dia LTF (*arrows on right*) following AIH; (2) T6, T7, T8, T9 EIC and RA muscles show no change in amplitude after AIH; (3) External oblique muscle shows decrease EMG amplitude during but no after AIH.

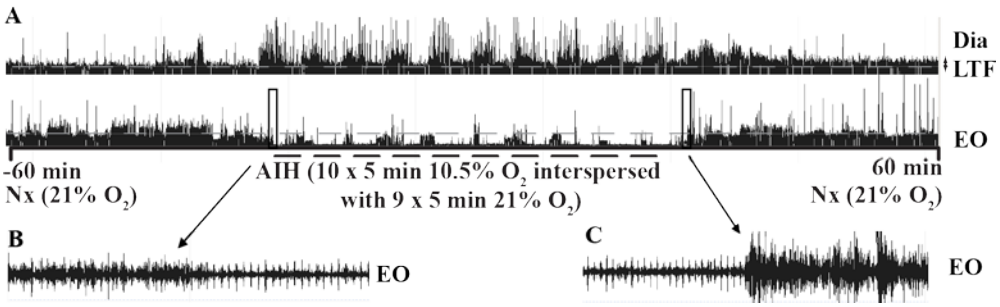

Figure 9. Representative traces of diaphragm and external oblique (EO) muscle activity in unanesthetized rats. Integrated Dia and external oblique EMG activity before, during and after acute intermittent hypoxia are presented (trace A). Raw EO signal in the transition between normoxia and hypoxia (trace B) and hypoxia and normoxia (trace C). Note: (1) long-lasting increase in diaphragm amplitude above baseline (*dotted white line*) corresponds to diaLTF (*arrows on right*) following AIH; (2) External oblique shows a decrease in EMG amplitude during AIH but no during normoxia before and after AIH.

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Chapter IV

Adenosine A_{2A} inhibition enhances acute intermittent hypoxia-induced diaphragm long term facilitation

A. Navarrete-Opazo, S Vinit and G.S. Mitchell

ABSTRACT

Acute intermittent hypoxia (AIH) elicits phrenic, diaphragm and second external intercostal (T2 EIC) long-term facilitation (LTF) in rats. Serotonin-dependent phrenic LTF is constrained by activation of adenosine A_{2A} receptors. After cervical (C2) spinal hemisection (C2HS), phrenic LTF is abolished at 2 weeks, but gradually returns by 8 weeks post-injury. In this study, we tested three hypotheses: 1) systemic adenosine A_{2A} receptor inhibition with istradefylline (KW6002) enhances both diaphragm and T2 EIC LTF in normal, unanesthetized rats; 2) rats with chronic (8 weeks), but not acute (1 week), cervical spinal hemisection (C2HS) express AIH-induced LTF in bilateral diaphragm and T2 EIC muscles; and 3) systemic adenosine A_{2A} receptor inhibition enhances diaphragm and T2 EIC LTF in rats with chronic (not acute) C2HS. Electromyography (EMG) radiotelemetry was used to record activity from diaphragm and T2 EIC during normoxia (baseline, 21% O2) and 60 min following AIH (10, 5-min episodes of hypoxia, 10.5%, 5-min normoxic intervals). Istradefylline significantly enhanced AIH-induced diaphragm LTF (diaLTF) versus AIH alone (33.1 ± 4.6% vs. 22.1 ± 6.4% above baseline, respectively; $p < 0.001$), but had no effect on T2 EIC LTF in normal rats ($p>0.05$). After acute C2HS, neither diaphragm nor T2 EIC LTF was observed. However, with chronic C2HS: 1) AIH-induced LTF was observed in contralateral (uninjured) diaphragm and T2 EIC muscles (18.7 ± 2.7% and 34.9 ± 4.9%, above baseline, respectively; $p<0.05$); and 2) istradefylline had no significant effects on contralateral AIH-induced diaLTF (AIH + KW6002: 23.2 ± 7.0% vs. AIH + vehicle: 18.7 ± 2.7% above baseline; $p= 0.447$) or T2 EIC LTF (AIH + KW6002: 36.1 ± 2.1 and AIH + vehicle: 34.9 ± 4.3% above baseline; $p=0.796$). The expression of ipsilateral (injured) diaLTF and T2 EIC LTF in chronic C2HS rats was highly variable. We conclude that moderate AIH alone or combined with adenosine A_{2A} antagonists could be a useful approach to promote spinal respiratory plasticity and, thus; represent a potential therapeutic strategy to restore respiratory function after chronic cervical spinal injuries.

INTRODUCTION

Plasticity is a fundamental property of the respiratory motor control system (Mitchell and Johnson, 2003). For example, moderate acute intermittent hypoxia (AIH) elicits plasticity in the phrenic motor system (Mahamed and Mitchell, 2007, Terada and Mitchell, 2011, Devinney et al., 2013). AIH-induced phrenic motor plasticity is expressed as a persistent increase in phrenic motor output in anesthetized rats, an effect known as phrenic long-term facilitation (pLTF). Increased diaphragm activity is also observed following AIH in unanesthetized rats instrumented with diaphragm electromyogram (EMG) telemeters (diaphragm long-term facilitation, diaLTF) (Terada and Mitchell, 2011). Similarly, facilitation is observed following AIH in inspiratory EMG activity from the second thoracic external intercostal (T2 EIC) muscle (T2 EIC LTF) (Navarrete-Opazo and Mitchell, 2013). Here we explore the hypothesis that AIH can induce diaphragm and T2 EIC LTF in chronic (not acute) cervical C2 hemisected (C2HS) rats. Further, we test the hypothesis that pretreatment with systemic adenosine A_{2A} receptor antagonist (Istradefylline) enhances diaphragm and T2 EIC LTF in normal and chronic C2HS rats.

Although pLTF requires spinal serotonin receptor activation (Baker-Herman and Mitchell, 2002, Feldman et al., 2003, Baker-Herman et al., 2004, Feldman et al., 2005), concurrent activation of spinal adenosine A_{2A} receptors constrains the expression of serotonin-dependent pLTF following moderate AIH (Hoffman et al., 2010). Thus, both systemic and spinal administration of an adenosine A_{2A} receptor antagonist enhances pLTF in normal anesthetized rats (Hoffman et al., 2010). It is not known if similar enhancement in either DiaLTF or T2 EIC LTF is observed following systemic A_{2A} receptor inhibition in normal unanesthezised rats.

Our lab has cumulative evidence suggesting that there is some potential for distinct mechanisms to give rise to DiaLTF and/or EIC LTF in rats with acute (<4 week) versus chronic (>8 weeks) SCI. For example, spinal serotonergic innervation is transiently reduced following C2HS (Golder et al., 2005), and serotonin-dependent pLTF is abolished at 2, but not 8 weeks post-injury (Golder et al., 2005). Interestingly, 1 week of daily AIH (dAIH, 10 episodes/day for 7 days), beginning 1 week post-C2HS restores breathing capacity in unanesthezised rats (Lovett-Barr et al., 2012), suggesting either: 1) a

mechanism that does not require serotonin receptor activation; or 2) that repetitive AIH is able to overcome the deficit in serotonergic innervation. Preliminary experiments from our laboratory demonstrate that the non-selective serotonin receptor antagonist, methysergide, fails to block functional recovery induced dAIH beginning one week post-injury (Terada, Vinit, MacFarlane and Mitchell, unpublished). Thus, it appears that the functional benefits of dAIH at this early time post-injury may arise from an alternate (possibly adenosinergic) mechanism. We do not yet know whether a single presentation of AIH is sufficient to elicit any degree of diaphragm or EIC LTF at this early time point (7 days), nor do we know if that ability recovers with chronic injuries (8 weeks). If it does, it is of considerable interest to know whether AIH-induced LTF is enhanced by systemic adenosine A_{2A} receptor inhibition (with Istradefylline) since that observation could have considerable therapeutic benefit.

The main purpose of the present investigation is to study, *in vivo*, the effect of systemic adenosine A_{2A} inhibition in AIH-induced diaphragm and T2 EIC LTF in normal and, acute and chronic C2HS unanesthezised rats, using a radiotelemetry system for electromyography (EMG). Specifically, we tested three hypotheses: 1) systemic adenosine A_{2A} receptor inhibition with istradefylline enhances both diaphragm and T2 EIC LTF in normal, unanesthetized rats; 2) rats with chronic (8 weeks), but not acute (1 week), cervical hemisection (C2HS) express AIH-induced LTF in bilateral diaphragm and T2 EIC muscles; and 3) systemic adenosine A_{2A} receptor inhibition enhances diaphragm and T2 EIC LTF in rats with chronic C2HS.

METHODS

Animals

All experiments were performed on 3-4 month old, male Sprague-Dawley rats (310-445 g, colony 211, Harlan, Indianapolis, IN). Animals were individually housed in a controlled environment (12-h light/dark cycle). The Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin approved all experimental procedures in this study.

Experimental preparation

Surgical preparation. For telemetry implantation and cervical hemisection procedures sterile surgery was performed under anesthesia induced with isoflurane in 100% O_2. The rats were injected with buprenorphine (0.03 mg/kg), carprofen (Rimadyl, 5 mg/kg) and enrofloxacin (Baytril, 4 mg/kg) subcutaneously to minimize potential post-operative pain and infection. Body temperature was maintained at 36.5-37.5°C using a rectal probe and external heating pad. A cannula was inserted into the trachea and the animals were artificially ventilated (tidal volume, 2.0-2.5 ml; Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA) with 1.5-2.5% isoflurane in 100% O_2 during surgery. Effective anesthesia was judged by abolition of pedal withdrawal and corneal blink reflexes. Oxygen saturation was monitored by pulse oximetry (model 8600; Nonin Medical Inc. Plymouth, MN) during the surgical procedures. At the end of surgery buprenorphine, carprofen and enrofloxacin at the same dose rats received prior to the surgery were administered at 12 h intervals for 48 h post-surgery. Rats were visually monitored and weighed daily. Additionally, in spinal cord injured rats, animal care included trimming nails after surgery; cleaning fur, eyes and mouth with warm water daily for 7 days to avoid accumulation of porphyrin. Rats had free access to pellets and high caloric nutritional gels inside their cages. In both telemetry implantation and spinal cord injury surgeries, the stainless steel staples were removed 7 days post-surgery. We report no post-surgery complications after telemetry implantation and spinal cord injury surgeries.

Telemetry transmitter implantation. After appropriate anesthesia and pre-operative care (see above), the rat was placed in supine position and the ventral surface of the abdominal muscle was exposed. A sterilized telemetry transmitter body (model 4ET-S1/2; Data Sciences International [DSI], St. Paul, MN) was inserted into the peritoneal cavity. The transmitter allowed simultaneous and continuous monitoring of electrical bio-potentials, body temperature and general locomotor activity. In experiments with normal rats, two bio-potential channels were used to record EMG from right diaphragm and T2 EIC muscle. In the SCI experiments, the four bio-potential channels were used to record EMG activity from bilateral diaphragm and second external intercostal (T2 EIC) muscles. Bilateral diaphragm and T2 EIC implantation technique was developed in this project. First, both right and left hemi-diaphragms were exposed through a midline incision following the Alba line. On both hemidiaphragms, two leads were implanted on the mid-costal area using a 23-G syringe needle guide and tissue adhesive (Vetbond 1469SB; 3M Animal care product, St. Paul, MN) as reported in previous studies (Terada and Mitchell, 2011). Next, right and left second external intercostal muscles were exposed through a 2.5 cm mid-sternum incision, starting in the upper edge of sternum, followed by retraction of pectoralis major and minor on the right and left side. The right and left second intercostal muscle were implanted 1.0 cm right and left from the sternum respectively and the second interspace was identified by counting from the first interspace. The bi-potential lead pairs targeting right and left external intercostal muscles were tunneled subcutaneously from the body of the transmitter placed in the peritoneal cavity. As used in the diaphragm, all the leads in T2 EIC were implanted using a 23-G syringe needle guide and tissue adhesive to keep the leads on place. Finally, abdominal muscles and pectoralis major were sutured in the midline with polysorb 3.0. Skin was closed with wound staples in both ventral thorax and abdomen.

Cervical C2 hemisection: One week after telemetry implantation surgery, spinal hemisections at the second cervical level (C2HS) were performed. The surgical technique was consistent with previous studies (Fuller et al., 2009, Vinit et al., 2009). After appropriate anesthesia and pre-operative care (see above), the spinal cord was exposed at the C2 level via a dorsal laminectomy. The duramater was cut and a left C2 hemisection (C2H2) lesion was induced 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 rats received only laminectomy.

Telemetry. For the AIH protocol (see below) rats were placed in custom-made Plexiglas chambers (see below) positioned on receivers (model RPC-2; DSI, St. Paul, MN). Signals from the implanted radiotelemetry transmitter were detected by the receivers and sent to a data exchange matrix (model ACQ-7700; DSI, St Paul, MN). Four channels of EMG (bilateral diaphragm and second external intercostal), body temperature and general locomotor activity in unanesthezised freely moving rats were monitored during the experimental protocol on a laboratory computer (data acquisition system: PONEMAH Physiology Platform; DSI, St. Paul, MN). EMG analysis was performed with Neuroscore software (DSI, St. Paul, MN) as described below.

Drug preparation. Istradefylline (KW-6002, Sigma-Aldrich) is a selective adenosine A_{2A} receptor ($A_{2A}R$) antagonist with a molecular weight of 384 and a Ki of 29.6 nM in rats. It has a half-life of 110 minutes, 97% availability after intraperitoneal injection and brain concentration of 500 uM after 4 hours (Yang et al., 2007), making it a suitable drug for our *in vivo* experiments. The drug was dissolved in DMSO at 9.3 mg/ml, sonicated and stored at 4°C in a dark vial protected from light. The day of the experiment the drug was administrated via intraperitoneal injection at a dose of 0.5 mg/kg. DMSO was used as vehicle in control rats.

Experimental groups. To investigate the effect of acute intermittent hypoxia (AIH) and adenosine A_{2A} inhibition, normal rats (n=28) were randomly allocated into the following groups: 1) AIH + KW6002, n=8; 2) AIH + vehicle, n=8; 3) Normoxia (Nx) + KW6002, n=6; 4) Nx + vehicle, n=6. Another cohort of rats with C2HS (n=16) was randomly assigned to the following groups 7 days post-surgery: 1) AIH + KW6002, n=4; 2) AIH + vehicle, n=4; 3) Nx + KW6002, n=3; 4) Nx + vehicle, n=3; 5) Sham, n=2. Finally, a third group of rats with of chronic C2HS (8 weeks post-surgery; n=16) were assigned to the

following groups: 1) AIH + KW6002, n=4; 2) AIH + vehicle, n=4; 3) Nx + KW6002, n=3; 4) Nx + vehicle, n=3; 5) Sham, n=2. Both acute and chronic C2HS rats did not receive any treatment before the beginning of the experiments.

Acute intermittent hypoxia protocol. Normoxic (21% O_2) and hypoxic (10.5 % O_2) conditions were established in custom-made chambers (Plexiglas cylinder, 12 x 4 inches id; 1 rat per chamber) by mixing O_2 and N_2 gas via a custom-made computer-controlled system to obtain the desired inspired oxygen concentrations. Within the chambers, CO_2 and O_2 levels were continuously monitored during the entire protocol (O_2 Analyzer, model 17518; CO_2 Analyzer, model 17515; VacuMed Inc, Ventura, CA). Gas flowed through the chamber at a rate of 4 L/min, keeping CO_2 concentration in the chamber less than 0.5% at all times. 95% of the change in O_2 levels within the chamber (i.e., AIH protocol) was achieved in 25 ± 5 sec. In normal rats, experiments were performed 7 days after telemetry implantation surgery. In C2HS animals, EMG amplitude was recorded during resting conditions (normoxia) for 20 minutes one day after C2HS, to assure a complete hemisection. For acute and chronic C2HS, experiments were done 7 days and 8 weeks after C2HS, respectively. At 8:00 am, on the experimental day, rats were placed in the chamber above the signal receivers for 2-hours acclimation, followed by a 1-hour baseline recording. Next, rats received an intraperitoneal (IP) injection of either A_{2A} antagonist (Istradefylline) or DMSO (vehicle) accordingly (see experimental groups above). Once all rats were in the chambers, the experimental groups received the AIH protocol (10, 5-min 10.5% O_2 interspersed with 5-min 21% O_2 intervals, for a total of 95 min, fig.1). The control and sham rats received the time control (TC) protocol (time matched continuous normoxia as AIH protocol). Finally, 1 hour post-treatment recording during normoxia was obtained in both TC and experimental groups. The chamber temperature was kept at 22.5-24.5°C during the entire protocol.

Tissue Processing. To prove the extent of cervical hemisections, each spinal cord was freshly removed after completion of experiments, immersed in paraformaldehyde (4%, overnight at 4°C) and

cryoprotected in increasing concentrations of sucrose (20–30%). Tissues were then frozen in isopentane (-45°C) and stored at -80°C. Longitudinal sections of the spinal cord (C1 to C6, 30 µm thick) were stained with cresyl violet and examined histologically using a light microscope to reconstruct the injury on the transversal plane (fig.4D,E), according to the Paxinos and Watson atlas (Paxinos and Watson, 1998) as was previously shown (Vinit et al., 2006). NIH ImageJ software (National Institute of Health; http://rsb.info.nih.gov/jj) was used to measure and compare the extent of the hemisection among groups.

Data analyses

EMG signal were analyzed with Neuroscore software. Raw signals were filtered (100-624 Hz), rectified, integrated (100 ms) and averaged for each muscle. EMG values during active locomotor activity were excluded in the analysis. Mean peak amplitude, respiratory frequency and calculated values of minute activity (amplitude x frequency) were averaged in all rats before and after AIH treatment for comparison. Data obtained during the pre-treatment period (1 hr.) were used to calculate their respective baseline values. For experiments assessing the spontaneous recovery in diaphragm and T2 EIC muscle after cervical hemisections, values were expressed as a percent change from normalized pre-injury baseline.

EMG amplitude, frequency and minute activity were compared among groups. Statistical comparisons were made for time (pre and post-treatment) and treatment groups (see experimental groups above) using 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 indicated as statistically significant were $p < 0.05$. All values are expressed as means ± SEM.

RESULTS

Enhanced long term facilitation with A2A receptor inhibition

Acute intermittent hypoxia (AIH) caused a sustained increase in diaphragm and second external intercostal (T2 EIC) EMG amplitude 0 to 60 min post AIH (22.1 ± 6.4% and 42.6 + 3.5% above baseline, respectively; $p < 0.001$, figs.2A, B), confirming previous reports of AIH-induced diaphragm and T2 EIC LTF (Navarrete-Opazo and Mitchell, 2013). Systemic adenosine A2A receptor inhibition (KW6002) significantly increases DiaLTF in normal rats (AIH + KW6002: 33.1 ± 4.6% vs. AIH + vehicle: 22.1 ± 6.4% above baseline; $p < 0.001$; fig.1, 2A), demonstrating that A2A receptors constrain diaphragm LTF in unanesthetized rats. However, adenosine A2A receptor inhibition does not increase T2 EIC LTF (AIH + KW6002: 44.1 ± 3.6% vs. AIH + vehicle: 42.6 ± 3.5% above baseline; $p = 0.238$, fig.1, 2B). Respiratory frequency appeared slightly higher in AIH plus KW6002-treated rats versus controls, but this effect was not statistically different from AIH plus vehicle-treated rats (18.7± 2.5% vs.15.3 ± 2.7% above baseline; $p = 0.365$, fig.2C). Finally, diaphragm minute activity LTF was significantly greater in AIH plus KW6002 versus AIH plus vehicle-treated rats (61.9 ± 3.9% vs. 40.4 ± 7.4% above baseline; $p < 0.001$; fig.3). In time controls, no differences in EMG amplitude, frequency or minute activity for were observed in diaphragm or T2 EIC muscles (fig.2).

Reduced ipsilateral (injured) motor activity one day after C2HS

To confirm the extent of C2HS, we photographed the C2 spinal cord through the microscope on the day surgery (fig.4C), and then reconstructed the cervical hemisection at the end of the experiments in perfused tissues (fig.4D, E). One the day after surgery, EMG recordings were recorded for 20 minutes post-surgery to confirm a lack of activity ipsilateral to injury (fig.4B). Reconstructions of cervical hemisections in perfused tissues demonstrated that similar areas (as a percent of total spinal cross-sectional area) were injured in all experimental groups (AIH + vehicle: 49.3 ± 2.9%, AIH + KW6002: 47.7 ± 1.3%, NX + vehicle: 50.2 ± 1. 7%, NX + KW6002: 45.3 ± 3.0%; $p=0.241$). For all groups, the left (injured) diaphragm had greatly reduced EMG signals versus pre-injury values (AIH + vehicle: 9.6 ± 2.4%, AIH +

KW6002: 7.1 ± 1.2%, NX + vehicle: 8.3 ± 2.1%, NX + KW6002: 8.0±2.0% of pre-injury values; $p < 0.001$; fig.4A, B). Similar reduced EMG activity was found in left (injured) second external intercostal muscle after hemisection (AIH + vehicle: 7.7 ± 2.7%, AIH + KW6002: 11.4 ± 1.3%, NX + vehicle: 8.4 ± 2.8%, NX + KW6002: 8.7 ± 1.0% of pre-injury values; $p < 0.001$; fig. 4A, B). Considering that these experiments were done in freely moving, unanesthezised animals, the presence of some small EMG signal after hemisection may correspond to contamination from nearby muscles, as described in similar preparations (Legrand and De Troyer, 1999). Interestingly, in all groups, one day post-injury, we found an increase in EMG amplitude in booth contralateral (uninjured) diaphragm (110.8 ± 1.4% of pre-injury values) and T2 EIC muscle (116.2 ± 0.9%), showing that compensatory mechanisms start early after C2HS.

Acute C2HS rats do not express AIH-induced long term facilitation

Seven days post-C2HS, rats were assigned to experimental groups (see methods). In all groups, contralateral (uninjured) diaphragm baseline (before AIH/NX) EMG amplitude was significantly greater than sham and pre-injury values in the same rats (AIH + vehicle: 121. 7 ± 9.4%, AIH + KW6002: 123.2 ± 7.9%, NX + vehicle, 120.5 ± 20.1%, NX + KW6002: 119.8 ± 6.2%, Sham: 100.94 ± 3.1% of pre-injury values; $p < 0.05$; fig. 6A), demonstrating compensatory plasticity of the uninjured hemi-diaphragm. Some small spontaneous recovery was observed in ipsilateral (injured) diaphragm, which was not different among groups (AIH + vehicle: 19.6 ± 5.3%, AIH + KW6002: 16.0 ± 2.8%, NX + vehicle: 16.9 ± 5.8%, NX + KW6002: 15.7 ± 2.2% of pre-injury values; $p > 0.05$; fig. 7A).

Unlike the contralateral diaphragm, uninjured T2 external intercostal muscle in all groups show no difference in baseline EMG amplitude versus pre-injury values and sham rats (AIH + vehicle: 104.6 ± 3.8%, AIH + KW6002: 102.9 ± 2.5%, NX + vehicle, 101.3 ± 4.5%, NX + KW6002: 100.5 ± 3.7%, Sham: 102.8 ± 5.7% of pre-injury values; $p > 0.05$; fig.6B). In contrast, the ipsilateral (injured) T2 EIC muscle shows a remarkable increase in EMG amplitude seven days post injury with no significant differences among groups (AIH + vehicle: 80.9 ± 4.7%, AIH + KW6002: 78.9 ± 1.0%, NX + vehicle: 83.4 ± 3.2%,

NX + KW6002: 85.0 ± 5.2% of pre-injury values; $p > 0.05$; fig. 7B), consistent with previous studies in anesthetized rats (Dougherty et al., 2012a). It is worth mentioning that compensatory plasticity of uninjured intercostal muscles, observed one day post-hemisection, returns to baseline 7 days post-injury, coinciding with the remarkable spontaneous recovery of injured external intercostal. The presence of early spontaneous compensatory plasticity in right diaphragm and T2 EIC muscle activity after cervical hemisection suggests that contralateral respiratory motorneurons are recruited and increase motor output to compensate for loss of ipsilateral activity. Once this activity is restored close to normal, as in T2 EIC, compensatory mechanisms may return to baseline conditions.

After acute spinal injury (7 days), we compared diaphragm and T2 EIC EMG amplitude before and after AIH (fig. 5B). AIH alone or combined with A2A receptor inhibition does not increase EMG amplitude, compared to baseline, on bilateral diaphragm and T2 EIC muscles (fig. 5,6,7). Thus, acute spinally injured rats do not exhibit diaphragm or T2 EIC long term facilitation.

Long term facilitation is restored with chronic C2HS

Eight weeks post-C2HS, a different cohort of rats assigned to four groups (see methods) was studied. Similar to acute spinal injury, contralateral (uninjured) diaphragm baseline shows EMG amplitudes above pre-injury values, compared to sham rats (AIH + vehicle: 119.7 ± 7.8%, AIH + KW6002: 122.7 ± 3.4%, NX + vehicle, 120.7 ± 9.3%, NX + KW6002: 122.8 ± 6.5%, Sham: 99.5 ± 1.6% of pre-injury values; $p < 0.05$; fig. 9A), showing a slightly stronger compensation versus acute (7 days) spinal injury. Right T2 EIC muscle in all groups showed no difference in baseline EMG amplitude compared to pre-injury values and sham rats (AIH + vehicle: 104.2 ± 4.9 %, AIH + KW6002: 102.7 ± 6.5%, NX + vehicle: 102.5 ± 5.8%, NX + KW6002: 100.4 ± 4.5%, Sham: 102.8 ± 5.7% of pre-injury values; $p > 0.05$; fig. 9B). For all experimental groups, the ipsilateral (injured) diaphragm shows small and similar increase of spontaneous recovery of EMG amplitude (AIH + vehicle: 22.4 ± 6.5%, AIH + KW6002: 24.4 ± 2.1%, NX + vehicle: 19.5 ± 1.3%, NX + KW6002: 18.3 ± 2.3% of pre-injury values; $p > 0.05$; fig. 10A). The ipsilateral (injured) T2 EIC muscles show complete recovery with no significant differences among groups (AIH +

vehicle: 95.2 ± 4.4 %, AIH + KW6002: 99.8 ± 3.0%, NX + vehicle: 99.5 ± 5.23%, NX + KW6002: 99.8 ± 6.4%, Sham: 103.5 ± 3.4% of pre-injury values; $p > 0.05$; fig. 10B), consistent with other studies (Dougherty et al., 2012a).

We compared diaphragm and T2 EIC EMG amplitude before and after AIH. AIH elicits a significant increase in EMG amplitude in both contralateral diaphragm and T2 EIC muscles (18.7 ± 2.7% and 34.9 ± 4.9% above baseline, respectively, i.e., diaphragm and T2 EIC long term facilitation; $p < 0.05$; fig.8, 9A, B). As in normal animals, AIH plus A2A inhibition elicits a greater, although not statistically significant increase in contralateral diaphragm EMG amplitude compared to AIH plus vehicle (23.2 ± 7.0% vs. 18.7 ± 2.7% above baseline, respectively; $p= 0.447$; fig. 9A) and do not have an effect in contralateral T2 EIC EMG amplitude (36.1 ± 2.1 vs. 34.91 ± 4.3% above baseline; $p=0.796$; fig. 9B). There is a great variability in ipsilateral (injured) diaphragm and T2 EIC muscle after AIH. Overall, there is no significant increase of ipsilateral diaphragm EMG amplitude after AIH, compared to controls (AIH + vehicle: 6.9 ± 6.1%, AIH + KW6002: 9.4 ± 7.9%, NX + vehicle: 7.9 ± 2.3%, NX + KW6002: 3.0 ± 2.3% above baseline, $p= 0.914$; fig. 10A). However, two rats from the AIH plus vehicle-treated group and one from the AIH plus A2A antagonist group showed increase in diaphragm EMG amplitude (i.e. diaLTF) (15.8%, 13.8% and 26.1 % above baseline, respectively). Similarly, ipsilateral T2 EIC showed no significant increase in EMG amplitude 0 to 60 minutes post-AIH compared to controls (AIH + vehicle: 11.2 ± 14.6%, AIH + KW6002: 4.1 ± 2.1%, NX + vehicle: 2.9 ± 0.8%, NX + KW6002: 3.0 ± 1.3% above baseline; $p = 0.604$; fig.10B), with the exception of one rat from the AIH plus vehicle treated group (49.2% above baseline, fig.8B).

DISCUSSION

The results of this study provide novel information regarding the effect of adenosine A2A receptor inhibition in AIH-dependent diaphragm and T2 EIC LTF in normal rats. Further, we demonstrate the impact of acute and chronic spinal injury on ipsilateral and contralateral diaphragm and T2 EIC LTF, and the impact of A2A receptor inhibition on that plasticity. We report four major findings: 1) A2A receptor inhibition has variable effects on AIH-induced respiratory muscle LTF in normal rats, enhancing diaphragm but not T2 EIC LTF; 2) spontaneous recovery of T2 EIC muscle activity below a C2HS is remarkable versus the modest recovery observed in diaphragm activity; 3) whereas persistent spontaneous increased motor activity is observed in contralateral diaphragm, only transient increased motor activity is observed in contralateral T2 EIC; and 4) AIH-dependent diaphragm and T2 EIC muscle LTF occurs following chronic (8 weeks) but not acute (1 week) C2HS, and the adenosine constraint to diaphragm LTF is partially restored following chronic C2HS. Collectively, these findings advance our understanding concerning the capacity for plasticity in different respiratory muscles, differential susceptibility of respiratory motor plasticity in distinct respiratory muscles to adenosinergic inhibition, the shifting balance of muscle contributions to breathing following spinal injury with time post-injury, and finally, time-dependent shifts in the capacity for respiratory motor plasticity following cervical spinal injury.

Enhanced diaphragm long term facilitation with A2A receptor inhibition

Enhanced diaphragm LTF following pre-treatment with istradefylline demonstrates that the A2A receptor imposed constraint to moderate AIH-induced phrenic LTF in anesthetized rats (Dale-Nagle et al., 2010, Hoffman et al., 2010) is also expressed in diaphragm LTF in unanesthetized rats. The lack of istradefylline effects on AIH-induced T2 EIC muscle LTF demonstrates that this motor pool is not subject to the same inhibitory interaction with adenosine receptors. Thus, just as with phrenic and hypoglossal LTF (Baker-Herman and Strey, 2011), there are both similarities and differences in factors modulating AIH-induced LTF in the phrenic versus inspiratory intercostal motor pools.

Istradefylline tends to increase diaLTF in rats with chronic C2HS which is consistent with our hypothesis that normal, serotonin-dependent LTF recovers with time post-injury (Golder and Mitchell, 2005). On the other hand, A2A receptor inhibition fails to reveal AIH-induced diaphragm LTF with acute C2HS, most likely since serotonergic innervation of the phrenic motor nucleus is greatly reduced at this time (Golder and Mitchell, 2005). In fact, at this early time post-injury, we suggest that A2A receptor inhibition may even limit the potential for alternative, adenosine dependent respiratory motor plasticity (Golder et al., 2008, Nichols et al., 2012). Thus, a treatment that has either no, or beneficial actions in terms of promoting plasticity in normal rats and rats with chronic SCI may even be detrimental with acute SCI. Something as simple as drinking coffee (a well-known A2A receptor antagonist) may have major, but different consequences with the progression of recovery from a spinal injury. We suggest that AIH elicits diaphragm plasticity via adenosine-dependent (serotonin-independent) mechanisms with early injury (7 days), but serotonin-dependent (adenosine-constrained) mechanism with chronic (8 weeks) cervical SCI.

Our working model of AIH-induced pLTF is that episodic hypoxia activates raphe serotonergic neurons that project to phrenic motor nuclei. Spinal serotonin release during hypoxic episodes subsequently activates Gq protein-coupled 5-HT2 receptors on or near phrenic motor neurons, and initiates intracellular cascades that underlie pLTF (Dale-Nagle et al., 2010). Moreover, activation of adenosine A2A receptors coupled to Gs protein gives rise to phrenic motor facilitation (PMF) (Dale-Nagle et al., 2010). We hypothesize that serotonin receptors coupled to Gq and adenosine receptor coupled to Gs proteins are both activated during AIH, but that Gq protein-coupled receptor signaling normally predominates. Further, we propose that AIH-induced pLTF (serotonin-dependent) and A2A receptor induced PMF (adenosine-dependent) are distinct mechanisms that interact via cross-talk inhibition (Hoffman et al., 2010). Recent evidence demonstrates that the inhibition from the A2A receptor-dependent pathway onto serotonin-dependent pLTF is via protein kinase A (PKA) activation (Hoffman and Mitchell, 2013). The specific mechanisms whereby PKA inhibits the serotonin-dependent pathway remain to be studied.

Ipsilateral (injured) versus contralateral (uninjured) plasticity

Spontaneous recovery of respiratory motor output below a cervical spinal injury can occur through a range of mechanisms, many representing forms of neuroplasticity. Compensation for cervical SCI, for example, can include recruitment of other respiratory muscles with spared innervation; in this case contralateral to the cervical hemisection. One possible mechanism for shifts in the balance of contributions from the different respiratory muscles is the removal of inhibitory sensory inputs onto uninjured (e.g. contralateral) respiratory motorneurons (Teitelbaum et al., 1993, Katagiri et al., 1994, Brichant and De Troyer, 1997). On the other hand, similar adaptations may also (at least partially) restore the contributions of affected motor pools (ipsilateral). One prominent example in the neural control of breathing is spontaneous expression of the crossed phrenic phenomenon (CPP), slowly and partially restoring phrenic/diaphragm activity below a cervical hemisection (Goshgarian, 2003). Here, we observed spontaneous compensatory increases in the motor output of the contralateral (uninjured/right) diaphragm, with a similar (but transient) increase in T2 EIC muscle activity contralateral to injury. Spontaneous recovery was remarkable in ipsilateral (injured/left) T2 EIC muscle, possibly reflecting greater crossed spinal pathways (versus phrenic) through interneurons (Dougherty et al., 2012a). As this spontaneous recovery occurred, contralateral (uninjured/right) T2 EIC activity returned towards normal, possibly in direct response to greater output from the injured side. Spontaneous recovery of diaphragm activity ipsilateral to injury was considerably less robust, and the contralateral activity remained elevated consistent with the idea that fundamental mechanisms shift the balance to intact motor pathways when other pathways are compromised by injury (or disease).

The amplitude and post-injury onset time of the CPP is variable across published reports (Nantwi et al., 1999, Fuller et al., 2006, Vinit et al., 2006, Dougherty et al., 2012b). In the present study, we found that the average of ipsilateral diaphragm amplitude was similar among groups, with a range between 18-22% of pre-injury values in both acute and chronic C2HS rats. Similar studies in anesthetized rats have shown spontaneous recovery of 29%, when expressed as a percent of activity in non-injured animals (Nantwi et al., 1999). In anesthetized rats, the CPP is thought to contribute approximately 16% to tidal

volumes generated after chronic C2HS (Dougherty et al., 2012). We did not explore spontaneous plasticity in the same animal over time to determine whether this recovery is time-dependent between 1 and 8 weeks post-injury. Unlike diaphragm activity on the injured side, we found robust spontaneous recovery of left T2 EIC EMG activity following C2HS. Time-dependent return of inspiratory intercostal activity has been reported previously; by 2 weeks post-injury inspiratory intercostal EMG activity ipsilateral to injury were similar to age-matched, uninjured controls, demonstrating nearly complete functional recovery (Dougherty et al., 2012a).

The CPP has been attributed to activation of a monosynaptic, bulbospinal pathway that crosses the spinal midline caudal to the injury (Goshgarian, 2003). This pathway is anatomically present, but functionally ineffective (Goshgarian, 2003). However, it can be revealed within minutes by depolarizing phrenic motor neurons pharmacologically (Ling et al., 1994). There are also latent, crossed-spinal neural pathways to intercostal motor neurons and their respective interneurons. Anatomical (Saywell et al., 2011) and neurophysiological (Kirkwood et al., 1988) data suggest that thoracic interneurons can relay respiratory synaptic drive across the spinal midline. Thus, spontaneous recovery of T2 EIC activity ipsilateral to C2HS may occur via a similar, but more extensive “crossed spinal” pathway relative to that innervating the phrenic motor nucleus. The greater T2 EIC versus diaphragm recovery suggests that inspiratory intercostal muscles may make relatively greater contributions to functional recovery of breathing capacity after cervical SCI.

Our finding suggests that contralateral respiratory motorneurons are recruited early after hemisection probably to compensate for loss of crossed phrenic and thoracic activity. When the ipsilateral phrenic nerve of C2HS rats is sectioned, contralateral phrenic neurogram amplitudes are elevated above control rats (Golder et al., 2003), suggesting that ipsilateral phrenic afferents post a tonic inhibitory constraint on contralateral phrenic activity. There are other possible mechanisms for these reciprocal relationships, such as changes in the neurochemical environment surrounding contralateral motor neurons due to injury (eg. microglial changes, changes in serotonergic modulation, etc.).

Long term facilitation occurs with chronic, not acute injury

Rats with acute C2HS do not express AIH-dependent diaphragm or T2 EIC LTF, most likely due to disruption of descending serotonergic inputs to spinal respiratory motor nuclei (Golder and Mitchell, 2005) and inadequate levels of hypoxemia to achieve serotonin-independent, adenosine-induced LTF (Nichols et al., 2012). Robust right diaphragm and T2 EIC LTF was observed in chronically hemisected rats, but variability in LTF expression ipsilateral to injury is less easily explained. Golder and Mitchell reported the return of ipsilateral pLTF 8 weeks post-C2HS in two rat strains (Golder and Mitchell, 2005); thus, we had expected similar recovery here. Possible differences include differences in the extent of hemisection (Fuller et al., 2009) variations due to the colony of rats used here (although of the same Sprague Dawley strain), or variable peripheral or spinal inflammation for unknown reasons (Huxtable et al., 2013).

Conclusion

There is a need for alternative approaches, such as pharmacological interventions to alleviate respiratory deficits after spinal injury and other clinical disorders that compromise breathing (Mitchell, 2007). AIH strengthens synaptic pathways to phrenic motoneurons and elicit diaphragm and inspiratory intercostal LTF in chronic SCI rats, offering a means of at least transient restoration of breathing capacity. The potential of using repetitive AIH exposures to achieve this goal has been demonstrated recently by Lovett-Barr and colleagues (2012). Furthermore, since systemic adenosine A_{2A} inhibition enhances diaphragm LTF, combined protocols of moderate AIH combined with A_{2A} receptor antagonists may amplify the therapeutic efficacy of repetitive AIH protocols. One relevant finding here is that this approach may work only with chronic spinal injury. Paradoxically, A_{2A} receptor antagonists may actually be counter-productive when administered too early following injury.

ACKNOWLEDGEMENT

This work was supported by NIH grants HL69064 and HL89209. A. Navarrete-Opazo was supported by Fulbright Scholarship. S. Vinit was supported by Craig H. Neilsen Foundation Fellowship. We thank Dr. Marie Pinkerton for assistance with the reconstruction of cervical spinal hemisection.

FIGURES

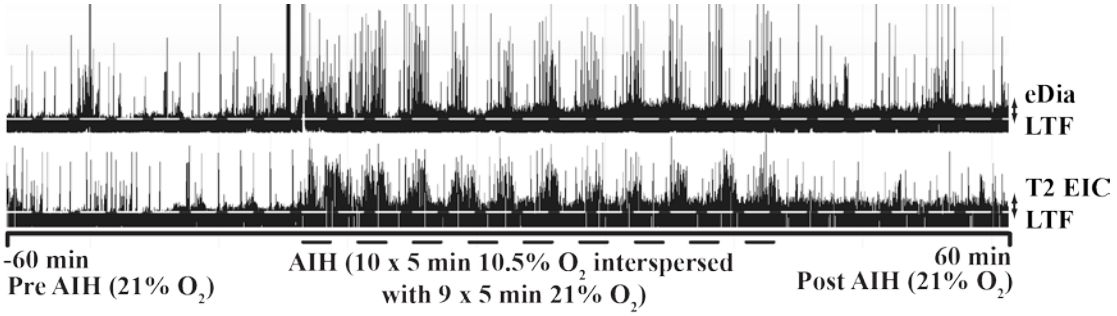

Figure 1: Representative integrated trace depicting right diaphragm (Dia) and right second thoracic external intercostal muscle (T2_EIC) before, during and after 10 episodes of acute intermittent hypoxia (AIH) and A2A antagonist (Istradefylline) in normal unanesthetized rats. Note the increase EMG activity above baseline in Dia, which is quantitatively higher than AIH alone (i.e., enhanced diaLTF, see fig. 2). T2 EIC muscle shows increase EMG amplitude above baseline (i.e., T2 EIC LTF) but not significantly different from AIH alone (see fig.2).

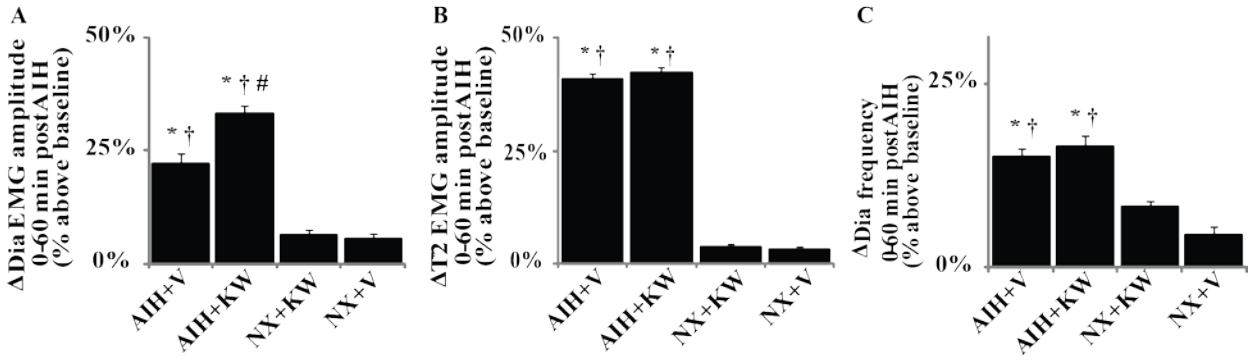

Figure 2: Changes in diaphragm (A), second external intercostal (T2 EIC) muscle (B) amplitude and respiratory frequency (C) expressed as percent change from baseline after acute intermittent hypoxia (AIH) and normoxia (NX) in unanesthetized normal rats. Significant increase in EMG amplitude was observed in diaphragm (A) and T2 EIC muscle (B) 0 to 60 min post AIH plus vehicle, but not in controls (normoxia plus vehicle) rats, indicative of robust diaphragm and T2 EIC long term facilitation (diaLTF and T2 EIC LTF, respectively). Significant increase in diaphragm amplitude was observed in AIH plus adenosine A2A antagonist (KW6002) compared to AIH plus vehicle (i.e., enhanced diaLTF) (A). Such enhancement was not observed in T2 EIC muscle (B). Respiratory frequency was significantly increased in AIH plus KW-6002 and AIH plus vehicle-treated rats, compared to controls (NX+KW6002 and NX+V). AIH: acute intermittent hypoxia; NX: normoxia; KW: KW6002 (istradefylline); V: vehicle (DMSO). Values are means ± SEM. *significantly different from TC, †significantly different from baseline, # significantly different from AIH plus vehicle-treated group; $p < 0.001$.

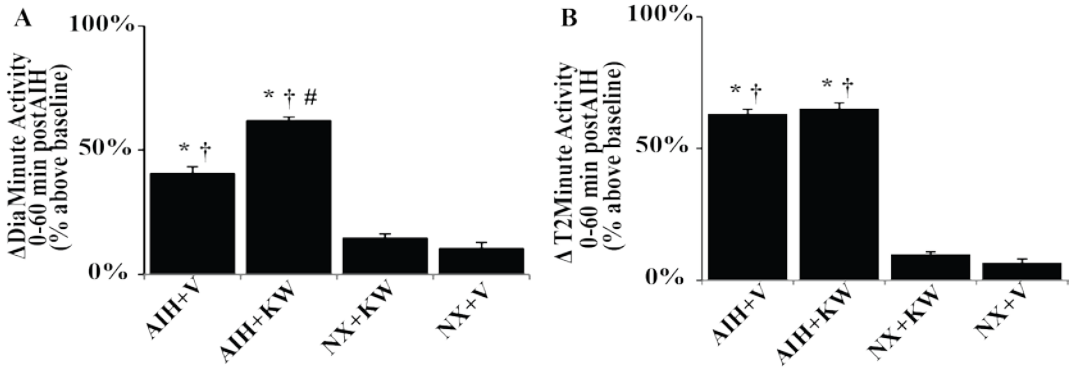

Figure 3: Changes in minute activity (amplitude x frequency) in diaphragm (A) and second external intercostal (T2 EIC) muscle (B) after normoxia (NX) and acute intermittent hypoxia (AIH) in normal unanesthetized rats. Note the significant increase in minute activity in both diaphragm and T2 EIC muscle after 10 episodes of acute intermittent hypoxia (AIH plus vehicle). Adenosine A_{2A} antagonist (KW6002) enhances diaphragm (A) but no T2 EIC (B) minute activity. AIH: acute intermittent hypoxia; NX: normoxia; KW: KW6002 (istradefylline); V: vehicle (DMSO). Values are means ± SEM. *significantly different from controls (NX + V, NX + KW6002), †significantly different from baseline, #significantly different from AIH plus vehicle; $p < 0.001$.

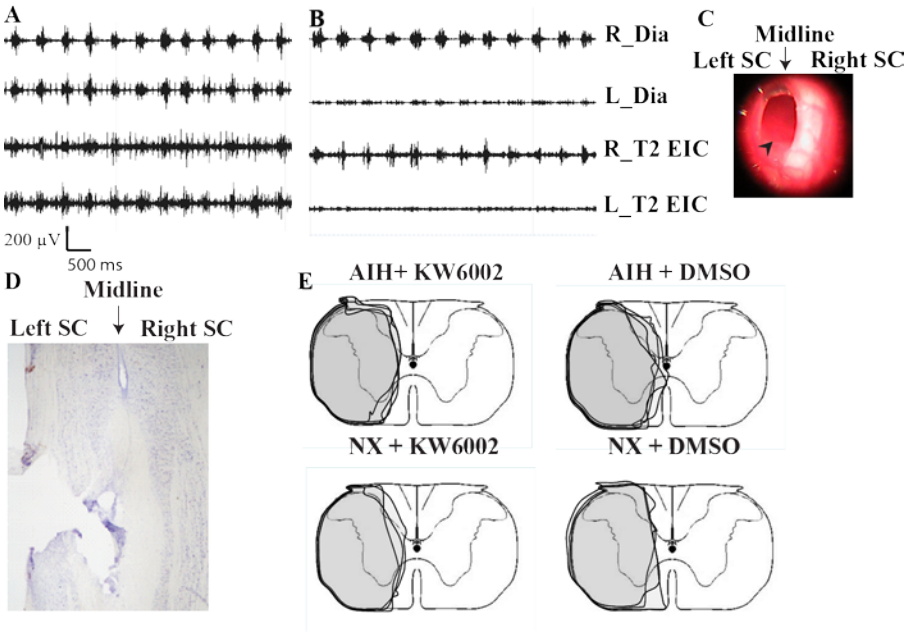

Figure 4: Representative raw traces of right/left diaphragm (R/L_Dia) and second thoracic external intercostal muscle (R/L T2 EIC) before (A) and one day after cervical hemisection (B). Note the significantly reduced EMG activity in left diaphragm and T2 external intercostal, confirming the cervical hemisection ($p<0.001$). (C) Representative photography of left C2 cervical hemisection through microscope 5x objective. (D) Representative longitudinal section of a cervical spinal cord (C1-C6) stained with cresyl violet. (E) Horizontal reconstruction of C2 left cervical hemisections of all studied groups showing no statistical difference among groups ($p=0.241$) AIH: acute intermittent hypoxia; Nx: normoxia; SC: spinal cord.

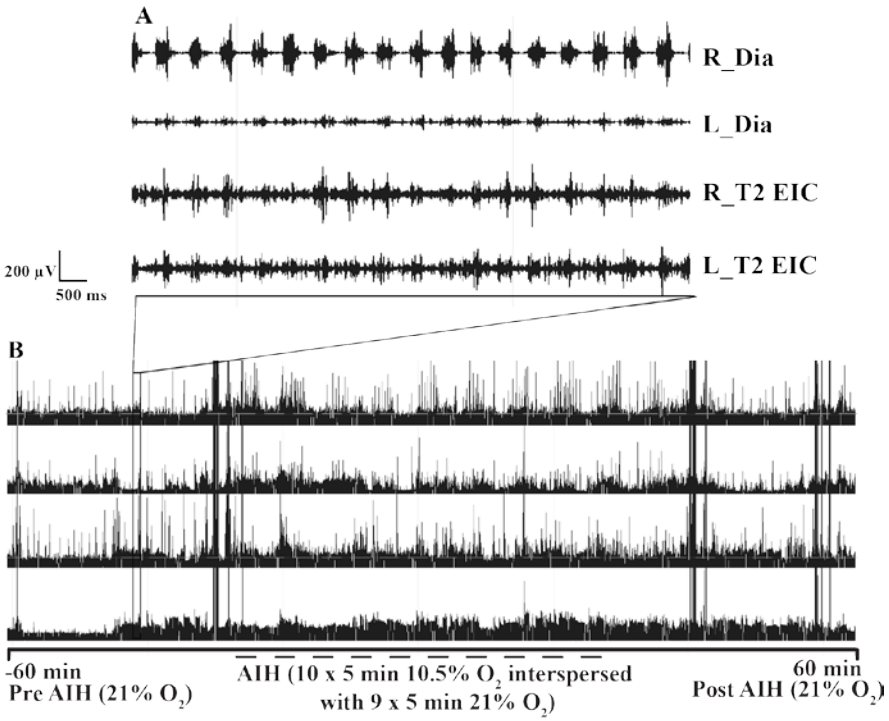

Figure 5: Representative raw (A) and integrated (B) traces of right/left diaphragm (R/L_Dia) and second thoracic external intercostal muscle (R/L_T2 EIC) at 7 days post-injury. Note the loss of AIH-dependent diaphragm and T2 external intercostal long term facilitation in (B). Raw traces show a remarkable left T2 EIC spontaneous recovery but a modest ipsilateral diaphragm activity.

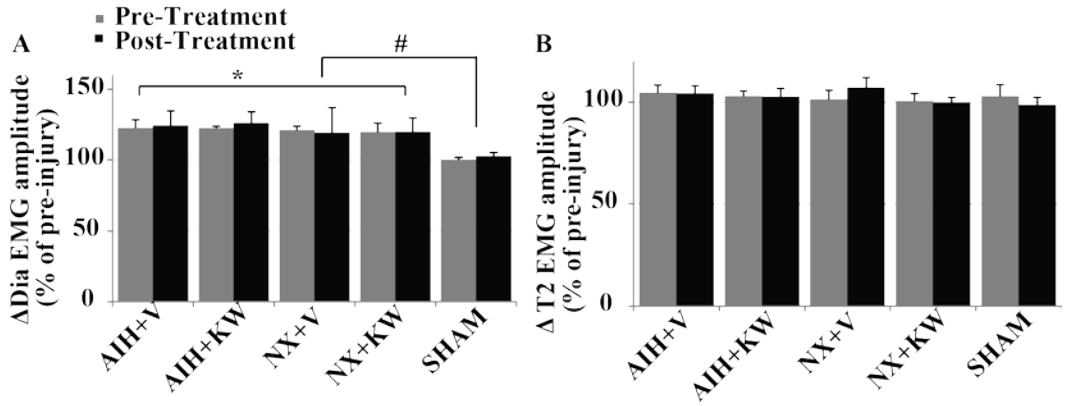

Figure 6: Changes in contralateral (uninjured) diaphragm (A) and second external intercostal (T2 EIC, B) EMG amplitude expressed as percent change of pre-injury values for pre-treatment (during baseline, grey bars) and post-treatment (after AIH/NX, black bars) at 7 days post-injury. In (A), all groups show significant increase in EMG amplitude compared to pre-injury values and sham, demonstrating compensatory plasticity. In (B) T2 EIC muscle show no different EMG amplitude compared pre-injury values and sham. No AIH-dependent diaphragm long term facilitation was observed 7 days post-injury in uninjured diaphragm and T2 EIC muscle. AIH: acute intermittent hypoxia; NX: normoxia; KW: KW6002 (istradefylline); V: vehicle (DMSO). Values are means ± SEM. * significantly different from pre-injury values, # significantly different from sham; $p < 0, 05$.

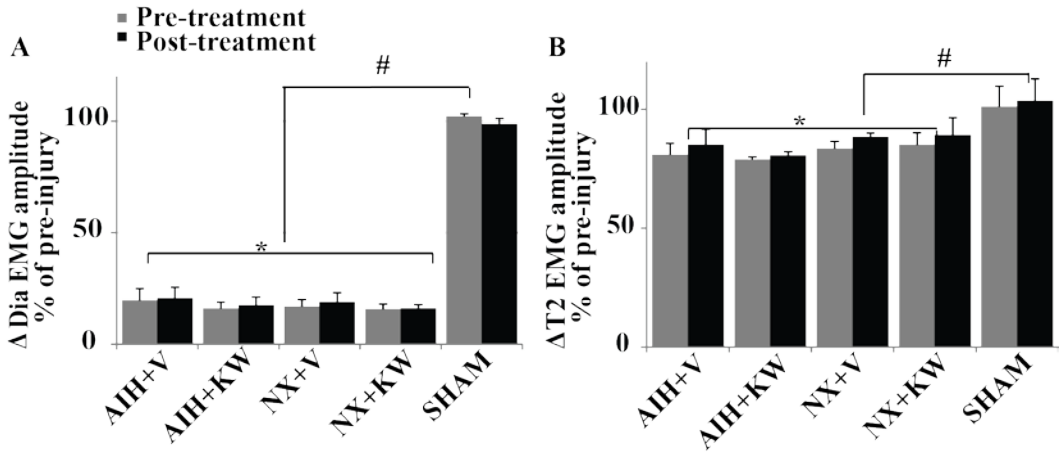

Figure 7: Changes in ipsilateral (injured) diaphragm (A) and second external intercostal (T2 EIC, B) EMG amplitude expressed as percent change of pre-injury values for pre-treatment (during baseline, grey bars) and post-treatment (after AIH/NX, black bars) at 7 days post-injury. Note: (1) in (A) all experimental groups show similar and small EMG peak amplitude (spontaneous recovery) which is significantly different from sham whereas in (B) all groups show remarkable spontaneous ipsilateral T2 EIC EMG peak amplitude; (2) no AIH-dependent diaphragm long term facilitation was observed 7 days post-injury in left diaphragm and T2 EIC muscle. AIH: acute intermittent hypoxia; NX: normoxia; KW: KW6002 (istradefylline); V: vehicle (DMSO). Values are means ± SEM. *significantly different from pre-injury values, #significantly different from sham; $p < 0, 05$.

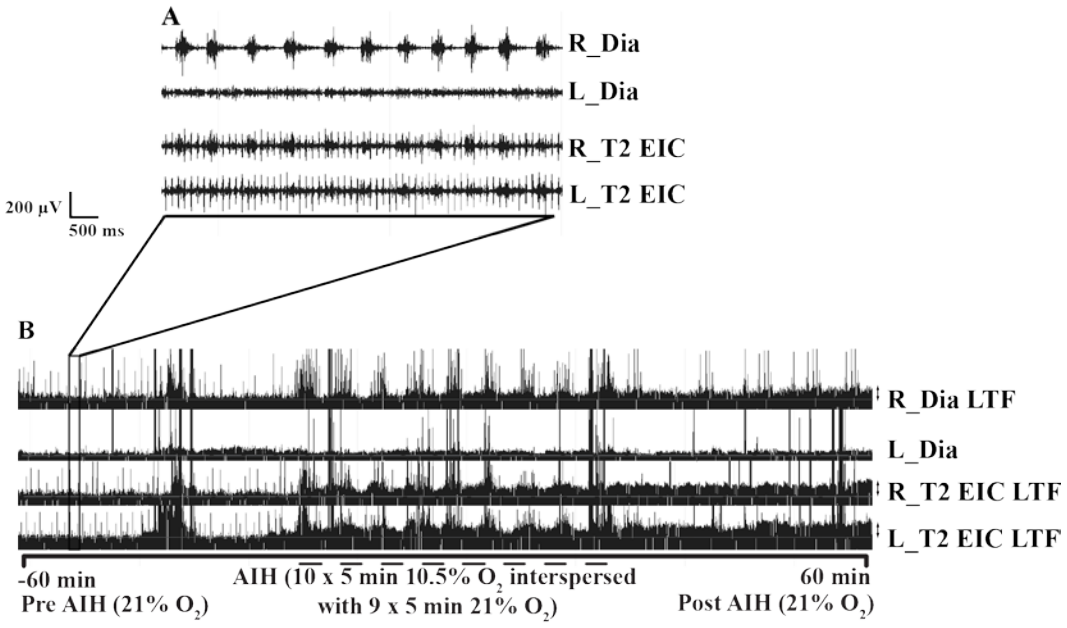

Figure 8: Representative raw (A) and integrated (B) traces of right/left diaphragm (R/L_Dia) and second thoracic external intercostal muscle (R/L_T2 EIC) at 8 weeks post-injury. Note: 1) AIH-dependent contralateral (uninjured) but not ipsilateral (injured) diaphragm long term facilitation (i.e. diaLTF); 2) Bilateral AIH-dependent T2 external intercostal long term facilitation (T2 EIC LTF). Raw traces (A) show complete ipsilateral (injured) T2 EIC spontaneous recovery but a modest ipsilateral diaphragm recovery.

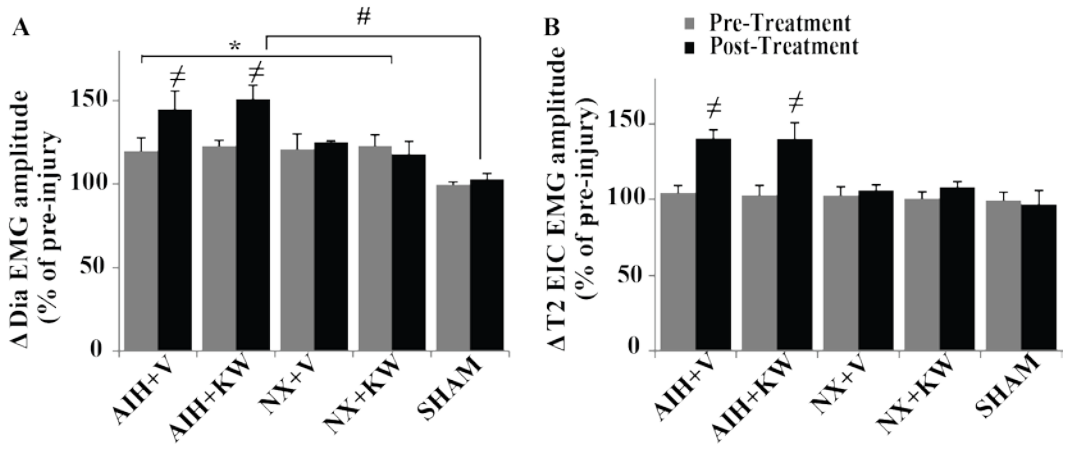

Figure 9: Changes in contralateral (uninjured) diaphragm (dia, A) and second external intercostal (T2 EIC, B) EMG amplitude expressed as percent change of pre-injury values for pre-treatment (baseline, grey bars) and post-treatment (after AIH/NX, black bars) at 8 weeks post-injury. In (A), all groups show significant increase in EMG amplitude compared pre-injury values and sham, demonstrating compensatory plasticity in diaphragm but not in T2 EIC muscle (B). Note: (1) AIH-dependent long term facilitation (LTF) was observed 8 weeks after injury in contralateral diaphragm and T2 EIC muscle (i.e., diaLTF and T2 EIC LTF, respectively); (2) Although adenosine A_{2A} inhibition (KW6002) tends to increase diaLTF, this effect was not statistically significant; (3) Adenosine A_{2A} inhibition did not enhance T2 EIC LTF. AIH: acute intermittent hypoxia; NX: normoxia; KW: KW6002 (istradefylline); V: vehicle (DMSO). Values are means ± SEM. * significantly different from pre-injury values, # significantly different from sham, ≠ significantly different from pre-treatment values; $p < 0.05$.

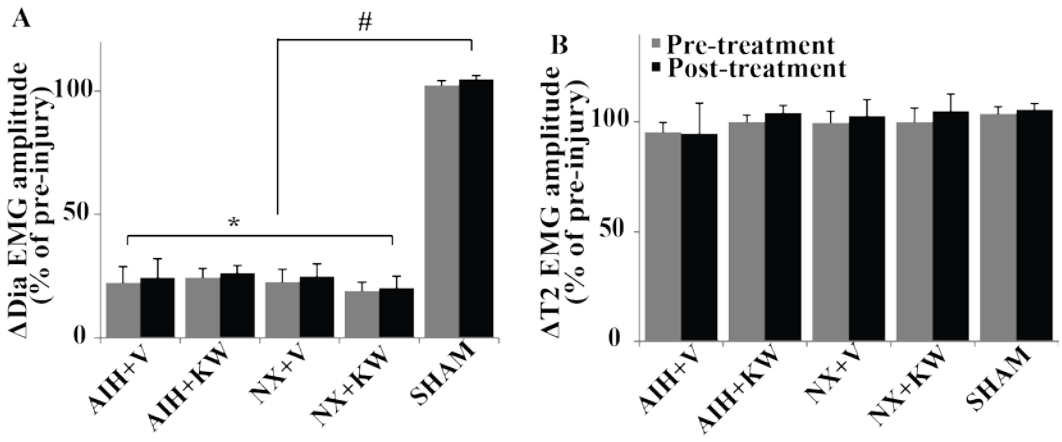

Figure 10: Changes in left (injured) diaphragm (A) and second external intercostal (T2 EIC, B) muscle EMG amplitude expressed as percent change of pre-injury values for pre-treatment (baseline, grey bars) and post-treatment (after AIH/NX, black bars) at 8 weeks post-injury. Note: (1) in (A), all experimental groups show modest ipsilateral diaphragm spontaneous recovery compared to pre-injury values and sham, whereas complete spontaneous ipsilateral T2 EIC recovery is observed in (B); (2) AIH-dependent diaphragm long term facilitation was not observed in injured diaphragm and T2 EIC muscle (see text). AIH: acute intermittent hypoxia; NX: normoxia; KW: KW6002 (istradefylline); V: vehicle (DMSO). Values are means ± SEM. * significantly different from pre-injury values, # significantly different from sham; $p < 0{,}001$.

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Chapter V

Daily acute intermittent hypoxia elicits functional recovery of diaphragm and inspiratory intercostal muscle activity after acute cervical spinal injury in unanesthezised rats

A. Navarrete-Opazo, S Vinit, B.J. Dougherty, and G.S. Mitchell

ABSTRACT

The major cause of mortality following spinal cord injury (SCI) is respiratory failure. Acute intermittent hypoxia (AIH) induces plasticity in respiratory motor system, expressed as diaphragm (Dia) and second external intercostal (T2 EIC) long term facilitation (LTF), DiaLTF is enhanced by systemic adenosine A_{2A} receptor inhibition in normal rats. Here, we studied the ability of repetitive AIH to restore lost activity in Dia and T2 EIC in rats with acute (7 days-7 weeks) cervical C2 hemisections (C2HS). Seven days post-C2HS, rats received daily AIH (dAIH: 7 days of 10, 5-min hypoxic episodes, 10.5% O_2, 5-min normoxic intervals) with or without intraperitoneal A_{2A} receptor antagonist (KW6002), followed by weekly presentations of AIH with/without KW6002. Weekly measurements included ventilation (plethysmography) and electromyography (EMG) from bilateral diaphragm and T2 EIC muscles during: 1) normoxia (Nx: 21% O_2), and 2) maximum chemoreceptor stimulation (MCS: 10.5% inspired O_2, 7% CO_2). dAIH significantly increased tidal volume (V_T) following C2HS during normoxia (dAIH + vehicle: 0.47 ± 0.02, dNX + vehicle: 0.40 ± 0.01ml/100 gr; $p<0.05$) and MCS (dAIH + vehicle: 0.83 ± 0.01, dNX + vehicle: 0.73 ± 0.01ml/100gr; $p<0.001$), which was not affected by Istradefylline. dAIH significantly enhances EMG activity in contralateral (uninjured) diaphragm; which was constrained by Istradefylline during normoxia (dAIH + vehicle: 136.3 ± 1.6%, dAIH + KW6002: 131.0 ± 0.8%, dNX + vehicle: 124.5 ± 1.2%, dNX + KW6002: 125.0 ± 1.5% of pre-injury values; $p<0.05$) and MCS (dAIH + vehicle: 97.1 ± 1.2%, dAIH + KW6002: 93.2 ± 1.0%, dNX + vehicle: 90.1 ± 1.1%, dNX + KW6002: 87.85 ± 1.3% of pre-injury values; $p< 0.05$). dAIH also enhances EMG activity in contralateral T2 EIC during normoxia but this effect was not affected by istradefylline (dAIH + vehicle: 110.5 ± 1.7%, dAIH + KW6002: 109.5 ± 1.9%, dNX + vehicle: 102.8 ± 1.7%, dNX + KW6002: 103.3 ± 1.1% of pre-injury values; $p< 0.001$). dAIH has no significant effect on ipsilateral (injured) diaphragm or T2 EIC EMG amplitude. We conclude that 1) dAIH enhances V_T during normoxia and MCS; which is attributed to an enhanced motor activity in contralateral diaphragm and T2 EIC; 2) dAIH-induced motor recovery is adenosine A_{2A} dependent in diaphragm but not in T2 EIC muscle. Repetitive AIH may be a useful therapeutic approach to enhance respiratory functional recovery with acute SCI.

INTRODUCTION

One of the major life-threatening consequences of cervical spinal cord injury (SCI) is the anatomic and functional disruption of the brainstem-spinal projections that give rise to spinal respiratory motor neuron activity and, thus, breathing (Winslow and Rozovsky, 2003). One approach to enhance respiratory motor function in SCI patients is to harness the capacity to induce plasticity in residual synaptic inputs to respiratory motor neurons (Mitchell, 2007). Intermittent hypoxia strengthens synaptic pathways to phrenic motorneurons (Fuller et al., 2003, Golder and Mitchell, 2005, Lovett-Barr et al., 2012), and induces respiratory motor plasticity (Mitchell et al., 2001a, Feldman et al., 2003, Mahamed and Mitchell, 2007, Devinney et al., 2013) with functional consequences expressed as persistent increases in: 1) phrenic nerve activity in anesthetized rats (phrenic long-term facilitation, pLTF) (Bach and Mitchell, 1996), 2) diaphragm (diaphragm long-term facilitation, diaLTF) (Terada and Mitchell, 2011) and second thoracic external intercostal muscles (T2 EIC LTF) muscle activity in unanesthetized rats (Navarrete-Opazo and Mitchell, 2013), and 3) tidal volume (ventilatory LTF, vLTF) in unanesthetized rats (Olson et al., 2001, Nakamura et al., 2010) and humans (Tester et al., 2014). Further, daily AIH (dAIH: 7 days of 10, 5-min hypoxic episodes, 10.5% O_2, 5-min normoxic intervals) induces recovery of respiratory (Lovett-Barr et al., 2012) and somatic function in rats (Lovett-Barr et al., 2012). We propose that dAIH is a viable option to restore ventilatory deficits and motor function in respiratory muscles (diaphragm/T2 EIC muscle) in acute cervical spinal cord injured rats.

Cervical C2 hemisection (C2HS) interrupts bulbospinal drive to all respiratory muscles distal to the lesion (e.g., ipsilateral diaphragm, intercostal). However, partial return of ipsilateral diaphragm/intercostal activity occurs over a period of weeks to months after C2HS (Nantwi et al., 1999, Fuller et al., 2006, Vinit et al., 2006, Dougherty et al., 2012a, Dougherty et al., 2012b, Mantilla et al., 2013). The extent that crossed phrenic phenomenon translates into a functional increases in respiratory capacity is questionable since three studies suggest that this spontaneous plasticity contributes minimally to tidal volume generating capacity up to 8 weeks post injury (Golder et al., 2003, Fuller et al., 2006, Dougherty et al., 2012b). In contrast, important ipsilateral recovery in rostral intercostal muscles has been reported

(Dougherty et al., 2012a), suggesting that accessory muscles might have a greater contribution to ventilatory capacity after C2HS. Plasticity in contralateral respiratory motor output has also been observed after SCI (Sherrey and Megirian, 1990, Teitelbaum et al., 1993, Katagiri et al., 1994, Brichant and De Troyer, 1997, Muir et al., 1998, Webb and Muir, 2002, Rowley et al., 2005, Mantilla et al., 2013). Thus, another goal of the present study is to investigate the relative contribution of ipsilateral/contralateral T2 EIC and diaphragm motor activity to ventilatory capacity during normoxia and maximum chemoreceptor stimulation (MCS) in acute C2HS rats.

In normal rats, acute intermittent hypoxia (AIH)-induced respiratory plasticity can be elicited by multiple, distinct cellular cascades that interact via cross-talk inhibition (Dale-Nagle et al., 2010, Nichols et al., 2012, Devinney et al., 2013). Thus, although phrenic pLTF induced by moderate AIH is serotonin-dependent (Bach and Mitchell, 1996, Baker-Herman and Mitchell, 2002), it is elicited by adenosine 2A (A_{2A}) receptors following severe AIH (Nichols et al., 2012). With moderate AIH, the normal, serotonin-dependent pLTF is actually constrained by concurrent activation of spinal A_{2A} receptors; thus, spinal or systemic A_{2A} receptor inhibition actually enhances pLTF in anesthetized rats (Hoffman et al., 2010), as well as diaLTF in unanesthetized normal rats (Navarrete Opazo et al., ibid).

In spinal cord injured rats, we hypothesize that different cellular mechanisms underlie acute vs. chronic AIH-induced functional recovery. Shortly after cervical spinal injury, serotonergic innervation of the phrenic motor nucleus is greatly disrupted (Saruhashi et al., 1996, Zhou and Goshgarian, 1999). Thus, serotonin-dependent pLTF cannot be elicited two weeks post cervical spinal hemisection in rats (Golder and Mitchell, 2005); however, with time post injury, both serotonergic innervation and the capacity for serotonin-dependent pLTF are restored (>8 weeks post-injury). Our lab has provided evidence that AIH-induced diaphragm and T2 EIC LTF is expressed 8 weeks but not 7 days post-C2HS (Navarrete-Opazo et al., ibid). Nevertheless, the capacity remains for A_{2A} receptor induced phrenic motor facilitation 2 weeks post-injury (Golder et al., 2008). Seven daily presentations of moderate AIH (dAIH; 10 episodes per day for 7 days, 10.5% O_2, 5 min intervals) at least partially restores breathing capacity 2 weeks post- C2HS (Lovett-Barr et al., 2012). At this early time point, functional recovery may have arisen from cumulative

effects of serotonin-dependent phrenic motor plasticity since there were more, and repeated hypoxic episodes relative to the study reporting that AIH-induced (3, 5 min episodes, 10.5% O_2) fails to elicit pLTF 2 weeks post-C2HS (Golder and Mitchell, 2005). On the other hand, it may be that the greater number of hypoxic episodes triggered instead an adenosine A2A receptor-dependent recovery of respiratory function. These alternatives can be discriminated by the response to systemic administration of an A2A receptor antagonist. The former case predicts that dAIH-induced functional recovery will be enhanced by A2A receptor inhibition, whereas the latter predicts that dAIH-induced functional gains will be attenuated or blocked by this treatment. The respiratory effects of combined A2A receptor inhibition and dAIH 2 weeks post-C2HS have not been explored. Our working hypothesis is that dAIH elicits plasticity and functional recovery via adenosine-dependent mechanisms with acute SCI, but reverts to serotonin-dependent (adenosine-constrained) mechanisms after chronic injury (see Chapter VI). Thus, we predict that systemic administration of an A2A receptor antagonist will impair dAIH-induced recovery of breathing capacity in acute (1-7 weeks) C2HS rats.

The duration of repetitive dAIH-induced functional recovery is not known. Previous studies demonstrated that dAIH-induced respiratory functional recovery last at least 1 day (Lovett-Barr et al., 2012) and preliminary data suggests that the effect of dAIH last one week post-exposure (Terada, Vinit and Mitchell, unpublished). Based on this latter observation, we proposed to extend dAIH-induced functional recovery by exposing our rats to a weekly presentation of AIH (remainder) to “remain” the system and maintain the functional gain elicited by dAIH, as it has been demonstrated in memory behavioral studies in rats (Martin et al., 2010) and humans (Rovee-Collier et al., 1980, Wiltgen and Tanaka, 2013).

Repetitive AIH also appears to affect non-respiratory motor systems. Recently, it has been reported that dAIH improves skilled forelimb function in a horizontal ladder-walking task in rats with cervical SCI (Lovett-Barr et al., 2012), increases leg-strength (Trumbower et al., 2012) and over-ground walking in persons with chronic SCI (Hayes et al., 2013). However, in these studies, intermittent hypoxia was most effective when paired with task-specific training (Hayes et al., 2013), which is an additional factor to

consider. The grooming test (Bertelli and Mira, 1993) utilizes brainstem mediated, stereotypical grooming actions as a means of evaluating somatic motor recovery after injury. Since this test relies on an innate behavior, it does not require pre-training, and the results are unaffected by repeated testing (Berntson et al., 1988, Bertelli and Mira, 1993).

The main purpose of the present investigation is to study the prolonged effects of combined dAIH and systemic adenosine A_{2A} receptor inhibition in unanesthetized rats with acute (1-7 weeks) C2HS. We used simultaneous plethysmography and electromyography (EMG) radiotelemetry from bilateral diaphragm and T2 EIC muscles to assess respiratory function, including time-dependent shifts in the relative contributions of ipsilateral versus contralateral muscles, as well as between diaphragm and inspiratory intercostal muscles. We also assessed grooming behavior to assess a non-respiratory motor behavior. The following hypothesis were tested: 1) dAIH improves breathing capacity (i.e. tidal volume, V_T) and grooming behavior; 2) T2 EIC muscle spontaneous motor recovery (amplitude) is greater than diaphragm motor activity ipsilateral to injury; 3) there is an increase in spontaneous motor activity in diaphragm and T2 EIC muscle contralateral to the injury; 4) dAIH enhances the ability to increase both diaphragm and T2 EIC motor activity during normoxia and maximal chemoreceptor stimulation; 5) systemic adenosine A_{2A} receptor inhibition before each AIH presentation impairs functional recovery of breathing capacity (V_T) and respiratory muscle activity; and 6) weekly AIH presentations for up to 7 weeks post-injury prolong the benefits of dAIH.

METHODS

Animals

All experiments began with 3-4 months old, male Sprague-Dawley rats (310-440 g, colony 211, Harlan, Indianapolis, IN). Rats were individually housed in a controlled environment (12-h light/dark cycle). The Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin approved all experimental procedures in this study.

Experimental preparation

Surgical preparation. For both telemetry implantation and C2 cervical hemisection, sterile surgery was performed under isoflurane anesthesia (in 100% O_2). The rats were injected with buprenorphine (0.03 mg/kg), carprofen (Rimadyl, 5 mg/kg) and enrofloxacin (Baytril, 4 mg/kg) subcutaneously to minimize potential post-operative pain and infection. Body temperature was maintained at 36.5-37.5°C using a rectal probe and external heating pad. A cannula was inserted into the trachea and the animals were artificially ventilated (tidal volume, 2.0-2.5 ml; Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA) with 1.5-2.5% isoflurane in 100% O_2 during surgery. Effective anesthesia was judged by abolition of pedal withdrawal and corneal blink reflexes. Oxygen saturation was monitored by pulse oximetry (model 8600; Nonin Medical Inc. Plymouth, MN) during surgery. At the end of surgery buprenorphine, carprofen and enrofloxacin (see above for dosage) were administered at 12 h intervals for 48 h post-surgery. Rats were visually monitored and weighed daily. Additionally, in spinally injured rats, animal care included trimming nails after surgery, and cleaning fur, eyes and snout with warm water daily for 7 days to avoid accumulation of porphyrin. Rats had free access to pellets and high caloric nutritional gels inside their cages. In both telemetry implantation and spinal cord injury surgeries, stainless steel staples closing the wound were removed 7 days post-surgery. We report no post-surgery complications after telemetry implantation or spinal cord injury surgeries.

Telemetry transmitter implantation. After induction of anesthesia, rats were placed in a supine position and the ventral surface of the abdominal muscle was exposed. A sterilized telemetry transmitter (model 4ET-S1/2; Data Sciences International [DSI], St. Paul, MN) was inserted into the peritoneal cavity. The transmitter allowed simultaneous and continuous monitoring of electrical bio-potentials, body temperature and general locomotor activity. In the present study, the four bio-potential channels were used to record electromyographic (EMG) activity from bilateral diaphragm and second external intercostal (T2 EIC) muscles. Implantations were performed as follow. First, both right and left hemi-diaphragms were exposed through a midline incision following the Alba line. On both hemidiaphragms, two leads were implanted on the mid-costal area using a 23-G syringe needle guide and tissue adhesive (Vetbond 1469SB; 3M Animal care product, St. Paul, MN) as reported in previous studies (Terada and Mitchell, 2011). Next, right and left T2 EIC muscles were exposed through a 2.5 cm mid-sternum incision, starting in the upper edge of sternum, followed by retraction of pectoralis major and minor on the right and left side. The right and left T2 EIC muscles were implanted 1.0 cm right and left from the sternum respectively and the second interspace was identified by counting from the first interspace. The bi-potential lead pairs targeting T2 EIC muscles were tunneled subcutaneously from the body of the transmitter placed in the peritoneal cavity. As used in the diaphragm, all the leads in T2 EIC were implanted using a 23-G syringe needle guide and tissue adhesive to keep the leads on place. Finally, abdominal muscles and pectoralis major were sutured in the midline with polysorb 3.0. Skin was closed with wound staples in both ventral thorax and abdomen.

Cervical C2 hemisection: One week after telemetry implantation, spinal hemisections at the second cervical level (C2HS) were performed. The surgical technique was consistent with previous reports (Fuller et al., 2009, Vinit et al., 2009, Dougherty et al., 2012b). After appropriate anesthesia and pre-operative care, the spinal cord was exposed at the C2 level via a dorsal laminectomy. The duramater was cut and a left C2 hemisection (C2HS) lesion was induced 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. The sham rats underwent a cervical laminectomy without having a spinal injury.

Whole-body plethysmography. Rats were placed individually in a 4 L DSI Plethysmography chamber (model 600-1211-001). Pressurized air flowed through the chamber at 4L/min, allowing control of inspired gas composition. The chamber was positioned onto a receiver (see below for telemetry signal acquisition) to measure EMG and plethysmography simultaneously. Compensated whole body plethysmography was used to assess tidal volume (V_T); compensation was based on chamber temperature (model P/N 60-1210-001) and humidity sensors (model P/N 600-1211-001). The system used a transducer (Buxco, model TRD5700) and a gas analyzer (CWE, Gemini). Plethysmography data were analyzed in 1 min bins during baseline (i.e. normoxia, 20 minutes) and during maximum chemoreceptor stimulation (hypoxia: 10.5% O_2 and hypercapnia: 7% CO_2; 20 minutes). To obtain a more accurate assessment of V_T, we used intraperitoneal temperature given by the telemetry system to compensate for changes in body temperature.

Telemetry signal acquisition. For the AIH protocol (see below), rats were placed in custom-made Plexiglas chambers positioned on receivers (model RPC-2; DSI, St. Paul, MN). Signals from the implanted radiotelemetry transmitter were detected by the receivers and sent to a data exchange matrix (model ACQ-7700; DSI, St Paul, MN). Four channels of EMG, body temperature and general locomotor activity in unanesthetized freely moving rats were monitored during the experimental protocol on a laboratory computer (data acquisition system: PONEMAH Physiology Platform; DSI, St. Paul, MN). EMG signals were sampled at 1200 Hz and analyzed with Neuroscore software (DSI, St. Paul, MN).

Grooming Test. Forelimb grooming function was assessed using a scoring system adapted from Bertelli and Mira (Bertelli and Mira, 1993), originally developed to examine recovery in a rat brachial plexus reconstruction model. Cool tap water was applied to the animal's head and back with soft gauze, and the

animal was returned to the chamber. Grooming activity was recorded with a video camera from the onset of grooming through at least two stereotypical grooming sequences (~2 min), which include (1) licking of the forepaws and face washing, (2) forelimb grooming of the face, (3) repetitive licking of the body, and (4) hindpaw scratching (Berntson et al., 1988). Scoring was done as illustrated in fig.10. Slow motion video playback was used to score each forelimb independently by the maximal contact made while initiating any part of the grooming sequence. The animals were tested after telemetry implantation, after spinal cord injuries and weekly after dAIH exposure until the end of the study.

Drug preparation. Istradefylline (KW-6002, Sigma-Aldrich) is a selective adenosine A_{2A} receptor antagonist with a molecular weight of 384 and a Ki of 29.6 nM in rats. It has a half-life of 110 minutes, 97% bio-availability after intraperitoneal injection, and ability to cross the blood brain barrier (Yang et al., 2007), make it a suitable drug for our *in vivo* experiments. The drug was dissolved in DMSO at 9.3 mg/ml, sonicated and stored at 4°C in a dark vial protected from light. The day of the experiment, the drug was administrated via intraperitoneal injection at a dose of 0.5 mg/kg.

Experimental design. Five days after telemetry implantation, grooming tests were performed, and then simultaneous plethysmography and EMG recordings were made during normoxia (Nx) and maximum chemoreceptor stimulation (MCS) to establish pre-C2HS values. Then, one day after C2HS, the same protocols were done to prove completeness of C2 hemisection.

Seven days post-C2HS, 32 rats were randomly allocated into the following groups: 1) dAIH + KW6002 (n=8), 2) dAIH + vehicle (n=8); 3) dNx + KW6002 (n=6); 4) dNx + vehicle (n=6); and 5) Sham (n=4). Rats in group 1 received a single dose of intraperitoneal injection (0.5 mg/kg) before AIH for 7 consecutive days (daily AIH) starting day 7 after C2HS (1-2 weeks post C2HS) and then weekly presentations of AIH and Istradefylline (3-7 weeks post-C2HS). Group 2 received the same protocol, but Istradefylline was replaced with vehicle (DMSO). During the same time window, rats in group 3 and 4 were exposed to normoxia and A_{2A} antagonist or vehicle, respectively. Sham rats were exposed to

normoxia and did not receive intraperitoneal injections. All groups were compared over time through assessment of weekly video recording of grooming test performance, and simultaneous plethysmography and EMG recordings during normoxia and MCS until the end of the study (fig.1).

Acute intermittent hypoxia protocol. Normoxic (21% O_2) and hypoxic (10.5% O_2) conditions were established in custom-made chambers (Plexiglas cylinder, 12 x 4 inches id; 1 rat per chamber) by mixing O_2 and N_2 gas with a custom-made computer-controlled mass-flow controller system to obtain the desired inspired oxygen concentrations. Within the chambers, CO_2 and O_2 levels were continuously monitored during the entire protocol (O_2 Analyzer, model 17518; CO_2 Analyzer, model 17515; VacuMed Inc, Ventura, CA). Gas flowed through the chamber at a rate of 4 L/min, keeping chamber CO_2 concentration less than 0.5% at all times. 95% of the change in O_2 levels within hypoxic episodes was achieved in 25 ± 5 sec. At 8:00 am, on the experimental day, rats were placed in the chamber for 2-hour acclimation. Next, intraperitoneal injections of either Istradefylline or vehicle were administrated accordingly (see experimental design). Once all rats were in the chambers, the experimental groups were administered the AIH protocol (10, 5-min 10.5% O_2 interspersed with 5-min 21% O_2, for a total of 95 min.). Control and sham rats were administered continuous normoxia (i.e. time controls; TC). Chamber temperature was kept at 22.5-24.5°C during the entire protocol.

Maximum chemoreceptor stimulation. Normoxic (21% O_2), hypoxic (10.5% O_2) and hypercapnic conditions (7% CO_2) were established in plethysmography chambers (see above) by mixing O_2, N_2 and CO_2 gas via a custom-made, computer-controlled mass flow controller system to obtain the desired inspired oxygen concentrations. After 30 min acclimation, baseline during normoxic condition was recorded for 20 minutes, followed by 20 minutes of maximum chemoreceptor stimulation (MCS) (10.5% O_2 and 7% CO_2, 20 min, fig.5A). This protocol enables assessment of maximal chemoreflex stimulated respiratory activity in unanesthetized rats, as demonstrated previously (Navarrete Opazo and Mitchell, ibid). EMGs and whole-body plethysmography were used to measure tidal volume (V_T) and breathing

frequency (f), along with EMG signals.

Tissue Processing. To verify the extent of cervical hemisections, each spinal cord was freshly removed after completion of experiments, immersed in paraformaldehyde (4%, overnight at 4°C) and cryoprotected in increasing concentrations of sucrose (20–30%). Tissues were then frozen in isopentane (-45°C) and stored at -80°C. Longitudinal sections of the spinal cord (C1 to C6, 30 µm thick) were stained with cresyl violet and examined histologically using a light microscope to reconstruct the injury on the transverse plane (Vinit et al., 2006) (fig.5E), according to Paxinos and Watson (Paxinos and Watson, 1998). NIH ImageJ software (National Institute of Health; http://rsb.info.nih.gov/jj) was used to measure and compare the extent of the hemisection among groups.

Data analyses

EMG signal were analyzed with Neuroscore software. Raw signal was filtered (100-624 Hz), rectified, integrated (100 ms) and averaged for each muscle (bilateral diaphragm/T2 EIC muscle). EMG values during active locomotor activity were excluded in the analysis. Absolute values of tidal volume and respiratory frequency were averaged in all rats. EMG amplitude of each muscle was expressed as a percent change from normalized pre-injury values. Grooming test score (0-5) was assessed separately in right and left forelimb.

All variables were compared among groups. Statistical comparisons were made for time (baseline and during MCS) and treatment (see experimental design) using 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 indicated as statistically significant were $P < 0.05$. All values are expressed as means ± SEM.

RESULTS

Significant reduction in ipsilateral motor activity and ventilatory capacity one day after C2HS

To establish pre-injury baseline values, uninjured rats (5 days post telemeter implantation) were exposed to 20 minutes of normoxia followed by 20 minutes of MCS (fig.5A) with simultaneous EMG and plethysmography recordings. One day after cervical hemisection all experimental groups show a significant reduction of tidal volume, compared to pre-injury values, during normoxia (dAIH + vehicle: 0.38 ± 0.01%, dAIH + KW6002: 0.39 ± 0.02%, dNX + vehicle: 0.40 ± 0.02%, dNX + KW6002: 0.40 ± 0.03, Sham: 0.55 ± 0.02 ml/100gr rats, $p>0.05$, fig.2A) and MCS (dAIH + vehicle: 0.74 ± 0.02%, dAIH + KW6002: 0.71 ± 0.02%, dNX + vehicle: 0.72 ± 0.03%, dNX + KW6002: 0.72 ± 0.02, Sham: 1.07 ± 0.02 ml/100gr rats, $p>0.05$, fig.2B).

Respiratory frequency significantly increased one day after C2HS compared to pre-injury values in all groups during normoxia (dAIH + vehicle: 105 ± 2, dAIH + KW6002: 101 ± 2, dNX + vehicle: 102 ± 2%, dNX + KW6002: 105 ± 3, Sham: 90 ± 2 breaths per minute, bpm, $p>0.05$, fig.3A) and MCS (dAIH + vehicle: 148 ± 2, dAIH + KW6002: 146 ± 2, dNX + vehicle: 148 ± 2%, dNX + KW6002: 147 ± 3, Sham: 138 ± 3 breaths per minute, bpm, $p>0.05$, fig.3B), with no significant differences among groups.

Left (ipsilateral/injured) diaphragm and second external intercostal (T2 EIC) motor EMG activity were measured one day post-C2HS during normoxia. There was a significant reduction in inspiratory motor amplitude, compared to pre-injury values, in left diaphragm (dAIH + vehicle: 16.9 ± 1.4%, dAIH + KW6002: 12.9 ± 1.3%, dNX + vehicle: 13.5 ± 2.8%, dNX + KW6002: 12.1 ± 1.6%, Sham: 103 ± 4.1% of pre-injury values; $p>0.05$, fig.8) and left T2 EIC (dAIH + vehicle: 11.0 ± 1.2%, dAIH + KW6002: 9.2 ± 1.6%, dNX + vehicle: 8.2 ± 2.0%, dNX + KW6002: 9.7 ± 3.0%, Sham: 101.2 ± 2.1 of pre-injury values; $p>0.05$, fig.6B), demonstrating the completeness of cervical hemisection (fig.5). Additionally, reconstruction of the left cervical hemisection (C2HS) demonstrated that all groups showed a similar area of injury, expressed as a percentage of the entire spinal cross sectional area at this level (dAIH + vehicle: 48.2 ± 1.3 %, dAIH + KW6002: 46.3 ± 1.0%, dNX + vehicle: 51.0 ± 1.7%, dNX + KW6002: 45.4 ± 1.5%; $p=0.053$, fig 5E).

Right (contralateral/uninjured) diaphragm and T2 EIC muscles show an increase in EMG amplitude (above pre-injury values), one day post-C2HS, demonstrating that compensatory mechanisms start early on after spinal injury. Spontaneous compensation was greater in right diaphragm (dAIH + vehicle: 125.6 ± 2.8 %, dAIH + KW6002: 128.0 ± 3.8%, dNX + vehicle: 123.5 ± 3.5%, dNX + KW6002: 128.2 ± 2.5% of pre-injury values; $p>0.05$, fig 4A) than in the right T2 EIC (dAIH + vehicle: 112.3 ± 1.5 %, dAIH + KW6002: 113.7 ± 2.7%, dNX + vehicle: 111.8 ± 2.5%, dNX + KW6002: 113.4 ± 2.2% of pre-injury values; $p>0.05$, fig 6A) with no statistical differences among groups.

Pre-injury EMG recordings done simultaneously with plethysmography showed a robust MCS response (fig.5A) in both diaphragm and second external intercostal muscle, as demonstrated previously (Navarrete and Mitchell, ibid). During MCS one day post-C2HS, left (ipsilateral/injured) diaphragm and T2 EIC muscle activity were completely abolished (fig.5B). Interestingly, MCS response was also abolished in right (contralateral/uninjured) T2 EIC, and it was significantly reduced in the right diaphragm one day post-injury (dAIH + vehicle: 91.63 ± 1.1%, dAIH + KW6002: 94.68 ± 1.93%, dNX + vehicle: 91.02 ± 1.58%, dNX + KW6002: 93.18 ± 2.34%, Sham: 101.2 ± 2.8% of pre-injury values; $p<0.05$, fig.4B,5B) with no significant differences among groups.

Daily acute intermittent hypoxia enhances ventilatory function

Three days post-dAIH, (i.e. 17 days post-C2HS), dAIH + vehicle and dAIH + KW6002-treated rats showed significant increases in V_T compared to controls and sham rats (dAIH + vehicle: 0.47 ± 0.01%, dAIH + KW6002: 0.46 ± 0.02%, dNX + vehicle: 0.40 ± 0.01%, dNX + KW6002: 0.38 ± 0.03, Sham: 0.56 ± 0.02% of pre-injury values, $p<0.05$, fig.2A) during quiet breathing conditions (normoxia). Although this effect is maintain 24 days post-injury, it was not statistically significant compared to time control groups. Time control rats (dNX + vehicle and dNX + KW6002) show considerable spontaneous recovery which become apparent 24 days post injury and progressively increased up to the 38 days post-injury; at this time, tidal volume of TC rats was not significantly different from sham (fig. 2A). During MCS, AIH-treated rats show a significant increase in V_T at 17 and 24 days post-C2HS and this effect was not

affected by Istradefylline (dAIH + vehicle: 0.83 ± 0.01%, dAIH + KW6002: 0.80 ± 0.02%, dNX + vehicle: 0.73 ± 0.01%, dNX + KW6002: 0.72 ± 0.01, Sham: 1.03 ± 0.02% ml/100gr; $p<0.001$, fig. 2B). At later time-points there were not significant differences among groups during normoxia and MCS.

Respiratory frequency (breaths per minute, bpm) remains significantly greater than pre-injury values at 17 days post-C2HS during normoxia (dAIH + vehicle: 109 ± 2, dAIH + KW6002: 107 ± 2, dNX + vehicle: 104 ± 2%, dNX + KW6002: 105 ± 2, Sham: 92 ± 2 breaths per minute, bpm, $p>0.05$, fig.3A) and then it shows a progressive decrease towards the end of the experiments so that at 45 days post-C2HS all groups were no different from sham rats (dAIH + vehicle: 100 ± 2, dAIH + KW6002: 97 ± 2, dNX + vehicle: 96 ± 2%, dNX + KW6002: 98 ± 2, Sham: 93 ± 2 breaths per minute, bpm, $p>0.05$, fig.3A). Respiratory frequency during MCS remains greater than pre-injury values 17 days post-C2HS (dAIH + vehicle: 149 ± 2, dAIH + KW6002: 151 ± 2, dNX + vehicle: 149 ± 2%, dNX + KW6002: 150 ± 3, Sham: 140 ± 2 breaths per minute, bpm, $p>0.05$, fig.3B) and it maintains similar magnitude up to the end of the study with no differences among groups (fig 3B).

Adenosine A2A receptor inhibition impairs AIH-induced motor activity in diaphragm

Right (contralateral/uninjured) diaphragm shows on average 20-30% increase in EMG amplitude (above pre-injury values) in all groups at all time-points after C2HS (fig.4A). Furthermore, dAIH-treated rats significantly improved motor activity in right diaphragm at 17 days post-C2HS when compared to dAIH + KW6002, controls and sham (dAIH + vehicle: 136.3 ± 1.6%, dAIH + KW6002: 131.0 ± 0.8%, dNX + vehicle: 124.5 ± 1.2%, dNX + KW6002: 125.0 ± 1.5%, sham: 103.3 ± 2.3% of pre-injury values; $p<0.05$, fig.4A). Thus, KW6002 impaired the functional benefits of dAIH at this early time post-injury, suggesting that respiratory motor recovery in diaphragm is A_{2A} receptor dependent.

Right T2 EIC muscle (contralateral/uninjured) shows a small increase in EMG amplitude (10-15% above pre-injury values) one day post-C2HS in all groups but it returns to pre-injury values 17 days post-injury in TC rats. In contrast, dAIH + vehicle and dAIH + KW6002-treated rats shows a significant greater EMG amplitude at 17 days post injury, compared to controls and sham animals (dAIH + vehicle:

110.5 ± 1.7%, dAIH + KW6002: 109.5 ± 1.9%, dNX + vehicle: 102.8 ± 1.7%, dNX + KW6002: 103.3 ± 1.1%, sham: 99.9 ± 1.7% of pre-injury values; $p < 0.001$, fig. 6A). Taken together, dAIH increases motor activity in both contralateral diaphragm and T2 EIC muscle but while adenosine A_{2A} receptor inhibition impaired motor recovery induced by dAIH in diaphragm, it did not have an effect in T2 EIC motor activity, suggesting that enhanced motor activity induced by dAIH is adenosine A_{2A} dependent in diaphragm but not in T2 EIC. Cellular differences between AIH-induced phrenic and thoracic respiratory plasticity remains to be investigated. Furthermore, a weekly presentation of AIH (reminder) was not capable of maintaining the increase in motor activity elicited by dAIH at later time-points in both right diaphragm and T2 EIC muscle.

Acute intermittent hypoxia does not induce ipsilateral motor recovery

Left (ipsilateral/injured) diaphragm shows a small EMG amplitude (~13% of pre-injury values) 17 days post-C2HS with a small increase (~20% of pre-injury values, fig.8) at the end of the study (52 days post-C2HS), consistent with previous studies showing a modest left (ipsilateral) phrenic activity after C2HS (Dougherty et al., 2012b). In contrast, left (ipsilateral/injured) T2 EIC muscle exhibits a remarkable spontaneous recovery (~74% of pre-injury values) 17 days post-C2HS with a small increase (~86% of pre-injury values) at the end of the experiments (52 days post-C2HS, fig.6B). Overall, dAIH alone or combined with adenosine A_{2A} inhibition did not have an effect in ipsilateral diaphragm (fig. 8) and T2 EIC (fig.6B) EMG amplitude in all time-points in acute (1-7 weeks) cervical hemisected rats.

Acute intermittent hypoxia does not enhance grooming behavior in injured forelimb

All rats exhibited normal grooming behavior (score 5, fig.11) after telemetry implantation and before spinal cord injury. One day after spinal injuries, all injured groups show complete paralysis of left (injured) hindlimb and forelimb; these rats were able to groom only with the right (uninjured) forelimb, but not the left forelimb (i.e. score = 0). This effect is consistent with a complete cervical hemisection. All animals showed normal grooming with the right forelimb at all time-points post-C2HS. After dAIH (17

days post-injury), half of the rats from all groups (dAIH + vehicle: n=4 rats, dAIH + KW6002: n=5, dNX + vehicle: n=2, dNX + KW6002: n=3) were able to touch the bottom of the snout with left forelimb (score of 1), which we attribute to a spontaneous improvement in balance of the trunk versus somatic functional recovery. The remaining rats in each group showed no improvement in grooming behavior with their left forelimb (i.e. score 0). Thus, dAIH has no demonstrable benefit in grooming behavior using the affected limb in rats with acute C2HS. The impact of dAIH following less severe injuries (and forelimb impairment) is unknown.

Variable maximum chemoreceptor response after acute spinal cord injuries

Regardless of the extensive spontaneous recovery of left (injured) T2 EIC muscle activity during normoxia, the MCS response was totally absent during the entire study, which may be explained due to a "ceiling effect" where the injured muscle is unable to further increase its motor activity. Similarly, MCS response in left diaphragm was completely abolished, except during the sixth measurement (fig.9B), which shows a variable response ranging from -13.4% to 23.5% of pre-injury values.

MCS response in right (uninjured) T2 EIC muscle is observed 38 days post-injury, with variable amplitude ranging from 13 to 70.8% of pre-injury values, with no significant differences among groups. Right diaphragm showed a reduced but consistent MCS response throughout the entire experiment (fig 4B). dAIH-treated rats significantly improve the MCS response in right diaphragm at 17 days post-injury (fig.4B) when compared to controls and sham rats (dAIH + vehicle: 97.1 ± 1.2%, dAIH + KW6002: 93.2 ± 1.0%, dNX + vehicle: 90.1 ± 1.1%, dNX + KW6002: 87.85 ± 1.3%, Sham: 102.9 ± 1.1% of pre-injury values; $p < 0.05$, fig.4B), effect that is impaired by istradefylline, suggesting that AIH-induced motor recovery in right diaphragm is adenosine A_{2A} dependent (vs. serotonin-dependent) in acute C2HS rats. At later time-points the MCS response in right diaphragm was no different among experimental groups (fig.9).

DISCUSSION

This study has six major findings: (1) spontaneous motor recovery was modest in ipsilateral (left) diaphragm but significant in left T2 EIC muscle during normoxia, suggesting a greater contribution of inspiratory intercostals muscles (vs. diaphragm) to ventilatory capacity during quiet breathing condition; (2) spontaneous increase in motor activity (above pre-injury values) is observed in contralateral (right) diaphragm and T2 EIC muscle beginning one day after C2HS, demonstrating that compensatory mechanisms start early after C2HS; (3) during MCS, only the contralateral (right) diaphragm shows a consistent, although reduced, response; suggesting a greater contribution of right diaphragm (vs. left diaphragm and T2 EIC muscles) to ventilatory capacity during maximum respiratory challenge; (4) dAIH improves breathing capacity (V_T) during normoxia and MCS, but this effect was transient and could not be maintained by weekly AIH "reminders"; (5) dAIH primarily improves contralateral (uninjured) but not ipsilateral (injured) diaphragm and T2 EIC motor activity; 6) adenosine A_{2A} receptor inhibition (istradefylline/KW6002) impairs the increase in motor activity induced by dAIH in contralateral diaphragm but not in T2 EIC muscle; demonstrating that AIH-induced recovery in diaphragm is adenosine A_{2A} dependent (vs. serotonin-dependent) in acute C2HS.

Altered breathing pattern after cervical C2 hemisection

After C2HS, paralysis of the ipsilateral diaphragm reduces tidal volume (V_T) and increases breathing frequency. This shift in breathing pattern is consistent with previous reports in unanesthetized (Fuller et al., 2005, Fuller et al., 2006) and anesthetized rats (Golder et al., 2001b). Changes in breathing pattern after SCI result initially from diminished pre-motor drive to respiratory motor neurons, thus constraining V_T. The frequency changes may either be indirect reflections of reduced vagal feedback, most likely arising lung stretch receptors, and/or altered afferent inputs to the central nervous system disrupted by the injury. A prominent role for the latter mechanism is revealed with rats with cervical spinal contusion undergoing vagotomy, yet still exhibit elevated breathing frequencies (Golder et al., 2011). After cervical SCI, a time-dependent increase in V_T occurs, reflecting the process of spontaneous spinal synaptic

plasticity (Dougherty et al., 2012b, Lovett-Barr et al., 2012). Similarly, we found that VT was substantially reduced one day post-C2HS, but exhibited remarkable spontaneous recovery from 24 days post-C2HS thereafter (fig.2A). In addition, although breathing frequency was significantly higher than sham rats, it exhibits a slow decline from 24 days post-C2HS thereafter (fig.3A). Thus, changes in breathing frequency are at least partially reversible as VT returns towards normal.

As reported previously (Fuller et al., 2005, Fuller et al., 2006, Fuller et al., 2009), SCI rats reveal deficits in ventilatory capacity when challenged with combined hypercapnia/hypoxia (i.e. MCS), confirming that C2HS causes a respiratory deficit characterized by the inability to respond appropriately to respiratory challenges. This deficit in breathing capacity is characterized by diminished ability to increase VT during MCS, with little recovery apparent over time, and minimal effect on breathing frequency (fig.2B).

Daily acute intermittent hypoxia induces respiratory functional recovery

Daily AIH improves VT at 17 days post-injury during normoxia and up to 24 days after injury during MCS (fig.2B). This effect could not be maintained by weekly AIH "reminders," suggesting that a greater frequency of repetitive AIH may be to preserve the functional benefits. To some extent, the initial dAIH-induced recovery was masked by spontaneous recovery during normoxia; thus, dAIH was an accelerant to restoring baseline breathing pattern. In contrast, respiratory frequency did not show significant difference across time, during either normoxia or MCS; thus, the spontaneous recovery of VT and frequency result from different mechanisms.

After C2HS, the ipsilateral (injured) phrenic nerve and/or hemidiaphragm show little inspiratory activity during normoxia (Golder and Mitchell, 2005, Dow et al., 2009) and we confirm this finding. Although spontaneous recovery of the injured diaphragm was small, the T2 EIC muscle showed remarkable recovery, reaching near normal levels consistent with previous studies (Sherrey and Megirian, 1990, Dougherty et al., 2012a). Thus, accessory respiratory muscles may make a relatively greater contribution to preserving/restoring breathing capacity after cervical spinal injury. Further studies are

warranted to determine the mechanisms underlying enhancement of intercostal muscle EMG activity after C2 spinal cord hemisection. The removal of inhibitory reflexes may excite intercostal motoneurons after spinal injury. In dogs, diaphragm paralysis rapidly increases activity of the inspiratory intercostal muscles in compensation (De Troyer, 1998). The increase may be due to the removal of sensory afferents arising from the phrenic nerve that normally inhibit inspiratory intercostal activity (De Troyer, 1998). This reflex is substantial, and bilateral stimulation of C5 phrenic afferents resulted in a 50% reduction in intercostal inspiratory activity (De Troyer, 1998). In our C2 hemisection model, one side of the diaphragm is paralyzed, most likely resulting in a disinhibition of inspiratory output of the intercostal muscles and, thus, indirect activation of these thoracic motoneurons.

Plasticity in contralateral (uninjured) respiratory motor output has been observed previously after spinal cord injury (Sherrey and Megirian, 1990, Teitelbaum et al., 1993, Katagiri et al., 1994, Rowley et al., 2005, Mantilla et al., 2013). With C2HS, right diaphragm and T2 EIC muscle activity increased, reflecting a form of compensatory respiratory plasticity. Presumably these muscles on the uninjured side now assume a much greater proportional role in breathing, at least shortly after injury. Right diaphragm EMG amplitude increases ~25% above pre-injury values and remains elevated throughout the duration of this study. We found similar compensatory plasticity in right T2 EIC muscle (fig.6A), but this compensation had declined by 24 days post-injury, coinciding with recovery of the left (injured) T2 EIC (fig. 6B). Although this reciprocal relationship suggests a causal relationship between these events, its mechanistic basis is not yet clear.

A previous study reported that AIH augmented crossed spinal synaptic pathways (phrenic long-term facilitation; pLTF) at 8 weeks, but not 2 weeks, post-injury (Golder and Mitchell, 2005). The timing of recovery in the ability to elicit pLTF below the injury coincides with recovery of serotonin terminal density in the phrenic motor nucleus below the injury. Consistent with this study, we now show that dAIH did not increase motor activity in ipsilateral diaphragm or T2 EIC. In contrast, dAIH contributed to further compensatory plasticity on the uninjured side (both diaphragm and T2 EIC muscles), where serotonergic innervation of phrenic and thoracic motor nuclei is intact.

Daily acute intermittent hypoxia does not improve automatic grooming behavior

The failure of dAIH to improve automatic grooming behavior differs in some respects from other studies demonstrating improved limb function in both rats and humans (Lovett-Barr et al., 2012, Trumbower et al., 2012). Even a single AIH presentation increases ankle strength (plantar flexion torque) in humans with motor incomplete chronic spinal injuries (Trumbower et al., 2012). Furthermore, dAIH (beginning 4 week post-C2HS) improves forelimb function in injured rats (Lovett-Barr et al., 2012). In this latter study the authors mention that it was somewhat difficult to discriminate the effects of AIH *per se* versus paired training. Thus, our study may differ from that of Lovett-Barr et al. (2012) in that the dAIH was applied shortly after C2HS (beginning 1 versus 4 weeks post-injury), the latter study used combined treatment (dAIH plus daily ladder walking) and the specific tasks differ in their demands on the animal (automatic grooming versus ladder walking). Considering that dAIH mainly increase contralateral motor activity rather than ipsilateral function in diaphragm and T2 EIC, the lack of ipsilateral forelimb recovery in acute C2HS rats is not completely surprising. However, we cannot rule out that the grooming test may not be sensitive enough to detect subtle somatic improvements elicited by dAIH.

Adenosine A_{2A} inhibition impairs dAIH-induced motor activity of diaphragm

We provide the first evidence that dAIH induces functional recovery via adenosine-dependent mechanisms with acute SCI since recovery of diaphragm EMG activity was abolished by daily pre-treatment with istradefylline (fig. 4A, B). In recent unpublished studies, we have also demonstrated that dAIH-induced functional recovery of breathing capacity after acute C2HS does not require serotonin receptor activation (Terada, Vinit, Mitchell, unpublished observations). Thus, we suggest that dAIH induced functional recovery occurs via adenosine-dependent, serotonin-independent mechanisms at this early time post-injury when serotonergic innervation below the site of injury is at its lowest (Golder and Mitchell, 2005). In contrast, we subsequently report that dAIH induced functional recovery is serotonin-dependent and adenosine constrained after chronic SCI (Navarrete-Opazo and Mitchell, ibid).

Following C2HS, single presentations of moderate AIH (3 episodes) restore ipsilateral phrenic nerve

activity, but only if delivered 8 weeks post-injury (Golder and Mitchell, 2005). This time-frame coincides with the return of serotonergic innervation to the phrenic motor nucleus below the injury (Golder and Mitchell, 2005). Interestingly, 1 week of dAIH, beginning 1 week post-C2HS restores ipsilateral phrenic motor output and breathing capacity in unanesthetized rats (Lovett-Barr et al., 2012), suggesting a different serotonin-independent mechanism. Confirming our hypothesis, we demonstrated that Istradefylline constrains dAIH-induced motor recovery at 17 days post-C2HS in contralateral diaphragm. Istradefylline does not have an effect in dAIH-induced motor recovery in T2 EIC, which is consistent with studies in normal rats showing that T2 EIC LTF is unaffected by adenosine A_{2A} receptor inhibition (Navarrete-Opazo et al., ibid).

Despite the effects of Istradefylline on functional recovery of diaphragm activity after acute C2HS, it does not constrain the enhanced tidal volume after dAIH which may be explained due to the compensatory effect of inspiratory accessory muscles, unaffected by adenosine A_{2A} receptor inhibition. Since scalenus medius and intercostal muscles of the cephalic spaces T1, T2, T3 are always inspiratory in the rat (Megirian et al., 1987), dAIH may increase their motor output (as in T2 EIC); which makes inspiratory accessory muscles important contributors to ventilatory capacity.

Variable MCS response after acute spinal injury

Numerous studies have shown that ipsilateral phrenic nerve activity (below chronic C2HS) increases during chemoreceptor challenge (Fuller et al., 2006, Fuller et al., 2009, Dougherty et al., 2012b). These observations are consistent with the hypothesis that the CPP primarily enables respiratory behaviors requiring large V_T, such as a sigh or augmented breath (Golder et al., 2003). However, we found a minimal and variable increase in ipsilateral diaphragm activity at 52 days post-injury. Surprisingly, the MCS response was also abolished in contralateral T2 EIC muscle for up to 38 days post-C2HS, and was significantly reduced in contralateral diaphragm versus pre-injury values. We speculate that contralateral respiratory motorneurons are recruited after hemisection to compensate for the loss of ipsilateral phrenic and thoracic activity and, therefore, they are subject to a “ceiling effect” where they cannot further

increase motor output. A similar effect may explain the lack of MCS response in ipsilateral T2 EIC muscle given its remarkable spontaneous recovery. Taken together, injured T2 EIC motor recovery may have a functional significance during quiet breathing, but not during respiratory challenge in acute C2HS. In contrast, uninjured diaphragm showed a reduced but consistent MCS response at all time-points, demonstrating that contralateral diaphragm has a great functional significance during respiratory challenge. Most importantly, dAIH was able to significantly improve MCS response (and breathing capacity) 17 days post-injury.

Collectively, we found that in freely moving, unanesthetized rats, dAIH is improves respiratory function; however, a weekly reminder of AIH is insufficient to increase functional recovery above control rats over time, suggesting that a greater frequency of AIH exposures is necessary to reach that effect. Moreover, this functional recovery is likely attributed to an increased EMG motor output in right (uninjured) diaphragm and injured T2 EIC muscle versus the injured diaphragm alone as is commonly (implicitly) assumed. Furthermore, Istradefylline impairs motor recovery induced by dAIH of the side contralateral to injury, suggesting that adenosinergic mechanisms play a key role in acute SCI. When systematically administered after SCI, an adenosine A_{2A} receptor-selective agonist (CGS21680) protects from tissue damage, locomotor dysfunction and different inflammatory readouts (Genovese et al., 2009, Paterniti et al., 2011), demonstrating that adenosine A_{2A} receptor activation is beneficial in early injury. Conversely, we hypothesize that adenosine A_{2A} receptor inhibition enhances dAIH-induced respiratory plasticity in chronic SCI rats, which will be explored in the next chapter of this thesis.

Acknowledgement

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FIGURES

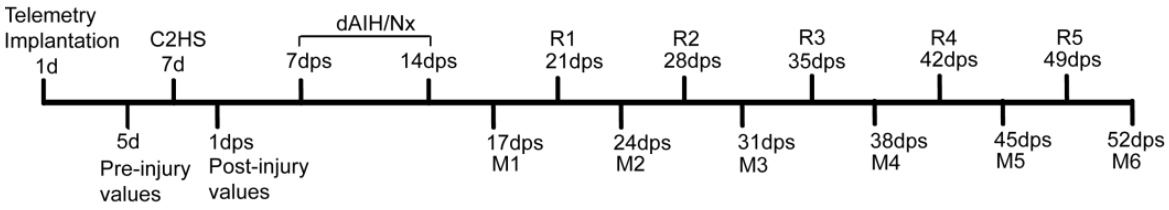

Figure 1: Timeline describing the entire study from day 1 (1d) until 52 days post-spinal cord injury (dps). C2HS: cervical hemisection in second segment, dAIH/dNx: daily acute intermittent hypoxia or normoxia for seven days depending on groups (see methods), R1-R5: weekly presentations “reminders” of acute intermittent hypoxia or normoxia, M1-M6: weekly measurements including grooming test and simultaneous plethysmography and electromyography of bilateral diaphragm and second external intercostal muscles.

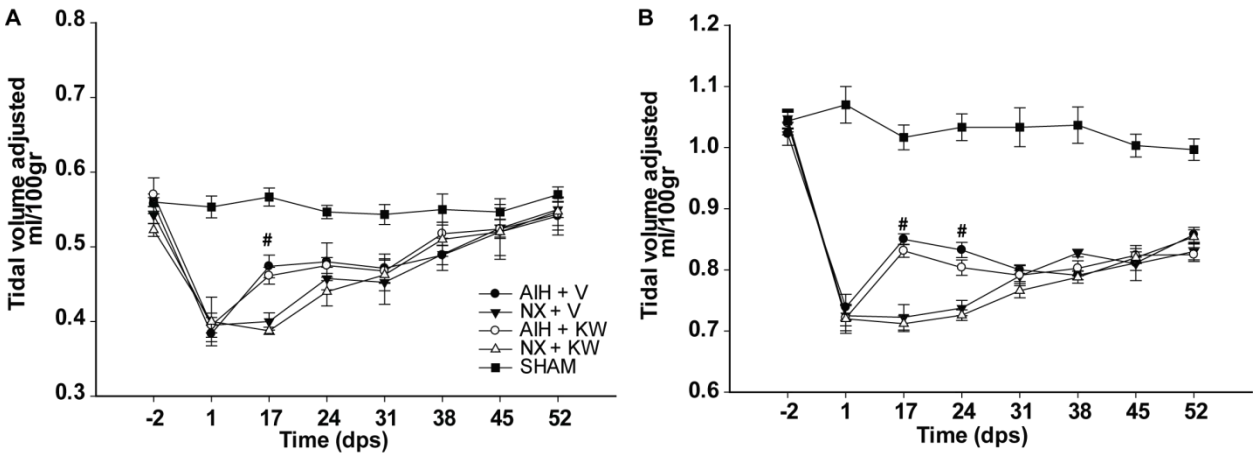

Figure 2: Absolute values of tidal volume (V_T) per 100 gr rat during normoxia (NX, A) and maximum chemoreceptor stimulation (MCS, B) in all groups 2 days before spinal injury and then, 1 day up to 52 days post spinal cord injury (dps). Note: 1) all groups show reduced V_T 1 dps in A and B compared to shams; 2) AIH elicits an increase in V_T during NX and MCS, which was not significantly affected by adenosine A_{2A} antagonist. AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002 (Istradefylline), NX (normoxia). Values are means ± SEM. # significantly different from controls (NX+V and NX+KW6002); $p < 0.05$.

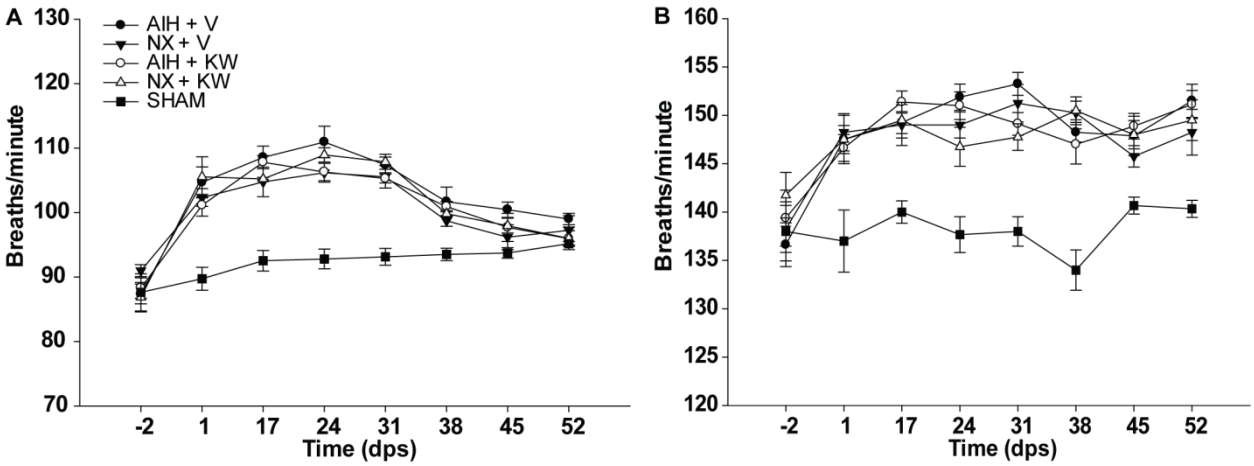

Figure 3: Absolute values of respiratory frequency during normoxia (NX, A) and maximum chemoreceptor stimulation (MCS, B) in all groups studied 2 days before spinal cord injury, and one day through 52 days post spinal cord injury (dps). Respiratory frequency is significantly increased in all groups, compared to sham during normoxia and MCS. AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002 (Istradefylline).

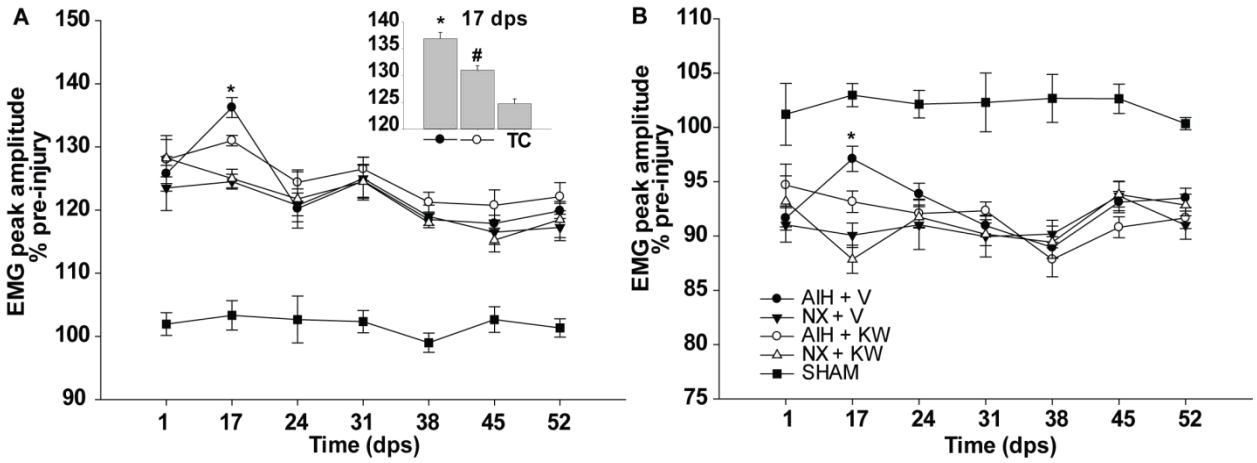

Figure 4 : Changes in contralateral (uninjured) diaphragm muscle amplitude during normoxia (Nx, A) and maximum chemoreceptor stimulation (MCS, B) expressed as percent change of pre-injury values one day up to 52 days post injury (dps). Note: 1) all groups show significant increase in EMG amplitude compared to sham and pre-injury values during Nx in A, demonstrating compensatory plasticity; 2) reduced MCS response in all groups in B; 3) dAIH significantly increases EMG amplitude at 17 dps during Nx and MCS and this effect is impaired by Istradefylline. AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002 (Istradefylline).Values are mean ± SEM.* significantly different from AIH + KW6002 and time controls (TC: NX+V and NX+KW6002), # significantly different from AIH + V and TC; p < 0, 05.

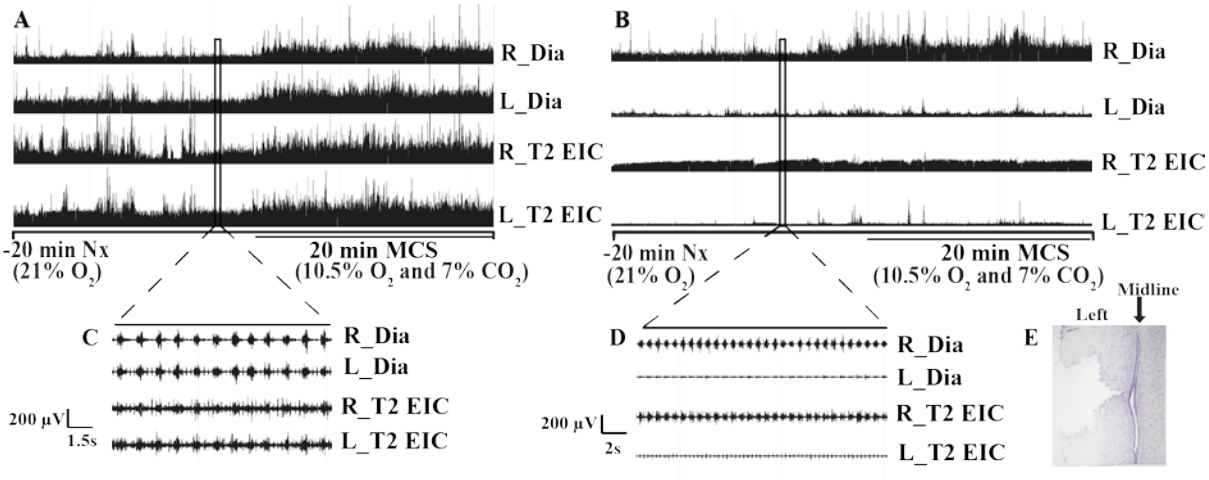

Figure 5: Representative integrated (A and B) and raw (C and D) EMG activity of right/left diaphragm (R/L_Dia) and second external intercostal muscle (R/L_T2 EIC) before (A) and one day after (B) spinal hemisection (C2HS) during maximum chemoreceptor stimulation (MCS). Note: 1) significantly reduced EMG activity in ipsilateral diaphragm and T2 external intercostal in (D), confirming the cervical hemisection; 2) absence of MCS response in injured (left) diaphragm and bilateral T2 EIC muscle in (B). (E) Representative longitudinal spinal cord slide (C1-C6) stained with Cresyl violet showing C2 hemisection.

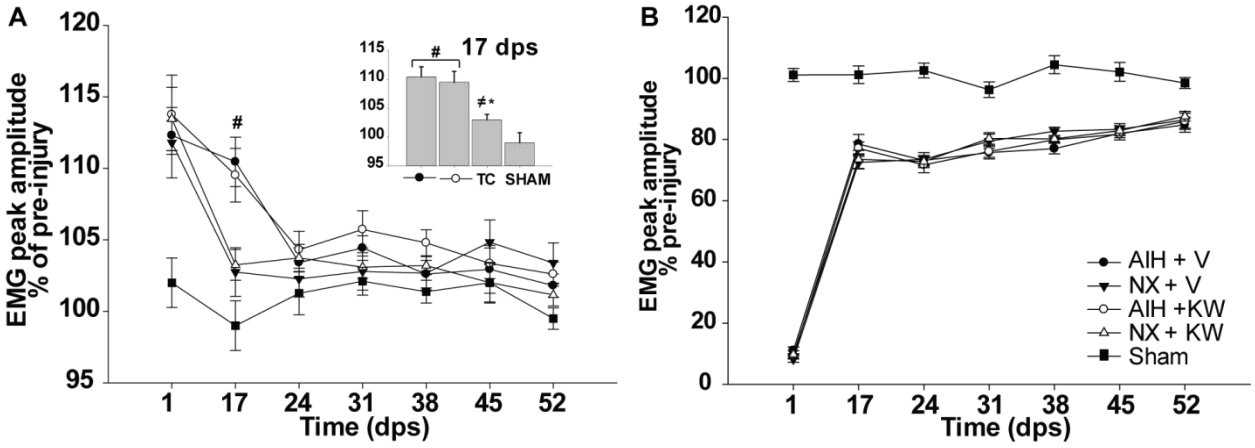

Figure 6: Changes in contralateral (uninjured, A) and ipsilateral (injured, B) second external intercostal muscle (T2 EIC) muscle depicting EMG amplitude expressed as percent change of pre-injury values one day up to 52 days post spinal cord injury (dps). Note: 1) right T2 EIC exhibits a transitory compensatory plasticity in all groups at 17 dps; 2) daily acute intermittent hypoxia (dAIH) increases motor activity at 17 dps in contralateral T2 EIC and this is not affected by adenosine A_{2A} inhibition; 3) spontaneous recovery is remarkable in ipsilateral T2 EIC and not affected by dAIH and A_{2A} inhibition. AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002 (Istradefylline).Values are mean ± SEM, # significant different from time controls (TC: NX+V and NX+KW6002) and sham rats, ≠ significantly different from dAIH-treated groups, * significantly different from sham; $p < 0.001$.

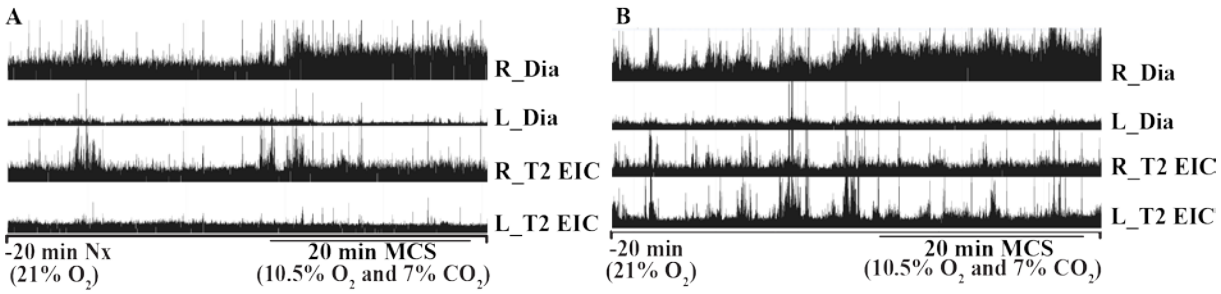

Figure 7: Representative integrated EMG activity of right/left diaphragm (R/L_Dia) and second external intercostal muscle (R/L_T2 EIC) at 17 (A) and 24 days post injury (dps) during maximum chemoreceptor stimulation (MCS). Note MCS response is abolished in left diaphragm and bilateral T2 EIC muscle.

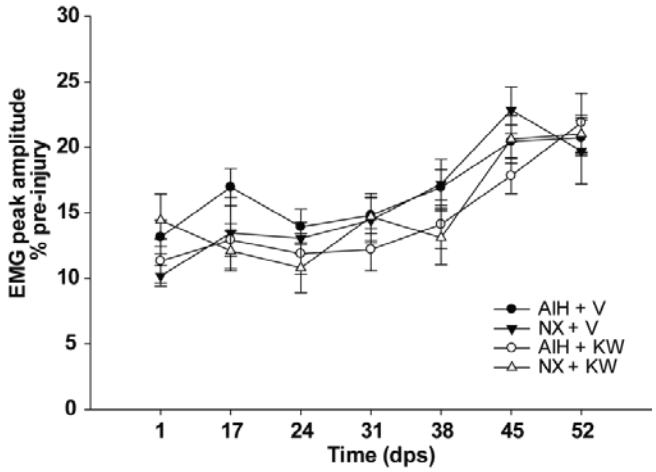

Figure 8: Changes in ipsilateral (injured) diaphragm EMG amplitude expressed as percent change of pre-injury values one day up to 52 days post spinal cord injury (dps). Note: 1) small the time-dependent spontaneous recovery in all groups, 2) daily AIH does not increase diaphragm motor activity. AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002 (Istradefylline).Values are mean ± SEM.

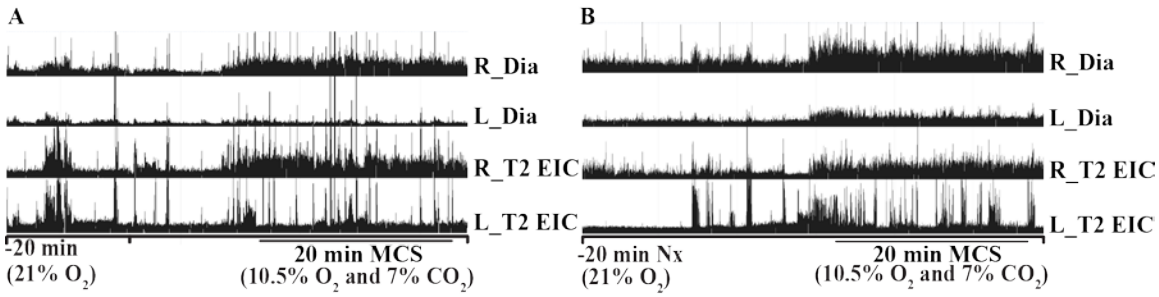

Figure 9: Representative integrated EMG activity of right/left diaphragm (R/L_Dia) and second external intercostal muscle (R/L_T2 EIC) at 45 (A) and 52 days (B) after spinal cord injury during maximum chemoreceptor stimulation (MCS). Note: 1) small and variable MCS response in left diaphragm and right T2 EIC; 3) the MCS response in left T2 EIC remains abolished at all this time-point studies.

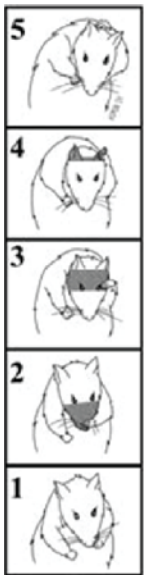

Figure 10: Grooming Test scoring.

5=animal is able to contact the back of the ears and/or the area behind the ears.

4= animal is able to contact the front, but not the back of the ears.

3=animal is able to contact the dorsal surface of the head from (and including) the eye to the front (but not including) the ears.

2=animal is able to contact the dorsal surface of the snout, below the level of the eye.

1=animal is able to reach the underneath of the snout.

0=animal does not contact head during grooming.

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Chapter VI

Repetitive acute intermittent hypoxia and adenosine A_{2A} receptor inhibition elicit functional recovery of breathing capacity with chronic cervical spinal injury

A. Navarrete-Opazo, B.J. Dougherty, and G.S. Mitchell

ABSTRACT

We recently demonstrated that daily exposure to acute intermittent hypoxia (dAIH) partially restores lost breathing capacity in rats two weeks post cervical spinal hemisections at C2 (C2HS). After acute C2HS, serotonin-dependent phrenic motor plasticity is no longer possible due to diminished serotonergic innervation below the injury; thus, dAIH elicits functional recovery of diaphragm (Dia) activity by a mechanism that is serotonin-independent, but adenosine-dependent. With time, serotonergic innervation below C2HS returns towards normal. Here, we tested the hypothesis that dAIH-induced respiratory functional recovery reverts to a serotonin-dependent, adenosine constrained mechanism with chronic C2HS. Rats with chronic C2HS received daily AIH for 7 days (dAIH: 10, 5-min hypoxic episodes, 10.5%, 5-min normoxic intervals) with or without systemic pretreatment with the A2A receptor antagonist Istradefylline (KW6002) starting 8 weeks post-C2HS. After dAIH exposure, rats received 3 times per week (3xw) presentations of AIH as "reminders" with/without Istradefylline for the duration of the experiment. We made weekly assessments of ventilation (tidal volume, V_T) and bilateral Dia and T2 external intercostal (EIC) electromyography during normoxia (Nx: 21% O_2) and maximum chemoreceptor stimulation (MCS: 10.5% O_2, 7% CO_2). dAIH increased V_T vs. controls during Nx (dAIH + vehicle: 0.57 ± 0.01, dNX + vehicle: 0.52 ± 0.01, dNX + KW6002: 0.53 ± 0.04 ml/100g, $p<0.05$), and adenosine A_{2A} inhibition significantly enhance such effect (dAIH + KW6002: 0.60 ± 0.08 vs. dAIH + vehicle: 0.57 ± 0.01 ml/100g, $p=0.031$). Adenosine A_{2A} inhibition significantly increased dAIH-induced motor recovery in contralateral Dia (dAIH + vehicle: 138.25 ± 5.38%, dAIH + KW6002: 157.50 ± 5.32%, dNX + vehicle: 127.0 ± 2.94%, dNX + KW6002: 119.25 ± 3.19% of pre-injury values; $p<0.05$) and ipsilateral Dia (dAIH + vehicle: 36.12 ± 4.76%, dAIH + KW6002: 56.0 ± 7.32%, dNX + vehicle: 21.0 ± 1.78%, dNX + KW6002: 16.25 ± 0.80% of pre-injury values, $p<0.001$), effect that was maintained by AIH 3xw for 12 weeks post-C2HS. We conclude that 1) dAIH improves respiratory function (V_T and diaphragm motor activity), which is enhanced by adenosine A_{2A} receptor inhibition; 2) AIH 3xw extends dAIH-induced functional recovery. Combined dAIH with A_{2A} receptor antagonists may be useful to enhance respiratory functional recovery with chronic cervical spinal injury.

INTRODUCTION

Since there are no approved therapies for chronic cervical SCI, there is a critical need for alternative approaches to alleviate respiratory deficits and improve the quality and duration of life for ventilator-dependent patients. Here we explore the hypothesis that repetitive exposure to acute intermittent hypoxia (AIH) is an effective strategy to elicit spinal respiratory motor plasticity and, thus, functional recovery of breathing capacity following chronic cervical spinal cord injuries in rats. Further, we test the hypothesis that pretreatment with systemic adenosine A_{2A} receptor antagonist enhances the extent of AIH-induced functional recovery.

Abundant literature demonstrates that single presentation of AIH strengthen spinal synaptic pathways to phrenic and thoracic motoneurons, and that these effects are revealed as AIH-induced long-term facilitation of diaphragm and second external intercostal (T2 EIC) motor output (DiaLTF and T2 EIC LTF, respectively) (Navarrete-Opazo and Mitchell, 2013). Although LTF in the phrenic nerve (pLTF) is serotonin-dependent in normal rats (Bach and Mitchell, 1996, Baker-Herman and Mitchell, 2002), it is constrained by adenosine A_{2A} receptor activation (Hoffman et al., 2010). Similar enhancement of DiaLTF is observed following systemic A_{2A} receptor inhibition in normal unanesthetized rats (Navarrete et al., ibid).

Following cervical C2 spinal hemisection (C2HS), AIH elicits functional recovery due to different cellular mechanisms in acute versus chronic spinal cord injuries. A single presentation of AIH can restore phrenic nerve activity below the injury, but only when AIH is presented at 8 weeks, not 2 weeks post-C2HS (Golder and Mitchell, 2005), which coincides with the return of serotoninergic innervation near phrenic motor nucleus (Golder and Mitchell, 2005), suggesting that at 8 weeks post-C2HS AIH-induced pLTF is serotonin-dependent. In contrast, chronic intermittent hypoxia (Fuller et al., 2003) or daily presentations of AIH for one week (dAIH) (Lovett-Barr MR et al., 2007) restore phrenic nerve activity and ventilatory capacity when dAIH was initiated one week post-C2HS. Paradoxically, this restoration of function when dAIH began one week post-C2HS appears to be due to a serotonin-independent (Terada, Vinit and Mitchell, unpublished), adenosine-dependent mechanism (Navarrete-Opazo and Mitchell, ibid).

The impact of repetitive AIH has not been investigated following chronic spinal injury, when serotonergic innervation of the spinal cord has had a chance to recover (Golder and Mitchell, 2005). Accordingly, we tested the hypothesis that the mechanism of dAIH-induced functional recovery of respiratory motor function reverts to a serotonin-dependent, adenosine constrained mechanism after chronic C2HS in unanesthetized rats. We predict that pretreatment with systemic A_{2A} receptor antagonist (Istradefylline/KW6002) would enhance dAIH-induced functional recovery of breathing capacity and diaphragm (not T2 EIC) motor output in chronic C2HS rats.

After C2HS, there is a slow, spontaneous increase in the strength of crossed-spinal synaptic pathways to ipsilateral phrenic motor neurons (Goshgarian, 2003, 2009). However, the contribution of crossed spinal pathway to tidal volume generation is small (~16%) (Dougherty et al., 2012b). Daily AIH (dAIH) increases EMG motor output in contralateral (uninjured) diaphragm but not ipsilateral (injured) diaphragm in acute (7-14 days post-injury) C2HS rats (Navarrete et al., ibid). Thus, another goal is to assess the relative contributions of injured vs uninjured respiratory muscles to dAIH-induced breathing functional recovery during normoxia and MCS with chronic C2HS rats.

Although chronic intermittent hypoxia (CIH: 5 min 11–12% O2 interspersed with 5 min air, 12 h/night, 7 nights) enhances ventilatory long-term facilitation (McGuire et al., 2003) and strengthens spinal synaptic pathways to phrenic motor neurons (Ling et al., 2001, Fuller et al., 2003), it also elicits morbidity, such as systemic hypertension (Zoccal et al., 2007, Tamisier et al., 2011), sleep fractionation (Perry et al., 2008), hippocampal neuron death and cognitive decline (Gozal et al., 2001, Xu et al., 2004). In contrast, dAIH elicits spinal plasticity without evidence for hypertension (Wilkerson and Mitchell, 2009) or hippocampal pathology (Lovett-Barr et al., 2012). Thus, an important question is how to maintain the beneficial (and safe) effects of daily AIH with more frequent presentations of intermittent hypoxia. We proposed that we could extend the functional benefits of dAIH by using a less-frequent protocol of repetitive AIH (rAIH: 3 times per week), which elicits robust neurochemical plasticity in the phrenic motor nucleus. Specifically, r AIH for 10 weeks increases expression of key molecules involved in AIH-induced pLTF, including serotonin, 5HT-2 receptors, BDNF, TrkB and ERK MAP kinases

(Satriotomo et al., 2012) without signs of hippocampal apoptosis or astrogliosis. Since rAIH upregulates pro-plasticity molecules without evidence for CNS pathology, it may be a useful therapeutic protocol to preserve, or even strengthen, functional recovery after dAIH with chronic SCI.

The main purpose of the present investigation is to study the long-term effects of dAIH with and without pretreatment with systemic adenosine A_{2A} receptor inhibition in chronic (8-16 weeks) C2HS rats using simultaneous plethysmography and EMG radiotelemetry to assess respiratory function in unanesthetized rats. We tested four specific hypotheses after chronic C2HS: 1) dAIH improves respiratory function during normoxia and MCS; 2) dAIH improves motor activity in contralateral (uninjured) and ipsilateral diaphragm and T2 EIC muscle; 3) Adenosine A_{2A} inhibition enhances dAIH-dependent respiratory motor activity in diaphragm but not T2 EIC; 4) AIH 3 times per week can maintain the effect of dAIH.

METHODS

Animals

All experiments began with 3-4 months old, male Sprague-Dawley rats (310-440 g, colony 211, Harlan, Indianapolis, IN). Rats were individually housed in a controlled environment (12-h light/dark cycle). The Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin approved all experimental procedures in this study.

Experimental preparation

Surgical preparation. For both telemetry implantation and C2 cervical hemisection, sterile surgery was performed under isoflurane anesthesia (in 100% O_2). The rats were injected with buprenorphine (0.03 mg/kg), carprofen (Rimadyl, 5 mg/kg) and enrofloxacin (Baytril, 4 mg/kg) subcutaneously to minimize potential post-operative pain and infection. Body temperature was maintained at 36.5-37.5°C using a rectal probe and external heating pad. A cannula was inserted into the trachea and the animals were artificially ventilated (tidal volume, 2.0-2.5 ml; Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA) with 1.5-2.5% isoflurane in 100% O_2 during surgery. Effective anesthesia was judged by abolition of pedal withdrawal and corneal blink reflexes. Oxygen saturation was monitored by pulse oximetry (model 8600; Nonin Medical Inc. Plymouth, MN) during surgery. At the end of surgery buprenorphine, carprofen and enrofloxacin (see above for dosage) were administered at 12 h intervals for 48 h post-surgery. Rats were visually monitored and weighed daily. Additionally, in spinally injured rats, animal care included trimming nails after surgery, and cleaning fur, eyes and snout with warm water daily for 7 days to avoid accumulation of porphyrin. Rats had free access to pellets and high caloric nutritional gels inside their cages. In both telemetry implantation and spinal cord injury surgeries, stainless steel staples closing the wound were removed 7 days post-surgery. We report no post-surgery complications after telemetry implantation or spinal cord injury surgeries.

Telemetry transmitter implantation. After induction of anesthesia, rats were placed in a supine position and the ventral surface of the abdominal muscle was exposed. A sterilized telemetry transmitter (model 4ET-S1/2; Data Sciences International [DSI], St. Paul, MN) was inserted into the peritoneal cavity. The transmitter allowed simultaneous and continuous monitoring of electrical bio-potentials, body temperature and general locomotor activity. In the present study, the four bio-potential channels were used to record electromyographic (EMG) activity from bilateral diaphragm and second external intercostal (T2 EIC) muscles. Implantations were performed as follow. First, both right and left hemi-diaphragms were exposed through a midline incision following the Alba line. On both hemidiaphragms, two leads were implanted on the mid-costal area using a 23-G syringe needle guide and tissue adhesive (Vetbond 1469SB; 3M Animal care product, St. Paul, MN) as reported in previous studies (Terada and Mitchell, 2011). Next, right and left T2 EIC muscles were exposed through a 2.5 cm mid-sternum incision, starting in the upper edge of sternum, followed by retraction of pectoralis major and minor on the right and left side. The right and left T2 EIC muscles were implanted 1.0 cm right and left from the sternum respectively and the second interspace was identified by counting from the first interspace. The bi-potential lead pairs targeting T2 EIC muscles were tunneled subcutaneously from the body of the transmitter placed in the peritoneal cavity. As used in the diaphragm, all the leads in T2 EIC were implanted using a 23-G syringe needle guide and tissue adhesive to keep the leads on place. Finally, abdominal muscles and pectoralis major were sutured in the midline with polysorb 3.0. Skin was closed with wound staples in both ventral thorax and abdomen.

Cervical C2 hemisection: One week after telemetry implantation, spinal hemisections at the second cervical level (C2HS) were performed. The surgical technique was consistent with previous reports (Fuller et al., 2009, Vinit et al., 2009, Dougherty et al., 2012b). After appropriate anesthesia and pre-operative care, the spinal cord was exposed at the C2 level via a dorsal laminectomy. The duramater was cut and a left C2 hemisection (C2HS) lesion was induced 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. The sham rats underwent a cervical laminectomy without having a spinal injury.

Whole-body plethysmography. Rats were placed individually in a 4 L DSI Plethysmography chamber (model 600-1211-001). Pressurized air flowed through the chamber at 4L/min, allowing control of inspired gas composition. The chamber was positioned onto a receiver (see below for telemetry signal acquisition) to measure EMG and plethysmography simultaneously. Compensated whole body plethysmography was used to assess tidal volume (V_T); compensation was based on chamber temperature (model P/N 60-1210-001) and humidity sensors (model P/N 600-1211-001). The system used a transducer (Buxco, model TRD5700) and a gas analyzer (CWE, Gemini). Plethysmography data were analyzed in 1 min bins during baseline (i.e. normoxia, 20 minutes) and during maximum chemoreceptor stimulation (hypoxia: 10.5% O_2 and hypercapnia: 7% CO_2; 20 minutes). To obtain a more accurate assessment of V_T, we used intraperitoneal temperature given by the telemetry system to compensate for changes in body temperature.

Telemetry signal acquisition. For the AIH protocol (see below), rats were placed in custom-made Plexiglas chambers positioned on receivers (model RPC-2; DSI, St. Paul, MN). Signals from the implanted radiotelemetry transmitter were detected by the receivers and sent to a data exchange matrix (model ACQ-7700; DSI, St Paul, MN). Four channels of EMG, body temperature and general locomotor activity in unanesthetized freely moving rats were monitored during the experimental protocol on a laboratory computer (data acquisition system: PONEMAH Physiology Platform; DSI, St. Paul, MN). EMG signals were sampled at 1200 Hz and analyzed with Neuroscore software (DSI, St. Paul, MN).

Drug preparation. Istradefylline (KW-6002, Sigma-Aldrich) is a selective adenosine A_{2A} receptor antagonist with a molecular weight of 384 and a Ki of 29.6 nM in rats. It has a half-life of 110 minutes, 97% bio-availability after intraperitoneal injection, and ability to cross the blood brain barrier (Yang et al., 2007), make it a suitable drug for our *in vivo* experiments. The drug was dissolved in DMSO at 9.3

mg/ml, sonicated and stored at 4°C in a dark vial protected from light. The day of the experiment, the drug was administrated via intraperitoneal injection at a dose of 0.5 mg/kg.

Experimental design. Five days after telemetry implantation simultaneous plethysmography and EMG recordings were done during normoxia (Nx) and maximum chemoreceptor stimulation (MCS) protocol to establish baseline values. Then, one day after spinal injuries same protocols were done to confirm C2 hemisection.

Eight weeks after C2HS, 27 rats were randomly allocated into the following groups: 1) dAIH + KW6002 (n=8); 2) dAIH + vehicle (n=8); 3) dNx + KW6002 (n=4); 4) dNx + vehicle (n=4); 5) Sham (n=3). Rats in group 1 received a single dose of intraperitoneal injection (0.5 mg/kg) before AIH for 7 consecutive days (daily AIH) starting day 8 weeks after C2HS (8-9 weeks post-C2HS) and then weekly presentations of AIH and Istradefylline (10-16 weeks post-C2HS). Group 2 received the same protocol, but Istradefylline was replaced with vehicle (DMSO). During the same time window, rats in group 3 and 4 were exposed to normoxia and A_{2A} antagonist or vehicle, respectively. Sham rats were exposed to normoxia and did not receive intraperitoneal injections. All groups were compared over time through assessment of weekly simultaneous plethysmography and EMG recordings during normoxia and MCS until the end of the study (fig.1).

Acute intermittent hypoxia protocol. Normoxic (21% O_2) and hypoxic (10.5% O_2) conditions were established in custom-made chambers (Plexiglas cylinder, 12 x 4 inches id; 1 rat per chamber) by mixing O_2 and N_2 gas with a custom-made computer-controlled mass-flow controller system to obtain the desired inspired oxygen concentrations. Within the chambers, CO_2 and O_2 levels were continuously monitored during the entire protocol (O_2 Analyzer, model 17518; CO2 Analyzer, model 17515; VacuMed Inc, Ventura, CA). Gas flowed through the chamber at a rate of 4 L/min, keeping chamber CO_2 concentration less than 0.5% at all times. 95% of the change in O_2 levels within hypoxic episodes was achieved in 25 ± 5 sec. At 8:00 am, on the experimental day, rats were placed in the chamber for 2-hour acclimation. Next,

intraperitoneal injections of either Istradefylline or vehicle were administrated accordingly (see experimental design). Once all rats were in the chambers, the experimental groups were administered the AIH protocol (10, 5-min 10.5% O_2 interspersed with 5-min 21% O_2, for a total of 95 min.). Time control and sham rats were administered continuous normoxia (i.e. time controls; TC). Chamber temperature was kept at 22.5-24.5°C during the entire protocol.

Maximum chemoreceptor stimulation. Normoxic (21% O_2), hypoxic (10.5% O_2) and hypercapnic conditions (7% CO_2) were established in plethysmography chambers (see above) by mixing O_2, N_2 and CO_2 gas via a custom-made, computer-controlled mass flow controller system to obtain the desired inspired oxygen concentrations. After 30 min acclimation, baseline during normoxic condition was recorded for 20 minutes, followed by 20 minutes of maximum chemoreceptor stimulation (MCS) (10.5% hypoxia together with 7% hypercapnia for 20 min, fig.5A). This protocol enables assessment of maximal chemoreflex stimulated respiratory activity in unanesthetized rats, as demonstrated previously (Navarrete-Opazo and Mitchell, ibid). EMGs and whole-body plethysmography were used to measure tidal volume (V_T) and breathing frequency (f), along with EMG signals.

Tissue Processing. To verify the extent of cervical hemisections, each spinal cord was freshly removed after completion of experiments, immersed in paraformaldehyde (4%, overnight at 4°C) and cryoprotected in increasing concentrations of sucrose (20–30%). Tissues were then frozen in isopentane (-45°C) and stored at -80°C. Longitudinal sections of the spinal cord (C1 to C6, 30 µm thick) were stained with cresyl violet and examined histologically using a light microscope to reconstruct the injury on the transverse plane (Vinit et al., 2006) (fig.5E), according to Paxinos and Watson (Paxinos and Watson, 1998). NIH ImageJ software (National Institute of Health; http://rsb.info.nih.gov/jj) was used to measure and compare the extent of the hemisection among groups.

Data analyses

EMG signal were analyzed with Neuroscore software. Raw signal was filtered (100-624 Hz), rectified, integrated (100 ms) and averaged for each muscle (bilateral diaphragm/T2 EIC muscle). EMG values during active locomotor activity were excluded in the analysis. Absolute values of tidal volume and respiratory frequency were averaged in all rats. EMG amplitude of each muscle was expressed as a percent change from normalized pre-injury values.

All variables were compared among groups. Statistical comparisons were made for time (baseline and during MCS) and treatment (see experimental design) using 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 indicated as statistically significant were $P < 0.05$. All values are expressed as means ± SEM.

RESULTS

Reduced tidal volume and ipsilateral motor activity after C2HS

Reconstruction of the cervical hemisections demonstrated that all groups showed similar areas of injury, expressed as a percentage of the total spinal cross sectional area (dAIH + vehicle: 49.01 ± 0.75 %, dAIH + KW6002: 47.07 ± 0.87%, dNX + vehicle: 49.36 ± 0.87%, dNX + KW6002: 48.72 ± 1.28%; p=0.283, fig.3B).

To establish baseline pre-injury values, normal rats were exposed to 20 minutes of normoxia followed by 20 minutes of maximum chemoreceptor stimulation (i.e. MCS, fig.2) for simultaneous EMG and plethysmography recordings five days after telemetry implantation. Combined hypercapnia (7% CO_2) and hypoxia (10.5% O_2) is a powerful stimulus to chemoreflexes, giving a standardized high drive to breathing for comparison among animals; however, this stimulus should not be regarded as the maximal respiratory muscle activity since yet higher levels can be achieved during respiratory defense reflexes such as a cough (Mantilla and Sieck, 2011).

One day after C2HS, tidal volume (V_T; ml/100g) is significantly reduced versus pre-injury values in all groups during normoxia (dAIH + vehicle: 0.37 ± 0.01, dAIH + KW6002: 0.36 ± 0.02, dNX + vehicle: 0.39 ± 0.01, dNX + KW6002: 0.35 ± 0.03 ml/100gr; p>0.05, fig.4A) and MCS (dAIH + vehicle: 0.70 ± 0.01, dAIH + KW6002: 0.72 ± 0.2, dNX + vehicle: 0.69 ± 0.03, dNX + KW6002: 0.72 ± 0.04 ml/100g; p>0.05, fig.4B), consistent with previous reports (Golder et al., 2001).

During normoxia, there was a significant reduction of EMG activity one day post-C2HS in ipsilateral diaphragm (dAIH + vehicle: 13.0 ± 1.6%, dAIH + KW6002: 10.4 ± 1.5%, dNX + vehicle: 9.0 ± 2.0%, dNX + KW6002: 11.5 ± 3.5% of pre-injury values, p >0.05) and T2 EIC muscle (dAIH + vehicle: 9.74 ± 1.7%, dAIH + KW6002: 8.6 ± 1.0%, dNX + vehicle: 6.6 ± 1.3%, dNX + KW6002: 8.6 ± 1.7% of pre-injury values; p >0.05), demonstrating the effectiveness of cervical hemisection (fig.3).

Adenosine A_{2A} inhibition enhances dAIH-induced breathing capacity with chronic C2HS

Nine weeks post-C2HS, (immediately after the last episode of dAIH/dNX), time control rats showed near normal V_T versus sham rats (fig.4A) during normoxia; confirming previous findings of a remarkable spontaneous recovery after cervical hemisection in rodents (Navarrete et al., ibid). At this time-point, dAIH + vehicle-treated rats show a significant increase in V_T (above normal values) compared to controls and sham rats (dAIH + vehicle: 0.57 ± 0.01, dNX + vehicle: 0.52 ± 0.01, dNX + KW6002: 0.53 ± 0.04, Sham: 0.53 ± 0.02 ml/100g, $p<0.05$, fig.4A). Istradefylline significantly enhanced this effect (dAIH + KW6002: 0.60 ± 0.08 vs. dAIH + vehicle: 0.57 ± 0.01 ml/100g, $p=0.031$, fig.4A) demonstrating that adenosine A_{2A} inhibition amplifies the effect of dAIH, as it was previously shown in anesthetized (Hoffman et al., 2010) and unanesthetized normal rats (Navarrete et al., ibid). At later time-points dAIH + V and dAIH + KW6002-treated rats show a greater V_T compared to TC and sham rats up to 12 weeks post-C2HS. From 13 up to 16 weeks post-C2HS, there are no statistical differences among groups.

During MCS, dAIH + KW6002 also increased V_T at 9 weeks post-C2HS versus dAIH + vehicle and control rats (dAIH + vehicle: 0.88 ± 0.02, dAIH + KW6002: 0.95 ± 0.01, dNX + vehicle: 0.84 ± 0.02, dNX + KW6002: 0.84 ± 0.02 ml/100g, $p < 0.001$, fig.4B). This increased V_T was not significantly different from sham rats (dAIH + KW6002: 0.95 ± 0.01 vs. Sham: 1.01 ± 0.02 ml/100g, $p= 0.068$, fig.4B) showing that Istradefylline combined with dAIH restored the MCS response to normal levels 9 weeks post-C2HS. dAIH + KW6002-treated rats maintained a greater TV than dAIH + vehicle-treated and control rats during MCS up to 12 weeks post-C2HS (dAIH + vehicle: 0.86 ± 0.01, dAIH + KW6002: 0.91 ± 0.02, dNX + vehicle: 0.82 ± 0.01, dNX + KW6002: 0.82 ± 0.03 ml/100g, $p < 0.05$, fig.4B), demonstrating AIH 3 times per week combined with A_{2A} antagonists maintain dAIH-induced functional recovery up to 12 weeks post-C2HS, after which there is no differences among groups.

As with previous reports (Golder et al., 2001a, Golder et al., 2001b, Fuller et al., 2006), C2HS decreases V_T and increases breathing frequency (breaths per minute, bpm) during normoxia (pre-injury: 84 ± 2 bpm vs. 1d post-C2HS: 106 ± 2 bpm; $p < 0.001$, fig.5A). Nine weeks post-C2HS, the increase in respiratory frequency returns to normal levels (~87 bpm, fig.5A) in all groups. During MCS, breathing

frequency significantly increases 1 d post injury (pre-injury: 137 ± 2 bpm vs. 1d post-C2HS: 148 ± 2 bpm, $p < 0.05$; fig. 5B). The increased breathing frequency remains greater than pre-injury values at 9 weeks post-C2HS (144 ± 3 bpm, fig.5B) and throughout the entire study, which may be compensating for the reduced V_T observed during MCS. dAIH and adenosine A2A inhibition did not affect respiratory frequency during normoxia and MCS in all time-points, suggesting that the mechanisms explaining the effect of AIH and Istradefylline are central rather than peripheral (chemoreceptor response).

A2A inhibition enhances dAIH-induced diaphragm motor recovery with chronic C2HS

Contralateral (uninjured) diaphragm increased EMG amplitude (above pre-injury values) one day post-C2HS during normoxia (dAIH + vehicle: 133.62 ± 2.84%, dAIH + KW6002: 128.87 ± 3.40%, dNX + vehicle: 127.75 ± 4.30%, dNX + KW6002: 130.25 ± 2.25% of pre-injury values, $p > 0.05$, fig.6A), demonstrating that compensatory plasticity start early after spinal injury.

During normoxia, A_{2A} receptor inhibition plus dAIH significantly enhanced EMG amplitude in contralateral diaphragm 9 weeks post-C2HS (immediately after last episode of dAIH) versus dAIH + vehicle, controls and sham rats (dAIH + vehicle: 138.25 ± 5.38%, dAIH + KW6002: 157.50 ± 5.32%, dNX + vehicle: 127.0 ± 2.9%, dNX + KW6002: 119.25 ± 3.19%, sham: 103.66 ± 4.05% of pre-injury values; $p<0.05$, fig.6A, 7). This effect is maintained up to 12 weeks post-C2HS, although at this time point it was not significantly different from dAIH + vehicle-treated rats (AIH + V:147.62 ± 8.14%, AIH + KW6002: 152.62 ±5.82% of pre-injury values, $p=0.437$, fig.6A,8). Our data demonstrates that 3xweek AIH reminders maintain the effects of dAIH in chronic C2HS rats. From 13 weeks post-C2HS up to the end of the study, there were no significant differences among groups which could be age-related.

Left (injured) diaphragm activity shows a similar spontaneous recovery in time control rats nine weeks post-C2HS (dNX + vehicle: 21.0 ± 1.7% vs. dNX + KW6002: 16.25 ± 0.75% of pre-injury values, $p= 0.531$; fig.6B) and throughout the entire study, with small improvement at 16 weeks post-C2HS (dNX + vehicle: 31.25 ± 2.83% vs. dNX + KW6002: 27.75 ± 4.60% of pre-injury values, $p= 0.644$; fig.7B). dAIH + KW6002-treated rats show a greater EMG amplitude compared to dAIH + vehicle and control

rats 9 weeks post-C2HS (dAIH + vehicle: 36.12 ± 4.76%, dAIH + KW6002: 56.0 ± 7.32%, dNX + vehicle: 21.0 ± 1.78%, dNX + KW6002: 16.25 ± 0.75% of pre-injury values, $p<0.001$, fig.6B,7), effect that is maintained at 10 weeks (dAIH + vehicle: 37.50 ± 3.85% vs. dAIH + KW6002: 57.12 ± 4.24% of pre-injury values, $p<0.001$), 11 weeks (dAIH + vehicle: 44.37 ± 3.21% vs. dAIH + KW6002: 54.87 ± 4.02%, $p<0.001$) and 12 weeks (dAIH + vehicle: 45.87 ± 2.54% vs. dAIH+ KW6002: 56.87 ± 4.75% of pre-injury values, $p=0.018$, fig.6B) post-C2HS. Although dAIH + vehicle-treated rats show a smaller increase in EMG amplitude than dAIH + KW6002-treated rats, the EMG amplitude was significantly greater than control rats from 9 weeks (dAIH + vehicle: 36.12 ± 4.76%, dNX + vehicle: 21.0 ± 1.78%, dNX + KW6002: 16.25 ± 0.75% of pre-injury values, $p<0.05$) up to 12 weeks post-C2HS (dAIH + vehicle: 45.87 ± 2.54%, dNX + vehicle: 23.75 ± 5.66%, dNX + KW6002: 29.75 ± 7.69% of pre-injury values, fig.6B).Taken together, 3xweek AIH with or without Istradefylline maintains the effect of dAIH in chronic C2HS rats up to 12 weeks post-C2HS with no differences among groups thereafter.

The contralateral (uninjured) T2 EIC muscle exhibit a small compensatory plasticity one day after C2HS (112.50 ± 1.97% of pre-injury values) that returns to normal 9 weeks post-C2HS with no significant differences among groups (dAIH + vehicle: 103.62 ± 2.67%, dAIH + KW6002: 101.12 ± 3.19%, TC: 101.37 ± 2.09%, sham: 100.7 ± 2.32% of pre-injury values, $p>0.05$; fig.9A). Left (uninjured) T2 EIC show complete recovery 9 weeks post-C2HS (dAIH + vehicle: 100.12 ± 2.19%, dAIH + KW6002: 99.87 ± 1.34%, TC: 102.25 ± 1.25%, sham: 98.66 ± 2.02% of pre-injury values, $p=0.76$; fig.9B). Regardless of the complete recovery of ipsilateral (left) T2 EIC muscle, dAIH alone or combined with adenosine A_{2A} antagonist did not have an effect in contralateral and ipsilateral T2 EIC motor activity, which may be due to a “ceiling” effect.

Daily AIH enhances diaphragm MCS response with chronic C2HS

Pre-injury EMG recordings done simultaneously with plethysmography showed a robust MCS response in both diaphragm and T2 EIC muscle (fig.2), as it was demonstrated previously (Navarrete and Mitchell, ibid). MCS response one day after C2HS was totally abolished in ipsilateral diaphragm and T2 EIC

muscle (fig.3). Interestingly, MCS was almost abolished in right T2 EIC and significantly reduced in the right diaphragm in all groups studied one day after C2HS (dAIH + vehicle: 85.50 ± 1.91%, dAIH + KW6002: 80.37 ± 4.80%, dNX + vehicle: 77.75 ± 4.64%, dNX + KW6002: 79.75 ± 4.27%, Sham: 107.0 ± 4.04% of pre-injury values; $p>0.05$, fig.10A).

Nine weeks post-C2HS, dAIH + KW6002-treated rats show an increase in EMG activity during MCS in right diaphragm (dAIH + vehicle: 108.0 ± 1.9%, dAIH + KW6002: 117.0 ± 3.74%, dNX + vehicle: 95.25 ± 2.78%, dNX + KW6002: 92.0 ± 2.79%, sham: 107.0 ± 4.04% of pre-injury values, $p<0.05$; fig.10A), effect that is maintained at 10 weeks post-C2HS. At 11 and 12 weeks post-C2HS, dAIH + vehicle and dAIH+KW6002-treated rats show an increase in EMG activity compared to control rats (dAIH + vehicle: 110.75 ± 5.93%, dNX + vehicle: 94.75 ± 4.09%, dAIH + KW6002: 105.75 ± 4.44%, dNX + KW6002: 88.50 ± 4.77% of pre-injury values, $p<0.05$; fig.10A). From 13 weeks post-C2HS up to the end of the study there are no significant differences among groups.

Left diaphragm show a small and variable MCS response ranging from 30 to 45% of pre-injury values throughout the entire study. dAIH + KW6002-treated rats show a greater, although not statistically significant, MCS response compared to dAIH + vehicle-treated and control rats throughout the entire study.

Surprisingly, regardless of the complete spontaneous recovery of left T2 EIC muscle, MCS response was totally absent in both right and left T2 EIC during the entire study (fig.11), which we attributed to a “ceiling” effect.

DISCUSSION

We provide novel information regarding the impact of daily acute intermittent hypoxia and adenosine A_{2A} receptor inhibition on breathing function in unanesthetized rats with chronic cervical spinal injuries. Our study has six major findings: 1) spontaneous motor recovery is modest in left (injured) diaphragm and complete in left T2 EIC muscle at 9 weeks post-C2HS; 2) dAIH increases respiratory functional recovery (tidal volume) during normoxia and MCS; 3) dAIH increases motor activity in contralateral (uninjured) and ipsilateral (injured) diaphragm during normoxia and MCS; 4) adenosine A_{2A} inhibition significantly enhances dAIH-induced respiratory functional recovery and motor activity in contralateral and ipsilateral diaphragm during normoxia; 5) AIH 3 per week remainders maintain the effect of dAIH-induced functional recovery up to 12 weeks post-C2HS; 6) from 13 weeks post-C2HS up to the end of the study there were no differences among groups, which we suggest could be due to age and/or sex hormone-related factors.

Altered breathing pattern after C2HS

After C2 spinal hemisection, rats maintain normal blood gases (Goshgarian et al., 1986, Goshgarian, 2009) and breathe with increased frequency and decreased V_T (Golder et al., 2001a, Golder et al., 2001b). This has been shown in unanesthetized (Fuller et al., 2005, Fuller et al., 2006) and anesthetized rats (Golder et al., 2001b). The changes in breathing pattern after SCI partially result from vagal feedback, most likely arising from changes in lung and chest wall compliance caused by the paralyzed ipsilateral diaphragm and intercostal muscles (Golder et al., 2001b). However, it may also reflect reorganization of the medullary respiratory control network (Golder et al., 2001b) and intrinsic plastic mechanisms initiated by the C2HS. In unanesthetized rats, the severity of the injury dictates post-injury breathing pattern, which correlates negatively with tidal volume and positively with breathing frequency (Golder et al., 2011).

There is remarkable time-dependent recovery of V_T starting one day to 7 week post-C2HS (Navarrete et al., ibid), consistent with other studies using cervical hemisection and contusion models (Golder et al.,

2011, Dougherty et al., 2012b, Lane et al., 2012, Lovett-Barr et al., 2012). As we predict, control rats show normal V_T 9 weeks post-C2HS which coincides with the reduced breathing frequency down to normal values during normoxia. In contrast, chronic C2HS rats exhibit ventilatory deficits when challenged with combined hypoxia/hypercapnia (Fuller et al., 2006, Fuller et al., 2009). During respiratory challenge there is a reduced V_T, and the respiratory frequency remains greater than pre-injury values up to the end of the study, with no differences among groups.

Spontaneous contralateral versus ipsilateral motor recovery

The recovery process can occur through a wide range of mechanisms, representing different types of neuroplasticity. Compensatory plasticity involves increased recruitment of other respiratory muscles via intact neural pathways (Teitelbaum et al., 1993, Katagiri et al., 1994, Brichant and De Troyer, 1997, Johnson and Mitchell, 2013), and may reflect the removal of contralateral inhibitory sensory inputs onto phrenic motorneurons (Goshgarian, 1981). Rats maintain normal blood gases after C2HS (Goshgarian et al., 1986), which suggests that spontaneous compensation is unlikely to result from persistent stimulation of chemoreceptors. On the other hand, plasticity in a neurologically impaired pathway can occur through changes in synaptic connections or other adaptations that increase the efficacy of neurotransmission to ipsilateral phrenic and thoracic motorneurons (Nantwi et al., 1999, Mitchell and Johnson, 2003, Golder et al., 2011, Johnson and Mitchell, 2013). In this scenario, the complete respiratory functional recovery observed in control chronic spinal cord injured rats may be attributed to the combined effect of spontaneous compensatory plasticity in right diaphragm (~ 20% increase in motor output, above pre-injury values in TC rats) as well as the important spontaneous motor recovery of inspiratory intercostal muscles. In our study, second external intercostal muscle shows complete recovery nine weeks post-C2HS.

After acute cervical hemisection, ipsilateral (injured) phrenic nerve and/or hemidiaphragm show comparatively little inspiratory activity during quiet breathing (i.e., eupnea). During eupnea, the phrenic nerve or hemidiaphragm ipsilateral to C2 hemisection is silent (i.e., no inspiratory bursting) in the days to

weeks after injury (Goshgarian, 1981, Nantwi et al., 1999, Golder et al., 2001a, Golder et al., 2001b). One to 2 months post-C2 hemisection, spontaneous inspiratory bursts during eupnea are observed in the phrenic nerve or hemidiaphragm ipsilateral to injury (Nantwi et al., 1999, Golder et al., 2001b, Fuller et al., 2006). In chronic C2HS rats, we also found a small and variable spontaneous recovery of injured diaphragm in control rats ranging from 16% up to 31% of pre-injury values at the end of the study (4 months post-C2HS), confirming the relatively small contribution of injured diaphragm to ventilatory recovery under quiet breathing conditions. In contrast, injured T2 EIC muscle shows complete recovery 9 weeks post-C2HS, suggesting that accessory inspiratory muscles contribute relatively more and compensate for respiratory deficits during quiet breathing conditions (Sherrey and Megirian, 1990, Dougherty et al., 2012a). Further studies are warranted to determine mechanisms underlying recovery of inspiratory intercostal EMG activity after C2HS. The removal of an inhibitory reflex may excite intercostal motoneurons after spinal injury since paralysis of the diaphragm in dogs increases activity of inspiratory intercostal muscles (De Troyer, 1998). This increase is due to the removal of sensory afferents arising from the phrenic nerve that normally inhibit inspiratory intercostal activity (De Troyer, 1998). This reflex is substantial and bilateral stimulation of C5 phrenic afferents resulted in a 50% reduction in intercostal inspiratory activity (De Troyer, 1998). Therefore, once left diaphragm is paralyzed, there may be a disinhibition of inspiratory intercostal muscle activity.

Spontaneous plasticity in neural pathways innervating respiratory muscles on the intact side of the spinal cord (i.e., compensatory plasticity) has been reported previously (Sherrey and Megirian, 1990, Brichant and De Troyer, 1997, Mantilla et al., 2013). Here we confirm a small, compensatory plasticity in contralateral (uninjured) diaphragm (~20% above pre-injury values) and T2 EIC muscle (12% above pre-injury values) in time control rats by one day post-C2HS, a limited compensatory mechanism aimed at preserving breathing capacity (Johnson and Mitchell, 2013). This compensation is maintained throughout the study in diaphragm but not in T2 EIC which coincides with the complete motor recovery of left T2 EIC, suggesting that there may be a cause effect relationship between compensatory plasticity in intact contralateral muscle and spontaneous recovery in injured ipsilateral intercostal muscles. The molecular

mechanisms explaining differences between inspiratory intercostal (thoracic) versus diaphragm (phrenic) plasticity remain to be explored.

Adenosine A2A inhibition enhances dAIH-induced ventilatory capacity

Istradefylline enhances dAIH-induced functional recovery of breathing capacity up to 12 weeks post-C2HS, demonstrating that adenosine A2A receptor inhibition amplifies the impact of repetitive AIH with chronic spinal injury, as it has been demonstrated in normal anesthetized (Hoffman et al., 2010) and unanesthetized rats (Navarrete Opazo et al., unpublished). However, this finding is in striking contrast to the response observed in acutely injured rats, where A2A receptor inhibition impairs dAIH induced functional recovery (Navarrete-Opazo et al., ibid). During MCS, dAIH and Istradefylline enhance VT up to sham values at 9 weeks post-C2HS. Taken together, enhanced ventilatory recovery is maintained up to 12 weeks post-C2HS during normoxia and MCS, demonstrating that presentation of AIH 3 times per week maintain dAIH-induced respiratory functional recovery. The relevance of this finding is that istradefylline combined with dAIH may increase ventilatory capacity during quiet breathing but also during a respiratory challenge like respiratory complications (i.e. atelectasis, pneumonia), frequently present in cervical spinal cord injuries (NSCISC, 2005). Since pulmonary complications increase ventilatory demand, combined dAIH and istradefylline may represent an effective way of increasing breathing capacity and perhaps reduce recovery time.

Adenosine A2A inhibition enhances dAIH-induced motor recovery in diaphragm

Our study demonstrates that daily AIH significantly increases motor activity in contralateral diaphragm, and this effect is maintained up to 12 weeks post-C2HS during normoxia and MCS. Furthermore, adenosine A2A receptor inhibition enhances dAIH-induced motor recovery 9 weeks post-C2HS (immediately after last episode of dAIH), coinciding with enhanced ventilation during normoxia, reflecting a possible cause-effect relationship. In contrast, dAIH alone or dAIH with Istradefylline did not increase contralateral T2 EIC muscle activity; we hypothesize that crossed synaptic inputs from right T2

EIC contribute to the spontaneous recovery in the left T2 EIC, thus, reaching a ceiling effect.

Although the extent of spontaneous functional recovery in ipsilateral diaphragm following cervical hemisection is small and limited, functional recovery can be enhanced by inducing additional plasticity with, for example, repeated intermittent hypoxia (Vinit et al., 2009, Lovett-Barr et al., 2012). Repetitive AIH improves left diaphragm motor output from 9 up to 12 weeks post-C2HS, an effect significantly enhanced by Istradefylline at the same time-points; thus, dAIH-induced plasticity is constrained by adenosinergic mechanisms at this time point (see discussion below). However, during MCS there were no differences among groups at any time-point. Despite complete recovery of the left T2 EIC, dAIH alone or with Istradefylline does not increase motor output further versus control rats at any time-point, which we speculate is due to a ceiling effect where the muscle cannot increase further the motor output.

Repetitive AIH 3 times per week increases the expression of key molecules involved in AIH-induced phrenic motor plasticity in normal rats (Satriotomo et al., 2012), and we now demonstrate that 3xw AIH presentations can maintain dAIH-induced plasticity in diaphragm activity for up to 12 weeks post-C2HS. From 13 weeks post-C2HS to the end of the study, there were no longer differences among groups, which we speculate is due to age-related factors.

AIH-induced respiratory plasticity is age-dependent

The lack of ipsilateral and compensatory plasticity at later time points (13-16 weeks post-C2HS) may be related to the advancing age (8 month-old) of the rats. Serotonin-dependent plasticity in phrenic motor output is diminished in an age- and sex-dependent manner. LTF decreases with advancing age in male rats; both phrenic and hypoglossal LTF are greater in young (3-4 month-old) than in middle-aged rats (13 month-old) (Zabka et al., 2001, Behan et al., 2002). Moreover, phrenic LTF is reduced and hypoglossal LTF almost eliminated in middle-aged (12 month old) male rats, but increased in female rats (Behan et al., 2002, Behan et al., 2003, Zabka et al., 2003). However, even in geriatric female rats where LTF is nearly abolished, pre-conditioning repetitive intermittent hypoxia is able to restore the capacity for respiratory motor plasticity. Thus, the capacity for intermittent hypoxia induced plasticity is expected to

be diminished in older rats, with at least some capacity to reverse that trend with repetitive/recurrent hypoxic exposures. We have no information suggesting that AIH 3x per week is sufficient to restore respiratory plasticity in middle-aged male rats.

Diminished LTF in aged male rats may result from decreases in androgen levels with advancing age (Zabka et al., 2001, Nelson et al., 2011). Gonadectomy in male adult rats has been reported to produce a decrease in testosterone levels associated with a reduction in LTF magnitude, which can be restored by subsequent testosterone replacement (Behan et al., 2003, Zabka et al., 2005). While testosterone can act through its specific steroid receptor, its effects on 5HT2A receptors appear to depend primarily on conversion to estrogen (Fink et al., 1998). Since phrenic and hypoglossal motor neurons express androgen and estrogen receptors, sex hormones could directly affect the function of respiratory motoneurons.

However, it is unlikely that the androgen/estrogen level difference can completely explain differential plasticity following dAIH in rats with chronic C2HS. Chronic intermittent hypoxia-induced ventilatory LTF is greater in neonatal (1 month-old) than in adult (2 month-old) rats (McGuire and Ling, 2005). Since neonatal rats have lower testosterone levels than adults rats, and testosterone appears to play a facilitative role for LTF (Behan et al., 2003) there may be additional factors explaining differential expression of plasticity. Different LTF is expected with age since serotonin modulation is different in young vs. adult rats (Hyttel, 1987, Gabriel et al., 1992). One remarkable feature of aging is a gradual decline of neurotransmitter receptor density, which is responsible for many functional deficits in older humans (Wang et al., 1995). For example, the density of 5-HT2 receptors declines substantially with aging in humans and rodents (Morgan, 1987). In rats, the density of 5-HT2 receptors also decreases from 1 to 7 month of age, and remains at this level up to 12 months of age (Hyttel, 1987). Furthermore, 5-HT decreases with age in the ventral horns of cervical spinal segments associated with the phrenic motor nuclei (Ko et al., 1997). We speculate that serotonin receptor density in the phrenic motor nuclei is greater in immature vs. young adult rats, and the density further declines with advancing age, thereby eliciting a greater LTF in immature, but smaller/no LTF in aged rats. Taken together, we may propose that decreased serotonergic innervation due to ageing and/or reduce levels of testosterone in the phrenic nuclei is at least

partially responsible for decreased serotonin-dependent AIH-induced plasticity in chronic spinal cord injured rats.

Adenosine A2A activation constrains AIH-induced respiratory plasticity with chronic C2HS

In normal rats, our working model of AIH-induced pLTF is that episodic hypoxia activates raphe serotonergic neurons that project to phrenic motor nuclei. Spinal serotonin release during hypoxic episodes subsequently activates Gq protein-coupled 5-HT2 receptors on or near phrenic motor neurons, and initiates intracellular cascades that underlie pLTF (Dale-Nagle et al., 2010). Adenosine A2A receptors, coupled to Gs proteins, give rise to phrenic motor facilitation (PMF) when activated alone, but constrain AIH-induced pLTF when co-activated with 5-HT2 receptors (Dale-Nagle et al., 2010). We proposed that AIH-induced pLTF (Gq protein-mediated) and A2A receptor induced PMF (Gs protein-mediated) are distinct mechanisms that interact via cross-talk inhibition (Hoffman et al., 2010). Cross-talk inhibition from the A2A receptor-dependent pathway constrains serotonin-dependent pLTF; therefore, systemic adenosine A2A antagonist enhances pLTF (Hoffman et al., 2010) and diaLTF (Navarrete et al., ibid) in normal rats.

We confirm our hypothesis that dAIH induces respiratory plasticity due to adenosinergic mechanisms in acute vs. serotoninergic mechanisms in chronic spinal cord injuries. With acute spinal cord injury, serotonin terminals innervating phrenic motor neurons have been disrupted (Golder and Mitchell, 2005), therefore, dAIH induces functional recovery due to adenosine-dependent mechanisms (<8 weeks post-C2HS) (Navarrete-Opazo, ibid). We now show that the impact of dAIH reverts to a serotonin-dependent and adenosine-constrained mechanism with chronic SCI (>8 weeks post-C2HS), a time-frame where serotonin terminals have been partially restored (Golder and Mitchell, 2005). Thus, Istradefylline, a highly specific adenosine A2A receptor antagonist, enhances (not diminishes as with acute C2HS) dAIH-induced compensatory and ipsilateral plasticity in unanesthetized chronic C2HS rats. However, this effect is limited to diaphragm activity since A2A receptors have no impact on the magnitude of AIH-induced plasticity in inspiratory intercostal muscles (Navarrete-Opazo. ibid).

Repetitive acute intermittent hypoxia elicits metaplasticity

Metaplasticity is the ability of prior experience to alter subsequent plasticity (Abraham and Bear, 1996, Hulme et al., 2013). Metaplasticity can be expressed as enhanced plasticity triggered by different stimuli such as dorsal cervical rhizotomy, chronic intermittent hypoxia (CIH), and repetitive AIH (rAIH). For example, bilateral cervical dorsal rhizotomy increases serotonergic innervation of the phrenic motor nucleus and augments serotonin-dependent long-term facilitation of phrenic motor output (Kinkead et al., 1998). Pretreatment with CIH (5 min 11–12% O2/5 min air, 12 h/night, 7 nights) enhances phrenic LTF in anesthetized rats (Ling et al., 2001) and ventilatory LTF in awake rats (McGuire et al., 2003). In spinal cord injured rats, CIH enhanced phrenic burst amplitude ipsilateral to the C2HS during normoxia, hypoxia and hypercapnia, but only when administered after chronic SCI (Fuller et al., 2003). The molecular mechanisms explaining CIH-induced plasticity and metaplasticity are not fully understood, but it has been proposed that CIH enhanced crossed phrenic pathways after SCI by a serotonin-dependent mechanism. Methysergide (a non-selective serotonin antagonist) abolishes CIH-enhanced phrenic LTF but the selective 5-HT2 antagonist ketanserin only partially reversed this effect (Ling et al., 2001), suggesting that enhanced LTF after CIH may involve up-regulation of a non-5-HT2 serotonin receptor subtype.

More subtle protocols of repetitive acute intermittent hypoxia (rAIH) elicit metaplasticity without detrimental effects elicited by CIH (Wilkerson and Mitchell, 2009, Lovett-Barr et al., 2012, Satriotomo et al., 2012). Rats exposed to rAIH (ten 5-min episodes/day, 3 days/week, 4 weeks) exhibit enhanced pLTF (MacFarlane et al., 2010, Vinit et al., 2010). Moreover rAIH for 10 weeks increase the expression of key molecules involves in the phrenic motor nucleus without signs of hippocampal apoptosis, astrogliosis, or hypertension (Wilkerson and Mitchell, 2009, Satriotomo et al., 2012). Daily AIH improves walking in humans with chronic, incomplete SCI, with no evidence for increased spasticity or autonomic dysreflexia (Hayes et al., 2013). Mechanisms underlying enhanced pLTF following rAIH are unknown. rAIH increases expression of 5-HT2A receptor, BDNF, TrkB, and phospho-ERK (Satriotomo et al., 2012). Therefore, rAIH alone or combined with A_{2A} antagonist may decrease adenosine activation and, thus,

cross-talk inhibition of the primary, serotonin-dependent mechanism.

Collectively, we found that in chronic C2HS rats, dAIH improves respiratory function during normoxia and MCS, and that AIH reminders 3 times per week maintain that effect. Repetitive AIH-induced functional recovery is likely attributed to an increased EMG motor output in uninjured and injured diaphragm rather than intercostal muscle, which plays a more important role during spontaneous recovery in control rats. Furthermore, adenosine A2A inhibition enhances dAIH-induced motor activity in diaphragm, confirming our hypothesis that serotonin-dependent AIH is constrained by adenosinergic mechanisms in chronic spinal cord injured rats; much in contrast to rats with acute spinal injuries (Navarrete-Opazo et al., ibid). The molecular mechanisms underlying interactions between adenosine and serotonin pathways as well as the long-term effect of adenosine A2A inhibition remain to be confirmed.

Significance

Repetitive AIH (rAIH) represents a potentially safe and effective means to enhance functional recovery of breathing capacity after chronic spinal cord injuries. Additionally, A2A receptor inhibition can enhance rAIH-induced respiratory functional recovery, suggesting that treatments as simple as combined rAIH with caffeine (a well-known A2A receptor antagonist) may amplify the extent of functional recovery, but only with chronic injury.

Currently, there are no approved therapies for chronic SCI; therefore, our approach represents a promising new strategy to enhance function in patients with chronic SCI, where the potential for further functional gains is limited. In our view, rAIH combined with adenosine A2A inhibition may represent a novel, non-invasive and effective treatment for chronic SCI-induced respiratory impairment.

Acknowledgement

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FIGURES

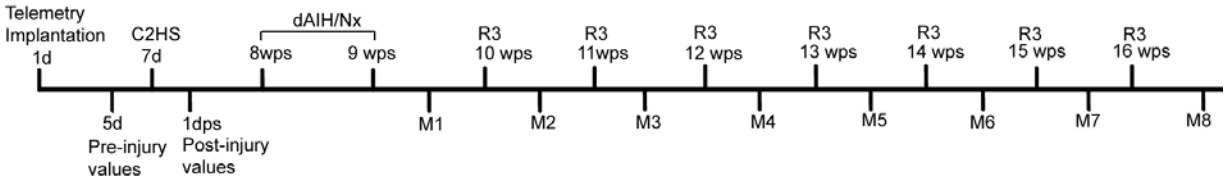

Figure 1. Timeline describing the entire study from day 1 (1d) until 16 weeks post-spinal cord injury (wps). C2HS: cervical hemisection in second segment, dAIH/Nx: daily acute intermittent hypoxia or normoxia for seven consecutive days (see methods), R3: 3 times per week presentations "reminders" of acute intermittent hypoxia/normoxia and vehicle (DMSO)/KW6002, M1-M8: weekly measurements of simultaneous plethysmography and electromyography of bilateral diaphragm and second external intercostal muscles.

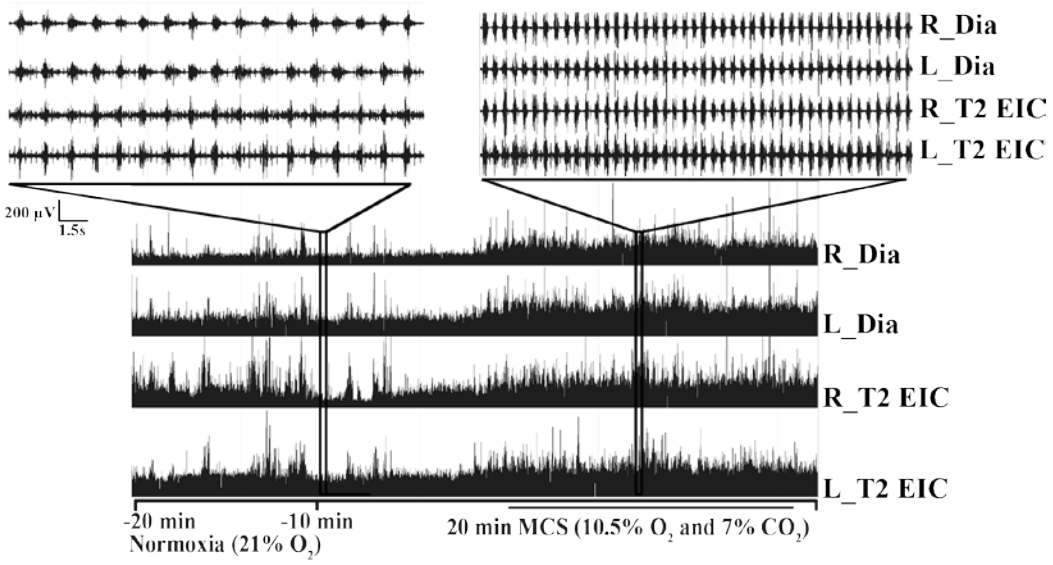

Figure 2. Representative raw (top) and integrated (bottom) EMG activity of right/left diaphragm (R/L_Dia) and second external intercostal muscle (R/L_T2 EIC) before cervical C2 hemisection (SCI) during 20 min normoxia and 20 min maximum chemoreceptor stimulation (MCS). Note the robust increase EMG amplitude during MCS in all muscles.

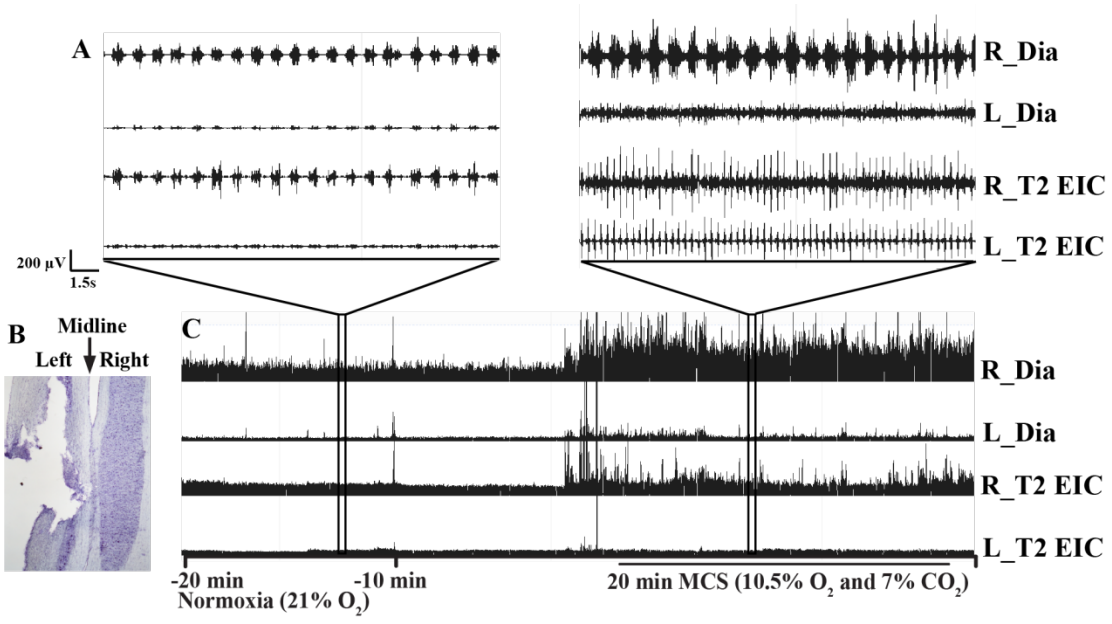

Figure 3. Representative raw (A, top traces) and integrated (C) EMG activity of right/left diaphragm (R/L_Dia) and second external intercostal muscle (R/L_T2 EIC) one day after cervical C2 hemisection (C2HS) during 20 min normoxia and 20 min maximum chemoreceptor stimulation (MCS). Note: 1) significantly reduced EMG activity in left diaphragm and T2 EIC muscle during normoxia and MCS, confirming the left C2HS; 2) Reduced MCS response in right T2 EIC muscle. 3) Increased MCS response in right diaphragm; 3) Representative longitudinal spinal cord slide (C1-C6) stained with cresyl violet showing left C2 hemisection (B).

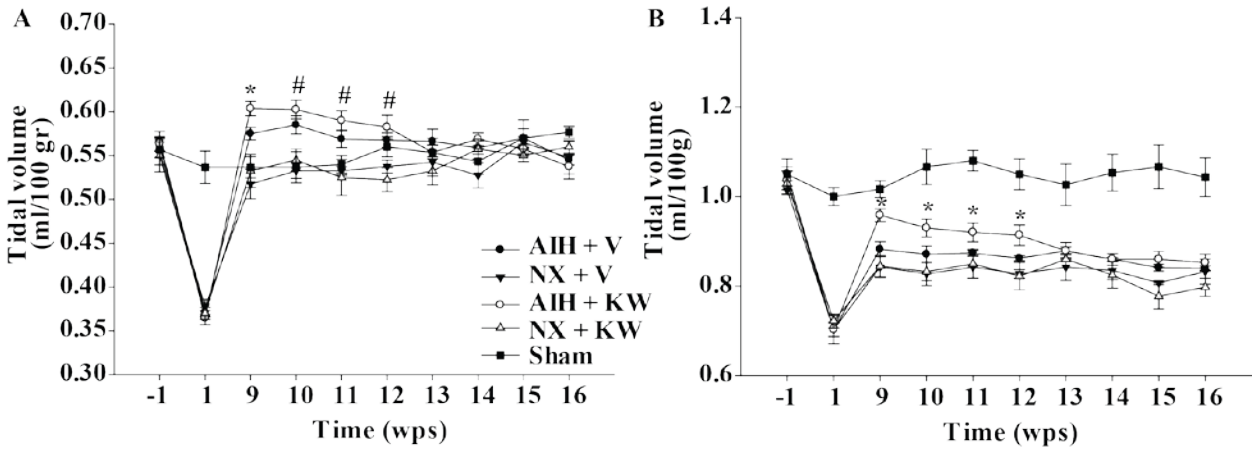

Figure 4. Absolute values of tidal volume (TV) per 100 gr rat during normoxia (Nx, A) and maximum chemoreceptor stimulation (MCS, B) in all groups one week before spinal injury and then, 1 up to 16 weeks post spinal cord injury (wps). Note: (1) time control rats show normal tidal volume at 9 wps during normoxia (A); (2) AIH + V-treated rats show a significant increase in TV compared to controls and sham rats demonstrating ventilatory long term facilitation (vLTF) up to 11 wps during Nx (A); (3) Istradefylline significantly enhances AIH-induced vLTF during normoxia (A) and MCS (B) up to 12 wps; (4) from 13 up to 16 wps there are no differences among groups. AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002 (Istradefylline), NX (normoxia, control rats). Values are means ± SEM. *significantly different from AIH+V and controls, #significantly different from controls; $p < 0.05$.

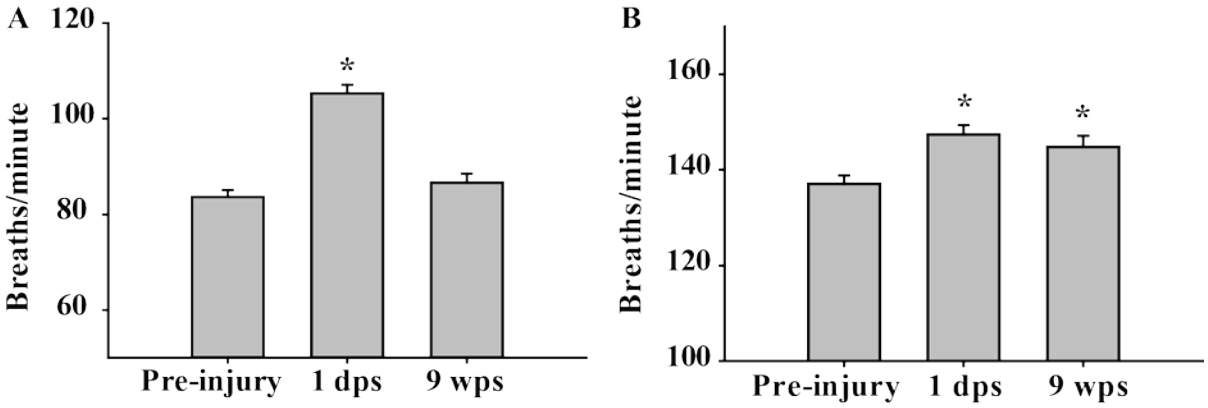

Figure 5. Absolute values of respiratory frequency during normoxia (Nx, A) and maximum chemoreceptor stimulation (MCS, B) in all groups studied before spinal cord injury, one day post spinal injury (1 dps) and 9 weeks post spinal injury (9 wps). Note: (1) significant increase in respiratory frequency 1 dps which return to normal 9 wps; (2) Respiratory frequency remains greater than pre-injury values during MCS.*significantly different from pre-injury values; $p<0.05$.

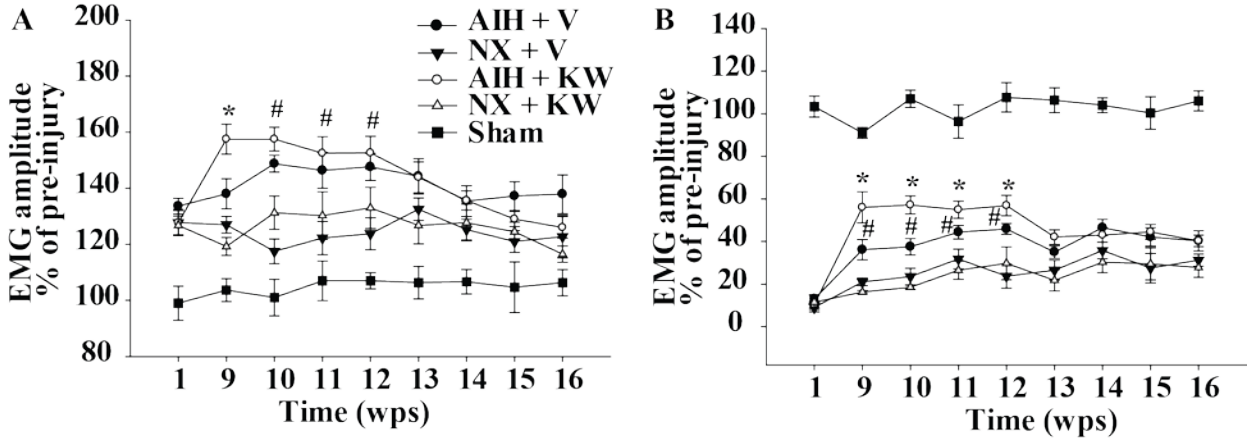

Figure 6. Changes in contralateral (uninjured, A) and ipsilateral (injured, B) diaphragm muscle amplitude during quiet breathing (normoxia) expressed as percent change of pre-injury values 1 up to 16 weeks post injury (wps). Note: (1) contralateral diaphragm shows an increase in motor output (i.e., compensatory plasticity) in all groups throughout the study; (2) small spontaneous recovery in ipsilateral diaphragm (i.e., ipsilateral plasticity); (3) dAIH significantly enhances ipsilateral plasticity up to 12 wps (B); 4) dAIH and adenosine A2A inhibition significantly enhance compensatory and ipsilateral plasticity up to 12 wps. 5) From week 13 up to the end of the study there was no significant difference among groups (see discussion). AIH: acute intermittent hypoxia, NX: normoxia, V: vehicle (DMSO), KW: KW6002 (Istradefylline).Values are mean ± SEM.* significantly different from AIH + V and controls, # significantly different from controls; $p < 0.05$.

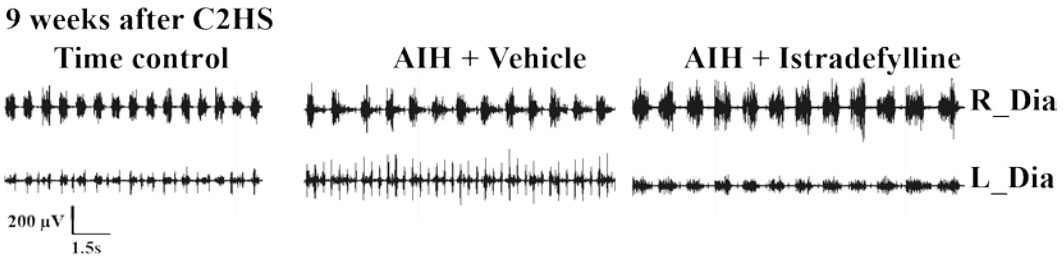

Figure 7. Representative raw EMG activity of right/left diaphragm (R/L_Dia) 9 weeks after cervical C2 hemisection (C2HS) during quiet breathing (normoxia). Notice the increased motor EMG amplitude in bilateral diaphragm in AIH + Istradefylline-treated rats, compared to AIH + vehicle (DMSO) and time control rats. AIH: acute intermittent hypoxia.

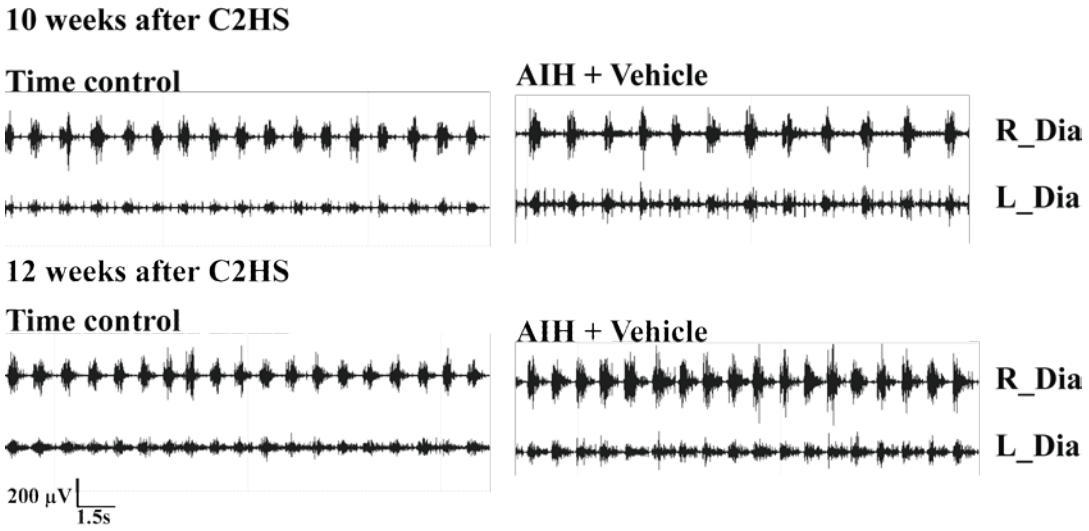

Figure 8. Representative raw EMG activity of right/left diaphragm (R/L_Dia) 10 and 12 weeks after cervical C2 hemisection (C2HS) during quiet breathing (normoxia). Notice the increase in motor EMG amplitude in AIH + vehicle (DMSO)-treated rats, compared to time control rats in bilateral diaphragm. AIH: acute intermittent hypoxia.

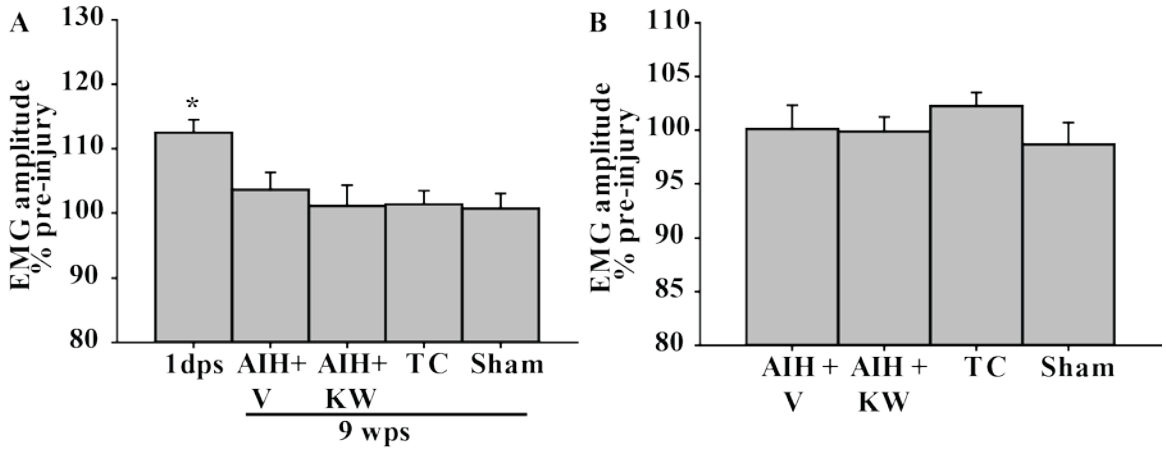

Figure 9: Changes in contralateral (uninjured, A) and ipsilateral (injured, B) second external intercostal (T2 EIC) muscle amplitude during quiet breathing (normoxia) expressed as percent change of pre-injury values 9 weeks post-injury (wps). Note: 1) contralateral T2 EIC muscle exhibits a small increase in motor output (i.e. compensatory plasticity) one day post-injury (1 dps) returning to normal 9 wps; 2) ipsilateral T2 EIC muscle shows complete recovery 9 wps; 3) there was no effect of AIH and/or adenosine A2A inhibition in bilateral T2 EIC muscle which may be due to a ceiling effect (see discussion). AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002 (Istradefylline).Values are mean ± SEM.* significantly different from values at 9 wps; $p < 0, 05$.

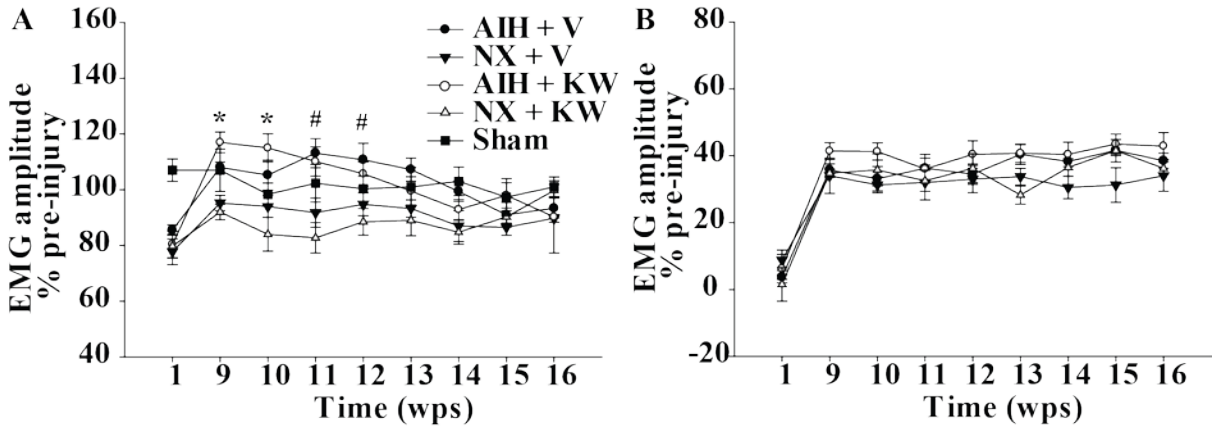

Figure 10. Changes in contralateral (uninjured, A) and ipsilateral (injured, B) diaphragm muscle amplitude during maximum chemoreceptor stimulation (MCS) expressed as percent change of pre-injury values 1 up to 16 weeks post injury (wps). Note: (1) AIH and adenosine A2A inhibition significantly increases EMG peak amplitude in contralateral diaphragm at 9 and 10 wps (A). (2) AIH alone or combined with KW6002 significantly increases EMG amplitude at 11 and 12 wps; (3) Small MCS response is observed in left diaphragm (B) throughout the entire study with no differences among groups. AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002 (Istradefylline). Values are mean ± SEM. *AIH+V significantly different from controls, #AIH+KW6002 significantly different from controls; $p < 0, 05$.

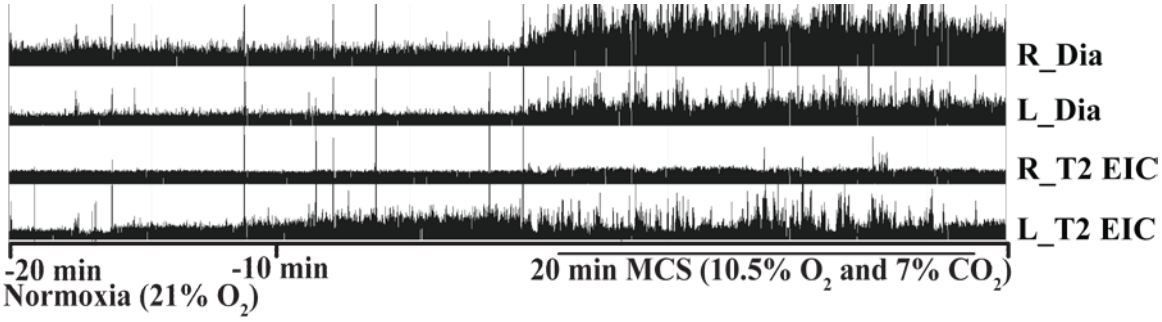

Figure 11. Representative integrated EMG activity of right/left diaphragm (R/L_Dia) and second external intercostal muscle (R/L_T2 EIC) at 16 weeks after cervical C2 hemisection during maximum chemoreceptor stimulation (MCS). Note: 1) small MCS response in left diaphragm. 2) Robust MCS response in right diaphragm. 3) MCS response in bilateral T2 EIC muscle remains abolished.

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CHAPTER VII

GENERAL DISCUSSION

The fundamental goal of this thesis was to test the hypothesis that repetitive acute intermittent hypoxia induces respiratory plasticity that confers functional benefits, compensating for functional deficits caused by acute and chronic cervical spinal injury in unanesthetized rats. The major conclusions of this work include: **(1)** during quiet breathing conditions there is a consistent inspiratory phasic activity in second external intercostal (T2 EIC) and variable inspiratory activity in mid-costal (T4-T7 EIC) muscles. Abdominal (rectus abdominis and external oblique) and caudal T8, T9 EIC muscles do not exhibit expiratory activity; **(2)** moderate acute intermittent hypoxia (AIH) elicits diaphragm as well as even greater inspiratory intercostal long term facilitation (LTF) in normal unanesthetized rats, suggesting that plasticity is a variable property of multiple inspiratory muscles; **(3)** adenosine A_{2A} inhibition enhances diaphragm but not inspiratory intercostal LTF, suggesting that different mechanisms influence phrenic versus thoracic respiratory motor plasticity; **(4)** daily AIH for 7 days improves breathing capacity and the capacity to increase diaphragm muscle activity following cervical spinal injury, but the diaphragm effects are primarily on the side contralateral to injury; **(5)** adenosine A_{2A} inhibition impairs dAIH-induced motor recovery in diaphragm in acute (1-7 weeks) C2 cervical hemisected (C2HS) rats, but enhance it with chronic hemisection (8-16 weeks), confirming our hypothesis that functional recovery in **acute** SCI is adenosine-dependent whereas it is serotonin-dependent (adenosine constrained) with **chronic** injury; **(6)** Although weekly presentations of AIH are not sufficient to retain the functional benefits of dAIH in acutely injured rats, AIH 3 times per week was able to maintain dAIH-induced functional recovery with chronic injury, thus extending the benefits of dAIH.

In this section, I will summarize the effects of moderate acute intermittent hypoxia in respiratory motor plasticity in normal and spinally injured unanesthetized rats. I will conclude with remarks on how this body of work contributes to the overall field of respiratory neurobiology and advances translation of our basic studies to possible clinical application.

Summary and Significance

In the **first chapter** of this thesis I established that the physiological effects of intermittent hypoxia (IH) are tightly associated with the dose of IH, including the severity of hypoxia (fraction of inspired oxygen), duration of the hypoxic stimuli, the number of cycles per day, the total time of exposures (days to weeks), and finally, the pattern of presentations across time (consecutive vs. alternating days). Extensive evidence indicates that moderate IH episodes (≥ 9% O_2) as well as lower cycles per day (≤15 cycles/day) are associated with therapeutic effects in several clinical conditions. In contrast, severe (<9% O_2) and/or more frequent (40-2400 cycles/day) IH cycles also elicit deleterious effects.

Moderate IH protocols (≥ 9% O_2, < 15 cycles/day) reduce hypertension (Serebrovskaya et al., 2008), strengthen innate immune responses while reducing inflammation (Serebrovskaya et al., 2011), reduce body weight, increase aerobic capacity (Urdampilleta et al., 2012), improve glucose tolerance (Chiu et al., 2004), increase bone mineral density (Guner et al., 2013), enhance spatial learning and memory (Zhang et al., 2005, Lu et al., 2009), rescue ischemia-induced memory impairment (Tsai et al., 2011, Tsai et al., 2013), reduce symptoms of depression (Basovich, 2010), improve post-ischemic recovery of myocardial contractile function (Wang et al., 2011), increase respiratory capacity in patients suffering from chronic obstructive pulmonary disease (Haider et al., 2009), and increase respiratory and non-respiratory somatic motor recovery following spinal injuries in rats and humans (Vinit et al., 2009, Lovett-Barr et al., 2012, Trumbower et al., 2012) without increasing inflammatory mediators (Tam et al., 2007, Querido et al., 2012). Thus, the moderate daily acute intermittent hypoxia protocol (dAIH: 10 episodes of 5 min 10.5% O_2, 5 min normoxic intervals, 7 days) studied here may have therapeutic potential to treat respiratory impairment with, for example, cervical spinal cord injuries or motor neuron disease. Moderate intermittent hypoxia could be universally applied in human patients since it is a safe, easy to administer, non-invasive and effective treatment strategy. Moreover, the frequent occurrence of negative side effects associated with most pharmacological therapies makes IH an attractive treatment option with great potential for therapeutic benefit in multiple clinical disorders.

In recent years, the rat has become an important animal model to study traumatic spinal cord injuries, not only because they are readily available but also because the morphological, biochemical, and functional changes that occur after SCI are similar to those seen in humans (Onifer et al., 2007). However, most of our understanding about the recruitment/use of inspiratory and expiratory muscle activity comes from studies in dogs, cats and humans (De Troyer et al., 1985, De Troyer and Wilson, 2002). Very few studies have characterized accessory respiratory muscle activity, including the external intercostal (EIC) and abdominal muscles in unanesthetized rats (Megirian et al., 1987). The lone study of Megirian and colleagues (Megirian et al., 1987) used a connecting cable to detect EMG signals, which may increase animal distress. Throughout this thesis, we used a radiotelemetry system, which allow EMG activity measurements in unanesthetized and unrestrained animals for long periods of time.

In the **third chapter** of this thesis I demonstrated that phasic respiratory activity in external intercostal muscles depends on the particular ribcage muscle under study, the animal's posture and the gas mixture it breathes. Consistent with previous studies in rats and dogs (Megirian et al., 1987, DiMarco et al., 1992) it was demonstrated that second thoracic (T2) EIC muscle always exhibit inspiratory activity during quiet breathing (eupnea). Considering the consistent inspiratory activity of diaphragm and T2 EIC muscles, these muscles were targeted in subsequent spinal cord injury experiments (chapters 3, 4 and 5). Mid-costal segments (T4-T7 EIC) show variable inspiratory activity superimposed to tonic activity. Interestingly caudal segments (T8-T9 EIC) and abdominal muscles show tonic activity exclusively without evidence for expiratory activity, consistent with studies done in unanesthetized dogs (De Troyer et al., 1989, de Almeida et al., 2010). Moreover, maximum chemoreceptor stimulation (MCS: 10.5% hypoxia and 7% hypercapnia) is a stronger stimulus than sustained poikilocapnic hypoxia (SH: 10.5% O_2) in diaphragm and rostral (T2, T4, and T5) EIC muscles for both EMG peak amplitude and ventilatory measurements. Considering the great spontaneous recovery of rats after spinal cord injuries (Beattie et al., 1997, Nantwi et al., 1999, Bareyre et al., 2004), a protocol of MCS was used in chapter 4 and 5 to assess and compare experimental vs. control spinal cord injured rats during a standardized, elevated level of respiratory drive.

Moderate acute intermittent hypoxia (AIH: 3, 5 min episodes of 10.5% O_2 interspersed with 5 min normoxic intervals) elicits plasticity in the phrenic motor system (Feldman et al., 2003, Mitchell and Johnson, 2003, Mahamed and Mitchell, 2007). AIH-induced plasticity has functional consequences, expressed as a persistent increase in phrenic motor output (phrenic long-term facilitation, pLTF), diaphragm activity (diaphragm long-term facilitation, diaLTF) and tidal volume (ventilatory LTF, vLTF) in unanesthetized, spontaneously breathing rats (Terada and Mitchell, 2011). **Chapter 3** of this thesis confirms that moderate AIH elicits robust diaLTF. Furthermore, it was demonstrated that AIH also elicits a greater relative LTF in the peak EMG amplitude in T2 EIC muscles (i.e., T2 EIC LTF) in unanesthetized rats. Interestingly, T2 EIC LTF is twice the coincident value of diaLTF, suggesting that plasticity in the inspiratory accessory muscles has considerable potential to increase their relative contributions to tidal volume generation with clinical disorders that cause respiratory insufficiency. The effect of AIH in mid-thoracic segments (T4, T5 EIC) displays high variability. Moderate AIH elicits either an increase (i.e., LTF) decrease (i.e., long term inhibition, LTI), or no change in EMG peak amplitude (i.e., LTF), which seems related to the posture of the animal at the moment of the exposure to AIH. A curled-up position during AIH favors LTF. In contrast, an extended position favors LTI, whereas a semi-curled posture elicits no change after AIH.

Our findings confirm that AIH-induced LTF is a property of inspiratory muscles, as originally reported by Fregosi and Mitchell (Fregosi and Mitchell, 1994). Repeated carotid sinus nerve (CSN) stimulation evokes a serotonin-dependent long-term facilitation (LTF) of both phrenic and inspiratory internal intercostal (IIC) nerve in anesthetized cats. Similarly, CSN stimulation-evoked LTF of IIC activity exceeds that of phrenic activity (Fregosi and Mitchell, 1994), consistent with a greater T2 EIC LTF versus DiaLTF in the present study. Although the physiological significance of LTF remains uncertain, it may stabilize breathing during sleep. Apneas, with consequent hypoxic episodes, are frequent during sleep; if multiple apneas occur in succession, intermittent hypoxia may induce LTF and prevent subsequent apneas by increasing respiratory motor output in inspiratory muscles (diaphragm, intercostals) as well as muscles of the upper airway (genioglossus), stiffening and increasing the patency of the upper

airway. This hypothesis is consistent with the observation that 15–25 s hypoxic episodes are sufficient to elicit LTF; a duration consistent with apnea-induced hypoxia. Regardless of its specific role, LTF indicates the capacity to alter synaptic strength of inputs to respiratory motoneurons. This capacity may impart the ability to shift the balance of motoneuron pools contributing to a breath. For example, during pregnancy, when the growing fetus compromises diaphragmatic function, a shift toward greater activation of accessory inspiratory muscles alleviates functional breathing deficits.

Our working model of AIH-induced pLTF is that episodic hypoxia activates raphe serotonergic neurons that project to phrenic motor nuclei. Episodic spinal serotonin release during hypoxic episodes subsequently activates Gq protein-coupled 5-HT2 receptors on or near phrenic motor neurons, and initiates intracellular cascades that underlie pLTF (Dale-Nagle et al., 2010). Moreover, activation of adenosine A_{2A} receptors coupled to Gs protein gives rise to phrenic motor facilitation (PMF) (Dale-Nagle et al., 2010) (Fig. 1). On the other hand, sub-threshold activation of adenosine 2A receptors constrains the expression of serotonin-dependent pLTF following AIH (Hoffman et al., 2010). Systemic and spinal adenosine A_{2A} receptor inhibition relieves this inhibitory constraint and enhances pLTF (Hoffman et al., 2010). **Chapter 4** of this thesis aimed to test the hypothesis that systemic A_{2A} receptor inhibition enhances AIH-dependent diaphragm and T2 EIC LTF in unanesthetized rats. Systemic A_{2A} receptor inhibition elicits enhanced diaLTF, but not T2 EIC LTF in normal unanesthetized rats, indicating that the neural control mechanisms underlying respiratory drive to these muscles is different. Mechanistic differences underlying phrenic vs. thoracic respiratory plasticity remain to be investigated.

We propose that AIH-induced pLTF (Gq protein-mediated) and A_{2A} receptor induced PMF (Gs protein-mediated) are distinct mechanisms that interact via cross-talk inhibition. We hypothesize that Gs protein-coupled A2A receptors constrain AIH-induced pLTF via PKA activation (Hoffman and Mitchell, 2013). One possibility is that PKA phosphorylates NADPH oxidase subunits and inhibits NADPH oxidase activity (Bengis-Garber and Gruener, 1996, Kim et al., 2007), thereby reducing ROS formation necessary for pLTF (MacFarlane and Mitchell, 2008) (Fig. 1). Reduced ROS formation would cause less inhibition of protein phosphatases known to constrain pLTF (MacFarlane et al., 2008, Wilkerson et al.,

2008). An alternative proposed mechanism is that activated PKA, increases protein kinase B (Akt) activation, which is an upstream positive regulator of the mammalian target of rapamycin (mTOR). MTOR activation leads to mitogen-activated protein kinases (MAPK) inhibition trough a negative feedback loop stemming from ribosomal protein S6 kinases (S6K1) (Carracedo et al., 2008). Since extracellular regulated MAP Kinases (ERK) are relevant downstream signaling molecules involved in AIH-induced pLTF (Wilkerson and Mitchell, 2009, Hoffman et al., 2012), its inactivation through mTOR may represent an alternative mechanism for cross-talk inhibition. Nevertheless, these possibilities are speculative; cellular mechanisms underlying cross-talk inhibition remain to be explored.

Following cervical C2 hemisection (C2HS), single presentations of moderate AIH (3 episodes) restore ipsilateral phrenic nerve activity, but only if delivered >8 weeks post-injury (Golder and Mitchell, 2005), which coincides with the return of serotonergic innervation to the phrenic motor nucleus below the injury (Golder and Mitchell, 2005). We hypothesized that acute (7 days) C2HS rats do not express serotonin-dependent diaphragm and T2 EIC LTF, which is consistent with our findings in chapter 4. In contrast, a robust diaphragm and T2 EIC LTF is observed in chronic (8 weeks) C2HS rats, a time-frame where serotonin terminals innervating phrenic motor nuclei have been partially restored (Golder and Mitchell, 2005).

In acute (<8 weeks) spinal cord injured rats, AIH-induced functional recovery appears to be adenosine-dependent. For example, daily AIH (dAIH: 10 episodes of 5 min 10.5% O_2 interspersed with 5 min normoxia, 7 days), beginning 1 week post-C2HS restores ipsilateral phrenic motor output and restores breathing capacity in unanesthetized rats (Lovett-Barr et al., 2012); this recovery is not blocked by methysergide, non-selective serotonin receptor antagonist, suggesting a serotonin-independent mechanism (Terada, Vinit, MacFarlane and Mitchell, unpublished). Accordingly, we hypothesized that adenosine A_{2A} antagonist (Istradefylline) impairs dAIH-induced functional recovery in acute C2HS unanesthetized rats (**chapter 5**). Indeed, although dAIH enhances EMG motor activity in contralateral diaphragm and T2 EIC muscle, this effect was greatly impaired by Istradefylline in diaphragm, but not in T2 EIC functional recovery (Fig.3). Since A_{2A} receptor inhibition does not impair dAIH-induced

ventilatory functional recovery, and T2 EIC muscles show significant spontaneous recovery, dAIH-induced respiratory plasticity in inspiratory intercostal muscles may be a critical contributor to functional recovery of ventilatory capacity, thus preventing inhibitory effects of Istradefylline on dAIH-induced ventilatory functional recovery.

Although dAIH significantly increases tidal volume in acute C2HS rats, it does not enhance automatic grooming behavior, which differs from other studies in rats and humans (Lovett-Barr et al., 2012, Trumbower et al., 2012). We may speculate that dAIH has a greater impact in contralateral intact muscles than ipsilateral forelimb; however, we cannot rule out that the grooming test may not be sensitive enough to detect subtle somatic improvements elicited by dAIH.

In **chapter 3**, we found that T2 EIC LTF is double that of diaLTF. Therefore, we hypothesize that there is a shift in the balance of diaphragm versus inspiratory intercostal contribution to breathing capacity, showing a greater relative contribution of inspiratory intercostal muscles after spinal cord injuries. Indeed, it was found a remarkable spontaneous recovery in left (ipsilateral) T2 EIC muscle at 7 days post-C2HS (**chapter 5 and 6**), consistent with studies done in anesthetized rats (Sherrey and Megirian, 1990, Dougherty et al., 2012a), and confirming that accessory muscles may significantly compensate for respiratory deficits during quiet breathing conditions. Further studies are warranted to determine the mechanisms underlying enhancement of intercostal muscle EMG activity after C2 spinal hemisection. Removal of a sensory inhibitory reflex arising from phrenic sensory nerves onto thoracic motor neurons increases inspiratory motor activity in intercostal muscles in dogs (De Troyer, 1998).

Cervical hemisection of the C2 spinal cord (C2HS) transiently paralyzes the hemi-diaphragm; however, partial return of ipsilateral phrenic motor neuron inspiratory bursting occurs and it has been termed the spontaneous crossed-phrenic phenomenon (CPP). Evidence suggests that the contribution of CPP to ventilatory capacity is negligible in anesthetized rats (Nantwi et al., 1999, Fuller et al., 2006, Vinit et al., 2006, Dougherty et al., 2012b). We confirmed those findings in unanesthetized rats. The average of ipsilateral diaphragm amplitude was similar among groups with a range between 18-30 % of pre-injury values in both acute and chronic C2HS rats during quiet breathing (eupnea). Similar studies in

anesthetized rats have shown spontaneous recovery of 28.75%, when expressed as a percent of activity in the homolateral nerve of non-injured animals (Nantwi et al., 1999).

Although external intercostal muscles may have a functional significance during quiet breathing conditions, they do not contribute during maximal chemoreceptor activation. We speculate that contralateral respiratory motor neurons are recruited early after hemisection to compensate for the loss of crossed phrenic and thoracic activity. Therefore, these muscles reach a "ceiling effect" where they cannot further increase their motor output during MCS. A similar effect may explain the lack of MCS response in ipsilateral T2 EIC muscle regardless of its remarkable spontaneous recovery in acute and chronic C2HS unanesthetized rats (chapter 5 and 6). In contrast, contralateral (uninjured) diaphragm showed a consistent MCS response at all time-points, demonstrating that compensatory plasticity in diaphragm has considerable functional significance during respiratory challenge. Although MCS responses in the ipsilateral (injured) diaphragm are absent in acute C2HS rats (1-7 weeks post-C2HS), this response is partially restored in chronic C2HS rats (8-16 weeks post-C2HS) reaching an average of roughly 30% of pre-injury values.

Since serotonin-dependent pLTF is constrained by coincident activation of A_{2A} receptors in normal rats (Hoffman et al., 2010), we hypothesized that systemic administration of an A_{2A} receptor antagonist (Istradefylline) would enhance dAIH-induced functional recovery in chronic C2HS rats. We confirmed this hypothesis. Istradefylline, a highly specific A_{2A} receptor antagonist enhances dAIH-induced functional recovery during normoxia and MCS, which is likely attributed to increased ipsilateral and contralateral plasticity in the diaphragm, demonstrating that serotonin-dependent AIH is once again constrained by an adenosine-dependent mechanism (Fig.3). The mechanisms underlying interactions between adenosine and serotonin and long-term effects of A_{2A} receptor remains to be explored.

Weekly presentations of AIH cannot increase functional recovery above time control rats in acute C2HS rats. Therefore, we modified the protocol with chronically injured rats studied in **chapter 6** by investigating a more robust protocol of repetitive AIH (rAIH), namely AIH 3 time per week (3xwAIH) which elicits robust neurochemical changes in the phrenic motor nucleus (Satriotomo et al., 2012).

Repetitive AIH for 10 weeks increases key molecules involved in AIH-induced pLTF, including serotonin, serotonin receptors, BDNF, TrkB and ERK MAP kinases (Satriotomo et al., 2012). Moreover, 3xwAIH induces metaplasticity (the ability of prior experience to alter subsequent plasticity). Rats exposed to 3xwAIH (ten 5-min episodes/day, 3 days/week for 4 weeks) exhibit enhanced pLTF (MacFarlane et al., 2010, Vinit et al., 2010). In chapter 6 it is demonstrated that repetitive AIH (3xw) maintains the effect of dAIH up to 12 weeks post-C2HS, which may represent a safe and effective protocol to extend the therapeutic benefits of an initial priming dose of dAIH. Mechanisms underlying rAIH-induced metaplasticity are not yet known.

The lack of ipsilateral and contralateral plasticity 13-16 weeks post-C2HS may be related with the age (8 month-old) of experimental and control rats at those time-points. Serotonin-dependent LTF decreases with advancing age in male rats (Zabka et al., 2001, Behan et al., 2002), which has being hypothesized is due to a decreased levels of testosterone with advancing age (Zabka et al., 2001, Nelson et al., 2011). Although the role of testosterone/estrogen in the control of breathing is not well understood, it has been proposed that sex hormones could have a neuromodulatory effect on serotonin receptors (Skatrud et al., 1978, White et al., 1983, Regensteiner et al., 1989). An additional factor explaining the lack of plasticity with age is the gradual decline of serotonin receptor density as it has been showed in rats and humans (Hyttel, 1987, Morgan, 1987, Ko et al., 1997).

Therapeutic implications

In the United States, there are more than 250,000 individuals living with spinal cord injury (SCI) (NSCISC, 2005). This PhD thesis demonstrates that repetitive AIH (rAIH) represents an effective protocol capable of enhancing AIH-induced plasticity in the spinal cord and thus, increase functional recovery in acute and chronic C2 cervical hemisected (C2HS) unanesthetized rats. Additionally, adenosine A_{2A} inhibition can enhance rAIH-induced respiratory functional recovery in chronic C2HS rats (Fig.3). Therefore, readily available drugs like adenosine 2A receptor antagonists (even caffeine) may represent a useful way to potentiate dAIH-induced functional gains in the treatment of chronic spinal

injuries and other disorders that compromise breathing. Currently, there are no approved therapies for thoracic or cervical SCI; therefore, our approach represents a promising new strategy to enhance function in patients with chronic SCI, where the potential for further functional gains is limited. However, the lack of plasticity observed on later time-points (13-16 weeks post-C2HS) may suggest that there is a limited functional gain with age. The decreased number of serotonin receptors with age may have converted the effect of AIH into an adenosine-dependent mechanism, as in early spinal cord injuries (Fig.2). Therefore, an adenosine A_{2A} antagonist would impair AIH-induced functional recovery after a long-term treatment. The clinical relevance of this hypothesis is that perhaps adenosine A_{2A} antagonists should be administrated only initially for a short period of time; whereas the weekly presentations of AIH (reminders) could be administrated throughout the entire treatment.

The novel technical approach used in this thesis included simultaneous ventilatory measurements and radiotelemetry system for electromyography (EMG). Radiotelemetry to study respiratory muscle activity in spinally injured animals offers several advantages: it allows simultaneous study of multiple muscles in unrestrained, unanesthetized rats with minimum discomfort; it permits continuous data collection (months), a key advantage in performing studies of chronic spinal injuries. Most importantly, since radiotelemetry permits investigation of drug effects in freely moving rats, it enables animal models that mimic real conditions and hence a more reliable clinical translation (Mogensen, 2011). It is recognized that the quality of physiological measurements collected from conscious, unstressed animals is superior since they are collected under conditions that best represent the normal state of the animal, are least influenced by chemical, stressful and psychological factors, and are most predictive of the results that would be achieved in human beings (FDA, 2001).

FIGURES

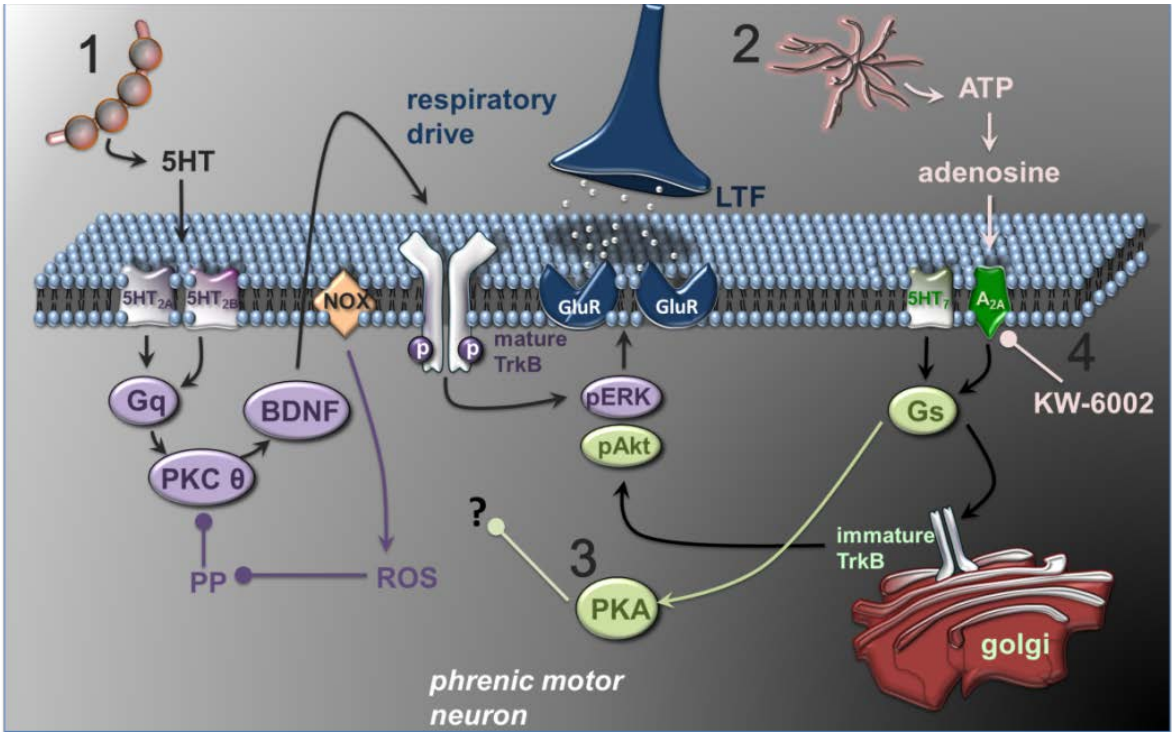

Figure 1. Mechanisms of AIH-induced LTF in normal rats. **(1)** Intermittent hypoxia (IH) stimulates serotonin release from raphe-spinal terminals near phrenic motoneurons. Serotonin activates serotonin type 2 receptors coupled to Gq-protein which in turn activates PKC Θ activity increasing BDNF synthesis from mRNA and NADPH oxidase (NOX) activity within phrenic motoneurons. NOX induce reactive oxygen species (ROS) formation which inhibit phosphatases that constrain LTF. BDNF is secreted extracellularly and binds to mature, fully glycosylated TrkB receptors expressed on the outer cell membrane. BDNF phosphorylates mature TrkB receptors, which in turn strengthen excitatory glutamatergic synapses onto phrenic motoneurons via activated MAP kinases (pERK1/2). **(2)** IH also induces the releases of ATP from astrocytes which extracellularly convert to adenosine. Adenosine activates A2A receptor coupled to Gs-protein increasing the synthesis and phosphorylation of intracellular, hypoglycosylated, immature TrkB protein without the need for BDNF (transactivation).

Phosphorylated intracellular TrkB strengthens excitatory glutamatergic synapses onto phrenic motoneurons via protein kinase B (pAkt). **(3)** A2A receptor-dependent pathway constrains serotonin-dependent LTF during AIH. Hypothetically, A2A receptor activation during AIH may inhibit NADPH-dependent ROS formation, through protein kinase A (PKA) activation, maintaining suboptimal ROS levels. **(4)** Systemic A2A receptor antagonist (KW-6002) prevents A2A receptor activation, enhancing LTF.

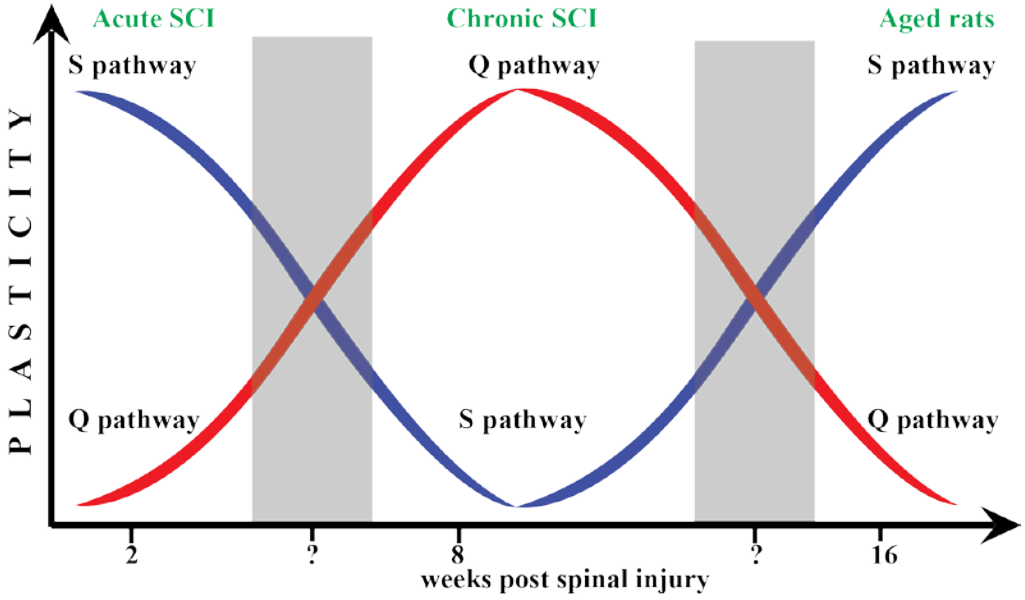

Figure 2. Schematic figure illustrating hypothetical mechanisms of acute intermittent hypoxia (AIH)-induced plasticity after spinal cord injuries (SCI). During early SCI (<8 weeks post-injury), serotonin terminals innervating phrenic motor nuclei have been disrupted and thus, AIH induce respiratory functional recovery due to an adenosine-dependent mechanism (S pathway, blue line). In a more chronic phase, once serotonin terminals are partially restored at 8 weeks post-injury, AIH induce functional recovery due to a serotonin-dependent mechanism (Q pathway, red line). In aged rats (~8 month-old), the number of serotonin receptors dramatically decrease, therefore AIH may induce functional recovery due to an adenosine-dependent mechanism (S pathway), as in early SCI. The time where the S and Q pathways cancel each other out is unknown (grey zone).

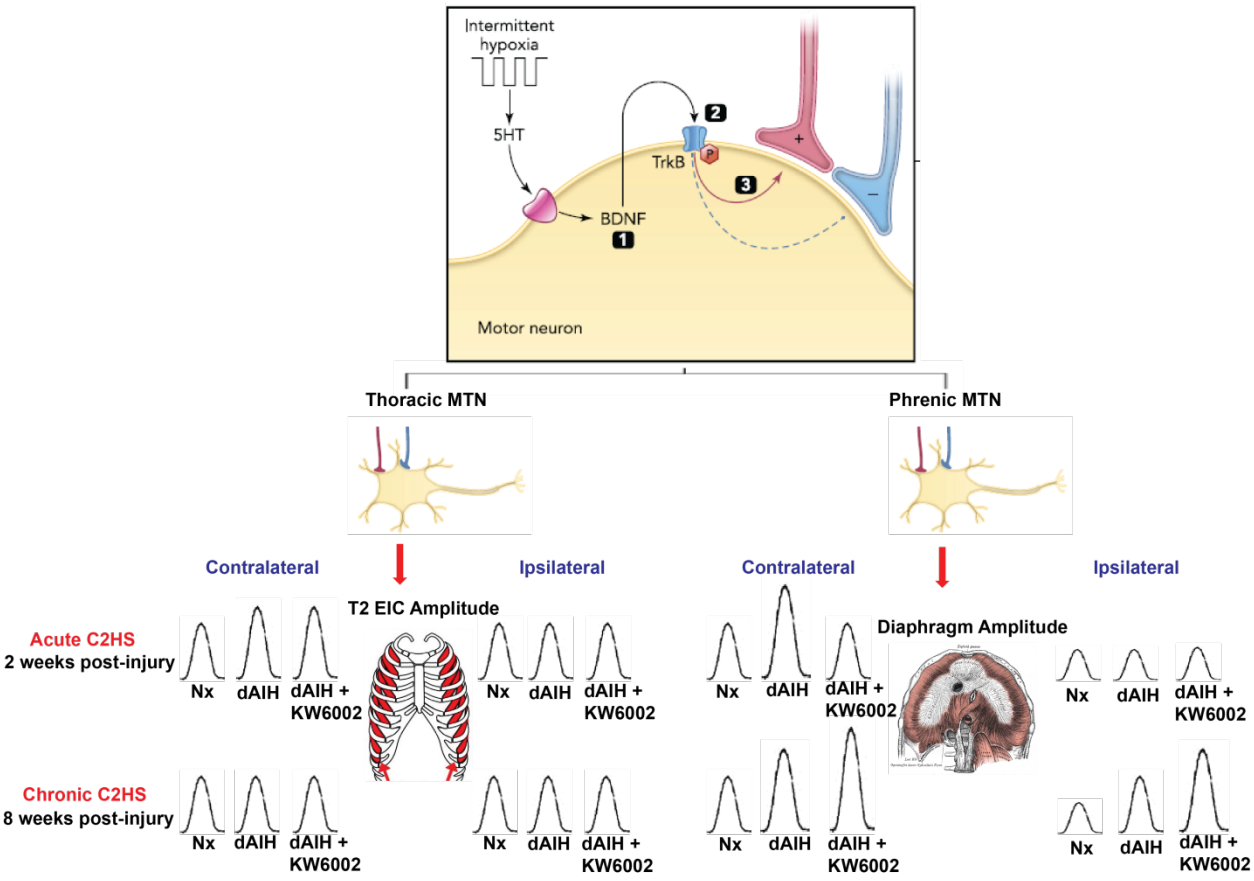

Figure 3. Mechanisms of acute intermittent hypoxia (AIH)-induced motor activity with acute versus chronic cervical C2 hemisection (C2HS). Intermittent hypoxia activates serotonin 5-HT2 receptors (serotonin-dependent, Q pathway) and adenosine A2A receptors (adenosine-dependent, S pathway). In normal rats the S pathway constrains the Q pathway; thus, an adenosine A_{2A} antagonist enhances AIH-induced long term facilitation. Daily AIH (dAIH) elicits functional recovery due to different mechanisms in acute versus chronic C2HS. In the **acute phase** (2 weeks post-C2HS), adenosine A_{2A} receptor inhibition (KW6002) impairs daily AIH (dAIH)-induced motor recovery (amplitude) in contralateral (uninjured) diaphragm but does not affect dAIH-induced motor activity in contralateral second external intercostal (T2 EIC). Ipsilateral (injured) diaphragm and T2 EIC are not affected by dAIH alone or combined with KW6002. At this time point there is a remarkable spontaneous recovery of ipsilateral T2 EIC muscle and a small recovery of ipsilateral diaphragm. In **chronic phase** (8 weeks post-C2HS),

adenosine A_{2A} receptor inhibition enhances dAIH-induced motor activity in contralateral and ipsilateral diaphragm. Ipsilateral T2 EIC muscle is completely recovered at this time-point. Both contralateral and ipsilateral T2 EIC are not affected by dAIH alone or combined with KW6002 which we attributed to a "ceiling effect" (see text). These findings suggest that dAIH-induced functional recovery is adenosine-dependent in the acute phase but serotonin-dependent (adenosine-constrained) in the chronic phase. Adenosine A_{2A} antagonist represents a way of amplifying dAIH-induced functional recovery with chronic spinal cord injuries.

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