SEROTONERGIC INNERVATION OF RESPIRATORY AND NON-RESPIRATORY MOTOR NUCLEI AFTER CERVICAL SPINAL CORD INJURY

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2020
To my family
ACKNOWLEDGMENTS

I would like to thank my family and friends for all their support over the past few years. I thank my parents, Edward P. and Carmen T. Ciesla, and siblings Lindsey T. and William B. Brezina and Edward N. Ciesla, for their unwavering support not only during my doctorate, but also through my whole life. I would also like to thank my mentors Drs. Gordon S. Mitchell and Elisa J. Gonzalez-Rothi who led and helped me grow as a scientist. I thank my committee Drs. Harry Nick, Ron Mandel, and Emily Fox for their guidance in my graduate work. I would like to thank the current and past members of the Mitchell and Gonzalez-Rothi labs for their support and friendship, specifically: Dr. Latoya L. Allen, Dr. Yasin B. Seven, Amy Poier, Kristin Smith, and Ashley Holland for their contributions to this dissertation. I would also like to thank all of the lab members and undergraduates I trained for reminding me how much I love mentoring. I want to thank my fellow PhD friends, Dr. Jess Dhillon, Dr. Melanie Shapiro, and Breanna Burkes who stood by me through the tough classes, presentations, volunteering, teaching, trivia nights and much more. Specifically, I want to thank Dr. Jolie Barter for keeping me sane and fun through all of our crocheting nights, collaborative projects, presentations, study groups, and SFN events. Lastly, I want to thank my boyfriend, Jason Conner for sticking with me, supporting me, always having chocolate, and being a constant ear throughout my graduate work. I could not have completed this work without my whole network of supporters.
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LIST OF ABBREVIATIONS

5-HT  5-hydroxytryptamine / serotonin
5-HT1  serotonin receptor 1
5-HT2  serotonin receptor 2
5-HT7  serotonin receptor 7
A2a  adenosine receptor 2a
AAV  adeno-associated virus
AIH  acute intermittent hypoxia
AKT  protein kinase B
BDNF  brain derived neurotrophic factor
BW  body weight
C2Hx  cervical hemisection at C2
cAMP  cyclic adenosine monophosphate
CIH  chronic intermittent hypoxia
CNS  central nervous system
CPG  central pattern generator
cSCI  cervical spinal cord injury
CtB  cholera toxin B fragment
cVRG  caudal ventral respiratory group
dAIH  daily acute intermittent hypoxia
DNA  deoxyribonucleic acid
ECM  extracellular matrix
EMG  electromyography
EPAC  exchange activation of adenylyl cyclase
ERK-MAP extracellular signal regulated-mitogen-activated protein
IH intermittent hypoxia
LTF long term facilitation
MAP mean arterial pressure
MAPK mitogen-activated protein kinase
Min minutes
mmHg millimeters of mercury
mTOR mammalian target rapamycin
NMDA N-methyl-D-aspartate receptor
NTD neural tube defects
Nx normoxia
PaCO₂ partial pressure of carbon dioxide in arterial blood
PaO₂ partial pressure of oxygen in arterial blood
PBS phosphate buffered saline
PFA paraformaldehyde
pLTF phrenic long-term facilitation
pMF phrenic motor facilitation
rAIH repetitive acute intermittent hypoxia
RMS root mean square
rVRG rostral ventral respiratory group
SBE standard base excess
SCI spinal cord injury
<table>
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<tr>
<td>SERT</td>
<td>serotonin transporter</td>
</tr>
<tr>
<td>TrkB</td>
<td>tropomyosin receptor kinase B</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VRC</td>
<td>ventral respiratory column</td>
</tr>
<tr>
<td>VRG</td>
<td>ventral respiratory group</td>
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Cervical spinal cord injury (cSCI) disrupts axonal projections between the brain and spinal cord, impairing motor functions, e.g. breathing. Since serotonin released by raphe bulbospinal axons promotes plasticity and functional recovery after cSCI, it is important to know the extent of serotonergic innervation, spontaneous re-innervation, and potential strategies to further enhance serotonergic innervation below the injury. In this dissertation, I focus on serotonergic innervation of motor nuclei in an experimental model of cSCI, C2 spinal hemisection (C2Hx), and investigate the impact of two experimental treatments to enhance re-innervation: intermittent hypoxia (IH) and folate supplementation. In Chapters 2-4, I test the hypotheses that different IH protocols increase serotonergic innervation in 1) phrenic and intercostal (AIM 1); 2) hypoglossal (AIM 2); and 3) axial and forelimb (AIM 3) motor nuclei. I assess serotonergic innervation via immunofluorescence in rats with and without C2Hx (12wks post-injury) exposed to 28 days of: 1) normoxia; 2) daily acute IH (dAIH28); 3) mild chronic IH (CIH); and 4) moderate CIH.
I observed spontaneous serotonergic re-innervation below C2Hx in phrenic and intercostal motor nuclei (Chapter 2). Although ipsilateral serotonergic structure number was decreased post-injury, they were larger, increasing total serotonergic area. Although IH had no effects on serotonergic innervation post-C2Hx, CIH increased serotonin structure size and area in phrenic motor nuclei of uninjured rats.

In the hypoglossal motor nucleus, dAlH28 increased serotonergic innervation in uninjured and injured rats (Chapter 3); C2Hx had no independent effect. Ipsilateral serotonergic structure number and total area were reduced post-C2Hx in axial and forelimb nuclei (Chapter 4); IH had no detectable effects. Lastly, I tested the hypothesis that folate supplementation/deprivation enhances/impairs serotonergic re-innervation of phrenic motor nuclei post-C2Hx (Chapter 5). Folate had no impact serotonergic innervation or diaphragm function post-C2Hx.

I conclude that C2Hx deferentially impacts serotonin re-innervation of respiratory versus non-respiratory motor nuclei. The functional impact of increased ipsilateral serotonergic structure size in phrenic nuclei post-C2Hx is unclear. These results indicate substantial spontaneous serotonergic re-innervation below C2Hx, without additional IH or folate effects. This spontaneous re-innervation may restore the capacity for serotonin-dependent plasticity and functional recovery after cSCI.
Spinal Cord Injury

Spinal cord injury (SCI) disrupts the integrity of nerve fibers that convey motor and sensory information from cortical centers and the brainstem to neurons in the spinal cord. Therefore, SCI impairs communication between the brain and the body. Removal of descending pathways, along with neuron loss due to the impact of the injury itself, impairs motor and sensory function below the injury site. The extent of impairment is dependent upon the location and severity of the injury. SCIs in more rostral spinal segments, or closer to the head, cause greater functional impairment, meaning that everything below the neck may be impaired. These high SCIs result in breathing impairments that ultimately lead to respiratory failure (Winslow and Rozovsky, 2003). In this dissertation, I study two highly novel approaches shown to improve motor function after SCI.

The demographics and causes of SCIs have been stable over the past 30 years. Currently in the United States of America, there are approximately 291,000 persons living with SCI, and about 17,700 new cases each year (NSCISC, 2020). Vehicular accidents are the leading cause of SCI, comprising about 39% of injuries; falls are a close second, comprising about 32% of SCI. The average age of the population suffering from SCI has increased from 29 in the 1970s to 43 years of age today. Males suffer more often from SCI, with about 78% of new injury occurring in men. The lifetime socioeconomic burden on people with SCI is estimated to be $4,000,000 per person. Thus, there is a critical need for new therapies to restore function, and quality of life, after SCI (Anderson, 2004; NSCISC, 2020).
Primary and Secondary Damage after Spinal Cord Injury

There are two stages of injury after SCI. The first stage is the initial insult to the spinal cord. Immediately after injury, there is breakdown of the blood spinal cord barrier, accompanied by edema and spinal inflammation that exacerbates the primary injury. These events extend well beyond the site of injury, and persist for weeks post-SCI (Alexander and Popovich, 2009; Windelborn and Mitchell, 2012). In the secondary stages of injury, demyelination, neuronal necrosis, tissue ischemia, edema, and glial scar formation occur. This second stage of injury can last years after the initial insult (Wang et al., 2017). This dissertation utilizes both sub-acute and chronic SCI models in rats. The sub-acute injury in this dissertation is 2 weeks post injury, during the secondary stage of injury. The chronic model of SCI in this dissertation is 8 or more weeks post injury, when damage from the primary and secondary stages of injury are presumably more stable.

An insidious cascade occurs after SCI, contributing to a hostile environment that hinders neural recovery. Disruption of the blood spinal cord barrier leads to infiltration of blood and debris into the spinal cord (Soderblom et al., 2013). Neuron death, periaxonal swelling, and myelin disruption occur within 15 minutes post-injury. Demyelination continues over time, growth cones are inhibited, and axons undergo Wallerian degeneration in rostral and caudal directions (Filous and Schwab, 2018). Apoptosis of cells such as oligodendrocytes begins about 6 hours after injury and continues for up to 3 weeks post-injury, and is thought to be a primary mediator of delayed demyelination and axonal degeneration (Crowe et al., 1997). The surviving post-mitotic oligodendrocytes cannot re-myelinate axons (Silver et al., 2014). In addition, microglia become activated, phagocytose debris, and release pro-inflammatory cytokines.
Astrocytes also become activated, proliferate, and migrate to the injured site (Filous and Schwab, 2018). Astrocytes release pro-inflammatory cytokines and chemokines, as well as growth-inhibitory extracellular matrix molecules (ECM) such as chondroitin sulfate proteoglycans. ECM molecules form a physical wall at the lesion penumbra called the glial scar (Fitch and Silver, 2008). The glial scar encapsulates the lesion, containing intra-lesion inflammatory and cytotoxic elements, preventing damage of nearby tissue (Sofroniew, 2009; Tom et al., 2004). There is debate on which components of the glial scar are responsible for preventing axonal growth.

Aside from the effects of primary and secondary injury, muscle wasting and chronic pain occur post-SCI (Abrams and Ganguly, 2015). People with chronic SCI are also susceptible to systemic infections, sepsis, urinary tract infections and infections from pressure ulcers (Cardenas et al., 2004; McCormick et al., 2013).

**Cervical Spinal Cord Injury**

About 58% of spinal injuries occur in the cervical region of the spinal cord (Anderson, 2004; NSCID, 2019). Cervical spinal cord injuries (cSCI) are devastating since they cause functional loss in the entire body. Functional losses include paralysis, impaired mobility, respiratory insufficiency, neuropathic pain, and loss of functional independence. Of the many impairments resulting from cSCI, compromised respiratory and upper extremity functions are of the utmost importance (Anderson, 2004) and are a focus of this dissertation.

**Neural Control of Breathing**

Breathing is often thought as an autonomic function, meaning that it occurs involuntarily via the sympathetic and parasympathetic nervous systems. However, breathing has little to do with parasympathetic or sympathetic function. In fact, a better description of this
somatic motor system is automatic. Although normally regulated by sub-conscious neural regulation, the normal patterns of breathing are interrupted during behaviors such as speaking, swallowing, laughing, coughing, or holding one’s breath. This section reviews the neural control of breathing including the cortex involved in voluntary breaths, brainstem centers involved in automatic respiration, and the motor neurons that project to respiratory muscles resulting in movements that expand the chest cavity filling the lungs with air.

**Elements of the Neural System Controlling Breathing**

Automatic control of breathing originates primarily in the ventral respiratory column (VRC) of the caudal pons and ventro-lateral medulla (Feldman et al., 2013). The VRC is a population of respiratory motor neurons that extends from the facial motor nucleus to the spinal-medullary junction/obex (Alheid and McCrimmon, 2008; Bianchi et al., 1973). This is where the respiratory rhythm generating neural network resides. Rhythmic signals are relayed from the VRC to spinal motor nuclei controlling respiratory muscles.

The pontine region of the VRC includes areas such as the Kölliker-Fuse nucleus and the parsabrachialis medialis. These regions are thought to modulate the respiratory cycle and play a role in the transition between respiratory phases (Alheid et al., 2004; BAXTER and OLSZEWSKI, 1955). Within the rostral VRC is the pre-Bötzinger complex, Bötzinger complex, retrotrapezoid nucleus, and parafacial respiratory group (Alheid and McCrimmon, 2008). The pre-Bötzinger complex is a critical nucleus for respiratory rhythm generation (Feldman et al., 2013). Rostral to the pre-Bötzinger complex is the Bötzinger complex which is critical for enforcing the expiratory phase of breathing by inhibiting inspiratory motor pools. The mutual constraint between these pools
maintain/enforce the alternating inspiratory and expiratory phases (Feldman et al., 2013). The retrotrapezoid nucleus senses increasing CO$_2$ and decreasing pH and in turn stimulates breathing (Alheid and McCrimmon, 2008). The function of the parafacial respiratory group is debated, but some believe it is involved in expiratory rhythm generation (Alheid and McCrimmon, 2008; Feldman et al., 2003).

Within the caudal VRC is the ventral respiratory group (VRG)(Alheid and McCrimmon, 2008). The VRG receives excitatory and inhibitory respiratory signals from the pre-Bötzinger and Bötzinger complexes. The rostral part off the VRG (rVRG) is known for its role in active inspiration. The rVRG receives excitatory input from the pre-Bötzinger complex during inspiration, and inhibitory input from the Bötzinger complex during expiration (Feldman et al., 2013; McCrimmon et al., 1995). Pre-inspiratory bulbospinal projections from the rVRG descend via the dorsolateral and ventromedial white matter to the ventral gray matter, where they synapse onto respiratory motor neurons within the spinal cord (Feldman et al., 2013; Lipski et al., 1994). The caudal part of the VRG (cVRG) is known for its role in active expiration, and receives excitatory input from the retrotrapezoid nucleus/parafacial respiratory group during expiration (Feldman et al., 2013). Pre-inspiratory bulbospinal projections from the cVRG cross the midline caudal to the obex and project to expiratory intercostal and abdominal spinal motor neurons (Fedorko and Merrill, 1984; Shiba et al., 1996).

Voluntary control of breathing interrupts the automatic respiratory rhythm to support behaviors such as swallowing, talking and holding one’s breath. Although little is known concerning the neuroanatomy of voluntary control of breathing, the corticospinal pathway directly innervates phrenic motor neurons (Butler, 2007;
Macefield and Gandevia, 1991). This suggests that the cite of voluntary breathing is in the spinal cord (Mitchell and Berger, 1975). In this proposed pathway, medullary centers are not bypassed but rather they communicate via afferent information (Butler, 2007).

**Phrenic diaphragm system**

The main group of spinal motor neurons responsible for breathing are the phrenic motor neurons in the mid-cervical spinal cord segments: C3-C6 in rats and C3-C5 in humans (Ellenberger and Feldman, 1988; Greer et al., 1992; Lane et al., 2008). Because of their importance in breathing, phrenic motor neurons are a major focus of this dissertation. Phrenic motor neurons receive bilateral VRG projections, with most crossed-spinal pathways decussating in the brainstem (Lipski et al., 1994). There are also “silent,” ineffective, contralateral projections from the VRG that decussate caudal to C2, known as crossed-phrenic pathways (Goshgarian et al., 1991). These latent pathways can be strengthened and transmit respiratory information to the contralateral spinal cord when respiration is disturbed (Fuller et al., 2006; Fuller et al., 2003; Golder and Mitchell, 2005; Goshgarian, 1979, 2009; Vinit et al., 2009). There are about 600 phrenic motor neurons in the cervical spinal cord (Boulenguez et al., 2007; Mantilla et al., 2009). These neurons project from the cord and innervate the diaphragm, a major inspiratory muscle. During inspiration, excitation of phrenic motor neurons causes the diaphragm to contract, creating negative pressure that allows air to enter the lungs. During expiration there is inhibition of phrenic motor neurons causing the diaphragm to relax and air to exit the lungs (Richter, 1982). A diagram of the phrenic diaphragm system is depicted in Figure 1-1.
Accessory respiratory pump muscles

Accessory respiratory muscles aid in breathing, particularly with elevated breathing demand or with injury or disease. Of particular interest to this dissertation are the intercostal muscles, located in the thorax in between adjacent ribs. They are innervated by intercostal motor neurons that reside throughout the thoracic spinal cord (T1-T11) (Monteau and Hilaire, 1991). Intercostal motor neurons receive information similar to phrenic motor neurons (Dobbins and Feldman, 1994; Merrill and Fedorko, 1984), from the descending brainstem respiratory centers. Intercostal motor neurons include the external, internal, and innermost intercostal motor neurons (Pilarski et al., 2019). External intercostal muscles lie on the exterior of the ribs and close to the sternum. External intercostal muscles are involved in expanding the chest cavity during inspiration. Internal and innermost intercostal muscles provide support of the chest wall and are responsible for expiratory efforts. Many other accessory pump muscles are involved in respiration and are not discussed in this dissertation including abdominal, obliques, pectoralis, clavicles, and accessory muscles of the back and neck, to name a few (Pilarski et al., 2019).

Upper airway muscles regulating airway resistance and patency

Upper airway muscles involved in breathing are the tongue, soft pallet, pharynx, larynx, and muscles of the head and neck (Pilarski et al., 2019). Humans must maintain a patent airway for air to flow, which requires activation of these upper airway muscles. Malfunctions in this patency can cause the airway to collapse resulting in an obstruction that disrupt air flow—an apnea (Dempsey et al., 2010).

This dissertation examines hypoglossal motor neurons, which innervate up to 7 tongue muscles. The hypoglossal motor nucleus comprises a column of motor neurons
approximately 3 mm long and is somatotopically organized based on location and function (Krammer et al., 1979). In short, this organization consists of lateral and medial branches of the hypoglossal nerve. The lateral hypoglossal branch comprises the styloglossus and hyoglossus nerve which lies on the dorsal most part of the nucleus and acts to retract the tongue. The medial branch of the hypoglossal nerve is comprised of the genioglossus and geniohyoid nerves which are the ventral part of the nuclei and act to protrude the tongue (Krammer et al., 1979). Hypoglossal motor neurons receive respiratory related input from the pre-Botzinger complex and VRG (Cheng et al., 2008; Fregosi, 2011; Lipski et al., 1994). During inspiration, the tongue moves out of the way to open the airway for a breath by moving down and anterior during inspiration (Cheng et al., 2008).

**Respiratory Consequences of Cervical Spinal Cord Injury**

High cSCI disrupts bulbospinal projections from the VRC to respiratory motor neurons (i.e. phrenic and intercostal). Indeed, the most devastating consequence of cSCI is impaired breathing function. A rapid, shallow breathing pattern, lack of drive to breathe, and lack of breathing coordination are all examples of breathing impairments after cSCI. People with cSCI often become dependent on mechanical ventilation to maintain adequate respiration (Brown et al., 2006). Airway protective behaviors are also impaired after cSCI. For example, people with cSCI have an ineffective cough that can lead to bronchial infections (De Troyer and Estenne, 1991). Unfortunately, the leading cause of death after cSCI is respiratory failure (Winslow and Rozovsky, 2003). Thus, there is a critical need to enhance respiratory function after cSCI. This dissertation addresses therapeutics with the potential to enhance breathing function in rodent models of cSCI.
Neural Control of Axial and Forelimb Motor Function

Axial motor neurons innervate muscles of the medial motor column which encompass many muscles important for neck and back stabilization, as well as lung ventilation (Mitchelle and Watson, 2016). Forelimb motor neurons innervate upper limb muscles such as the biceps and deltoids (Tosolini et al., 2013; Tosolini and Morris, 2012). There are similar components involved in the neural control of axial and forelimb motor function. Although these muscles have different functions, they must work together by maintaining balance, posture and limb coordination during walking. This section reviews the neural control of axial and forelimb functions ranging from the cortex to spinal motor neurons.

Elements of the Neural System Controlling Axial and Limb Movement

Spinal control of axial and limb function

Axial motor neurons are located in along the whole spinal cord and comprise the medial motor column. This dissertation specifically focuses on the axial motor neurons in the cervical spinal cord which innervate muscles of the head and neck. The medial motor column can be subdivided into epaxial and hypaxial muscles. Epaxial muscles extend the head and trunk and include muscles such as erector spinae (Mitchelle and Watson, 2016). Hypaxial muscles flex the head and trunk, and include the prevertebral muscles of the neck (Standring, 2016). Important for this dissertation are scalene motor neurons of the axial motor pool since scalene muscles are important for inspiratory behaviors (Pilarski et al., 2019; Shinozaki et al., 2019). Forelimb motor neurons are located in cervical spinal segments C2-C8. In the rat, forelimbs are involved in tasks such as locomotion, reaching and grasping. These motor neurons are located in the lateral ventral gray matter of the spinal cord and include motor neurons of the biceps
(Tosolini et al., 2013; Tosolini and Morris, 2012). There is an extensive network that communicates with one another to ensure proper control of axial and forelimb function. Motor neurons controlling axial and forelimb function receive input from cortical areas including the premotor, primary motor and supplementary motor cortex (corticospinal tract), as well as the red nucleus (rubrospinal tract) and reticular nuclei (reticulospinal tract).

**Supra-spinal control of axial and limb function**

Neurons in the primary motor cortex are the initial site of voluntary control. The primary motor cortex is somatotopically organized so that specific areas of the motor cortex control specific muscles. These neurons have afferent and efferent connections to the associate motor areas such as the basal ganglia, cerebellum, sensory and visual cortex. Neurons of the motor cortex also communicate with the brain stem and spinal cord. The projections from the primary motor cortex to brainstem and spinal cord neurons are part of the corticospinal tract. A majority of the descending corticospinal projections cross the midline at the pyramidal decussation (Van Wittenbergh and Peterson, 2020). The crossing corticospinal projections comprise the lateral corticospinal tract while the remaining ipsilateral projections comprise the anterior corticospinal tract. The lateral corticospinal tract controls limb movement while the anterior corticospinal tract controls axial muscle movements (Van Wittenbergh and Peterson, 2020). Spinal projections from cortical and bulbospinal areas have similarities and differences in humans and rats. For example, the projections from the corticospinal tract descend into the ventral and lateral white matter tracts in humans and dorsal white matter tracts in rats (Armand, 1982).
In addition to corticospinal control, axial and forelimb motor neurons receive input from the rubrospinal tract. The rubrospinal tract originates in the red nucleus and sends projections down the dorsolateral white matter where they make connections onto motor neurons in humans and rats (McCurdy et al., 1987). Rubrospinal projections are involved in coordinated, whole arm movements, posture, and orientation of the body in space (Alstermark et al., 1984; Gibson et al., 1985; Levesque and Fabre-Thorpe, 1990; van Kan and McCurdy, 2001). Motor neurons of the upper limbs also receive inputs from the reticulospinal tract which arises in the reticular formation and project via the ventromedial white matter tracts in humans and rats. Reticulospinal control of axial and forelimb functions are also involved in stepping coordination and has been shown to be important postural functions (Prentice and Drew, 2001; Schepens and Drew, 2004), locomotion (Matsuyama and Drew, 2000) and reaching (Schepens and Drew, 2004, 2006).

**Axial and Limb Motor Consequences of Cervical Spinal Cord Injury**

The motor neurons controlling the neck, arms, and hands are in the cervical region of the spinal cord. A major functional consequence of cSCI is impaired upper limb function (Tosolini et al., 2013; Tosolini and Morris, 2012). Arm and hand functions are necessary to perform simple daily tasks. People living with chronic tetraplegia indicate that regaining arm and hand function would improve their quality of life (Anderson, 2004). In animal models of cSCI, the impaired forelimb never fully recovers, leaving lasting deficits in outcomes such as grip strength (Anderson et al., 2004). This partial recovery is thought to correlate with sparing of the dorsal cortical spinal tract (Anderson et al., 2005). In addition, information to cervical axial motor neurons is disrupted after cSCI which could result in impairments in neck control and breathing (Pilarski et al., 2019; Shinozaki et al., 2019). This is especially important in animal
models of cSCI when neck muscles are directly injured to attain access to the spinal column. Spinally injured rats have difficulty raising their head for food and water acutely after cSCI, however this behavior is recovered within a week (unpublished observations). In addition, breathing function may further be affected when respiratory muscles of neck, such as scalene muscles, are impaired after cSCI (Shinozaki et al., 2019).

**Experimental Models of Cervical Spinal Cord Injury**

To identify treatments and rehabilitation strategies to improve respiratory function after cSCI, animal models of cSCI are needed. Several experimental models are commonly used to address certain aspects of injury and therapeutic focus. Common models include: contusion, compression, distraction, dislocation, hemisection or transection injuries (Cheriyan et al., 2014). All injury models vary in neural pathway sparing and can be used to answer specific scientific questions.

A commonly used model of cSCI in rats is a cervical hemisection at C2 (C2Hx) which provides a precise unilateral lesion that interrupts descending bulbospinal pathways as seen in Figure 1-2. C2Hx is often used to study breathing impairments since the lesion disrupts all descending inputs to phrenic motor neurons on the ipsilateral (“same”) side while leaving contralateral (“opposite”) projections intact. This lesion silences the ipsilateral phrenic nerve and paralyzes the ipsilateral diaphragm (Goshgarian, 2003). C2Hx is a well-defined and surgically reproducible lesion that produces a prolonged and stable functional deficit in breathing capacity (Fuller et al., 2006). C2Hx exhibits a respiratory phenotype of rapid, shallow breathing, equivalent to the persistent respiratory deficits in individuals with cSCIs. C2Hx also causes forelimb
paralysis by disrupting descending inputs to ipsilateral forelimb motor neurons (Anderson et al., 2004; Anderson et al., 2005; Gonzalez-Rothi et al., 2016).

Models of spinal compression and contusion are also used to model cSCI and are often described as a more representative “clinically relevant model” of SCIs (Sharif-Alhoseini et al., 2017). However, unilateral contusions are more variable to perform and result in inconsistent spinal pathology and outcome measures. In addition, it is difficult to localize contusion injuries because spinal edema usually spreads in unpredictable ways. Contusions injuries are less consistent and often recover to uninjured levels within weeks (Golder et al., 2011). Currently, ways to prolong spontaneous recovery after contusion models of SCI include increasing dwell time of the impact (Streijger et al., 2013).

Although hemisection injuries are rare in people (Brown-Séquard syndrome), C2Hx enables precise removal of premotor synaptic input to tease apart mechanisms of respiratory dysfunction, spontaneous spinal plasticity following injury, as well as therapeutic interventions aiming to restore respiratory function and neuron re-innervation. All experiments presented in this dissertation utilize the C2Hx model of cSCI for reasons mentioned above. Specifically, this dissertation chose C2Hx because of its ability to produce precise injuries with consistent breathing impairments.

**Serotonin**

*Overview of the Serotonergic Nervous System*

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a well-known monoaminergic neurochemical first discovered in 1937 by Vialli and Erspamer. Initially, serotonin was known for its ability to constrict smooth muscle in the gut (Perrin and Noristani, 2019; RAPPORT et al., 1948; Vialli and Erspamer, 1937). Although serotonin
is concentrated in the gut, it has profound influences in the central nervous system (CNS). The effects of serotonin are dependent on the type of receptors to which they bind, and secondary messengers involved. Serotonin plays important roles in neuromodulation (Perrier and Cotel, 2015), brain development (Whitaker-Azmitia, 2001), neurogenesis (Alenina and Klempin, 2015), synaptic plasticity (Marinesco et al., 2006), thermoregulation (Myers, 1981), breathing (Hilaire et al., 2010), pain (Cortes-Altamirano et al., 2018), as well as a laundry list of neurological disorders (Di Giovanni, 2013; Fuller, 1991). One interesting property of serotonin is that it can act as a neurotransmitter, neuromodulator, or neurotrophic factor (Perrin and Noristani, 2019), and is an important regulator of plasticity (Bach et al., 1993; Dayer, 2014; Lesch and Waider, 2012).

Strong serotonergic innervation surrounds alpha motor neurons in the spinal cord, including phrenic, intercostal, axial and limb motor neurons (Holtman et al., 1984; Lipski et al., 1994). Serotonin projections throughout the spinal cord arise mainly from the raphe pallidus and obscurus (Bowker et al., 1982). Serotonin plays many roles within the spinal cord, including regulation of motor neuron excitability (Perrier 2013). Serotonin is also involved in locomotion by regulating rhythm and coordination through central pattern generators (Ballion et al., 2001; Feraboli-Lohnherr et al., 1997) and in respiratory plasticity (Fuller et al., 2001b). Because of its impact on motor neurons, the distribution of serotonergic innervation of spinal motor neurons is addressed throughout this dissertation. The sections below describe the unique properties of serotonin and how it contributes to motor neuron function.
Anatomy of Serotonergic Neurons: Brainstem and Spinal Cord

Ramon y Cajal was the first to identify serotonin neurons in the brainstem in the early 1900s (Perrin and Noristani, 2019; Ramón y Cajal, 1904). However, the anatomical distribution of serotonin neurons in the brainstem was not well understood until the 1960’s. It was then that Dahlstrom and Fuxe classified 9 serotonin neuron clusters in the raphe nucleus of the medulla (Dahlström and Fuxe, 1964). The raphe nuclei can be divided into two major subsections: the rostral nuclei, which are located in the midbrain and in the rostral pons, and the caudal nuclei, which are located in the caudal pons and medulla. Rostral raphe nuclei project primarily to structures in the forebrain, whereas caudal raphe send projections to the spinal cord (Törk, 1990).

Nearly all serotonin in the spinal cord descends from the caudal, ventral raphe nuclei (Jacobs and Azmitia, 1992; Skagerberg and Björklund, 1985; Steinbusch, 1981). The caudal raphe extends from the pons to the spinomedullary border and consists of the raphe pallidus, the raphe obscurus, the raphe magnus, the rostral ventrolateral medullary nucleus, and the lateral paragigantocellularis reticularis (Törk, 1990). Certain spinal regions receive a higher density of serotonin projections including lamina I of the dorsal horn, the gray matter adjacent to the central canal, the ventral horn motor nuclei, and the intermediolateral cell column of the thoracic cord (Bowker et al., 1982). Raphe magnus projects to lamina I and II of the dorsal horn, raphe obscurus projects to lamina IX of the ventral horn, and raphe pallidus projects to the intermediate zone (Bowker et al., 1982; Törk, 1990). In addition to serotonin, raphe neurons are contain other neuro-active substances, such as substance P, TRH and glutamate (Schmidt and Jordan, 2000).
Serotonin receptors

Serotonin exhibits both excitatory and inhibitory effects on motor neurons depending on the receptors to which it binds (Beato and Nistri, 1998; Di Pasquale et al., 1997; Lindsay and Feldman, 1993; Nurrish et al., 1999; Perrier, 2016). There are seven families of serotonin receptors (5-HT1-7), and 14 different receptor sub-types (Hochman et al., 2001). Serotonin receptors can be expressed both pre- and post-synaptically and in extra-synaptic regions (Hochman et al., 2001; Hodges and Richerson, 2008). Many serotonin receptors are expressed on motor neuron membranes (Perrier, 2013). Of particular interest to this dissertation are the 5-HT2 and 7 receptors, which are involved in respiratory plasticity (Baker-Herman and Mitchell, 2002; Hoffman and Mitchell, 2011).

5-HT2 receptors. The 5-HT2 receptor family is comprised of three different receptor subtypes (A, B & C). They activate Gq protein, generating inositol trisphosphate and activating protein kinase C (Conn and Sanders-Bush, 1986), generally increasing neuronal excitability through multiple mechanisms, including phosphorylation of glutamate receptors (Bocchiaro and Feldman, 2004; Fuller et al., 2000; McGuire et al., 2008) and enhancing persistent sodium currents (Heckman et al., 2008; Peña and Ramirez, 2002). 5-HT2 receptors are located in the brainstem where they play an important role in respiratory rhythm generation (Günther et al., 2006; Peña and Ramirez, 2002). They are highly expressed in the ventral spinal neurons, including phrenic motor neurons where they regulate neuron excitability and are involved in phrenic motor plasticity (Fuller et al., 2005; MacFarlane et al., 2011; Tadjallli and Mitchell, 2019). 5-HT2 receptors are also implicated in locomotor control (Fouad et al., 2010) and regulating coordinated movements (Sławińska et al., 2014).
**5-HT7 receptors.** The 5-HT7 receptor family are Gs protein coupled receptors that increase cyclic adenosine monophosphate (cAMP) production (Perrier et al., 2013), increasing neuronal excitability. 5-HT7 receptors are located in the brainstem pre-Bötzinger neurons, where they may play a role in formation of the respiratory network (Manzke et al., 2008). Spinal 5-HT7 receptors are expressed on motor neurons (Doly et al., 2005), and are involved in phrenic motor plasticity (Baker-Herman and Mitchell, 2002; Hoffman and Mitchell, 2011; MacFarlane and Mitchell, 2009). In addition, 5-HT7 receptors are implicated in the central pattern generator (CPG) activity for locomotion (Landry et al., 2006; Liu et al., 2009). 5-HT7 agonists such as 8-hydroxy-2-(di-N-propylamino)-tetralin can induce locomotor like movements in paralyzed mice (Landry et al., 2006).

**Synaptic versus volume neurotransmission**

In the CNS, serotonin boutons, or terminals, can either synapse onto neurons where they release serotonin in the synapse, or can release serotonin from *en passant* varicosities located next to synapses (Hodges and Richerson, 2008). Non-synaptic varicosities release serotonin in the vicinity of motor neurons where they diffuse to extra-synaptic receptors or receptors on nearby motor neurons. This form of transmission is termed volume neurotransmission (Agnati et al., 1986). Volume neurotransmission is a less specific interaction that allows serotonin to act as a paracrine neuromodulator that alters ongoing synaptic activity. The receptors to which serotonin binds determine if serotonin will act as a fast neurotransmitter or a slow neuromodulator. Nature seems to favor the neuromodulatory behavior of serotonin because six of the seven receptor subtypes favor neuromodulation (Bockaert et al., 2006).
One factor contributing to whether or not a serotonergic neuron forms a synapse is based on its location. In the fronto-parietal cortex, only about 5% of serotonergic projections form synapses (Descarries et al., 1975). In the sensorimotor cortex, only about 3% form synapses (DeFelipe and Jones, 1988). In the superior colliculus, about 80% form synapses (Dori et al., 1998). In the dorsal horn of the spinal cord, about 40% of serotonin projections form synapses (Hentall et al., 2006; Marlier et al., 1991; Ridet et al., 1993). In the ventral horn and intermediate zone of the spinal cord, more serotonin projections form classical axo-dendritic synapses versus other spinal regions, although the exact number is unknown (Privat et al., 1988).

Serotonin transmission is regulated by two transporters. The first transporter is the vesicular monoamine transporter which packages serotonin from the pre-synaptic cytoplasm to vesicles, thus controlling its release. The second transporter is the serotonin transporter (SERT), which re-absorbs serotonin from the extracellular space back into the serotonergic neuron (Iversen, 1971; Murphy et al., 2004). The transmission of serotonin is dependent on the density and location of SERT.

**Trophic effects of serotonin**

A unique feature of serotonin is its ability to act as a neurotrophic factor. Neurotrophic factors support survival, development and function of neurons. Many trophic effects of serotonin occur in development, but some effects endure in the mature nervous system. Serotonin has trophic effects on neural proliferation (Lauder et al., 1981), synaptogenesis (Lipton and Kater, 1989), and can influence synaptic formation (Chubakov et al., 1986; Haydon et al., 1984) and maintenance (Chen et al., 1994). Serotonin regulates the maturation of target cells directly and indirectly by eliciting release of glial S100β (Azmitia, 1999). When serotonin is removed from the CNS, the
structure and function of that region is compromised, which impacts the region’s ability to respond to the changing environment (Azmitia, 1999).

**Serotonergic Functions in Motor Control**

Serotonin is distributed throughout the brain and spinal cord where it modulates motor activity, acting to both inhibit and excite neurons. Although little is known about the role of serotonin in certain brain regions, serotonergic neurons project to the basal ganglia, substantia nigra, subthalmic nucleus, caudate nucleus, putamen, globus pallidus and the cerebellum. Brain regions innervated by serotonin are necessary for initiation and coordination of movement (Kawashima, 2018). Serotonin modulate firing rate and pattern in the motor cortex. Motor cortex receptors consist mostly of 5-HT1, inhibiting neuronal activity, and 5-HT2, stimulating neuronal activity (Vitrac and Benoit-Marand, 2017). In addition, serotonin in the motor cortex is involved in learning and memory. Serotonin increases overall excitability of neurons facilitating long-term potentiation (Vitrac and Benoit-Marand, 2017). In the spinal cord, serotonin affects motor control by acting on interneurons and motor neurons as described below.

**Neuromodulatory Effects of Serotonin on Motor Neurons**

Serotonin demonstrates important neuromodulatory effects on motor neurons (Hochman et al., 2001). A neuromodulator is most commonly defined as a substance that alters the response of a target neuron to the traditional neurotransmitters without directly leading to action potentials (Hodges & Richerson, 2008). For example, serotonin modulates motor neuron excitability in the following ways: 1) activating potassium and sodium channels that depolarize motor neurons towards activation threshold, 2) inhibiting calcium activated potassium conductance, and 3) inducing persistent inward currents, mediated by voltage gated calcium and sodium channels leading to continued
motor neuron depolarization and amplified synaptic inputs (Lindsay and Feldman, 1993; Perrier et al., 2013). These effects can be dependent on the amount of serotonin release and the receptor activated (Ciranna, 2006). Low amounts of serotonin increase excitability, whereas high amounts block action potentials (Hannon and Hoyer, 2008; Li and Zhuo, 1998).

**Serotonin as a Regulator of Plasticity**

Serotonin is important for many forms of plasticity including plasticity during development, synaptic plasticity and spinal respiratory plasticity. Plasticity in serotonergic fibers are also seen after injury where they express a regenerative sprouting response and can form new connections (Azmitia et al., 1978; Nobin et al., 1973). Just as serotonin’s effects are dependent on the receptors to which they bind, serotonin receptors determine the type of plasticity that occurs. For example, in some model systems, 5-HT2A is involved in synaptic plasticity whereas 5-HT7 enhances neurite length (Kraus et al., 2017). Important for this dissertation is that serotonin is sufficient to elicit long-term facilitation (LTF) of motor neurons (Dale-Nagle et al., 2010a). Serotonin dependent plasticity will be described in more detail in later sections.

**The Role of Serotonin in Control of Breathing**

Serotonergic projections from the caudal raphe nuclei terminate within the brainstem and spinal cord onto respiratory neurons. In addition, serotonin receptors are located on respiratory motor neurons in the brainstem and spinal cord. Microinjection of exogenous serotonin to the brainstem increases respiratory frequency (Lindsay and Feldman, 1993). Spinal respiratory motor neurons, phrenic motor neurons in particular, are highly innervated by serotonin, receiving projections from the caudal raphe nucleus, primarily from the raphe pallidus and raphe obscurus nuclei (Steinbusch, 1981;
Hochman et al., 2001). Microinjection on exogenous serotonin influences phrenic nerve output. For example, serotonin can increase the excitability of phrenic motor neurons as well as inhibit phrenic output (Di Pasquale et al., 1997; Lindsay and Feldman, 1993). In addition, serotonin is sufficient to elicit spinal respiratory neuroplasticity (Baker-Herman and Mitchell, 2002; Gonzalez-Rothi et al., 2015b).

**The Role of Serotonin in Control of Limb Function**

Serotonin's effects on motor neurons that control the limbs are described in previous sections, such as its neuromodulatory effects on locomotion. Locomotor function arises from a network of motor neurons and interneurons that are organized to make up CPGs. CPGs are a network of neurons with spontaneous, synchronous activities that produce a rhythmic motor pattern without afferent or efferent input, such as walking (Marder and Bucher, 2001). CPGs are regulated, in part, by descending serotonergic projections (Cazalets et al., 1992; Kiehn and Kjaerulff, 1996). Application of serotonin and N-methyl-D-aspartate (NMDA) to the spinal cord initiates axial locomotor patterns, indicating serotonin modulates locomotor CPGs (Branchereau et al., 2000). Serotonin deficiency alone does not impair limb motor neuron function (Van den Bergh et al., 1996; Van den Bergh et al., 1987). However, after an insult, such as with spinal cord injury, serotonin is necessary for recovery. When serotonin is depleted, motor neurons are slower to recover compared to controls (Van den Bergh et al., 1988). In addition, recovery of limb function after SCI correlates with levels of serotonin, indicating that serotonin is important for normal limb function (Hashimoto and Fukuda, 1991).
Serotoninergic Projections after Spinal Cord Injury

Anatomical Changes after Spinal Cord Injury

Spinal serotoninergic projections from the brainstem are disrupted after SCI. However, serotonin within the spinal cord spontaneously recovers with time after injury (Camand et al., 2004; Golder and Mitchell, 2005; Saruhashi et al., 1996). Only 30 days after C2Hx, the number of serotoninergic terminals in the ipsilateral phrenic motor nucleus increases compared to spinally intact controls (Tai et al., 1997). In addition, the number of serotonin terminals with multiple synapses onto phrenic motor neurons increases after C2Hx. Electron microscopy identification of these serotonin synapses on phrenic motor neurons show the active zones of the serotonin terminals increased in number and length 30 days after C2Hx (Tai et al., 1997). Although, serotoninergic neurons show profound plasticity after chronic SCI, other components of the serotoninergic system change as well. For example, 5-HT2A receptors are upregulated following C2Hx (Fuller et al., 2005). In addition, when serotonin levels are reduced due to SCI, pericytes known for their role in capillary tone turn amino acids into trace amines, which bind to serotonin receptors (Li et al., 2017). Thus, compensation including anatomical changes in serotonin, as well as changes in receptors and their affinities, occur after SCI.

Serotonin-Dependent Function after Spinal Cord Injury

The first study to show serotonin’s importance for functional recovery after spinal injury was Hashimoto and Fukuda (Hashimoto and Fukuda, 1991). In this study, they utilized a compression model of thoracic SCI at T11, and treated rats with molecules known to deplete serotonin or kill serotoninergic neurons. They found that rats had less recovery when serotonin was depleted versus SCI rats with normal serotonin recovery. Since then, many studies have shown that serotonin administration improves motor
output after SCI (Perrin and Noristani, 2019). In the context of breathing function after cSCI, Golder and colleagues showed that the amplitude baseline of ipsilateral phrenic output correlates with serotonin innervation of phrenic motor neurons (Golder and Mitchell, 2005). In addition, administration of serotonin after C2Hx elicits recovery of respiratory activity in the initially paralyzed ipsilateral phrenic nerve in a dose dependent manner that was reversed with methysergide, a serotonin receptor antagonist (Zhou and Goshgarian, 2000). Thus, it is commonly believed that increased serotonergic availability below the SCI correlates with functional recovery.

**Intermittent Hypoxia Induced Motor Plasticity**

The nervous system is capable of profound plasticity. Plasticity helps maintain function in response to an event that may impair normal function such as injury or disease. Examples of plasticity include changes in morphology and function, such as synapse number, synaptic strength, or receptor expression. Intermittent hypoxia (IH) elicits spinal motor plasticity. In addition, IH is a promising therapeutic tool that restores respiratory and non-respiratory somatic motor function in rodent models and humans with chronic incomplete SCI (Gonzalez-Rothi et al., 2015b; Trumbower et al., 2012). The sections below describe IH’s effects on plasticity and functional recovery.

**Important Intermittent Hypoxia Definitions**

To discuss IH induced motor plasticity, we must define terminology. The temporary response to a stimulus is termed modulation. When the stimulus is removed, the nervous system rapidly returns to normal function (Mitchell and Johnson, 2003). An increase in motor neuron firing in the presence of a neuromodulator is an example of modulation. In contrast, plasticity is a persistent response to a stimulus lasting long after the stimulus is removed. Specifically, plasticity is defined as a persistent change in the
neural control system based on experience (Mitchell and Johnson, 2003). Long-term facilitation (LTF) of phrenic motor neurons after acute intermittent hypoxia (AIH) is a well-studied example of plasticity. Metaplasticity is plastic plasticity, or a change in the capacity to express plasticity (Mitchell and Johnson, 2003). For example, repeated AIH preconditioning enhances AIH-induced LTF (Ling et al., 2001; Wilkerson and Mitchell, 2009).

IH is the cycling between normoxia and hypoxia. IH increases phrenic motor output via spinal plasticity and can be harnessed as a therapy to restore breathing capacity following SCI (Dale-Nagle et al., 2010b; Gonzalez-Rothi et al., 2015b). When intermittent hypoxia is administered more than once, it is called repetitive acute intermittent hypoxia (rAIH). rAIH is an umbrella term for any protocol that involves repeated IH exposure. For example, daily acute intermittent hypoxia (dAIH), or exposure AIH on successive days is one form of rAIH.

There are many forms of respiratory plasticity. Phrenic motor facilitation (pMF) is an umbrella term for the long-term increase in phrenic nerve activity. pMF can be elicited by administration of pharmacological agents to the cervical spinal cord, such as a serotonin agonist or by exposure to AIH (Navarrete-Opazo and Mitchell, 2014b). When the long-term increase in phrenic nerve activity is elicited by acute intermittent hypoxia it is termed phrenic long-term facilitation (pLTF) (Dale-Nagle et al., 2010a; Mitchell et al., 2001).

**The Concept of Intermittent Hypoxia “Dose”**

IH protocols differ in their potential for plasticity. For example, IH treatment varies from a few bouts within a single treatment session, to repetitive episodes, 8 to 12 hours per day, over several days or weeks. Thus, one must be conscious of particular IH
“dose” based on: 1) level of inspired oxygen during hypoxia, 2) duration of the hypoxic episodes, 3) duration of the normoxic episodes, 4) number of hypoxic/normoxic cycles, 5) duration of exposure period, 6) pattern of presentation (daily or multiple times a week), and 7) total time period of IH exposure (one day or 4 weeks) (Navarrete-Opazo and Mitchell, 2014b). As the “dose” of IH becomes more chronic (i.e. lower inspired oxygen or longer hypoxic exposure), the effects of IH shift from therapeutic to pathological.

**Acute Intermittent Hypoxia and Plasticity in the Respiratory Motor System**

AIH is a modest form of hypoxia exposure that triggers spinal respiratory motor plasticity. Much of this plasticity has been studied in the phrenic motor system which responds to AIH with progressively enhanced motor output, known as pLTF (Bach and Mitchell, 1996). pLTF is a prolonged increase in phrenic motor output lasting hours after AIH (3 to 15 episodes) by strengthening synapses onto respiratory motor neurons (Dale-Nagle et al., 2010a; Devinney et al., 2013; Mahamed and Mitchell, 2007; Mitchell et al., 2001).

The proposed mechanism of AIH is that episodic hypoxia repeatedly activates the carotid body; repeated chemoafferent neuron activation activates neurons in the raphe nuclei, intermittently releasing serotonin near phrenic motor neurons and triggering pLTF (Dale-Nagle et al., 2010a; Gonzalez-Rothi et al., 2015b). It has been shown that AIH induced pLTF is serotonin dependent and requires activation of 5-HT2A receptors on or near phrenic motor neurons for its induction, but not maintenance (Baker-Herman and Mitchell, 2002; Fuller et al., 2001b; MacFarlane et al., 2011).

In addition to plasticity in phrenic motor nuclei, AIH also elicits plasticity in the intercostal (Fregosi and Mitchell, 1994; Navarrete-Opazo and Mitchell, 2014a) and
hypoglossal motor nuclei (Bach and Mitchell, 1996; Bocchiaro and Feldman, 2004). Although less studied, the mechanisms of plasticity in these motor pools are thought to be similar to the mechanisms involved in pLTF (Baker-Herman and Strey, 2011).

**Pathways of acute intermittent hypoxia**

There are currently 5 known intracellular pathways to pMF. These include: 1) vascular endothelial growth factor (VEGF) - induced pMF, 2) erythropoietin - induced pMF, 3) inactivity-induced pMF, 4) “Q pathway” of IH induced pLTF, and 5) “S pathway” of IH induced pLTF. Since this dissertation focuses on IH induced motor plasticity, we will focus on the Q and S pathways represented in Figure 1-3.

**Q pathway.** The “Q pathway” is mediated by metabotropic 5-HT2 receptor activation. It is named for the Gq proteins to which 5-HT2 receptors are coupled. Episodic serotonin release and 5-HT2 receptor activation are necessary and sufficient to elicit pMF without AIH (MacFarlane and Mitchell, 2008). AIH-induced pLTF is predominantly driven by the Q pathway. The current working model of the Q pathway is that repetitive, moderate hypoxia triggers intermittent serotonin release in the vicinity of the phrenic motor nucleus. Serotonin then binds and activates 5-HT2 receptors, triggering an intracellular Gq signaling cascade (Baker-Herman and Mitchell, 2002). This signaling includes extracellular signal regulated-mitogen-activated protein (ERK-MAP) kinase activity (Hoffman et al., 2012; Wilkerson and Mitchell, 2009), new synthesis of brain-derived neurotrophic factor (BDNF), activation of its high-affinity receptor, tropomyosin receptor kinase B (TrkB) (Baker-Herman et al., 2004; Dale et al., 2017), and downstream activation of protein kinase C-theta (Agosto-Marlin et al., 2017; Devinney et al., 2015). Although this pathway includes 5-HT2 receptors, there seems to be a differential mechanism between 5-HT2A and 5-HT2B receptors which is still being
elucidated. 5-HT2B receptor-mediated pMF is dependent on nicotinamide adenine dinucleotide phosphate oxidase activity and nitric oxide generation by neuronal nitric oxide synthase whereas 5HT2A is not (MacFarlane et al., 2011, 2014). The link between the Q pathway and NMDA receptors is unclear (Turner et al., 2018).

**S pathway.** The “S pathway” is mediated by 5-HT7 receptor or adenosine 2a (A2a) receptor activation. It is named for the Gs proteins to which 5-HT7 and A2a receptors are coupled. The S Pathway is triggered by severe AIH which ranges from 25 to 35 millimeters of mercury (mmHg) pressure of oxygen in arterial blood (PaO2) (Nichols et al., 2012). The S pathway is initiated by either 5-HT7 (Hoffman and Mitchell, 2011) or A2a receptors (Nichols et al., 2012) that signals the Gs signaling cascade that includes activation of adenylyl cyclase, cAMP signaling (Fields and Mitchell, 2017) via exchange protein activated by cAMP (EPAC)(Fields et al., 2015), activation of protein kinase B (AKT) (Golder et al., 2008), mammalian target rapamycin (mTOR) signaling (Dougherty et al., 2015), and then new synthesis of immature TrkB isoform (Hoffman and Mitchell, 2013). As with the Q pathway, the exact link between the S pathway and NMDA receptors has yet to be determined (Turner et al., 2018).

**Repetitive acute intermittent hypoxia preconditioning**

rAIH encompasses several low dose IH paradigms ranging from daily, given on consecutive days, to multiple times a week. It is important to identify the proper AIH paradigm to optimize the therapeutic potential. rAIH (10, 5 minute episodes 3x/week, 10 weeks) enhances spinal respiratory plasticity in intact rats by upregulating growth and trophic factors in phrenic motor neurons that are critical for spinal respiratory motor plasticity including BDNF, TrkB, phosphorylated ERK, VEGF, and VEGF receptor 2 (Satriotomo et al., 2012; Wilkerson and Mitchell, 2009). rAIH also increases plasticity-
related proteins in non-respiratory motor neurons (Satriotomo et al., 2016). rAIH also increases serotonin terminal density and 5-HT2A receptors within phrenic motor neurons (Satriotomo et al., 2012). rAIH paradigms appear to have the most functional benefit when paired with task-specific training. For example, rodents with SCI exposed to daily rAIH for 7 consecutive days and underwent ladder walking training had more functional recovery than rats that only received rAIH or ladder walking training alone (Lovett-Barr et al., 2012; Prosser-Loose et al., 2015).

**Functional recovery of breathing and walking after acute intermittent hypoxia**

The spinal cord is capable of spontaneous and induced functional recovery after SCI. An example of spontaneous functional recovery of motor output below an injury is the contribution of crossed-phrenic pathways (Goshgarian, 2003; Raineteau and Schwab, 2001). Induced functional recovery below an injury can be from interventions that trigger spinal plasticity (Lovett-Barr et al., 2012). One intervention of particular interest is AIH, which strengthens the contribution of spared neural pathways to enhance function (Dale et al., 2014; Gonzalez-Rothi et al., 2015a).

Our lab discovered that therapeutic AIH has impacts beyond the respiratory motor system, amplifying limb function in rodents (Lovett-Barr et al., 2012) and humans with chronic, incomplete cSCI (Hayes et al., 2014; Navarrete-Opazo et al., 2017a; Trumbower et al., 2012). Thus, there is considerable potential to harness AIH induced spinal motor plasticity to improve respiratory and non-respiratory somatic motor function in rodent models and humans with clinical disorders that compromise breathing, including chronic incomplete SCI (Dale et al., 2014; Gonzalez-Rothi et al., 2015a, b; Trumbower et al., 2012).
pLTF appears in spinally intact rats, and in rats with C2Hx (Fuller et al., 2000; Doperalski & Fuller, 2006), possibly due to upregulation of 5-HT2A receptors following C2Hx (Fuller et al., 2005) or a switch to an adenosine-dependent mechanism (Navarrete-Opazo et al., 2015; Navarrete-Opazo et al., 2014). AIH induced pLTF does not appear in the ipsilateral phrenic nerve until weeks to months later (Golder & Mitchell, 2005), therefore AIH may be useful as a therapeutic intervention to enhance functional capacity after chronic SCI (Dougherty et al., 2018; Fuller et al., 2003). Because functional recovery after SCI correlates with spinal serotonin concentration, the reduction in serotonergic innervation to the ipsilateral phrenic motor nucleus shortly after C2Hx may be a limiting factor in AIH induced pLTF after acute SCI (Golder & Mitchell, 2005).

In rats, even a single AIH presentation increases respiratory output after chronic (8 weeks), but not acute (2 weeks), C2Hx (Golder and Mitchell, 2005). dAIH (10, 5 minutes 10.5% O₂, 5 minutes 21% O₂, 1 hour 40 minutes/day) for one week partially restores lost breathing capacity following C2Hx (Lovett-Barr et al., 2012; Navarrete-Opazo et al., 2015). In people with chronic SCI (years post-injury), 10 days of exposure to dAIH (8, 2 minutes 8% O₂, 2 minutes 21%O₂) showed significantly improved breathing function, specifically minute ventilation (Tester et al., 2014).

AIH also enhances leg strength and walking ability in humans with chronic SCI (Hayes et al., 2014; Tester et al., 2014; Trumbower et al., 2012). A single presentation of AIH (15, 1 minute 9% O₂, 1 minute 21% O₂) significantly improves ankle plantar flexor strength in humans after chronic SCI, an effect that lasted for 90 minutes post-exposure (Trumbower et al., 2012). In yet another study, people with chronic SCI
exposed to dAIH for 5 days (15, 90 seconds 9% O2, 90 seconds 21%O2) improved
over-ground walking speed and distance, and this recovery was enhanced when paired
with walking training (Hayes et al., 2014). In all of these studies using AIH to improve
function in humans with chronic SCI, there is no evidence for increased spasticity or
autonomic dysreflexia (Hayes et al., 2014; Trumbower et al., 2012).

AIH has been shown to be safe with no indication of pathological consequences,
no increase in neuroinflammation, reactive gliosis or hippocampal cell death (Lovett-
Barr et al., 2012; Satriotomo et al., 2012). In a recent study, 3 months of rAIH exposures
was shown to be safe and did not elicit heart, liver, or brain pathology in uninjured or
C2Hx rats (Gonzalez-Rothi et al., unpublished data). Other outcomes of AIH include:
decreased blood pressure, decreased inflammation, increased aerobic capacity and
neurogenesis (Navarrete-Opazo and Mitchell, 2014b). Collectively, available evidence
suggests that AIH represents a safe and effective means to restore motor function with
chronic SCI.

**Inflammation impairs acute intermittent hypoxia efficacy**

Inflammation, such as that due to SCI, may pose a serious threat to AIH efficacy
as a treatment for respiratory insufficiency. Even mild inflammation undermines
serotonin dependent AIH-induced plasticity (Huxtable et al., 2013; Vinit et al., 2011).
When low-grade systemic inflammation is induced with lipopolysaccharide (LPS)
injections, AIH-induced plasticity is impaired (Vinit et al., 2011). Plasticity is restored
when pre-treated with the nonsteroidal anti-inflammatory drug ketoprofen (Huxtable et
al., 2013). A recent study by Huxtable et al. showed a prominent role for p38 mitogen-
activated protein kinase (MAPK) in the cellular cascade linking inflammation and
impaired AIH-induced plasticity (Huxtable et al., 2015). However, systemic inflammation
has no effect on the S pathway-mediated pMF (Agosto-Marlin et al., 2017). Therefore, it is thought that in the acute stages of SCI, with profound inflammation and little serotonin, the S pathway of plasticity will dominate. In the chronic phases of SCI, when there is less inflammation and serotonin is stabilized, the Q pathway of plasticity will dominate.

**Chronic Intermittent Hypoxia**

Chronic intermittent hypoxia (CIH) protocols use frequent episodes of moderate hypoxia, about 2 minutes each, for 8-12 hours per day, mimicking hypoxia experienced during moderate sleep apnea. These more robust CIH protocols may overcome loss of serotonin innervation to caudal motor neurons in the acute stages of SCI recovery, for example by strengthening the crossed phrenic phenomenon (Fuller et al., 2003), or undermine its effects by eliciting inflammation. CIH enhances serotonergic innervation of hypoglossal motor nuclei (Rukhadze et al., 2010), enhancing the potential for plasticity.

**Chronic intermittent hypoxia and plasticity in the control of breathing**

CIH can facilitate or constrain plasticity of the respiratory control system (Dale-Nagle et al., 2010a). CIH consisting of 5 minutes interval of 11% O2 interspersed with normoxia for 12 hours, administered for 7 nights, enhances serotonin dependent pLTF (Ling et al., 2001). This CIH protocol was also shown to enhance ipsilateral phrenic motor output in rats with SCI (Fuller et al., 2003). On the other hand, one night of CIH for 8 hours, 2 minutes 10.5% O2 interspersed with 2 minutes of normoxia, abolishes pLTF 16 hours after CIH exposure. pLTF can be restored with administration of ketoprofen, a non-steroidal anti-inflammatory drug (Huxtable et al., 2015). This study supports that neuroinflammation caused by CIH blunts plasticity. Differences between
protocols of 2 and 5 minute intervals on serotonergic innervation of motor pools are unknown.

**Pathogenesis of chronic intermittent hypoxia and relationship to sleep apnea**

Sleep apnea is defined as periods where there is a disruption in airflow during sleep causing the oxygen in the blood to drop. When this occurs, the body awakens and breathing resumes. Thus, there are two components to SA, intermittent periods of hypoxia and disturbances in sleep. Apneic episodes can result from airway obstruction, termed obstructive sleep apnea, or cessation of brainstem respiratory activity, termed central sleep apnea (Dempsey et al., 2010).

CIH mimicking sleep apnea causes chronic systemic inflammation (Kheirandish-Gozal and Gozal, 2019), which can blunt serotonin induced plasticity. Thus, the potential benefits of IH induced plasticity and future translation into clinical practice may be negated by deleterious side-effects in people with sleep apnea. CIH and sleep apnea side effects include hypertension (Fletcher et al., 1992), impaired baroreflex control of heart rate (Gu et al., 2007), neurocognitive deficits (Row, 2007), metabolic syndrome (Tasali and Ip, 2008), and tumor metastasis (Almendros et al., 2014). However, it is important to understand links between CIH and recovery after SCI since people with SCI have a high comorbidity of sleep apnea (Sankari et al., 2014a).

**Characteristics of Sleep Apnea after Spinal Cord Injury**

About 80% of people with cSCI have comorbid sleep disordered breathing (Sankari et al., 2014a). A majority of people with this co-morbidity did not have sleep apnea prior to the injury (Berlowitz et al., 2005). So, it appears that SCI itself increases susceptibility to sleep apnea. Whether sleep apnea is obstructive or central cause is
debated. Regardless, whatever the cause of apnea, the apneas appear by two weeks post injury (Berlowitz et al., 2005).

Some studies supporting that sleep apnea after cSCI is central in nature; they also show that individuals with tetraplegia are more susceptible to central sleep apnea versus paraplegia (Sankari et al., 2014c; Sankari et al., 2019), possibly due to the respiratory impairments in those with cSCI. One hypothesis is the disruption of the ventilatory control system consequent to injury, such as hypoventilation, leads to central apneas during sleep (Sankari et al., 2014c). The resulting CIH causes sensory LTF, increased chemoreflex sensitivity, and an overshoot of ventilation. This ventilatory overshoot tells the body to hypoventilate, which restarts the cycle (Sankari et al., 2019). However, in cases of central sleep apnea cSCI, there are often other contributing factors (Burns et al., 2000).

Studies supporting the idea that the comorbidity of SCI and sleep apnea is due to an obstructive nature hypothesize increased collapsibility of the upper airway (Sankari et al., 2014b). A review by Fuller and colleagues describe multiple mechanisms that may contribute to obstructive sleep apnea after SCI including: 1) body mass, 2) reduced lung volumes and loss of traction forces, 3) increased time sleeping in the supine position, 4) altered parasympathetic and sympathetic tone, 5) changes in brainstem and chemosensitivity, and 6) medications (Fuller et al., 2013). While there is debate on the mechanism of sleep apnea after SCI, there is little doubt that sleep apnea is a major problem after SCI. Although serotonin is important in regulating the tone of upper airway muscles (Behan and Brownfield, 1999; Kubin et al., 1992), little is known concerning how serotonergic innervation to upper airway motor nuclei, such as the hypoglossal
motor nuclei, change after cSCI. Thus, this dissertation addresses changes in serotonergic innervation of the hypoglossal motor nuclei with CIH mimicking that of sleep apnea and cSCI.

**Potential Impact of Sleep Apnea on Functional Outcomes after Spinal Cord Injury**

People with SCI have increased chemosensitivity which has the potential to enhance AIH plasticity and functional recovery (Bascom et al., 2016; Lusina et al., 2006). Thus, it is hypothesized that sleep apnea might be a way that nature is preconditioning people with SCI for therapeutic interventions such as AIH. In a recent study, people with SCI who had mild to moderate sleep apnea had a better response to AIH than those without sleep apnea, suggesting that people with this co-morbidity may benefit more from AIH therapy (Vivodtzev et al., 2020). On the other hand, due to the chronic systemic inflammation that results from both sleep apnea and SCI, people with both conditions may have reduced potential for functional recovery and AIH induced plasticity. In this case, combinatorial treatments to enhance function and reduce inflammation must be investigated. For example, combining AIH with an anti-inflammatory medication might release this constraint and allow functional improvements. Further research is needed to understand the potential benefits versus disadvantages of SCI and sleep apnea for AIH interventions.

**Folate in the Central Nervous System**

Folate is a water-soluble vitamin, also known as vitamin B9 or folic acid. Although folate can be obtained from foods such as leafy greens, supplemental folate has been shown to be protective to the CNS (Kronenberg and Endres, 2010). Folate received its fame for its importance in embryonic development of the CNS, aiding in the closure of the neural tube. Low folate levels in pregnant mothers increases the risk of fetal neural
tube defects, such as from improper closure of the neural tube (Meethal et al., 2013). This realization of folate’s role in neural tube defects (NTD) was discovered in the mid-1960s. In the early 1990s, after about 30 years of research, the American Center for Disease Control and Prevention recommended that women who previously had a child with NTD consume supplemental folate to prevent NTD in subsequent pregnancies. Shortly after, the recommendation for supplemental folate was extended to all women of childbearing age. By the late 1990’s foods were fortified with folate in the United States (Crider et al., 2011) to ensure that all women were consuming recommended amounts.

**Functional Importance of Folate**

In the adult, folate is a primary methyl donor for most reactions in the body. Importantly, it methylates deoxyribonucleic acid (DNA), regulating gene expression (Friso et al., 2017). Folate also methylates homocysteine (Bailey and Gregory, 1999), an amino acid associated with cardiovascular disease (Selhub, 1999). Circulating homocysteine is cytotoxic to cells and promotes DNA breakage, oxidative stress, and apoptosis (Rathor et al., 2015; Wang et al., 2012; Xu et al., 2005). By methylating homocysteine to methionine, folate reduces these cytotoxic effects. Folate is involved in the synthesis of nucleotides and neurotransmitters, such as serotonin (Gospe et al., 1995; Moore et al., 2017; Subramaniapillai et al., 2017), and is protective against glutamate and NMDA-mediated excitotoxicity in the brain (Lin et al., 2004). In addition, supplemental folate enhances function after SCI and other neurological conditions such as Amyotrophic lateral sclerosis (Iskandar et al., 2004; Iskandar et al., 2010; Zhang et al., 2008).

Deficiencies in folate correlate with depression (Bottiglieri, 2005), cognitive decline (Reynolds, 2006), and Alzheimer's Disease (Serot et al., 2001). Low folate
levels also reduce neurotransmitter metabolism (Gospe et al., 1995), increase oxidative stress (Rathor et al., 2015), and increase the risk of cancer (Friso et al., 2017; Lubecka-Pietruszewska et al., 2013). Interestingly, persons with SCI consume less folate than the daily recommended value by up to 75% (Groah et al., 2009; Perret and Stoffel-Kurt, 2011; Walters et al., 2009). However, no studies have been conducted on SCI and folate deficiency.

**Folate Manipulation after Spinal Cord Injury**

Studies by Iskandar et al. report improved locomotor function in rats who received supplemental folate 6 weeks after thoracic contusion injuries (Iskandar et al., 2004; Iskandar et al., 2010; Miranpuri et al., 2017; Stewart et al., 2019). In addition, Iskandar’s group report that supplemental folate stimulates axonal regeneration after SCI. In this model, they paired a sciatic nerve injury with a cervical dorsal transection injury in which the sciatic nerve was grafted into the cervical dorsal injury. Using retrograde tracing techniques, the authors visualized the percentage of dorsal root ganglia neurons that regenerated into the graft in animals with and without folate treatment. Animals that were supplemented with folate had more fluorescently labeled neurons than the control group, suggesting folate stimulated axonal growth into the graft (Iskandar et al., 2004). The lab performed this experiment using various doses of folate to obtain a dose response curve. The response was biphasic and dose dependent with an optimal dose response at 80ug/kg body weight resulting in the maximum number of regenerated spinal neurons (Iskandar et al., 2004). No toxic effects were observed in the animals who received high folate concentrations (Iskandar et al., 2004).

Iskandar’s group investigated the mechanism by which folate enhances neural regeneration after SCI. They blocked multiple steps in the folate pathway: 1) the folate
receptor using folate receptor knock-down mice to prevent folate from entering the cell, 2) methotrexate to block the activation of folate into 5-tetrahydrofolate, 3) nitric oxide to block the methylation of homocysteine to methionine, and 4) a DNA methyltransferase inhibitor to block methylation of DNA. When these steps were blocked, axonal regeneration did not occur (Iskandar et al., 2010). This suggests that DNA methylation is necessary for increased axonal growth after injury. Using a global DNA analysis of the spinal cord, Iskandar found that spinal and peripheral nerve injury suppress spinal cord DNA methylation and when supplemented with folate, methylation levels return to baseline (Iskandar et al., 2010). In addition, the group used nitric oxide to inhibit the effects of folate to improve locomotor score and axon regeneration after spinal contusion injury (Stewart et al., 2019).

A study by Zhang et al. combined folate with fetal stem cells as a treatment to enhance function after thoracic contusion injuries in rats. Limb function was improved in rats treated with the combined treatment than with folate or stem cells alone (Zhang and Shen, 2015). In addition to enhancing function and axonal regeneration after SCI, folate supplementation also reduces matrix metalloproteinase-2 expression, a critical player in ECM breakdown, and alleviated neuropathic pain (Miranpuri et al., 2017). In addition, folate may be beneficial after SCI by reducing apoptosis (Zhang and Shen, 2015), activated microglia, and reactive astrocytes (Zhang et al., 2008); thus reducing SCI-induced inflammation and promoting recovery.

**Goals of Dissertation**

This dissertation explores the distribution of serotonergic innervation of spinal and brainstem motor pools after chronic incomplete cSCI. We aimed to utilize two novel interventions, acute intermittent hypoxia (AIH; chapters 2-4) and folate supplementation
(chapter 5), to enhance serotonergic re-innervation of motor pools after C2Hx. cSCI disrupts serotonergic innervation below the injury. Since serotonin underlies motor functional recovery following cSCI, it is important to know the extent of serotonergic re-innervation after incomplete cSCI and to understand factors that accelerate that process. rAIH is one treatment known to elicit serotonin-dependent respiratory and limb motor plasticity, partially restoring breathing and limb functions after chronic cSCI. Conversely, CIH similar to that experienced during sleep apnea elicits neuropathology, and potentially undermines the capacity for AIH-induced motor plasticity. Although rAIH was previously reported to increase serotonergic innervation of the phrenic motor nucleus in intact rats, the impact of different IH protocols on serotonergic innervation of respiratory motor nuclei in rats with or without chronic cSCI has not been investigated.

In Aim 1 we tested the hypothesis that various protocols of IH administered daily for 28 days will increase serotonergic innervation of phrenic and intercostal motor nuclei cervical spinal hemisection at C2 (C2Hx; chapter 2). In addition, the impact of C2Hx and various IH protocols on serotonergic innervation of motor pools rostral to injury have never been investigated. In Aim 2 we tested the hypothesis that various protocols of IH administered daily for 28 days will increase serotonergic innervation of hypoglossal motor nuclei after C2Hx (chapter 3). Further, the impact of IH on serotonergic innervation of axial and forelimb cervical motor nuclei has never been investigated. In Aim 3 we tested the hypothesis that various protocols of IH administered daily for 28 days will increase serotonergic innervation of axial and forelimb nuclei after C2Hx (chapter 4). For these three aims we investigated serotonergic innervation 20 weeks after injury. We assessed serotonergic innervation via immunofluorescence in male
Sprague Dawley rats with and without 2Hx (12 weeks post-injury) exposed to 28 days of: 1) normoxia; 2) daily AIH (10, 5 minutes 10.5% O₂ episodes per day; 5 minutes normoxic intervals); 3) mild CIH (5 minute 10.5% O₂ episodes; 5 minute intervals; 8 hours/day); and 4) moderate CIH (2 minute 10.5% O₂ episodes; 2 minute intervals; 8 hours/day).

Lastly, in Aim 4 we investigated the effects of folate manipulation on serotonergic re-innervation and recovery of breathing function after C2Hx. The effects of folate supplementation and deprivation after cSCI have not been investigated. We tested the hypotheses that 1) folate supplementation enhances, and deprivation hinders, breathing recovery; and 2) folate supplementation stimulates, and deprivation blunts, serotonergic innervation of phrenic motor neurons after C2Hx (chapter 5). We assessed serotonergic innervation of phrenic motor nuclei and recorded diaphragm electromyography during spontaneous breathing, spontaneous sighs, and tracheal occlusion 2 and 8 weeks after C2Hx.

Overall, this dissertation maps out serotonin innervation of various motor pools depicted in Figure 4. The motor neurons focused in this dissertation are the brainstem hypoglossal, cervical spinal phrenic, axial, and forelimb, and thoracic spinal intercostal motor neurons cord. Serotonergic innervation of these motor pools is assessed at 2, 8, and 20 weeks after C2Hx.
Figure 1-1. Phrenic Diaphragm System. Phrenic motor neurons reside in the cervical spinal cord C3-C6. They receive inputs from brainstem respiratory centers, depicted as green projections. Serotonin from the raphe nucleus, depicted as red projections, also innervates phrenic motor neurons. Phrenic motor neurons project out of the ventral spinal cord and innervate the diaphragm. During inspiration, excitation of phrenic motor neurons causes the diaphragm to contract, creating negative pressure that allows air to enter the lungs. During expiration there is inhibition of phrenic motor neurons causing the diaphragm to relax and air to exit the lungs.
Figure 1-2. Animal Model of Cervical Spinal Hemisection at C2. A commonly used model of cSCI in rats is a cervical hemisection at C2 (C2Hx) which provides a precise unilateral lesion that interrupts descending bulbospinal pathways. C2Hx disrupts all descending inputs to phrenic motor neurons on the ipsilateral side while leaving contralateral projections intact. This lesion results in silencing the ipsilateral phrenic nerve and paralysis of the ipsilateral diaphragm. C2Hx also results in forelimb paralysis by disrupting descending inputs to ipsilateral forelimb motor neurons.
This dissertation focuses on two distinct pathways to intermittent hypoxia induced respiratory plasticity, Q and S pathways. The “Q pathway” (left) is serotonin depend and acts via 5-HT2a and 5-HT2b receptor activation. The current working model of the Q pathway is that repetitive, moderate hypoxia triggers intermittent serotonin release in the vicinity of the phrenic motor nucleus. Serotonin then binds and activates 5-HT2 receptors, triggering an intracellular Gq signaling cascade. This signaling includes extracellular signal regulated-mitogen-activated protein (ERK-MAP) kinase activity, new synthesis of brain-derived neurotrophic factor (BDNF), activation of its high-affinity receptor, tropomyosin receptor kinase B (TrkB), and downstream activation of protein kinase C-theta (PKCθ). The S pathway is initiated by either 5-HT7 or adenosine 2a (A2a) receptors that signals the Gs signaling cascade that includes activation of adenylyl cyclase, cAMP signaling via exchange protein activated by cAMP (EPAC), activation of protein kinase B (AKT), mammalian target rapamycin (mTOR) signaling, and then new synthesis of immature TrkB isoform. The exact link between the Q and S pathways and NMDA receptors has yet to be determined.
Figure 1-4. Goals of this Dissertation. The goal of this dissertation is to understand the changes in serotonergic innervation of motor neurons after cervical spinal injury and various protocols of intermittent hypoxia or folate manipulation. We tested the hypotheses that various protocols of intermittent hypoxia (IH) administered daily for 28 days will increase serotonergic innervation of 1) phrenic and intercostal motor nuclei cervical spinal hemisection at C2 (aim 1, Chapter 2); 2) hypoglossal motor nuclei (aim 2, Chapter 3); and 3) axial and forelimb nuclei (aim 3, Chapter 4) in spinally intact rats and 8 weeks after C2 spinal hemisection (C2Hx). We also tested the hypotheses that 1) folate supplementation enhances, and deprivation hinders, breathing recovery; and 2) folate supplementation stimulates, and deprivation blunts, serotonergic innervation of phrenic motor neurons after C2Hx (aim 4, Chapter 5).
CHAPTER 2
SEROTONERGIC INNERVATION OF RESPIRATORY MOTOR NUCLEI AFTER CERVICAL SPINAL INJURY: IMPACT OF INTERMITTENT HYPOXIA

Abstract

Although cervical spinal cord injury (cSCI) disrupts bulbo-spinal serotonergic projections, partial recovery of spinal serotonergic innervation below the injury site is observed after incomplete SCI. Since serotonin contributes to functional recovery post-injury, treatments to restore or accelerate serotonergic innervation are of considerable interest, such as intermittent low oxygen (intermittent hypoxia, IH). IH was previously shown to increases serotonin innervation near respiratory motor neurons in intact rats and improves function after SCI. Here, we tested the hypotheses that spontaneous serotonergic reinnervation of key respiratory (phrenic and intercostal) motor nuclei: 1) is substantially restored 12 weeks post C2 hemisection (C2Hx); 2) is further enhanced by IH; and 3) results from sprouting of crossed-spinal serotonergic projections that were spared by the injury. Serotonin was assessed via immunofluorescence in male Sprague Dawley rats with and without C2Hx (12wks post-injury); individual groups were exposed to 28 days of: 1) normoxia; 2) daily acute IH (dAIH28: 10, 5 minute 10.5% O₂ episodes per day; 5 minute normoxic intervals); 3) mild chronic IH (IH28-5/5: 5 minute 10.5% O₂ episodes; 5 minute intervals; 8 hours/day); or 4) moderate chronic IH (IH28-2/2: 2 minute 10.5% O₂ episodes; 2 minute intervals; 8 hours/day), simulating the IH experienced with moderate sleep apnea. After C2Hx, the number of ipsilateral serotonergic structures was decreased in both motor nuclei, regardless of IH protocol. In both motor nuclei, serotonergic structures were larger after C2Hx and total area of serotonin immunolabeling was increased in the phrenic motor nucleus. Both chronic IH protocols increased serotonin structure size and total area in phrenic motor nuclei of
uninjured rats but had no detectable effects after C2Hx. Although the functional implications of reduced, but larger, serotonergic structures are unclear, our findings confirm that serotonergic innervation is substantially restored following injury. Our results indicate substantial spontaneous serotonergic reinnervation below cSCI, yet intermittent hypoxia had minimal effect.

**Overview**

Spinal cord injury (SCI) impairs both motor and sensory function. Over half of all SCI occur in the cervical region (cSCI; (NSCISC, 2020) and can lead to severe respiratory impairment. In fact, respiratory failure is the major cause of death in people with cSCI (Winslow and Rozovsky, 2003). Since most cSCI are incomplete, one promising strategy to restore breathing function is to harness intrinsic mechanisms of spinal plasticity, strengthening spared neural pathways to respiratory motor neurons (Dale-Nagle et al., 2010b; Goshgarian, 2003). One key bulbo-spinal pathway necessary to enable certain forms of respiratory motor plasticity (and presumably functional recovery) is from serotonergic raphe nuclei of the medulla (Bowker et al., 1982; Jacobs and Azmitia, 1992; Skagerberg and Björklund, 1985; Steinbusch, 1981). Thus, following injury, preservation and restoration of serotonergic innervation to respiratory motor nuclei are of considerable interest.

Serotonin modulates motor neuron activity (Lindsay and Feldman, 1993; Perrier, 2016; Perrier et al., 2013) and initiates/orchestrates novel forms of spinal motor plasticity (Baker-Herman and Mitchell, 2002; MacFarlane and Mitchell, 2009). Since all CNS serotonergic neurons are located in the brainstem (Dahlström and Fuxe, 1964; Törk, 1990), cSCI disrupts descending serotonergic projections to respiratory motor nuclei (Golder and Mitchell, 2005; Perrin and Noristani, 2019; Tai et al., 1997). With
time post-injury, serotonergic reinnervation partially recovers, correlating with at least some functional improvements (Camand et al., 2004; Golder and Mitchell, 2005; Hashimoto and Fukuda, 1991; Saruhashi et al., 1996; Tai et al., 1997; Zhou and Goshgarian, 2000). Despite the functional significance of this modulatory system for preservation and restoration of respiratory function, we do not yet know the time course or extent of spontaneous serotonergic reinnervation of phrenic or intercostal motor nuclei after cSCI. Further, it is not known if spontaneous serotonergic reinnervation of ipsilateral respiratory motor nuclei after cSCI results from axon terminal sprouting in spared serotonergic projections or represents growth of new serotonergic axons into these motor nuclei. Understanding the extent, time course and mechanisms of serotonergic reinnervation in key respiratory motor nuclei (e.g. phrenic and intercostal) after chronic cSCI may help guide our development of therapeutic strategies to restore breathing function by harnessing serotonin-dependent respiratory motor plasticity, such as therapeutic acute intermittent hypoxia (AIH; (Dale et al., 2014; Gonzalez-Rothi et al., 2015b).

AIH has emerged as a potential therapeutic tool, triggering neuroplasticity in spared neural pathways, thereby restoring respiratory and non-respiratory motor function in both rodent models and in humans with chronic, incomplete SCI (Dale et al., 2014; Gonzalez-Rothi et al., 2015b; Hayes et al., 2014; Lovett-Barr et al., 2012). One mechanism whereby AIH enhances breathing function after SCI requires spinal serotonin receptor activation (Baker-Herman and Mitchell, 2002; Dougherty et al., 2018). Since repetitive AIH (3x per week for 10 weeks) is reported to increase serotonergic innervation of the phrenic and other cervical spinal motor nuclei in
uninjured rats (Satriotomo et al., 2012; Satriotomo et al., 2016), AIH itself may accelerate/enhance serotonergic reinnervation of phrenic and intercostal motor nuclei after cSCI, enabling functional recovery. Although “low-dose” AIH elicits functional benefits, more intense protocols of intermittent hypoxia simulating episodes experienced during sleep apnea (chronic intermittent hypoxia; CIH) elicit dose-dependent pathology (Dale et al., 2014; Navarrete-Opazo and Mitchell, 2014b) and can undermine (Huxtable et al., 2015) or facilitate the potential for AIH to elicit serotonin dependent plasticity (Vivodtzev et al., 2020). Such “high-dose” CIH is quite prominent in people with cSCI since nearly 80% exhibit sleep apnea (Berlowitz et al., 2005; Sankari et al., 2014a; Sankari et al., 2019). To optimize therapeutic IH protocols to treat those with cSCI, it is essential to know the extent and time course of spontaneous serotonergic reinnervation and the impact of IH on this process.

Here we investigate the impact of three, commonly studied IH protocols that range from low-dose, therapeutic daily AIH to more severe, pathogenic protocols simulating mild to moderate sleep apnea (Navarrete-Opazo and Mitchell, 2014b). We tested the hypotheses that: 1) spontaneous serotonergic reinnervation of phrenic and intercostal motor nuclei is nearly complete 12 weeks post C2 hemisection (C2Hx); 2) IH further enhances serotonergic reinnervation of phrenic and intercostal motor nuclei; and 3) serotonergic reinnervation is due to sprouting of existing/spared crossed-spinal serotonergic projections. We report that C2Hx reduces the number but increases the size of serotonergic structures innervating phrenic and intercostal motor neurons, resulting in equal (intercostal) or even increased (phrenic) total areas of serotonergic structures within the respective motor nuclei. Reinnervation was due to sprouting of
spared crossed-spinal serotonergic projections to the phrenic motor nucleus. Although no IH protocol affected innervation recovery after C2Hx, both CIH protocols increased serotonergic innervation of the phrenic motor nucleus in uninjured rats. Collectively, we demonstrate that serotonergic reinnervation of phrenic and intercostal motor nuclei is considerable after C2Hx, suggesting the potential to elicit (and harness) serotonin-dependent respiratory motor plasticity with chronic cSCI (Dougherty et al., 2018).

Methods

Animals

All experiments were conducted with adult male (initially 350-450 grams) Sprague-Dawley rats (Envigo, Indianapolis IN, Colony 208A). Rats were housed in pairs in a controlled environment (12 hour light/dark cycles) with food and water ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida Health Sciences Center. A total of 79 rats were studied, beginning at 11-12 weeks of age. Spinal intact and C2 hemisection (C2Hx) rats were randomly assigned to intermittent hypoxia exposure groups at 8 weeks post-injury (or equivalent time for uninjured rats), and exposed for 28 consecutive days to either: 1) normoxia (Nx28; n = 8 intact, n = 9 with C2Hx); 2) daily acute intermittent hypoxia (dAIH28, n=10 intact, n=8 with C2Hx), 3) mild chronic intermittent hypoxia (CIH; 8 hours per day) with an equivalent desaturation index of 6 (IH28-5/5, n=10 intact, n=12 with C2Hx); and 3) moderate CIH at an equivalent desaturation index of 15 (IH28-2/2, n=10 intact, n=12 with C2Hx). The experimental timeline and details of the IH protocols are illustrated in Figure 2-1.
**Phrenic Motor Neuron Labeling**

Anesthesia was induced with 3.5% isoflurane (in 100% O₂) in a plexiglass chamber, and then maintained at 2-2.5% isoflurane via nose cone. Adequate anesthetic depth was confirmed by the absence of toe pinch and palpebral responses. To retrogradely label phrenic motor neurons, rats received bilateral intrapleural injections of Cholera toxin B fragment (CtB; 0.2% w/v CtB; dissolved in sterile H₂O; Calbiochem, Billerica, MA) at least 14 days prior to C2Hx (Dale-Nagle et al., 2011; Guenther et al., 2010; Mantilla et al., 2009). 25 µL of CtB was loaded into a 25 µL Hamilton syringe attached to a 9.5 mm sterile needle for bilateral injections (2 × 12.5 µL = 25µL total per animal) at the 5th intercostal space ~6 mm deep.

**C2Hx Injury**

Anesthesia, C2Hx, and animal care were performed as described previously (Fuller et al., 2008; Gonzalez-Rothi et al., 2015c). Rats were induced with 3.5% isoflurane (in 100% O₂) in a chamber and then maintained (2-2.5% in O₂) via nose cone throughout the surgery; adequate anesthetic depth was confirmed by the absence of toe pinch and palpebral responses. Body temperature was maintained at 36.5–37.5°C with a heating pad. Artificial tears were applied to prevent eye damage (Rugby, NDC 0536-1086-91). Toenails were clipped on all limbs. The surgical site was shaved and cleaned (chlorhexidine scrub, 58829-140-01, First Priority, Inc., Elgin, IL). C2Hx was performed by making a dorsal incision on the neck and dissecting muscle to expose the C2 lamina. Following C2 laminectomy and durotomy, the left spinal cord was hemisected with a micro-knife caudal to the C2 dorsal roots. A gap (~1 mm) at the injury site was created by aspiration. The dura was closed with 9-0 ethilon nylon suture, the overlaying muscles
were closed with 3-0 Polysorb absorbable suture and the skin was closed with 9 mm stainless steel wound clips. Post-operative care included pain management with an analgesic (buprenorphine, 0.03 mg/kg, s.q., Hospira, IL) and an anti-inflammatory drug (meloxicam, 2 mg/kg., s.q. Portland, ME) given at 12 and 24-hour intervals for 2 days post-surgery. Rats received lactated Ringer’s solution (5 ml 2x/day, s.q.) and were manually fed a nutritional supplement (Diet Gel Boost; Clear H2O; Westbrook, ME) until adequate volitional drinking and eating were observed. Visualization of C2Hx showed anatomically complete lesions with the absence of white and gray matter from the left lateral edge to the spinal cord midline.

**Intermittent Hypoxia Protocols**

Eight weeks post-C2Hx (or 6 weeks post-CtB injections for uninjured rats), rats were housed in PlexiGlass exposure cages with free access to food and water (Therapeutiq, Kansas City, MO). IH protocols were administered to the cages daily for 28 days. Normoxia control rats received continuous air flow (21% O2, 8 hours per day; Figure 2-1B). Rats assigned to the dAIH groups received 10, 5 minute episodes of 10.5% O2, alternating with 5 minute normoxic intervals for ten episodes per day (~1.5 hours/day; Figure 2-1C). Rats assigned to IH28-5/5 groups received 5 minute episodes of 10.5% O2, alternating with 5 minute normoxic intervals for 8 hours/day (48 episodes per day; Figure 2-1D). Five minute hypoxic/normoxic intervals yield 6 hypoxic episodes per hour, somewhat equivalent to mild sleep apnea (5 to 10 events per hour)(American Academy of Sleep MedicineAmerican and medicine, 1999; Young et al., 2008). Rats assigned to IH28-2/2 received 2 minute episodes of 10.5% O2, with 2 minute normoxic intervals for 8 hours/day (120 hypoxic episodes per day; Figure 2-1E). This protocol consisting of 15 hypoxic episodes per hour is equivalent to clinical criteria for the
transition to moderate sleep apnea (American Academy of Sleep Medicine and medicine, 1999; Young et al., 2008).

**Tissue Harvesting, Histology & Analysis**

One day after their final exposure, rats were perfused intracardially with cold 0.1M phosphate buffer saline (PBS), followed by paraformaldehyde (4% paraformaldehyde w/v in 0.1M PBS, pH 7.4). The spinal cord was harvested and then: 1) post-fixed overnight in paraformaldehyde (4% in 0.1M PBS, pH 7.4); and 2) cryoprotected at 4°C in 20% sucrose solution in 0.1M PBS for 3 days followed by 30% sucrose solution in 0.1M PBS for 3 days. To verify injury, cervical spinal segments C1-C3 were embedded in paraffin, cut at 7 µm, mounted on positively charged slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA), and allowed to dry overnight. Sections were stained with Luxol Fast Blue and cresyl violet. Sections were deparaffinized by heating at 60°C for 20 minute and submerged 3 times in xylene for 5 minute each. Sections were rehydrating by graded alcohol (100%-70%) for 2 minutes each and then stained overnight using Luxol Fast Blue (0.1g Luxol Fast Blue in 100mL of 95% ethanol and 0.5mL 10% acetic acid). The following day, tissues were rinsed with 95% ethanol for 2 minutes and distilled water for 5min. Differentiation was achieved by dipping the tissue in 0.5% lithium carbonate and 70% ethanol, then rehydrated by distilled water for 5min. Tissues were placed in 0.1% cresyl violet for 15min. Cresyl violet was rinsed by dipping tissue into distilled water twice. Tissue was then dehydrated through graded alcohol (70–100%) for 1 minute each, cleared in Histoclear twice for 2 minutes each (National Diagnostics, Atlanta, GA) and cover-slipped (Eukitt, Electron Microscope Science, PA). Injury sites were imaged using bright field at 10x zoom level (BZ-x710, Keyence Co., Osaka, Japan) to confirm lateral C2Hx.
Cervical (C3-C5) and thoracic (T4-T6) spinal segments were transversely sectioned (40 µm thickness) using a freezing microtome (Leica SM 200R, Buffalo Grove, IL). Tissues were stored in antifreeze solution at -20°C until processed (30% glycerol, 30% ethylene glycol, 40% 0.1 M PBS, pH 7.4). Every 12th section was selected and stained for serotonin and CTB-labeled phrenic and intercostal motor neurons. Free-floating sections were washed with 0.1M PBS (pH 7.4), then incubated in heat induced epitope retrieval (TissuePro, cat#: HIER01-32R) for 30 minutes at 85°C. Tissues were washed again with 0.1M PBS-Triton (0.1%, pH 7.4) and then incubated in a blocking solution (5% normal donkey serum (NDS, GeneTex) in 0.1M PBS-Triton (0.1%, pH 7.4) at room temperature for 60 minutes. Primary antibody staining was performed by incubating tissue sections in 5% NDS in 0.1M PBS-Triton (0.1%, pH 7.4) with anti-5-HT (1/2000, rabbit serum, Immunostar #20080) and anti-CTB (1/2500, goat serum, Millipore #227040) in 4°C overnight. The following day, tissues were washed with 0.1M PBS-Triton (0.1%, pH 7.4). Secondary antibody staining was performed by incubating tissues in 5% NDS in 0.1M PBS-Triton (0.1%, pH 7.4) with secondary antibodies conjugated to Alexa Fluor® 594 (donkey anti-rabbit 594; 1:500, Invitrogen, Ref# A11055) and Alexa Fluor® 488 (donkey anti-goat 488; 1:1000, Invitrogen, Ref# A21207) in a dark box at room temperature for 2 hours. Sections were washed with 0.1M PBS-Triton (0.1%, pH 7.4) and mounted on positively charged slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. Slides were cover slipped with VectaShield Antifade Hard Set Mounting Medium (Cat# H-1400). Fluorescently labeled sections were captured using an epifluorescent microscope with 20x magnification (Keyence BZ-X700, Keyence Corporation of America, Itasca, IL).
Spinal cord sides were marked prior to tissue sectioning to ensure accurate side determination during imaging. Phrenic and intercostal motor neurons were determined by CtB-positive cell labeling within the ventral horn.

**Data Analysis**

**Serotonin immunofluorescence**

Serotonin immunofluorescence was quantified using a custom MATLAB (MathWorks, Natick, MA, USA) code. Thresholds were determined using a custom adaptive threshold algorithm in MATLAB as previously described (Allen et al., 2019). The adaptive threshold was calculated by constructing a pixel intensity histogram from the image. The pixel intensity corresponding to the 99th percentile was selected as the adaptive threshold. Injury (or intact) and intermittent hypoxia exposure protocol remained blinded throughout analyses. We observed serotonin-positive boutons and fibers; however, we cannot differentiate between serotonin boutons and fibers using our analysis. Thus, we call these “serotonin structures”. Independent serotonin structures were determined by being larger than 4 pixels and being at least 1 pixel apart. Using these parameters, we quantified:

**Number of structures.** The number of structures was determined using a representative image from each tissue section and then averaged to obtain the average number of immunolabeled structures per rat.

**Area per structure.** The area per structure was determined by the pixel size of each structure. The pixel numbers were multiplied by the aspect ratio of pixel per micron (0.377) to determine the area per structure using a representative image from each tissue section, and then averaged to obtain the average area of each immunolabeled structure per rat.
Total area. The total area of serotonin-positive immunolabeling was calculated by multiplying the number of structures by the area per structure within a representative image from each tissue section and then averaged to obtain the average total area of serotonin immunolabeling per rat.

Intensity per structure. The fluorescent intensity in each immunolabeled structure was determined within a representative image from each tissue section and then averaged per rat to obtain the intensity of each structure.

Identification of phrenic motor neurons

Within the cervical C3-C5 spinal sections, the custom MATLAB code first identified CtB-labelled phrenic motor neurons versus non-positive areas and assigned them binary values (CtB-positive=1, CtB-negative=0) to determine phrenic vs. non-phrenic areas. Then, the center of gravity of the CtB-labelled phrenic motor neurons was calculated. The final region of interest was defined by a circular area with a 50 µm radius centered at the center of gravity of CtB-positive phrenic motor neurons (Seven et al., 2018b). The center of gravity equation was defined as:

\[
\tilde{g}(x, y) = \frac{\sum (p_i \times \vec{r}_i^* (x, y))}{\sum p_i}
\]  

(2-1)

Center of gravity was calculated based on the distance from a standardized reference point (image origin) and binary values. One rat in the intact+Nx28 groups was excluded in the phrenic motor neuron analysis because there were no sections with CtB-labeled phrenic motor neurons.

Identification of serotonin immunolabeling in the central commissure

The same cervical spinal tissue used to identify serotonin innervation around phrenic motor neurons was used to quantify the total area of serotonin positive pixels in
the central commissures, including the dorsal and ventral commissures (lamina X). A point at the center of the central canal was selected and a circular region of interest was created with a radius of 100 µm.

**Identification of intercostal motor neurons**

Within thoracic T4-T6 spinal segments, the MATLAB code first identified CtB-labelled intercostal motor neurons. Then, regions of interest were defined by a circular area with a 35 µm radius centered at the center of gravity of each CtB-positive intercostal motor neuron. If there were overlapping areas of interest within in a tissue section, serotonin positive pixels in the overlap were not counted twice. We report the raw values for serotonergic innervation of intercostal motor neurons, but we also normalized the number of serotonin structures and total area to the number of labeled intercostal motor neurons per section. Results of normalized serotonin immunolabeling around intercostal motor nuclei were consistent to the raw data, so we only the raw data is presented.

**Statistics**

Statistical calculations were performed using JMP 11.0 (SAS Institute, Cary, NC). Serotonin immunofluorescence data were analyzed using a mixed model with repeated measures design. The independent variables were injury (intact vs. C2Hx), IH protocol (Nx28, dAIH28, IH28-5/5, and IH28-2/2), and spinal cord side relative to injury (ipsilateral vs. contralateral as the repeated measure). Due to the large number of comparisons within the mixed model, we were concerned that significance would be hidden within intact and C2Hx groups. To avoid type 2 statistical errors, the intact and C2Hx groups were analyzed separately. In the intact groups, we averaged the left and right sides of spinal cord and ran a one-way ANOVA with IH protocol as the
independent variable (Nx28, dAIH28, IH28-5/5, and IH28-2/2). In C2Hx groups, we ran a two-way repeated measures ANOVA with IH protocol (Nx28, dAIH28, IH28-5/5, and IH28-2/2) and spinal cord side relative to injury (ipsilateral vs. contralateral as the repeated measure) as the independent variables. Serotonin immunofluorescence crossing the central commissure was analyzed using a two-way ANOVA. The independent variables were injury (intact vs. C2Hx) and protocol (Nx28 vs. dAIH28 vs. IH28-5/5 vs. IH28-2/2). A significance level of 0.05 was set for all statistical comparisons. When significant differences were observed, individual comparisons were made based on the Fisher Least Significance Difference post hoc test. All data are displayed as mean ± standard error of the mean.

Results

Serotonergic Innervation of Phrenic Motor Nuclei

Serotonin innervation was quantified within a 50 µm radius around CtB-labeled phrenic motor neurons. We observed prominent differences in the size of serotonergic structures after C2Hx, although there were no obvious differences in the total area of immunoreactive pixels in the phrenic motor nucleus. Serotonin projections in intact rats and contralateral to C2Hx had a thin and continuous appearance, somewhat like “beads on a string.” Ipsilateral to C2Hx, serotonin structures were less abundant but larger, regardless of IH protocol (Figure 2-2).

Regardless of IH protocol, there was a significant reduction in serotonergic structure number in the phrenic motor nucleus after injury (Figure 2-3A); reduced by 30% in the ipsilateral (p<0.0001) and 12% in the contralateral phrenic nucleus versus intact rats (p<0.05). Within C2Hx rats, the number of serotonergic structures ipsilateral to injury was 20% less than the contralateral side (p<0.0001). IH protocol had no effect
on the number of serotonin structures near CtB-labeled phrenic motor neurons in injured or uninjured rats (p=0.1377).

Next, we quantified the average area of each serotonin labeled structure near CtB-labeled phrenic motor neurons (Figure 2-3B). Ipsilateral to C2Hx, serotonin structures were significantly enlarged; the average size of each structure was nearly double (90% increase) that in uninjured rats, regardless of IH protocol (intact vs. C2Hx p<0.0001). Within the intact groups, we averaged the left and right spinal cord sides and found that IH28-5/5 and IH28-2/2 had significantly greater areas per structure (~13%) versus dAlH28 and Nx28 (one-way ANOVA by IH protocol; IH28-2/2 vs. Nx28 p=0.0024, IH28-2/2 vs. dAlH p=0.0034, IH28-5/5 vs. Nx28 p=0.0087, IH28-5/5 vs. dAlH28 p=0.0134). In injured rats alone, there was no similar effect of IH on the size of serotonin structures (two-way repeated measures ANOVA p=0.9623).

The total area of serotonin immunolabeling actually increased post-C2Hx compared to uninjured rats (p<0.0001; Figure 2-3C). Ipsilateral to C2Hx, the total area of serotonergic immunolabeling was 30% greater versus intact (p<0.0001). Ipsilateral structures were also significantly larger versus contralateral side (~41%; p<0.0001). However, IH had no effect on the total area of serotonin immunolabeling after C2Hx (two-way repeated measures ANOVA, p=0.6323). In the uninjured rats, when the left and right sides were averaged and analyzed via one-way ANOVA (by IH protocol), IH28-5/5 and IH28-2/2 had significantly greater total areas versus Nx28, about a 25% increase (IH28-2/2 vs. Nx28 p=0.0179, IH28-5/5 vs. Nx28 p=0.0262).

**Serotonin Positivimmunolabeling in the Central Commissure**

Immunofluorescent labeling was used to identify serotonergic labeled pixels at the cervical central commissure in the same sections used to determined serotonergic
reinnervation in the phrenic motor nucleus. This analysis was used as a proxy to determine if ipsilateral serotonin structures observed after C2Hx were due to sprouting of existing (spared) crossed spinal pathways versus possibly new contralateral projections crossing the midline. All spinal sections studied had positive serotonin immunolabeling in this midline region. On visual inspection, there were no obvious differences in serotonin labeling between groups (Figure 2-4A). We quantified the area of serotonin-positive pixels in a region of interest around the central canal (Figure 2-4B). There was no statistically significant difference in the number of serotonin positive pixels surrounding the central commissures of injured versus uninjured rats, regardless of IH protocol (two-way ANOVA p=0.4648). Although there were no effects of IH protocol (p=0.3487), there were slightly more serotonin positive pixels in the central commissures of uninjured rats treated with IH28-2/2.

**Serotonergic Innervation of Intercostal Motor Nuclei**

Serotonin innervation was quantified within a 35 µm radius around CtB-labeled intercostal motor neurons. Upon first glance, we did not notice obvious differences in serotonergic innervation between IH protocols in either uninjured or injured rats. However, we did observe obvious differences in serotonergic innervation after C2Hx, similar to the phrenic motor pool. In intact rats, or contralateral to C2Hx in injured rats, serotonin projections appeared normal (i.e. thin and continuous; “beads on a string”). Ipsilateral to C2Hx, serotonin structures were less abundant and thicker/larger, regardless of IH protocol (Figure 2-5).

The number of serotonin structures around intercostal motor neurons was reduced 12 weeks post-C2Hx (Figure 2-6A). There were significantly fewer structures (~44%) ipsilateral to injury versus the contralateral side or uninjured rats (both
p<0.0001). The number of serotonin structures was unaffected by IH in injured or uninjured rats (p=0.2507). The size of serotonin structures was significantly larger on the ipsilateral side post-C2Hx versus the contralateral side or uninjured rats (~56%; Figure 2-6B; p<0.0001). There was no effect of IH protocol on the average size of serotonergic structures in uninjured or C2Hx rats (p=0.3473).

The total area of serotonin immunolabeling around intercostal motor neurons (Figure 2-6C) exhibited complex differences post-injury. There was an interaction of protocol vs. injury vs. side on total area of serotonin immunolabeling (p=0.0344). The total area ipsilateral to C2Hx was 16% lower versus the contralateral side or in intact rats (both p<0.0001), regardless of IH protocol. Although not statistically significant, ipsilateral to C2Hx, exposure to dAIH28 increased (~10%) total serotonin immunolabeling area of intercostal motor nuclei versus Nx28 (p=0.063). There were no effects IH protocol on total ipsilateral serotonin area neuron versus the contralateral side or uninjured rats (p>0.05).

Intercostal motor nuclei are spaced along the thoracic spinal cord and do not cluster like phrenic motor neurons. Thus, to identify possible differences, and bias, in intercostal motor neurons, we counted the number of CtB-labeled intercostal motor neurons in each section each thoracic spinal section. The average number of CtB-labeled intercostal motor neurons varied from 1-5 neurons per 40 µm section of thoracic spinal tissue (data not displayed). We found no differences in intercostal motor neuron numbers between injured and uninjured rats (p=0.4213), and there were no effects of IH protocol (p=0.9556). In addition to quantifying the raw serotonin structure numbers and total area, we normalized serotonin structure number and total area to the average
number of CtB-labeled intercostal motor neurons per thoracic section to account for slight variations in the number of intercostal motor neurons per section. The overall results were comparable between the raw and normalized data, so we only report the raw data.

Serotonin Immunofluorescent Intensity around Phrenic and Intercostal Motor Nuclei

We quantified staining intensity in serotonergic structures in the phrenic and intercostal motor nuclei (Figure 2-7) despite known limitations in using immunofluorescence as an indicator of antigen concentration (Fritschy, 2008; Matos et al., 2010). In the phrenic motor nucleus, average serotonin staining intensity within serotonin-positive structures was decreased by C2Hx (Figure 2-7A). Ipsilateral to C2Hx, serotonin structures had 36% lower optical density compared to the contralateral side or intact rats (both p<0.0001). IH had no impact on serotonin labeling intensity after C2Hx (two-way repeated measures ANOVA p=0.4431). In uninjured rats, we averaged left and right sides and ran a one-way ANOVA for IH protocol; in this analysis, dAIH28 had significantly greater structure intensity (27%) versus Nx28 (p=0.0444). There were no significant effects of mild (IH28-5/5) or moderate (IH28-2/2) CIH protocols in intact rats.

In the intercostal region, serotonin positive structures had ~59% lower average intensities ipsilateral to C2Hx compared to the contralateral side and intact rats (Figure 2-7B; both p<0.0001). There was no effect of any IH protocol on the intensity of immunoreactivity within serotonin structures around intercostal motor nuclei of injured or uninjured rats (p=0.0988). In the intact group alone, there was no effect of IH protocol (one-way ANOVA; p=0.254). After C2Hx, ipsilateral intensities were significantly less
than contralateral and there were no effects of IH protocols (two-way repeated measures ANOVA; \( p<0.0001 \) and \( p=0.286 \) respectively).

**Discussion and Conclusion**

We investigated spontaneous serotonergic reinnervation of the phrenic and intercostal motor nuclei following cervical spinal hemisection, a frequently studied model of cSCI. Further, we investigated the impact of multiple, frequently studied IH protocols on serotonergic innervation in both injured and uninjured rats. After C2Hx, spontaneous reinnervation of the ipsilateral phrenic and intercostal motor nuclei was impressive, although it was characterized by reduced numbers of larger serotonergic structures. Total area of serotonin immunolabeling represented by serotonergic structures was either increased (phrenic) or maintained (intercostal) in these critical motor nuclei. Thus, with time post-injury, serotonergic reinnervation of respiratory motor nuclei is sufficiently robust to contribute meaningfully to serotonin-dependent respiratory motor plasticity. This knowledge is essential given recent attempts to harness such plasticity as a therapeutic modality to restore breathing ability after cSCI.

CIH simulating mild (IH-5/5) or moderate (IH-2/2) for 28 days increased the size of serotonergic structures within the phrenic motor nucleus of uninjured rats, although the IH protocol with therapeutic promise, dAIH28, had no effect. In injured rats, IH had no effects on serotonergic innervation of the phrenic motor nucleus. However, in intercostal motor nuclei, dAIH for 28 days increased serotonergic innervation, suggesting some potential of dAIH to promote serotonin-dependent intercostal motor plasticity following cSCI. The extent of serotonergic innervation below a cSCI has profound implications for the potential to harness IH as a therapeutic modality to treat those with chronic SCI.
Serotonergic Reinnervation after C2Hx

We report profound changes in serotonergic structures around phrenic and intercostal motor nuclei due to the injury alone. Spinal serotonin arises from the raphe nucleus in the medulla, which descends mostly to the ipsilateral cord, with some contralateral projections that decussate at multiple spinal levels (Bowker et al., 1981; Liang et al., 2015). C2Hx severs descending axons from medullary serotonergic neurons innervating the ipsilateral phrenic and intercostal motor nuclei (Golder and Mitchell, 2005; Perrin and Noristani, 2019; Tai et al., 1997). Thus, the nearly complete restoration of total serotonin immune-reactive area within these motor nuclei 12 weeks post injury is impressive. The functional significance of fewer, but larger serotonin immunoreactive structures is not clear. Nevertheless, this extent of recovery is indicative of the potential for recovery of serotonin-dependent plasticity with time post-injury (Dougherty et al., 2018; Navarrete-Opazo et al., 2017b; Navarrete-Opazo et al., 2015).

The size of serotonin terminals in the phrenic motor nucleus of cats ranges from 1-3µm (Pilowsky et al., 1990). In the present study, the size of serotonin structures around phrenic motor neurons was ~1.5 µm in intact rats. However, after C2Hx, ipsilateral serotonin structures nearly doubled in size to ~3 µm. These data support literature accounts that serotonin axons enlarge and that their terminals swell during sprouting (Mamounas et al., 2000; Mamounas et al., 1995). Other accounts suggest that indirect injuries enhance serotonergic innervation of the phrenic motor nucleus; for example, 26 days after bilateral cervical dorsal rhizotomy, the number of serotonergic terminals in the phrenic motor nucleus is increased (Kinkead et al., 1998).
Thirty days following an incomplete C2 lateralized hemisection, the number of serotonergic terminals in the phrenic motor nucleus was reported to increase, as was the number of active zones between serotonin terminals and phrenic motor neurons (Tai et al., 1997). Although we found reduced numbers of serotonergic terminals 12 weeks post-injury, possibly due to our complete spinal hemisections, increased size post-C2Hx enhanced the overall area of serotonin innervation within the phrenic motor nucleus. Considerably less is known about serotonin re-innervation of the intercostal motor nuclei, but our data suggests a similar pattern to the phrenic motor nucleus with the exception that the swelling of serotonin terminals is less robust and is not sufficient to offset the persistent reductions in structure numbers.

The source of serotonin structures reinnervating the phrenic motor nucleus is not completely clear, but our suggestive evidence leads us to conclude that spared terminals ipsilateral to injury sprout and hypertrophy. Specifically, there was no change in pixel density of serotonin immunoreactive fibers crossing the spinal midline, presumably ruling out invasion of new serotonergic axons across the midline to reinnervate the phrenic motor nucleus ipsilateral to injury. Our findings confirm a previous report that new serotonergic projections fail to cross the spinal midline after SCI (Saruhashi et al., 1996), and are consistent with an electron microscopy study demonstrating sprouting of serotonin terminals, and the formation of new synapses with phrenic motor neurons after C2Hx (Tai et al., 1997).

Because this study was only on anatomical characteristics of serotonin innervation of phrenic and intercostal nuclei, the functional significance of the abnormal number and size of serotonin immunoreactive structures remains uncertain. These
results suggest that spontaneous return of at least some serotonergic function occurs post-injury. However, further studies are needed to further define the time course of recovery, and to verify function in these abnormal structures.

**Effects of IH on Serotonergic Innervation in Uninjured Rats**

The observations that IH28-5/5 and IH28-2/2, but not dAlH28, increased the size of serotonergic structures in the phrenic motor nucleus of uninjured rats has important implications concerning the ability of IH preconditioning to enhance serotonergic function. For example, one week of moderate CIH (5 minute intervals for 12 hours/night) enhances AIH-induced, serotonin-dependent phrenic long-term facilitation in normal rats (Ling et al., 2001). Although the mechanism of enhancement was not determined in that study, enhanced phrenic long-term facilitation remained serotonin-dependent. Our finding that similar CIH (5 minute intervals for 8 hours/day) enlarges serotonin structures in the phrenic motor nucleus of uninjured rats could suggest enhanced serotonin release, thereby triggering greater serotonin-induced plasticity (Ling et al., 2001; MacFarlane and Mitchell, 2009). To our knowledge, this finding has not been reported previously.

Lower doses of repetitive AIH (rAIH; 10, 5 minute episodes 3x/week, 10 weeks) are also reported to enhance serotonergic innervation in the phrenic motor nucleus (Satriotomo et al., 2012) and AIH-induced phrenic long-term facilitation (MacFarlane et al., 2018). However, in the present study of daily AIH for 28 days, we did not observe similar anatomical findings (i.e. no increase in serotonergic innervation). These prior studies differed in the total duration of repetitive AIH exposure (10 vs. 4 weeks), details of AIH exposure (intermittent vs. continuous exposure days), and substrains of Sprague Dawley rats used (Envigo versus Taconic). Our observation that
dAIH28 did increase serotonin staining intensity within phrenic motor nucleus structures of uninjured rats may also explain differences in reported (this study versus Satriotomo et al., (2012). In that earlier paper, there were differences in quantification; greater serotonin-concentrations per structure (as we report here) could increase the number of detected structures, leading to the conclusion that the number of anatomical structures had increased. Although optical density measurements do correlate with serotonin concentration, it is not a linear relationship (Matos et al., 2010). Thus, we cannot differentiate among possible explanations for our divergent results.

In contrast to the phrenic motor nucleus, IH had no apparent impact on serotonergic innervation of intercostal motor nuclei in uninjured rats. Reasons for these differences between intercostal versus phrenic motor nuclei are unclear.

**Lack of IH Effects on Serotonergic Reinnervation 12 Weeks Post-C2Hx**

Although we originally predicted that CIH would further enhance and/or accelerate serotonergic recovery after C2Hx, CIH had no significant impact on serotonergic innervation of phrenic or intercostal motor nuclei in injured rats. Since serotonin reinnervation is already robust at this time frame, CIH could still accelerate recovery. Why IH increases serotonergic innervation in intact, but not injured rats is unclear.

Although the serotonergic structures were larger in ipsilateral intercostal motor nuclei post-C2Hx, the total area of serotonin immunolabeling was reduced. One exciting observation in our study was that dAIH28 restored serotonergic innervation to normal, suggesting that dAIH preconditioning may enhance serotonergic function. Thus, serotonin targeted therapies may be effective post-SCI, and may be enhanced by the cumulative effects of repeated AIH exposures. Indeed, repeated AIH does improve
intercostal activity after cSCI (Navarrete-Opazo et al., 2015), and elicits profound intercostal plasticity (Navarrete-Opazo and Mitchell, 2014a). Our current results verify that serotonin-dependent recovery of intercostal function is possible after cervical SCI.

We report substantial spontaneous serotonergic reinnervation of the phrenic and intercostal motor nuclei 12 weeks post-C2Hx, with reduced numbers of larger serotonergic varicosities surrounding phrenic and intercostal motor neurons. Although the functional impact of increased serotonergic structure size is unclear, our results nevertheless demonstrate that substantial serotonergic function may be possible after chronic cervical SCI. Thus, serotonin-targeted therapies may be possible to enhance respiratory function after SCI, for example with appropriate AIH exposures. Indeed, repetitive AIH elicits spinal respiratory motor plasticity and functional recovery in both rodent models and humans with chronic SCI (Dale et al., 2014; Gonzalez-Rothi et al., 2015b).
Figure 2-1. Experimental time-line and intermittent hypoxia protocols. A. Rats were injected with Cholera toxin subunit B (CtB) 2 weeks prior to C2 spinal hemisection (C2Hx). 8 weeks post C2Hx rats began intermittent hypoxia exposures for 28 days. B. Normoxia protocol (Nx28) consisted of 28 days of 21% O\textsubscript{2} for 8 hours. C. Daily acute intermittent hypoxia (dAIH28) consisted of 10, 5 minute episodes of 10.5% O\textsubscript{2} alternating with 5 minute normoxic intervals (~1.5 hours/day) for 28 days. D. Mild chronic intermittent hypoxia (IH28-5/5) consisted of 8 hours per day of 5 minute episodes of 10.5% O\textsubscript{2} alternating with 5 minute normoxic intervals. E. Moderate chronic intermittent hypoxia (IH28-2/2) consisted of 2 minute episodes of 10.5% O\textsubscript{2} alternating with 2 minute normoxic intervals for a total duration of 8 hours/day. Rats were randomly assigned to groups: 1) spinal intact+Nx28 (n=8); 2) spinal intact+dAIH28 (n=10); 3) spinal intact+IH28-5/5 (n=10); 4) spinal intact+IH28-2/2 (n=10); 5) C2Hx+Nx28 (n=9); 6) C2Hx+dAIH28 (n=8); 7) C2Hx+IH28-5/5 (n=12); and 8) C2Hx+IH28-2/2 (n=12).
B. Nx28: Normoxia for 8 hours

\[ \begin{array}{c}
O_2 \\
21\% \\
10.5\%
\end{array} \]

8hrs

C. dAlH28: 5min episodes of 10.5%O₂ for 10 cycles

\[ \begin{array}{c}
O_2 \\
21\% \\
10.5\%
\end{array} \]

5min

8hrs

D. IH28-5/5: 5min episodes 10.5%O₂ for 8 hours

\[ \begin{array}{c}
O_2 \\
21\% \\
10.5\%
\end{array} \]

5min

8hrs

E. IH28-2/2: 2min episodes 10.5%O₂ for 8 hours

\[ \begin{array}{c}
O_2 \\
21\% \\
10.5\%
\end{array} \]

2min

8hrs

Figure 2-1. Continued
Figure 2-2. Representative images of serotonergic innervation in the phrenic motor nucleus. Representative images from uninjured rats, and rats 12 weeks post-C2Hx exposed to 28 days of normoxia. Images from illustrate serotonin (red), often described as looking like “beads on a string”, and Cholera toxin subunit B-labeled phrenic motor neurons (green). Images from left to right are representative samples from the left and right side of the cervical spinal cord of intact rats, and the ipsilateral and contralateral sides of the cervical spinal cord from rats 12 weeks post-C2Hx. Images were taken at 20x magnification with the phrenic motor nucleus at the center. The white scale bars represent a distance of 100 µm.
Figure 2-3. Serotonergic innervation of the phrenic motor nucleus: effects of injury and IH protocol. Serotonin innervation in the phrenic motor nucleus was quantified in uninjured rats (left) and rats 12 weeks post-C2 hemisection (C2Hx; right) exposed to 28 days of intermittent hypoxia (IH) protocols (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). A. Number of serotonin structures. C2Hx significantly reduced the number of serotonin structures ipsi- and contralateral to C2Hx versus uninjured rats (p<0.0001 and p<0.05, respectively). Post-C2Hx, ipsilateral serotonin structure number was also significantly lower vs. contralateral side (p<0.0001). B. Size of serotonin structures. C2Hx significantly increased area of serotonin structures ipsilateral to injury versus uninjured or contralateral side (p<0.0001). There were no IH effects after C2Hx. We averaged the left and right sides of uninjured rats and ran a one-way ANOVA by IH protocol. IH28-5/5 and IH28-2/2 rats had significantly greater areas per structure vs. dAIH28 or Nx28 (IH28-2/2 vs. Nx28 p=0.0024, IH28-2/2 vs. dAIH28 p=0.0034, IH28-5/5 vs. Nx28 p=0.0087, IH28-5/5 vs. dAIH28 p=0.0134). C. Total area of serotonin immunolabeling. C2Hx significantly increased the total area of serotonin immunolabeling ipsilateral to injury versus uninjured rats or contralateral side (p<0.0001). There were no IH effects after C2Hx. We averaged the left and right sides of uninjured rats and ran a one-way ANOVA by IH protocol. IH28-5/5 and IH28-2/2 protocols had significantly greater areas per structure than Nx28 (IH28-5/5 vs. Nx28 p=0.0179, IH28-5/5 vs. Nx28 p=0.0262). All other data were analyzed via repeated measures Mixed Model ANOVA; injury (intact vs. C2Hx) and IH protocol (Nx28, dAIH28, IH28-5/5 and IH28-2/2) were between subject variables; side (left/ipsilateral vs. right/contralateral) was the within subject repeated measure. Differences were considered significant if p<0.05. Bars denote means ± 1 SEM with the individual rats denoted as data points.
Figure 2-3. Continued
Figure 2-4. Total serotonin immunoreactive pixel density at cervical central commissures. A. Representative images of serotonergic fibers near central commissure of cervical spinal sections (left). Spinal intact (top) and C2 hemisection (C2Hx; bottom) rats were exposed to 28 consecutive days of normoxia. Dotted white lines represent gray and white matter border. Serotonin (red) mostly occurs in gray matter and exhibits the typical “beads on a string” morphology. Images were taken at 20x magnification with the central canal at the center. The white scale bars represent a distance of 100µm. B. Quantitative analysis of serotonergic fibers at central commissures (right). Neither C2Hx nor any of IH protocol (Nx28, white; dAlH28, light gray; IH28-5/5, dark gray; IH28-2/2, black) had any effect on pixel density of serotonergic innervation in cervical central commissures. Data were analyzed via two-way ANOVA (p=0.4648) with injury (intact vs. C2Hx) and IH protocol (Nx28, dAlH28, IH28-5/5, and IH28-2/2) as independent variables. Bars denote means ± 1 SEM with individual rats as individual data points.
Figure 2-5. Representative images of serotonergic innervation in intercostal motor nuclei. Representative images from uninjured rats, and rats 12 weeks post-C2Hx exposed to 28 days of normoxia. Images illustrate serotonin (red), often described as looking like “beads on a string”, and cholera toxin subunit B-labeled phrenic motor neurons (green). Images from left to right are representative samples from the left and right side of the thoracic spinal cord of intact rats, and the ipsilateral and contralateral sides of the thoracic spinal cord from rats 12 weeks post-C2Hx. Images were taken at 20x magnification with the phrenic motor nucleus at the center. The white scale bars represent a distance of 100 µm.
Serotonin innervation in intercostal motor nuclei was quantified in uninjured rats (left) and rats 12 weeks post-C2 hemisection (C2Hx; right) exposed to 28 days of IH protocols (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). A. Number of serotonin structures. C2Hx significantly reduced the number of serotonin structures ipsilateral to injury versus uninjured rats and contralateral side (p<0.0001). No IH protocol significantly affected the number of serotonin structures innervating intercostal motor nuclei in uninjured rats or rats 12 weeks post-C2Hx. B. Size of serotonin structures. C2Hx significantly increased the area of individual 5-HT structures ipsilateral to injury versus uninjured rats and contralateral side (p<0.0001). IH had no effects on uninjured rats or rats 12 weeks post-C2Hx. C. Total area of serotonin immunolabeling. C2Hx reduced the total area of serotonin immunolabeling ipsilateral to injury versus uninjured rats or the contralateral side (p<0.0001). There were no IH effects on total area of intercostal motor nuclei in uninjured rats. All data were analyzed with a repeated measures Mixed Model ANOVA; injury (intact vs. C2Hx) and IH protocol (Nx28, dAIH28, IH28-5/5 and IH28-2/2) were between subject variables; side (left/ipsilateral vs. right/contralateral) was the repeated measure. Differences were considered significant if p<0.05. Bars denote means ± 1 SEM with the individual rats as individual data points.
Figure 2-6. Continued
Figure 2-7. Immunoreactive serotonin staining intensity within immunopositive structures. Optical density in immunopositive structures of the phrenic motor nucleus was quantified in uninjured rats (left) and rats 12 weeks post-C2 hemisection (C2Hx; right) exposed to 28 days IH protocols (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). A. Serotonin labeling intensity in phrenic motor nucleus. C2Hx significantly reduced the intensity of serotonin positive structures ipsilateral to injury versus uninjured rats and contralateral side (p<0.0001). There was no IH effect on staining intensity after C2Hx. We averaged the left and right sides of uninjured rats and ran a one-way ANOVA by IH protocol. In uninjured rats, dAIH28 significantly increased staining intensity per structure versus Nx28 (p=0.0444). B. Serotonin labeling intensity in intercostal motor nuclei. C2Hx significantly reduced the intensity of serotonin staining within structures ipsilateral to C2Hx versus uninjured rats or contralateral side (p<0.0001). There were no IH effects on serotonin structure intensity in uninjured rats or rats post- C2Hx. All data were analyzed with a repeated measures Mixed Model ANOVA; injury (intact vs. C2Hx) and IH protocol (Nx28, dAIH28, IH28-5/5 and IH28-2/2) were between subject variables; side (left/ipsilateral vs. right/contralateral) was the within subject repeated measure. Differences were considered significant if p<0.05. Data are displayed in arbitrary units (A.U.). Bars denote means ± 1 SEM with the individual rats as individual data points.
Figure 2-7. Continued
Although low-dose, acute intermittent hypoxia (AIH) elicits serotonin-dependent plasticity in hypoglossal (XII) motor neurons, high-dose chronic intermittent hypoxia (CIH) mimicking that experienced during obstructive sleep apnea elicits neuroinflammation that can inhibit AIH-induced plasticity. On the other hand, daily AIH and mild CIH protocols amplify XII motor plasticity. Thus, intermittent hypoxia (IH) effects on XII motor plasticity are protocol dependent. IH pre-conditioning could enhance serotonin-dependent plasticity by altering serotonergic innervation of the XII motor nuclei. Thus, one major goal of this study was to test the hypothesis that IH protocols of different intensity differentially affect XII serotonergic innervation in rats. Sleep apnea (and associated CIH) is highly prevalent in people with cervical spinal cord injuries (cSCI), potentially impacting XII responses to AIH in this population. Since repetitive AIH is emerging as a promising therapeutic strategy to improve respiratory and non-respiratory motor function after cSCI, we tested the hypotheses that XII serotonergic innervation is increased by repetitive AIH and/or mild (but not moderate) CIH in rats with cSCI. Serotonergic innervation was assessed via immunofluorescence in male Sprague Dawley rats, with and without cervical C2 hemisection (C2Hx; 8 weeks post-injury) exposed to 28 days of: 1) normoxia; 2) daily AIH (10, 5min 10.5% O2 episodes per day; 5min intervals); 3) mild CIH (5min 10.5% O2 episodes; 5min intervals; 8 hrs/day); and 4) moderate CIH (2min 10.5% O2 episodes; 2min intervals; 8 hrs/day). Daily AIH, but neither CIH protocols, significantly increased serotonergic
innervation of XII motor nuclei in spinally intact and injured rats, C2Hx did not affect serotonergic innervation of XII motor nuclei. Because daily AIH enhances serotonergic innervation of XII motor nuclei, it may be possible to enhance the capacity for AIH-induced, serotonin-dependent plasticity in upper airway muscles after cSCI, or in other clinical disorders that compromise upper airway patency.

Overview

Acute intermittent hypoxia (AIH) elicits serotonin-dependent respiratory plasticity in both respiratory pump (e.g. phrenic, inspiratory intercostal) and upper airway (e.g. hypoglossal, XII) motor neurons. Although this phenomenon is best studied in the phrenic motor system (Devinney et al., 2013; Feldman et al., 2003; Fields and Mitchell, 2015; Mitchell et al., 2001), it has also been explored in the XII motor neurons that innervate the tongue (Bach and Mitchell, 1996; Baker-Herman and Strey, 2011). Serotonin receptor activation near phrenic motor neurons is both necessary (Baker-Herman and Mitchell, 2002) and sufficient (MacFarlane and Mitchell, 2009) for AIH-induced phrenic motor plasticity, most often referred to as phrenic long-term facilitation (pLTF). Treatments or conditions that increase serotonergic innervation in the phrenic motor nucleus enhance the capacity for AIH-induced pLTF (Kinkead et al., 1998; Ling et al., 2001).

Preconditioning with repetitive AIH was reported to increase serotonergic innervation of the phrenic motor nucleus (Satriotomo et al., 2012), and enhance AIH-induced pLTF (MacFarlane et al., 2018) through unknown mechanisms. Both repetitive AIH (Wilkerson and Mitchell, 2009) and mild chronic intermittent hypoxia (CIH) (Zabka et al., 2003) enhance XII LTF; however, it is unknown if intermittent hypoxia (IH) preconditioning enhances XII serotonergic innervation. One major goal of this study was
to test the hypothesis that (low-dose) IH preconditioning increases XII serotonergic innervation.

Therapeutic AIH is emerging as a promising treatment to improve breathing (and non-respiratory motor) function in people with cervical spinal cord injuries (cSCI) (Gonzalez-Rothi et al., 2015b). Unfortunately, sleep apnea is highly prevalent in people with cSCIs (Berlowitz et al., 2005; Fuller et al., 2013; Sankari et al., 2014a). CIH simulating that experienced during sleep apnea elicits neuropathology, and can undermine (Huxtable et al., 2015) or enhance (Ling et al., 2001) AIH-induced respiratory motor plasticity, or improved limb function in people with chronic SCI (Vivodtzev et al., 2020). One study suggests that cSCI diminishes serotonergic innervation of the XII motor nucleus (Golder et al., 2001), but there are conflicting reports concerning the impact of CIH on XII serotonergic innervation (Rukhadze et al., 2010; Wu et al., 2017), depending on the specific CIH protocol. Since it is of considerable interest to know if cSCI per se impacts XII serotonergic innervation, and if IH preconditioning normalizes (or even increases) that innervation, a secondary goal of this study was to investigate the impact of cSCI and repetitive (low and high dose) IH preconditioning on XII serotonergic innervation in rats.

Since the impact of different IH protocols ranging from therapeutic AIH to pathogenic CIH, and their ability to restore (or even enhance) serotonergic innervation of XII motor nuclei after cSCI has not been investigated, we tested the hypotheses that serotonergic innervation of XII motor nuclei: 1) increases with low- but not high-dose IH protocols in normal rats; 2) decreases following C2 spinal hemisection (C2Hx); and 3) is restored after C2Hx by low-dose IH protocols. We utilized immunofluorescence to
detect serotonin varicosities within the XII motor nucleus of intact rats, or 12 weeks post-C2Hx, exposed to 3 commonly studied protocols of repetitive IH.

Methods

Animals

All experiments were conducted with adult male (350-450g) Sprague-Dawley rats (Colony 217, ENVIGO Laboratories) housed in pairs in a controlled environment (12 hour light/dark cycles) with food and water ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida. A total of 79 rats were studied (11-12 weeks of age). Spinally intact and C2 hemisection (C2Hx) rats were randomly assigned to IH groups at 8 weeks post-injury and exposed for 28 consecutive days to: 1) normoxia (Nx28); 2) daily AIH (dAIH28), 3) mild CIH (IH28-5/5); and 3) moderate CIH (IH28-2/2). Experimental groups were: 1) spinal intact+Nx28 (n=8); 2) spinal intact+dAIH28 (n=10); 3) spinal intact+IH28-5/5 (n=10); 4) spinal intact+IH28-2/2 (n=10); 5) C2Hx+Nx28 (n=9); 6) C2Hx+dAIH28 (n=8); 7) C2Hx+IH28-5/5 (n=12); and 8) C2Hx+IH28-2/2 (n=12).

C2Hx Injury

Anesthesia, C2Hx and animal care were performed as described previously (Fuller et al., 2008; Gonzalez-Rothi et al., 2015c). Rats were anesthetized with 3.5% isoflurane (in 100% O2) and maintained at 2-2.5% isoflurane (in 100% O2) via nose cone throughout surgery; adequate anesthesia was verified by the absence of toe pinch and palpebral responses. Body temperature was maintained at 36.5–37.5°C with a heating pad. Artificial tears (Rugby, NDC 0536-1086-91) were applied to prevent eye damage. Nails were clipped from forelimbs and hind limbs to minimize scratching injury, and the surgical site was shaved and cleaned (chlorhexidine scrub, 58829-140-01, First
Priority, Inc., Elgin, IL). C2Hx was performed by making a dorsal incision on the neck and dissecting muscle to expose C2 laminae. Following C2 laminectomy and durotomy, the left C2 spinal cord was hemisected with a microknife caudal to the C2 dorsal roots. A gap (~1 mm) at the injury site was created by gentle aspiration. The overlying dura was closed with 9-0 ethilon nylon suture, overlaying muscles were sutured with 3-0 Polysorb absorbable suture, and skin was closed with 9mm stainless steel wound clips. Each rat received post-operative care post-surgery including pain management with an analgesic (buprenorphine, 0.03 mg/kg, s.q., Hospira< IL) and anti-inflammatory drug (meloxicam, 2 mg/kg, s.q. Portland, ME) given at 12 and 24-hour intervals for 2 days post-surgery. Rats received lactated Ringers solution (5 ml 2x/day, s.q.) and were fed manually with a nutritional supplement (Diet Gel Boost; Clear H2O; Westbrook, ME) until adequate volitional drinking and eating resumed.

**Intermittent Hypoxia Protocols**

Starting 8 weeks post-C2Hx, rats were housed in a custom-designed PlexiGlass gas exposure cages with free access to food and water. Respective IH protocols were administered to the cages daily for 28 days. Normoxia control rats received continuous gas flow of air (21% O2, 8hrs per day). Rats assigned to the dAIH28 groups received 10, 5min episodes of 10.5% O2 alternating with 5min normoxic intervals for a total of 1.5hrs/day (10 hypoxic episodes per day). Rats assigned to IH28-5/5 groups received 5min episodes of 10.5% O2 with 5min normoxic intervals for a total of 8hrs/day (6 hypoxic episodes per hour for 8hrs, 48 episodes per day). The 5min hypoxia/normoxia protocols results in 6 hypoxic episodes per hour, semi-equivalent to clinical criteria for mild sleep apnea (American Academy of Sleep MedicineAmerican and medicine, 1999; Young et al., 2008). Rats assigned to IH28-2/2 groups received 2min episodes of 10.5%
O2 with 2min normoxic intervals for a total duration of 8hrs/day (15 hypoxic episodes per hour for 8hrs; 120 episodes per day). This protocol results in 15 episodes per hour, semi-equivalent to clinical criteria for diagnosing moderate sleep apnea (American Academy of Sleep Medicine and medicine, 1999; Young et al., 2008).

**Tissue Histology and Analysis**

Rats were perfused intracardially with cold 0.1M phosphate buffer saline (PBS), followed by paraformaldehyde (4% paraformaldehyde w/v in 0.1M PBS, pH 7.4). The brainstems and spinal cords were harvested, post-fixed overnight in paraformaldehyde (4% paraformaldehyde w/v in 0.1M PBS, pH 7.4) and cryoprotected at 4°C in 20% sucrose solution in 0.1M PBS for 3 days, followed by 30% sucrose solution in 0.1M PBS for 3 days. To verify extent of injury, cervical spinal segments C1-C3 were embedded in paraffin, cut at 7µm, directly mounted on positively charged slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. Sections were stained with Luxol Fast Blue and cresyl violet. Sections were first deparaffinized via heating at 60°C for 20min, then submerged three times in xylene for 5min each. Sections were rehydrated by graded alcohol (100%-70%) for 2min each. Sections were stained overnight using Luxol Fast Blue (0.1g Luxol Fast Blue in 100mL of 95% ethanol and 0.5mL 10% acetic acid). The following day, tissues were rinsed with 95% ethanol for 2min and distilled water for 5min. Differentiation was achieved by dipping the tissue in 0.5% lithium carbonate and 70% ethanol, then rehydrated with distilled water for 5min. Tissues were placed in 0.1% cresyl violet for 15min and rinsed by dipping tissue into distilled water twice. Tissue was then dehydrated through graded alcohol (70–100%) for 1min each, cleared in Histoclear twice for 2min each (National Diagnostics, Atlanta, GA) and cover-slipped (Eukitt, Electron Microscope Science, PA). Injury sites were imaged.
using bright field at 10x zoom level (BZ-x710, Keyence Co., Osaka, Japan) to confirm lateral C2Hx.

Brainstems were sectioned in transverse plane (40µm thickness) using a freezing microtome (Leica SM 200R, Buffalo Grove, IL). Tissues were stored in antifreeze solution at -20°C until processed (30% glycerol, 30% ethylene glycol, 40% 0.1 M PBS, pH 7.4). Every 6th section was selected and stained for serotonin and neurons (NeuN). Free-floating sections were washed with 0.1M PBS (pH 7.4), then incubated in heat induced epitope retrieval (TissuePro, cat#: HIER01-32R) for 30min at 85°C. Tissues were washed again with 0.1M PBS-Triton (0.1%, pH 7.4) and incubated in a blocking solution (5% normal donkey serum (NDS, GeneTex) in 0.1M PBS-Triton (0.1%, pH 7.4) at room temperature for 60min. Primary antibody staining was performed by incubating tissue sections in 5% NDS in 0.1M PBS-Triton (0.1%, pH 7.4) with anti-5-HT (1/2000, rabbit serum, Immunostar #20080) and anti-NeuN (1/500, mouse serum, Millipore MAB377) at 4°C overnight. The following day, tissues were washed with 0.1M PBS-Triton (0.1%, pH 7.4). Secondary antibody staining was performed by incubating tissue in 5% NDS in 0.1M PBS-Triton (0.1%, pH 7.4) with secondary antibodies conjugated to Alexa Fluor® 594 (donkey anti-rabbit 594; 1:500, Invitrogen, Ref#A11055) and Alexa Fluor® 488 (donkey anti-mouse 488; 1/500, Invitrogen, Ref#A21202) in a dark box at room temperature for 2hrs. Sections were washed with 0.1M PBS-Triton (0.1%, pH 7.4) and mounted on positively charged slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. Slides were cover slipped with VectaShield Antifade Hard Set Mounting Medium (Cat#:H-1400). Fluorescently labeled sections were captured using an epifluorescent microscope with 20x magnification (Keyence BZ-X700,
Keyence Corporation of America, Itasca, IL). Sides of the brain stem were marked prior to tissue sectioning to ensure accurate side determination during imaging and analyses. XII motor neurons were determined by size and anatomical location (bregma).

**Data Analysis**

Serotonin immunofluorescence was quantified using a custom MATLAB (MathWorks, Natick, MA, USA) code. We quantified the average number of immunolabeled structures (structure number), average area of immunolabeling per structure (structure area), average total innervation area of serotonin-positive immunolabeling within a region of interest (bouton number multiplied by bouton area; total innervation area); average intensity per structure (structure intensity) was also assessed optical density, despite limitations in making inferences about serotonin concentration based on measures of immunofluorescent (Fritschy, 2008; Matos et al., 2010). Individual serotonin structures were identified as immunopositive structures larger than 4 contiguous pixels, with a gap of at least 1 pixel between structures. Thresholds were determined using a custom adaptive threshold algorithm in MATLAB as previously described (Allen et al., 2019). Adaptive threshold was calculated by constructing a pixel intensity histogram from the image. Pixel intensity above the 99th percentile was selected as the adaptive threshold. Investigators remained blinded to injury status and IH exposure protocol throughout analyses.

Regions containing NeuN labeled XII motor neurons were hand selected for analysis based on known anatomical location. The MATLAB code first displayed green to identify motor neurons and bregma level. A region of interest was selected, outlining the XII motor pool based on bregma level, creating a polygon shape that encompassed all observed NeuN positive XII motor neurons that were >19µm in cross section.
MATLAB code was then used to identify 5-HT immunoreactive pixels within this region of interest. In this report, we distinguish caudal XII nuclei as between bregma 12.72mm and 14.6mm, and XII column as all XII neurons, including pre-XII neurons, from bregma 10.8mm to 14.6mm. We report serotonergic innervation of both regions with emphasis on the caudal XII nuclei since this region contains neurons involved in maintaining upper airway patency, such as the genioglossus (Cori et al., 2018; Krammer et al., 1979).

**Statistics**

Statistical inferences were made using JMP 11.0 software (SAS Institute, Cary, NC). Serotonin immunofluorescence was analyzed using a mixed model with a repeated measures design. Independent variables were IH protocol (Nx28, dAIH28, IH28-5/5, IH28-2/2), injury (intact vs. C2Hx), and side relative to injury (ipsilateral vs. contralateral as the repeated measure). We also performed a separate analysis of uninjured and C2Hx groups separately. In uninjured rats, we averaged left and right sides and ran a one-way ANOVA with IH protocol as the independent variable. In C2Hx groups, we ran a two-way repeated measures ANOVA with the independent variables IH protocol (Nx28, dAIH28, IH28-5/5, and IH28-2/2) and side (ipsilateral vs. contralateral as the repeated measure). A significance level of 0.05 was set for all statistical comparisons. When significant differences were observed, individual comparisons were made based on the Fisher Least Significance Difference post hoc tests. All data are displayed as mean ± standard error of the mean.
Results

Representative Images of Serotonergic Innervation in Hypoglossal (XII) Motor Nuclei

All medullary sections were positive for NeuN and serotonin. During image acquisition, bregma level was determined based on anatomical landmarks. XII motor nuclei were identified using well-defined anatomical locations, and presence of large, NeuN positive cells >19µm (putative motor XII neurons). Six representative sections selected for imaging and analysis ranged from bregma level 10.8mm to 14.6mm, which contains pre-XII and XII motor pools. The analysis divided sections into the caudal XII motor pool from bregma 12.72mm to 14.6mm, versus the entire XII motor pool (all sections). Representative images of serotonergic innervation from each group are shown in Figure 3-1.

Serotonergic Reinnervation of Caudal Hypoglossal (XII) Nuclei

There were no C2Hx effects on any outcome measured on either side of the medulla. The number of serotonergic structures innervating caudal XII motor nuclei was significantly affected by IH protocol (p<0.0001; Figure 3-2). Intact dAlH28 rats had the greatest number of serotonergic structures compared to other intact groups. Serotonergic structures in intact dAlH28 rats increased 50% versus intact rats exposed to Nx28 (p=0.0028); 48% versus intact I H28-5/5 (p=0.0001); and 51% versus I H28-2/2 (p<0.0001). In contrast, there was a non-significant trend towards fewer serotonin structures after CIH versus Nx28, decreases of 20% in I H28-5/5 (p=0.3632) and 28% in I H28-2/2 (p=0.2371). After C2Hx, dAlH28 again had the highest number of serotonergic structures by 37% versus C2Hx Nx28 (p=0.001) and 22% versus I H28-5/5 (p=0.0209). There was a non-significant trend towards more structures in dAlH28 versus I H28-2/2
exposed rats (p=0.0826), a 17% increase. After C2Hx, a progressive increase in serotonin structures was apparent (versus Nx28) from 19% in mild (IH28-5/5 p=0.1282) to 24% in moderate CIH (IH28-2/2 p=0.0374).

IH had no significant effects on serotonergic structure area in caudal XII motor nuclei (p=0.1622; Figure 3-3). However, IH affected total serotonergic innervation area (p<0.0001; Figure 3-4). Spinally intact rats exposed to dAIH28 had greater total serotonergic innervation versus Nx28 (p=0.0169) by 34%, IH28-5/5 (p=0.0009) by 46%, and IH28-2/2 (p=0.0005) by 49%. Although not statistically significant, total serotonergic innervation area tended to be reduced in uninjured rats exposed to CIH (IH28-5/5 p=0.3652; IH28-2/2 p=0.2609) versus Nx28. After C2Hx, dAIH28 treated rats exhibited greater total innervation area versus Nx28 by 35% (p=0.0035), and IH28-5/5 by 23% (p=0.0375). C2Hx dAIH28 rats had only 19% increase in serotonergic innervation area than C2Hx IH28-2/2 (p=0.0722). CIH tended to enhance total serotonergic innervation area after C2Hx versus Nx28, although these effects were only not statistically significant (C2Hx Nx28 vs. C2Hx IH28-5/5 p=0.2390, 16%; vs. C2Hx IH28-2/2 p=0.1250, 19%).

Since there was no effect on staining intensity of serotonergic structures (p=0.576; Figure 3-5), the effects of IH protocol on serotonin structure number and total innervation is not due to group differences in immunofluorescence.

**Serotonergic Reinnervation of Entire Hypoglossal (XII) Column**

When serotonergic innervation was analyzed in the entire XII column, similar results were obtained (data not shown); we present caudal XII column data in greater detail since this region is mostly involved in maintaining upper airway patency during normal conditions (Cori et al., 2018; Krammer et al., 1979). Although there was no
C2Hx effect on serotonergic innervation in any outcomes, IH still had significant effects on the number of serotonergic structures (p<0.0001). dAlH28 exposed intact rats had 39% greater serotonergic structure number versus IH28-5/5 (p=0.0019) and IH28-2/2 (p=0.0022). There was no effect of dAlH28 versus Nx28 (p=0.1457) on serotonin structure number in uninjured rats. Although not statistically significant, there was a trend towards less serotonergic structure numbers in intact rats exposed to CIH versus Nx28, decreases of 25% in IH28-5/5 (p=0.1016) and 20% in IH28-2/2 (p=0.1113). In C2Hx rats, serotonin structure number was higher in dAlH28 treated versus Nx28 (p=0.0004), IH28-5/5 (p=0.0014) and IH28-2/2 (p=0.0267) treated rats, increases of 33%, 28%, and 18% respectively. In each case, CIH rats appeared to have more structures versus Nx28, although this trend was not statistically significant (IH28-5/5 p=0.4920, 8%; IH28-2/2 p=0.0602, 15%).

There were no significant differences in serotonergic structure area (p=0.1993) in the entire XII column. IH still affected total serotonergic innervation area (p=0.0001). Spinally intact rats exposed to dAlH28 had 40% greater total serotonergic innervation area versus IH28-5/5 (p=0.0042) and IH28-2/2 (p=0.0067), but only 17% versus Nx28 (p=0.2275). There was also a trend towards less total innervation area in uninjured rats after CIH compared to Nx28; 28% in IH28-5/5 (p=0.1047) and 38% in IH28-2/2 (p=0.1450). Rats with C2Hx exposed to dAlH28 had greater total innervation area versus Nx28 by 34% (p=0.0005), IH28-5/5 by 31% (p=0.0007), and IH28-2/2 by 19% (p=0.242). There were non-significant trends towards increased total serotonergic innervation area after C2Hx with CIH exposures versus Nx28 (C2Hx Nx28 vs. IH28-5/5
p=0.7004, 15%, and vs IH28-2/2 p=0.0752, 5%). There was no effect on structure staining intensity in any group (p=0.9272).

**Discussion and Conclusion**

We demonstrate that daily AIH for 28 days increases serotonergic innervation within the XII motor nucleus of rats. This effect was observed both in spinally intact rats, and in rats with lateralized cervical spinal hemisections. In contrast, neither mild nor moderate CIH had a similar effect, again demonstrating that low-dose repetitive AIH is unique in its effects on neural structures involved in the neural control of breathing.

dAIH effects on XII serotonergic innervation suggests a potential mechanism to explain enhanced XII long-term facilitation following AIH preconditioning (Wilkerson and Mitchell, 2009). We did not verify a previous report that C2Hx decreases XII serotonergic innervation (Golder et al., 2001). Our findings must be considered in the context of the specific IH protocols used (28 days), and time post-injury (3 months).

Based on our findings, we suggest that dAIH28 has potential as a therapeutic intervention to increase XII serotonergic innervation, enhance XII motor plasticity and preserve airway patency, potentially reducing the incidences of obstructive sleep apnea.

Literature reports suggest that CIH effects on XII serotonergic innervation may depend on the specific CIH protocol studied. For example, rats exposed to 3min normoxia/hypoxia cycles at 6.9% O2, 10 hrs per day for 40 days exhibited increased XII serotonergic innervation (Rukhadze et al., 2010). In striking contrast, rats exposed to 1min cycles of 7% O2 alternating with 27% O2, 10 hrs per day for 3 to 5 weeks (Wu et al., 2017) exhibited decreased XII serotonergic innervation (Wu et al., 2017). Here, we report two distinct CIH protocols (IH28 5/5 and IH28 2/2) have no statistically significant impact on serotonergic innervation. Factors to consider when comparing results include:
1) details of the CIH protocol such as severity, number and length of hypoxic episodes, interval length, and the total exposure duration (hours per day and number of days); 2) rat strain and sub-strain (Golder et al., 2005; Wilkerson and Mitchell, 2009); and 3) age and sex (Zabka et al., 2003). Based on our findings and literature accounts (Rukhadze et al., 2010; Wu et al., 2017), IH effects on XII serotonergic innervation likely exhibit a hormetic dose response curve, with increases at low dose and decreases at high dose IH (Navarrete-Opazo and Mitchell, 2014b). Since, in the present study, we emphasized mild to moderate IH preconditioning protocols, we detect clear enhancement with low-dose IH, with minimal effect in the moderate range. It is unclear if more severe IH preconditioning protocols would decrease serotonergic innervation.

In addition to serotonin, other neurochemicals play a major role in regulating XII nerve activity and plasticity, such as norepinephrine (Horner, 2008; Kubin, 2014; Lui et al., 2018; Tadjalli and Peever, 2010). Indeed, phrenic and XII motor neurons express both serotonin- and norepinephrine-dependent plasticity (Bach and Mitchell, 1996; Bocchiaro and Feldman, 2004; Huxtable et al., 2014; Lui et al., 2018; Neverova et al., 2007; Tadjalli and Peever, 2010). Changes in XII norepinephrine innervation and the impact of IH preconditioning remain unknown, a topic worthy of further investigation.

Contrary to a prior report (Golder et al., 2001), we did not detect any independent effects of C2Hx on XII serotonergic innervation. Although we cannot explain these discrepant results, we do demonstrate that dAIH might be useful as a therapeutic modality to increase serotonergic innervation, and the capacity for serotonin-dependent XII motor plasticity in those with cSCI. Since most people with chronic cSCI exhibit sleep disordered breathing (Bernhardt et al., 2017; Sankari et al., 2014a; Sankari et al.,
2019), this property may be useful in preserving/restoring upper airway patency in people with chronic cSCI, thereby minimizing the incidence of obstructive sleep apnea (Lim and Veasey, 2010; Mateika and Komnenov, 2017; Veasey, 2003).

Overall, we report that low-dose (therapeutic), but not high-dose (pathogenic), IH enhances serotonergic innervation of XII motor nuclei in rats with and without cervical spinal injury. This daily AIH effect may be useful to preserve/restore serotonergic function in upper airway motor pools in diverse neuromuscular disorders that compromise upper airway patency and non-respiratory tongue functions, such as swallowing. Conversely, mild/moderate IH experienced by many patients with neuromuscular disorders may represent a form of spontaneous compensation, increasing respiratory motor output in ways that offset sleep disordered breathing and/or dysphagia associated with disease or injury (e.g. SCI, ALS, stroke).
Figure 3-1. Representative images of serotonergic innervation in XII motor nuclei. Representative images of brainstem sections of the XII motor pool immunolabeled for NeuN (green), serotonin (red), and their overlay (green, red). Top four rows represent left (left) and right (right) XII motor pools of spinally intact rats that received Nx28, dAIH28, IH28-5/5 or IH28-2/2 (top to bottom). Lower four rows represent ipsilateral (left) and contralateral (right) XII motor pools of rats 12 weeks post-C2Hx that received Nx28, dAIH28, IH28-5/5 or IH28-2/2 (top to bottom). All images are 20x magnification. Scale bar (white): 100µm.
Figure 3-2. Number of serotonin immune-positive structures in caudal XII nuclei. Serotonergic structures around the caudal XII motor nucleus (bregma 12.72mm to 14.6mm) were counted in rats that were spinally intact (left) or 12 weeks post-C2Hx (right) exposed to 28 days of IH (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). In intact rats, dAIH28 exposed rats had the greatest number of serotonergic boutons versus intact Nx28 (p=0.0028), IH28-5/5 (p=0.0001) and IH28-2/2 (p<0.0001). After C2Hx, dAIH28 had the highest serotonergic innervation versus C2Hx Nx28 (p=0.001) and IH28-5/5 (p=0.027). C2Hx IH28-2/2 had greater serotonergic innervation versus C2Hx Nx (p=0.0374). Data were analyzed via repeated measures mixed model with an IH protocol (Nx28, dAIH28, IH28-5/5, and IH28-2/2) and injury (intact vs. C2Hx) as between subject variables, and side (left/ipsilateral vs. right/contralateral) as repeated measure. Differences were considered significant if p<0.05. Data are displayed as mean ±1 SEM.
Figure 3-3. Area of serotonin immune-positive structures innervating the caudal XII motor nuclei. Serotonergic structure size around the caudal XII motor nucleus (bregma 12.72mm to 14.6mm) were counted in rats that were spinally intact (left) or 12 weeks post-C2Hx (right) exposed to 28 days of IH (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). There were no significant effects of IH protocol or C2Hx on the size of each serotonergic structure. Data were analyzed via repeated measures mixed model with an IH protocol (Nx28, dAIH28, IH28-5/5, and IH28-2/2) and injury (intact vs. C2Hx) as between subject variables, and side (left/ipsilateral vs. right/contralateral) as repeated measure. Differences were considered significant if p<0.05. Data are displayed as mean ±1 SEM.
Figure 3-4. Total serotonin innervation area in the caudal XII motor nuclei. Serotonergic innervation area around the caudal XII motor nucleus (bregma 12.72mm to 14.6mm) were counted in rats that were spinally intact (left) or 12 weeks post-C2Hx (right) exposed to 28 days of IH (Nx28, white; dAlH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). In intact rats, dAlH28 exposed rats had the greatest serotonergic innervation area versus intact Nx28 (p=0.0169), IH28-5/5 (p=0.0009), and IH28-2/2 (p=0.0005). After C2Hx, dAlH28 had the highest serotonergic innervation area versus C2Hx Nx28 (p=0.0035) and IH28-5/5 (p=0.0375) but not IH28-2/2 (p=0.072). Data were analyzed via repeated measures mixed model with an IH protocol (Nx28, dAlH28, IH28-5/5, and IH28-2/2) and injury (intact vs. C2Hx) as between subject variables, and side (left/ipsilateral vs. right/contralateral) as repeated measure. Differences were considered significant if p<0.05. Data are displayed as mean ±1 SEM.
Figure 3-5. Intensity of serotonin immune-positive structures around the caudal hypoglossal (XII) motor nuclei. Serotonergic structure intensity around the caudal XII motor nucleus (bregma 12.72mm to 14.6mm) were counted in rats that were spinally intact (left) or 12 weeks post-C2Hx (right) exposed to 28 days of IH (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). There were no significant effects of IH protocol or C2Hx on the size of each serotonergic intensity. Data were analyzed via repeated measures mixed model with an IH protocol (Nx28, dAIH28, IH28-5/5, and IH28-2/2) and injury (intact vs. C2Hx) as between subject variables, and side (left/ipsilateral vs. right/contralateral) as repeated measure. Differences were considered significant if p<0.05. Data are displayed as mean ±1 SEM.
CHAPTER 4
SEROTONERGIC INNERVATION OF AXIAL AND FORELIMB MOTOR NUCLEI
AFTERCERVICAL SPINAL INJURY, WITH AND WITHOUT INTERMITTENT HYPOXIA

Abstract

Cervical spinal cord injury (cSCI) disrupts serotonergic innervation below the site of injury. Since serotonin contributes to motor recovery after cSCI, it is important to know the extent of serotonergic reinnervation after chronic cSCI. Repetitive acute intermittent hypoxia (rAIH) is a treatment reported to partially restore spinal serotonergic innervation. On the other hand, chronic intermittent hypoxia (CIH) comparable to that experienced during sleep apnea, elicits neuropathologies that undermine rAIH benefits. Here, we tested the hypotheses that C2 hemisection (C2Hx) causes persistent reductions in serotonergic innervation of axial and forelimb motor nuclei in rats, and that AIH and CIH exert differential effects on serotonergic reinnervation of these motor nuclei in uninjured rats and rats after chronic C2Hx. Serotonergic innervation was assessed via immunofluorescence in the axial and forelimb motor nuclei of male Sprague Dawley rats with and without C2Hx (12wks post-injury) exposed to 28 days of: 1) normoxia; 2) daily AIH (dAIH28; 10, 5min 10.5% O$_2$ episodes; 5min normoxic intervals); 3) mild CIH (5min 10.5% O$_2$ episodes; 5min intervals; 8 hrs/day); and 4) moderate CIH (2min 10.5% O$_2$ episodes; 2min intervals; 8 hrs/day). In both motor nuclei ipsilateral to C2Hx: 1) there were significant reductions in serotonergic structure number and total innervation area; 2) serotonin structure size was unaffected; and 3) serotonin staining intensity was increased. Neither AIH nor CIH affected serotonergic innervation in either intact or C2Hx rats. Although serotonin innervation of axial and forelimb motor nuclei was reduced 12 weeks post-C2Hx, and AIH did not enhance innervation,
substantial residual serotonergic innervation may support therapies targeting serotonin to enhance function after cSCI.

**Overview**

Spinal cord injury (SCI) results in motor and sensory impairments at or below the site of injury. Over half of all SCIs occur in the cervical region (NSCISC, 2020), resulting in widespread paralysis, including the muscles required for breathing and arm/forelimb function. One major priority for people with tetraplegia is to regain arm and hand function (Anderson, 2004). Two motor neuron pools supporting neck, arm and hand (or forelimb and paw) function are the axial and forelimb motor pools. Cervical axial motor neurons innervate accessory respiratory muscles and stabilize the head and neck, such as the scalene muscles (Mitchelle and Watson, 2016; Pilarski et al., 2019). Forelimb motor neurons innervate muscles such as the biceps and deltoids (Tosolini et al., 2013; Tosolini and Morris, 2012). Although these motor neuron pools have distinct functions, they work together by maintaining balance, posture and limb coordination (Carrier, 1990; Peterson, 1989).

One strategy to enhance motor function after incomplete cervical SCI (cSCI) is to strengthen spared neural pathways to harness intrinsic mechanisms of recovery, such as serotonin dependent spinal plasticity (Dale et al., 2014; Dougherty et al., 2018; Gonzalez-Rothi et al., 2015b). Despite the importance of spinal serotonin for motor function (Di Pasquale et al., 1997; Lindsay and Feldman, 1993), all spinal serotonergic neurons are located in the brainstem (Bowker et al., 1982; Dahlström and Fuxe, 1964; Törk, 1990). Therapies targeting serotonin dependent recovery are complicated by the fact that cSCI itself removes descending ipsilateral spinal serotonin projections. However, with time, serotonergic innervation below the site of injury recovers,
correlating with spontaneous functional recovery and serotonin-induced plasticity (Camand et al., 2004; Golder and Mitchell, 2005; Hashimoto and Fukuda, 1991; Saruhashi et al., 1996; Tai et al., 1997). However, the extent of serotonergic reinnervation of axial and forelimb motor nuclei after chronic cSCI has not been described. Understanding the extent of spinal serotonergic reinnervation around these motor pools after SCI is necessary for serotonin dependent treatments to improve lost neck and forelimb motor function and overall quality of life for people with cSCI.

Acute intermittent hypoxia (AIH) is a therapeutic modality that elicits plasticity in spared synaptic pathways to restore somatic motor function after chronic, incomplete SCI (Dale et al., 2014; Gonzalez-Rothi et al., 2015b; Lovett-Barr et al., 2012; Trumbower et al., 2012). One mechanism by which AIH elicits spinal respiratory motor plasticity is by spinal serotonin receptor activation (Bach and Mitchell, 1996; Baker-Herman and Mitchell, 2002). In one report, AIH increased serotonergic innervation of the phrenic motor nucleus in spinally intact rats (Satriotomo et al., 2012). It is suspected that axial and forelimb motor neurons undergo similar mechanisms in rats with and without cSCI. In fact, AIH improves limb function in rodents with chronic SCI (Lovett-Barr et al., 2012; Prosser-Loose et al., 2015), and these findings have been translated to human studies in which AIH improves leg strength, walking ability, and hand function in people with chronic incomplete SCI (Hayes et al., 2014; Navarrete-Opazo et al., 2017a; Trumbower et al., 2017; Trumbower et al., 2012). On the other hand, chronic intermittent hypoxia (CIH), mimicking that experienced during sleep apnea, elicits pathology that undermines the beneficial effects of AIH. Recently, the optimal IH protocol to enhance function after SCI has been a top priority. The impact of various
intermittent hypoxia (IH) protocols on serotonergic innervation of axial forelimb motor nuclei after a cSCI has not been investigated.

Here we investigate the impact of commonly studied IH protocols that range from therapeutic daily AIH through more severe, possibly pathogenic, protocols simulating mild to moderate sleep apnea (Navarrete-Opazo and Mitchell, 2014b). We hypothesized in the motor nuclei containing axial and cervical forelimb motor neurons: 1) serotonergic reinnervation is reduced by C2 spinal hemisection (C2Hx); and 2) various IH protocols exert differential effects on serotonergic innervation in intact rats and rats 12 weeks post-C2Hx. In both axial and forelimb motor nuclei, serotonin structure number and innervation area were reduced 12 weeks post-C2Hx, but there were no changes in the size of serotonergic structures as reported previously in the phrenic motor nucleus (Ciesla et al., in ibid). None of the IH protocols studied affected serotonergic innervation in either axial or forelimb motor nuclei in intact rats or rats 12 weeks after C2Hx. Understanding the extent of spinal serotonin loss and reinnervation of these motor nuclei after SCI is necessary to devise treatments that improve lost motor function and overall quality of life for people with SCI.

**Methods**

**Animals**

All experiments were conducted with adult male (initially 350-450 grams) Sprague-Dawley rats (Envigo, Indianapolis IN, Colony 208A, University of Florida) housed in pairs in a controlled environment (12 hour light/dark cycles) with food and water *ad libitum*. All experimental protocols were approved by the University of Florida Institutional Animal Care and Use Committee. A total of 79 rats were studied, beginning 11-12 weeks of age. Spinally intact and C2 hemisection (C2Hx) rats were randomly
assigned to intermittent hypoxia exposure groups at 8 weeks post-injury, and exposed for 28 consecutive days to either: 1) normoxia (Nx28; n = 8 intact, n = 9 with C2Hx); 2) daily acute intermittent hypoxia (dAIH28, n=10 intact, n=8 with C2Hx), 3) mild chronic intermittent hypoxia (CIH; 8 hours per day) with an equivalent desaturation index of 6 (IH28-5/5, n=10 intact, n=12 with C2Hx); and 3) moderate CIH at an equivalent desaturation index of 15 (IH28-2/2, n=10 intact, n=12 with C2Hx).

**C2Hx Injury**

Anesthesia, C2Hx and animal care were performed as described previously (Fuller et al., 2008; Gonzalez-Rothi et al., 2015c). Rats were induced with 3.5% isoflurane (in 100% O2) in a chamber and then maintained (2-2.5% in O2) via nose cone throughout the surgery; adequate anesthetic depth was confirmed by the absence of toe pinch and palpebral responses. Body temperature was maintained at 36.5–37.5°C with a heating pad. Artificial tears were applied to prevent eye damage (Rugby, NDC 0536-1086-91). Toenails were clipped on all limbs. The surgical site was shaved and cleaned (chlorhexidine scrub, 58829-140-01, First Priority, Inc., Elgin, IL). C2Hx was performed by making a dorsal incision on the neck and dissecting muscle to expose the C2 lamina. Following C2 laminectomy and durotomy, the left spinal cord was hemisected with a micro-knife caudal to the C2 dorsal roots. A gap (~1 mm) at the injury site was created by aspiration. The dura was closed with 9-0 ethilon nylon suture, the overlaying muscles were closed with 3-0 Polysorb absorbable suture and the skin was closed with 9 mm stainless steel wound clips. Post-operative care included pain management with an analgesic (buprenorphine, 0.03 mg/kg, s.q., Hospira, IL) and an anti-inflammatory drug (meloxicam, 2 mg/kg., s.q. Portland, ME) given at 12 and 24-hour intervals for 2 days post-surgery. Rats received lactated Ringer’s solution (5 ml 2x/day, s.q.) and were
manually fed a nutritional supplement (Diet Gel Boost; Clear H₂O; Westbrook, ME) until adequate volitional drinking and eating were observed. Visualization of C2Hx showed anatomically complete lesions with the absence of white and gray matter from the left lateral edge to the spinal cord midline.

**Intermittent Hypoxia Protocols**

8 weeks post-C2Hx (or 6 weeks post-CtB injections for uninjured rats), rats were housed in PlexiGlass exposure cages with free access to food and water (Therapeutiq, Kansas City, MO). IH protocols were administered to the cages daily for 4 weeks. Normoxia control rats received continuous air flow (21% O₂, 8 hours per day; Figure 1B). Rats assigned to the dAIH groups received 10, 5-minute episodes of 10.5% O₂, alternating with 5 minute normoxic intervals for ten episodes per day (~1.5 hours/day; Figure 1C). Rats assigned to IH28-5/5 groups received 5-minute episodes of 10.5% O₂, alternating with 5 minute normoxic intervals for 8 hours/day (48 episodes per day; Figure 1D). Five minute hypoxic/normoxic intervals yield 6 hypoxic episodes per hour, somewhat equivalent to mild sleep apnea (5 to 10 events per hour) (American Academy of Sleep MedicineAmerican and medicine, 1999; Young et al., 2008). Rats assigned to IH28-2/2 received 2-minute episodes of 10.5% O₂, with 2 minute normoxic intervals for 8 hours/day (120 hypoxic episodes per day; Figure 1E). This protocol consisting of 15 hypoxic episodes per hour is equivalent to clinical criteria for the transition to moderate sleep apnea (American Academy of Sleep MedicineAmerican and medicine, 1999; Young et al., 2008).

**Tissue Histology**

One day after their final exposure, rats were perfused intracardially with cold 0.1M phosphate buffer saline (PBS), followed by paraformaldehyde (4%
paraformaldehyde w/v in 0.1M PBS, pH 7.4). The spinal cord was harvested and then: 1) post-fixed overnight in paraformaldehyde (4% in 0.1M PBS, pH 7.4); and 2) cryoprotected at 4°C in 20% sucrose solution in 0.1M PBS for 3 days followed by 30% sucrose solution in 0.1M PBS for 3 days. To verify injury, cervical spinal segments C1-C3 were embedded in paraffin, cut at 7µm, directly mounted on positively charged slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. Sections were stained with Luxol Fast Blue and cresyl violet. Sections were deparaffinized by heating at 60°C for 20min and submerged 3 times in xylene for 5 min each. Sections were rehydrated by graded alcohol (100%-70%) for 2min each and then stained overnight using Luxol Fast Blue (0.1g Luxol Fast Blue in 100mL of 95% ethanol and 0.5mL 10% acetic acid). The following day, tissues were rinsed with 95% ethanol for 2min and distilled water for 5min. Differentiation was achieved by dipping the tissue in 0.5% lithium carbonate and 70% ethanol, then rehydrated by distilled water for 5min. Tissues were placed in 0.1% cresyl violet for 15min. Cresyl violet was rinsed by dipping tissue into distilled water twice. Tissue was then dehydrated through graded alcohol (70–100%) for 1min each, cleared in Histoclear twice for 2min each (National Diagnostics, Atlanta, GA) and cover-slipped (Eukitt, Electron Microscope Science, PA). Injury sites were imaged using bright field at 10x zoom level (BZ-x710, Keyence Co., Osaka, Japan) to confirm lateral C2Hx.

Cervical (C3-C5) spinal segments were sectioned in transverse plane (40µm thickness) using a freezing microtome (Leica SM 200R, Buffalo Grove, IL). Tissues were stored in antifreeze solution at -20°C until processed (30% glycerol, 30% ethylene glycol, 40% 0.1 M PBS, pH 7.4). Every 12th section was selected and stained for
serotonin. Free-floating sections were washed with 0.1M PBS (pH 7.4), then incubated in heat induced epitope retrieval (TissuePro, cat#: HIER01-32R) for 30min at 85°C. Tissues were washed again with 0.1M PBS-Triton (0.1%, pH 7.4) and then incubated in a blocking solution (5% normal donkey serum (NDS, GeneTex) in 0.1M PBS-Triton (0.1%), pH 7.4) at room temperature for 60 min. Primary antibody staining was performed by incubating tissue sections in 5% NDS in 0.1M PBS-Triton (0.1%, pH 7.4) with anti-5-HT (1/2000, rabbit serum, Immunostar #20080) in 4°C overnight. The following day, tissues were washed with 0.1M PBS-Triton (0.1%, pH 7.4). Secondary antibody staining was performed by incubating tissue in 5% NDS in 0.1M PBS-Triton (0.1%, pH 7.4) with secondary antibodies conjugated to Alexa Fluor® 594 (donkey anti-rabbit 594; 1:500, Invitrogen, Ref#A11055) in a dark box at room temperature for 2 hours. Sections were washed with 0.1M PBS-Triton (0.1%, pH 7.4) and mounted on positively charged slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. Slides were cover slipped with VectaShield Antifade Hard Set Mounting Medium (Cat#:H-1400). Fluorescently labeled sections were captured using an epifluorescent microscope with 20x magnification (Keyence BZ-X700, Keyence Corporation of America, Itasca, IL). The sides of the spinal cord were marked prior to tissue sectioning to ensure accurate side determination during imaging and analyses. Regions containing putative axial and forelimb motor neurons were hand selected for analysis based on known anatomical location.

**Serotonin Immunofluorescence Analysis**

Serotonin immunofluorescence was quantified using a custom MATLAB (MathWorks, Natick, MA, USA) code. We quantified the average number of immunolabeled structures (number of structures), average area of immunolabeling per
structure (area per structure), average total area of serotonin-positive immunolabeling (number of boutons multiplied by the area of each structure; total area), and average intensity of each structure (intensity per structure). Independent serotonin structures were determined by being larger than 4 pixels and being at least 1 pixel apart. Thresholds were determined using a custom adaptive threshold algorithm in MATLAB as previously described (Allen et al., 2019). The adaptive threshold was calculated by constructing a pixel intensity histogram from the image. The pixel intensity corresponding to the 99th percentile was selected as the adaptive threshold. Injury (or intact) and intermittent hypoxia exposure protocol remained blinded throughout analyses.

Regions containing putative axial and forelimb motor neurons were hand selected for analysis based on known anatomical location. The MATLAB code first displayed the green channel for identification of gray vs white matter from the background autofluorescence. Points were selected along the border of the medial-ventral gray to include the known anatomical locations of the axial forelimb motor neurons. A line of best fit along the gray/white matter border was determined. Then, an ellipse was formed along the line of best fit with $r1 = 50$ microns and $r2 = 100$ microns within the gray matter to include all the axial and forelimb motor neurons. The MATLAB code then identified 5-HT immunoreactive pixels within this region of interest.

**Statistics**

Statistical calculations were performed using JMP 11.0 (SAS Institute, Cary, NC). Serotonin immunofluorescence data was analyzed using a repeated measures mixed design. The independent variables were injury (intact vs. C2Hx), IH protocol (Nx28, dAIH28, IH28-5/5, and IH28-2/2), and spinal cord side relative to injury (let/ipsilateral vs.
right/contralateral as the repeated measure). A significance level of 0.05 was set for all statistical comparisons. When significant differences were observed, individual comparisons were made based on the Fisher Least Significance Difference post hoc test. All data are displayed as mean ± 1 standard error of the mean.

Results

Serotonin Labeling in Putative Axial and Forelimb Motor Nuclei

All cervical spinal sections were labeled positive for serotonin (Figure 1). The putative axial and forelimb motor neurons were determined using defined anatomical locations in the medio-ventral and lateral-ventral gray matter of the cervical spinal cord, respectively. An elliptical region of interest with radii of 50µm and 100µm was drawn around the putative axial and forelimb motor nuclei and positive serotonergic pixels within this region were analyzed. Serotonin appeared thin and continuous, somewhat like “beads on a string” in both regions of all rats. Upon first observance, we did not notice an obvious effect of C2Hx compared to the spinally intact groups in either region. In both regions, we also did not notice obvious differences in serotonergic innervation between the IH various protocols in either spinally intact rats or after C2Hx.

Serotonergic Innervation of the Putative Axial Motor Nucleus

The number of serotonergic structures innervating axial motor nuclei (Figure 2A) was reduced following C2Hx. Ipsilateral to C2Hx, there were 63% less structures versus spinally intact groups (p<0.0001). The number of ipsilateral structures was also reduced 57% versus the contralateral side post-C2Hx (p<0.0001). There was no IH protocol effect on the number of serotonergic structures in spinally intact or C2Hx rats. The size of serotonergic structures near putative axial neurons (Figure 2B) was not affected by C2Hx or IH protocol (p>0.05). Because serotonin structure number was reduced post-
injury, there was less overall serotonergic innervation area, unlike the phrenic and intercostal motor nuclei where increased structure size offset decreased numbers (Ciesla et al., in *ibid*). Total serotonergic innervation area in axial motor nuclei was reduced post-C2Hx (Figure 3C). Ipsilateral to C2Hx total area of serotonergic innervation was 65% reduced versus spinally intact groups, and 60% less versus the contralateral side (p<0.0001 for all). Again, there was no IH protocol effect on total area of serotonergic innervation in this region in either spinally intact rats or injured rats.

**Serotonergic Innervation of the Putative Forelimb Motor Nucleus**

The number of serotonergic structures in the region of interest around putative forelimb motor neurons (Figure 3A) was significantly reduced following C2Hx. Ipsilateral to C2Hx, there were 64% fewer structures versus spinally intact rats, and 64% less structures versus the contralateral side (p<0.0001 for all). There was no IH protocol effect on the number of serotonergic structures innervating putative forelimb neurons in intact or injured rats. The size of serotonergic structures in this region was not affected by C2Hx (Figure 3B) or IH protocol in intact rats or injured rats (p>0.05). Due reduced serotonin structure number, the total serotonergic innervation area in forelimb motor nuclei was significantly reduced after C2Hx (Figure 3C). Ipsilateral to C2Hx there was a 65% reduction in area versus spinally intact rats, and a 57% reduction versus the contralateral side (p<0.0001 for all). The serotonin total innervation area in forelimb motor nuclei was not affected by IH protocol in intact or C2Hx rats.

**Serotonin Immunofluorescence Intensity in Putative Axial and Forelimb Motor Nuclei**

The intensity of staining in each serotonergic structure around phrenic and intercostal motor nuclei was also quantified (Figure 7), despite limitations in using
optical density as an indicator of antigen concentration (Fritschy, 2008; Matos et al., 2010). In both regions of interest, there was a significant C2Hx effect since ipsilateral serotonin structures expressed higher optical densities versus those in intact rats or the contralateral side (p<0.0001 for both). Around putative axial motor neurons there was an 11% increase in staining intensity versus contralateral structures and intact rats (Figure 7A). Serotonin intensity was 25% greater in putative forelimb motor neurons versus intact rats, and 21% greater than contralateral structures after C2Hx (Figure 7B). In both regions of interest, there were no IH effects on staining intensity.

**Discussion and Conclusion**

We investigated the impact of chronic C2Hx on serotonergic reinnervation of axial and forelimb motor nuclei, and the ability of commonly studied IH protocols to enhance reinnervation. We report that C2Hx is associated with reduced serotonergic structure numbers, similar to other nearby motor nuclei, such as the phrenic motor nucleus (Ciesla et al., ibid). However, in the motor nuclei studied here, there was an overall decrease in serotonergic innervation area since the bouton size remained the same, This contrasts with the phrenic and intercostal motor pools where the serotonergic structures are enlarged after C2Hx, preserving (intercostal) or increasing (phrenic) total serotonergic innervation area in these respiratory motor pools (Ciesla et al., ibid). However, in contrast to our guiding hypothesis, there were no effects of any IH protocol on serotonergic innervation of the axial or forelimb motor nuclei, regardless of spinal injury status. Persistent loss of serotonergic innervation 12 weeks post-C2Hx has implications concerning the potential for spontaneous or induced serotonin-dependent functional recovery with chronic SCI (Gonzalez-Rothi et al., 2015b).
Changes in Serotonergic Reinnervation after C2Hx

Overall, we report reduced serotonergic innervation of ipsilateral putative axial and forelimb motor nuclei 12 weeks post-C2Hx. Since C2Hx disrupts descending serotonergic axons from the caudal raphe, some loss of serotonin innervation in cervical motor nuclei below the injury is not unexpected, although the extent of recovery in prior reports can be substantial (Camand et al., 2004; Golder and Mitchell, 2005; Saruhashi et al., 1996; Tai et al., 1997). It is well documented that spinal serotonin is reduced with acute injury, correlating with loss of motor function (Golder and Mitchell, 2005; Hashimoto and Fukuda, 1991). With time post injury, spinal serotonin spontaneously and partially recovers, in correlation with improved motor function (Camand et al., 2004; Ghosh and Pearse, 2014; Hashimoto and Fukuda, 1991; Saruhashi et al., 1996). The time-course and extent of serotonergic loss and recovery in axial and forelimb motor nuclei has not been reported. Although there was less serotonin innervation in these regions ipsilateral to injury, the intensity of ipsilateral structures increased. This semi-quantitative increase in serotonin concentration (Fritschy, 2008; Matos et al., 2010) in axial and forelimb motor nuclei may suggest partial compensation for the loss in boutons, increasing the amount of serotonin available for release from each spared structure. We did not observe a similar increase in serotonin staining intensity in other motor pools, such as the phrenic and intercostal (Ciesla et al., ibid).

Axial motor neurons in the medial and hypaxial motor columns innervate muscles of the neck and shoulders (Mitchelle and Watson, 2016). Some of these axial muscles are also accessory respiratory muscles, such as the scalenus (Pilarski et al., 2019; Shinozaki et al., 2019). Forelimb motor neurons are supplied by the lateral motor column (Mitchelle and Watson, 2016) and include the biceps brachii,
acromideodeltoideus, spinodeltodieus and extensor carpi radialis (Tosolini and Morris, 2012). Although these muscle groups have different anatomical locations and functions, the extent of serotonergic reinnervation of these motor pools after cSCI is similar.

Muscles of the neck innervated by axial motor neurons are surgically separated during C2Hx surgery to access the spinal cord. Thus, it is possible that surgical procedure may have affected serotonergic innervation, or other aspects of axial motor neurons. We do not think this is the case since if serotonergic innervation of the axial motor nucleus was impacted by the C2Hx surgery, we would’ve seen bilateral changes versus ipsilateral changes exclusively. Although, there are not many reports of the axial motor pool post-cSCI, rats appear to regain lost function; they are able to lift their head to eat and drink. Similarly, there is substantial (not complete) recovery of forelimb function after C2Hx (Gonzalez-Rothi et al., 2015c). We did not perform a time-course of spinal serotonin levels after C2Hx, but we suspect that there was greater loss of serotonin initially after C2Hx, followed by slow, spontaneous recovery to the levels observed here 12 weeks post-injury. This transient decrease would account for observed and documented recovery of neck and forelimb function after cSCI. Future work needs to address time-course of deficits and recovery of serotonin post-C2Hx.

**Effects of IH on Serotonergic Innervation in Spinal Intact Rats**

This was the first study to compare the effects of multiple IH protocols on serotonergic innervation of axial and forelimb motor nuclei. In this study, none of the IH protocols used affected serotonergic innervation of axial and forelimb motor nuclei in spinal intact or injured rats. Our lab previously reported that repetitive AIH (rAIH; 10, 5 min episodes 3x/week, 10 weeks) enhanced serotonergic innervation in the phrenic motor nucleus of intact rats (Satriotomo et al., 2012). Because axial and forelimb motor
neurons neighbor phrenic motor neurons in the cervical spinal cord, we had originally predicted that 28 days of daily AIH would similarly increase serotonergic innervation of these regions, but that was not the case. These studies differed in the total duration of rAIH exposure (10 versus 4 weeks), the intermittent vs continuous days of AIH exposure, and the specific sub-strain of Sprague Dawley rats investigated (Taconic versus Envigo). Additionally, the regions of interest quantified in these two studies varied (200µm x 200µm square versus 50µm x 100µm ellipse). Unfortunately, we do not know the reasons for these apparently conflicting results at this time.

**Effects of IH on Serotonergic Innervation after C2Hx**

Serotonin is transiently reduced after C2Hx, and spontaneously, partially recovers with time post-C2Hx (Camand et al., 2004; Golder and Mitchell, 2005; Saruhashi et al., 1996). This study began IH exposures 8 weeks after C2Hx, as serotonergic innervation recovers, in order to elucidate the impact of AIH. We aimed to utilize the various IH protocols to further enhance serotonergic innervation after chronic C2Hx. Just as with our companion study of phrenic and intercostal motor pools (Ciesla et al., ibid), none of the IH protocols tested here impacted serotonergic innervation of axial or forelimb motor nuclei in chronic C2Hx rats. It was previously reported that AIH (7 days beginning 1 week post-injury) enhances limb function and walking ability in rats 2 weeks after cSCI (Lovett-Barr et al., 2012). In addition, 7 days of AIH paired with task specific training (starting 4 weeks after SCI) improved locomotor function in rats up to 8 weeks after SCI (Prosser-Loose et al., 2015). These studies differ in the time of AIH exposure post-injury (1 vs 4 vs 8 weeks post- injury), the study end point post-injury (2 vs 12 weeks post injury), duration of AIH (1 vs 4 weeks), and the combination of task specific motor training. People with chronic, incomplete SCI have improved walking
ability and hand use after AIH exposure (Hayes et al., 2014; Trumbower et al., 2017; Trumbower et al., 2012). This improvement in people with SCI is enhanced when AIH is paired with task specific training, such as over ground walking (Hayes et al., 2014). Thus, it is possible that AIH paired with task specific training may further enhance serotonin innervation of targeted motor nuclei. A detailed hypothetical model has been proposed to account for the synergy between AIH and task specific training (Welch et al., 2020). However, from another perspective, there may be sufficient serotonergic innervation to underlie AIH-induced functional recovery of motor behaviors.

Overall, we conclude that serotonergic innervation of axial and forelimb motor nuclei remains reduced 12 weeks after a cervical spinal hemisection, unlike known respiratory motor nuclei where the size of each structure increases, thereby offsetting some of the loss of structure number. In contrast, serotonin structures around the axial/forelimb pools do not swell, but they do appear to increase their serotonin concentration, possibly a sign of compensation to preserve serotonergic function. Contrary to expectations, no IH protocol studied affected serotoninergic innervation of axial or forelimb motor nuclei, with or without spinal injury. The substantial spontaneous reinnervation of these motor pools suggest that there is considerable substrate for serotonergic function at this point post-injury, although it may be impaired to some extent. Thus, results from this study suggest that therapeutics targeting spared serotonin pathways are possible.
Figure 4-1. Representative images of serotonergic innervation of putative axial and forelimb motor nuclei. Serotonin (red) immunofluorescent labeling was observed in all cervical sections. Serotonin labeling of the left/ipsilateral and right/contralateral ventral horns of a spinal intact rat and a rat 12 weeks after C2 hemisection (C2Hx) exposed to 28 consecutive days of normoxia. The putative axial (top) and forelimb (bottom) motor nuclei were selected based on anatomical locations depicted in the cervical spinal schematics above respective fluorescent images. Images taken at 20x magnification. Scale bar: 100µm.
Figure 4-2. Serotonergic innervation of the putative axial motor nucleus. Serotonin innervation around the putative axial motor nucleus was quantified in spinal intact rats (left) and 12 weeks after C2 hemisection (C2Hx; right) exposed to 28 days of varied intermittent hypoxia (IH) protocols (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). A. Number of serotonin structures. C2Hx significantly reduced the number of serotonin structures innervating the putative axial motor nucleus ipsilateral to C2Hx versus contralateral C2Hx and versus spinal intact rats (p<0.0001). There were no effects of IH in spinal intact rats or after C2Hx B. The size of serotonin structures. There were no significant effects of C2Hx or IH protocol on the size of serotonin structures innervating the putative axial motor nucleus. C. Total serotonin innervation area. C2Hx alone significantly reduced the total area of serotonin innervation of putative axial motor nuclei ipsilateral to C2Hx compared to contralateral structures and spinal intact rats (p<0.0001). There were no significant effects of IH after C2Hx or in spinal intact rats. All data were analyzed with a repeated measures Mixed Model ANOVA; injury (intact vs. C2Hx) and IH protocol (Nx28, dAIH28, IH28-5/5 and IH28-2/2) were between subject variables; side (left/ipsilateral vs. right/contralateral) was the within subject repeated measure. Differences were considered significant if p<0.05. Bars denote means ± 1 SEM with the individual rats denoted by individual data points.
Figure 4-2. Continued
Figure 4-3. Serotonergic innervation of the putative forelimb motor nucleus. Serotonin innervation around the putative forelimb motor nucleus was quantified in spinal intact rats (left) and 12 weeks after C2 hemisection (C2Hx; right) exposed to 28 days of varied intermittent hypoxia (IH) protocols (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). A. Number of serotonin structures. C2Hx significantly reduced the number of serotonin structures innervating the putative forelimb motor nucleus ipsilateral to C2Hx versus contralateral C2Hx and versus spinal intact rats (p<0.0001). There were no effects of IH in spinal intact rats or after C2Hx. B. The size of serotonin structures. There were no significant effects of C2Hx or IH protocol on the size of serotonin structures innervating the putative forelimb motor nucleus. C. Total serotonin innervation area. C2Hx alone significantly reduced the total area of serotonin innervation of putative forelimb motor nuclei ipsilateral to C2Hx compared to contralateral structures and spinal intact rats (p<0.0001). There were no significant effects of IH after C2Hx or in spinal intact rats. All data were analyzed with a repeated measures Mixed Model ANOVA; injury (intact vs. C2Hx) and IH protocol (Nx28, dAIH28, IH28-5/5 and IH28-2/2) were between subject variables; side (left/ipsilateral vs. right/contralateral) was the within subject repeated measure. Differences were considered significant if p<0.05. Bars denote means ± 1 SEM with the individual rats denoted by individual data points.
Figure 4-3. Continued
Figure 4-4. Intensity of 5-HT immune positive structures around putative axial and forelimb motor neurons. Serotonin innervation around the putative axial and forelimb motor nuclei were quantified in spinal intact rats (left) and 12 weeks after C2 hemisection (C2Hx; right) exposed to 28 days of varied intermittent hypoxia (IH) protocols (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). In both putative axial (A) and forelimb (B) motor nuclei, C2Hx significantly reduced the intensity of serotonin positive structures ipsilateral to injury compared to contralateral to C2Hx and spinal intact rats (p<0.0001). There was no effect of IH on the intensity of serotonin structures in after C2Hx or in spinal intact rats in neither the putative axial nor forelimb motor neurons. All data were analyzed with a repeated measures Mixed Model ANOVA; injury (intact vs. C2Hx) and IH protocol (Nx28, dAIH28, IH28-5/5 and IH28-2/2) were between subject variables; side (left/ipsilateral vs. right/contralateral) was the within subject repeated measure. Differences were considered significant if p<0.05. Data are displayed in arbitrary units (A.U.) as mean±SEM. Bars denote means ± 1 SEM with the individual rats denoted by individual data points.
Figure 4-4. Continued
CHAPTER 5
IMPACT OF DIETARY FOLATE ON RESPIRATORY RECOVERY AFTER CERVICAL SPINAL HEMISECTION

Abstract

Folate supplementation enhances spinal sensory axon growth and has been reported to improve locomotion after spinal injury. Since folate supplementation and deprivation effects on breathing function after cervical spinal injury are not known, we tested the hypotheses that: 1) folate supplementation enhances and deprivation impairs breathing recovery after C2 spinal hemisection (C2Hx); and 2) folate supplementation stimulates and deprivation impairs serotonergic reinnervation of the phrenic motor nucleus after C2Hx. We compared the effects of folate deprivation and supplementation (80µg/kg/day) after sub-acute (2 week) and chronic (8 week) C2Hx. We recorded diaphragm electromyography during spontaneous breathing, spontaneous sighs and tracheal occlusion. Folate manipulation did not impact diaphragm function or serotonergic reinnervation after sub-acute or chronic C2Hx. At both time points, ipsilateral serotonergic varicosities were larger versus contralateral. At the times and doses studied, folate manipulations had no effect on serum folate or homocysteine levels, demonstrating the essential need to measure plasma levels in future studies. C2Hx alone reduced serum folate, and increased homocysteine. Although folate manipulations did not impact breathing function or serotonergic innervation after C2Hx, we cannot discount potential roles for folate in mediating recovery in other CNS injury models.
Overview

Cervical spinal cord injury (cSCI) causes respiratory muscle paralysis, ventilator dependence and death from respiratory failure. Thus, strategies to restore respiratory function after cervical SCI are essential. Recent studies suggest that folate supplementation may support functional recovery and axon regeneration after SCI (Iskandar et al., 2004; Iskandar et al., 2010; Miranpuri et al., 2017; Zhang et al., 2008). In fact, persons with spinal cord injuries (SCI) report reduced dietary folate intake of up to 75% of the daily recommended value (Groah et al., 2009; Perret and Stoffel-Kurt, 2011; Walters et al., 2009). The extent to which circulating folate levels correlate with the extent of injury/functional recovery after SCI is unknown. Thus, folate deficits could impair functional recovery and axonal regrowth following spinal cord injury.

Folate is a vital nutrient for proper development of the central nervous system (CNS), facilitating neural tube closure and reducing the incidence of fetal malformations (Imbard et al., 2013; van der Put et al., 2001). Folate’s role in maintaining the health and functionality of the adult CNS is less clear. Following thoracic spinal contusion, folate supplementation elicits small but statistically significant improvements in locomotor function in rats (Iskandar et al., 2004; Miranpuri et al., 2017; Zhang and Shen, 2015). In support of these reports of functional recovery, folate supplementation also: 1) reduces matrix metalloproteinase-2 expression, a critical player in extracellular matrix breakdown; and 2) enhances spinal sensory axon regrowth into a peripheral nerve graft after dorsal column injury (Iskandar et al., 2004; Iskandar et al., 2010). Here, we hypothesize that preserving/restoring normal folate levels may be beneficial in promoting functional recovery in other motor systems following SCI, such as the motor system responsible for breathing.
We investigated the effects of folate manipulation on respiratory function using a well-studied experimental model of cSCI, cervical spinal hemisection at C2 (C2Hx). C2Hx removes input from descending respiratory neurons resulting in defined respiratory impairments (Fuller et al., 2006). We tested the specific hypothesis that folate supplementation enhances functional recovery of breathing capacity after C2Hx, and that dietary folate deprivation hinders spontaneous functional recovery of breathing capacity after C2Hx (Sandhu et al., 2009; Vinit and Kastner, 2009). Further, the impact of folate supplementation and deprivation on neural regeneration after cSCI is unknown. Since C2Hx disrupts descending serotonergic projections, and folate is important for axonal growth and neurotransmitter synthesis, we also tested the hypothesis that folate supplementation stimulates, and folate deprivation blunts, recovery of diaphragm function and serotonergic innervation in the phrenic motor nuclei necessary for adequate diaphragm function at two time points post-C2Hx. For unknown reasons, our experimental manipulations of folate (dietary and injections) failed to manipulate serum levels of folate, demonstrating that direct measurement of folate and its metabolites is critical in studies concerning the impact of folate on functional recovery following cSCI. In accordance, we found no effect of attempted folate manipulations on ipsilateral or contralateral diaphragm EMG function, or serotonergic innervation of the phrenic motor nucleus after sub-acute and chronic C2Hx. However, we do report that C2Hx alone reduces ipsilateral diaphragm EMG amplitude at these times post-injury.

Methods

Animals

All experiments were conducted with adult male (11-12 weeks of age; 350-450 g) Sprague-Dawley rats (Harlan, Indianapolis IN, Colony 208A). Rats were housed in pairs
in a controlled environment (12 h light/dark cycles) with food and water *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida. Animals were randomly assigned to one of six groups: 1) folate deprived (n=5); 2) folate control (n=5); or 3) folate supplemented (n=9) after sub-acute C2Hx; or 4) folate deprived (n=7); 5) folate control (n=4); or 6) folate supplemented (n=9) after chronic C2Hx.

**Folate Manipulation**

Attempts to manipulate folate levels included both changes in dietary intake and intraperitoneal folate injections (I.P.; time-line depicted in Figure 5-1A). Rats on the control diet and those receiving folate supplementation via injections received custom chow containing 4mg/kg folate, a standard dose in rat chow (Envigo teklad custom diet TD. 160607). Rats on the folate deprived diet received custom chow containing 0mg/kg folate (Envigo teklad custom diet TD. 160606). All other ingredients in the chow were the same. Rats were acclimated to the respective diets for 4 weeks before C2Hx and continued these diets until the end of the study. This period was considered optimal to ensure folate depletion based on literature accounts (Durand et al., 1996; Lu et al., 2015; Pravenec et al., 2013), although we did not observe similar efficacy based on ELISA measurements of serum folate levels (see below). The amount of food consumed and rat weights were monitored twice weekly. There were no differences in food consumption or weight gain between groups on custom diets. Folate was supplemented via daily I.P. injections of 5-methyltetrahydrofolic acid disodium salt (Sigma-Aldrich; 80μg/kg body weight), starting 3 days before and continuing for 2 weeks post injury similar to prior reports (Iskandar et al., 2004; Iskandar et al., 2010). Control
and deprived rats received I.P. injections containing saline vehicle 3 days before injury until 2 weeks post injury.

Retrograde labeling of Phrenic Motor Neurons

Rats were anesthetized with 3.5% isoflurane (in 100% O₂) and maintained at 2-2.5% isoflurane (in 100% O₂) via nose cone throughout the surgery (Allen et al., 2019). All rats were injected intrapleurally with Cholera toxin B fragment (CtB) subunit bilaterally (0.2% w/v CtB; dissolved in sterile H₂O; Calbiochem, Billerica, MA) to retrogradely label phrenic motoneurons (Dale-Nagle et al., 2011; Guenther et al., 2010; Mantilla et al., 2009) 14 days prior to C2Hx. 25μL of CtB was loaded into a 25μL Hamilton syringe attached to a 9.52 mm sterile needle for bilateral injections (2 × 12.5μL = 25μL total per animal) at the 5th intercostal space ~6 mm deep.

C2Hx Injury

Anesthesia, C2Hx and post-surgical animal care were performed as described previously (Fuller et al., 2008; Gonzalez-Rothi et al., 2015c). Rats were anesthetized with 3.5% isoflurane (in 100% O₂) and maintained at 2-2.5% isoflurane (in 100% O₂) via nose cone throughout surgery; anesthesia depth was confirmed by the absence of toe pinch and palpebral responses (Allen et al., 2019; Dougherty et al., 2012). Body temperature was maintained at 36.5–37.5°C with a heating pad. Artificial tears (Rugby Artificial, NDC 0536-1086-91) were applied to prevent eye globe damage. Nails were clipped from forelimbs and hind limbs, and the surgical site was shaved and cleaned (chlorhexidine scrub, 58829-140-01, First Priority, Inc., Elgin, IL). The C2Hx was performed by making a dorsal incision on the neck of the animal and dissecting the muscle to expose the C2 lamina. Following C2 laminectomy and durotomy, the left side of the C2 spinal cord was hemisected with a microknife, caudal to the C2 dorsal roots. A
gap (~1 mm) at the injury site was then created by gentle aspiration. The overlying dura was subsequently sutured with 9-0 ethilon nylon suture, the overlaying muscles were sutured with 3-0 Polysorb absorbable suture and the skin was closed with 9 mm stainless steel wound clips. Each rat received post-operative care following surgery which included pain management with an analgesic (buprenorphine, 0.03 mg/kg, s.q., Hospira< IL) every 12 hours and an anti-inflammatory drug (meloxicam, 2 mg/kg., s.q. Portland, ME) every 24 hours for 2 days post-surgery. Rats received lactated ringers solution (5 ml 2x/day, s.q.) and were manually fed via syringe containing a nutritional supplement deprived of folate (folic acid deficient Nutra-Gel™, BioServ, lot #197773) until adequate volitional drinking and eating resumed (Allen et al., 2019).

**Diaphragm EMG Recordings**

Diaphragm EMG recordings were used to assess breathing/diaphragm function in anesthetized, spontaneously breathing rats after acute (2 weeks) or chronic (6-8 weeks) C2Hx. Anesthesia was induced with 3.5% isoflurane (in 100%O₂) and body temperature was maintained at ~37 °C with a custom-made heating table and a rectal thermometer (Physitemp, model 700 1H). Anesthesia was maintained at 2–2.5% isoflurane initially via nose cone and then via tracheal cannula. A tail vein catheter was placed for intravenous delivery of urethane and fluids. Rats were slowly converted (6 ml/h; Harvard Apparatus syringe pump) to urethane anesthesia (1.6 g/kg, i.v.) and isoflurane was withdrawn. After conversion, the depth of anesthesia was confirmed by the absence of toe-pinched and palpebral response.

A femoral arterial catheter was placed to monitor blood pressure and sample blood gases. Blood gases and pH were measured in samples taken from a femoral arterial catheter (~0.3 ml; ABL800, Radiometer, Copenhagen, Denmark). Inspired
oxygen was controlled to maintain blood PaO$_2$ of 85-95mmHg (balanced N$_2$). Following laparotomy, diaphragm muscle was bilaterally implanted into the mid costal region with two pairs of Teflon-coated, multistring stainless-steel electrodes (AS631, Cooner Wire, Chatsworth, CA). Electrodes were uninsulated for ~2 mm at the point of contact with in the mid-costal regions of the diaphragm (Seven et al., 2014; Seven et al., 2018a). Rats were given ample time to clear isoflurane from the body before recordings commenced (i.e. the same amount of time that they were on isoflurane)(Seven et al., 2018a).

EMG signals were amplified (1000×) and band-pass filtered (10–1000 Hz) using an analog amplifier (Model 1800, A-M Systems, Carlsborg, WA, USA). They were digitized at 1 kHz and recorded (Powerlab, AD Instruments, Colorado, United States). EMG amplitude was estimated using root-mean-squared (RMS) EMG calculated over a 50-ms sliding window. RMS EMG value is a measure of the power of a signal and well correlated with force generated by various muscles including diaphragm muscle (Mantilla et al., 2010).

Bilateral diaphragm EMG activity was recorded during three different behaviors: 1) spontaneous breathing (arterial PaO$_2$ >85mmHg, PaCO$_2$ ~47mmHg; 10 min), 2) spontaneous sighs during a 5 minute hypoxia/hypercapnia challenge (10.5%O$_2$, 7%CO$_2$), and 3) the last 5s of ~40s sustained tracheal occlusion via forced closure of the airway at end-expiration (Mantilla et al., 2010; Mantilla and Sieck, 2011; Seven et al., 2018a). Standard base excess was kept between −3 and 3mq/l. In these anesthetized, spontaneously breathing rats, slightly elevated inspired O$_2$ (21–30% O$_2$) was necessary to attain PaO$_2$ >85mmHg. Spontaneous sighs and the response to sustained tracheal occlusion were measured as an indication of near-maximal
respiratory muscle activation (Mantilla and Sieck, 2011; Seven et al., 2014). Spontaneous sighs were considered if RMS EMG amplitudes were more than twice spontaneous breathing RMS EMG amplitude and were followed by a post-sigh apnea. Ipsilateral diaphragm EMG recordings were included in the analysis if EMG signal was rhythmic, reflecting inspiratory activity in phase with contralateral diaphragm activity, persistent across multiple inspiratory bursts, and comprised more than one motor unit. Figure 5-1C shows a trace over three breaths representing the paralyzed hemi-diaphragm with no activity compared with the contralateral diaphragm.

**Tissue Histology**

After terminal diaphragm EMG recordings, rats were perfused intracardially with cold 0.1M phosphate buffer saline (PBS), followed by paraformaldehyde (PFA, 4% paraformaldehyde, w/v in 0.1M PBS, pH 7.4). The spinal cord was harvested and post-fixed overnight in 4% PFA and cryoprotected at 4°C in 20% sucrose solution in 0.1M PBS for 3 days, followed by 30% sucrose solution in 0.1M PBS for 3 days. To verify completeness of the lesion, cervical spinal segments C1-C3 were parfinized, cut longitudinally at 7µm, directly mounted on positively charged slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. Sections were stained using 0.1% cresyl violet and dehydrated through graded alcohol (70–100%), cleared in Histoclear (National Diagnostics, Atlanta, GA) and cover-slipped (Eukitt, Electron Microscope Science, PA). Injury sites were imaged using brightfield at 10x zoom level (BZ-x710, Keyence Co., Osaka, Japan) to confirm lateral C2Hx (Figure 5-1B).

Cervical spinal segments containing phrenic motor neurons (C3-C5) were sectioned in transverse plane (40µm thickness) using a freezing microtome (Leica SM 200R, Buffalo Grove, IL). Tissues were stored in antifreeze solution at -20°C until
processed (30% glycerol, 30% ethylene glycol, 40% 0.1 M PBS, pH 7.4). Every 12th section was selected and stained for serotonin and CtB. Free-floating sections were washed with 0.1M PBS (pH 7.4), then incubated in heat induced epitope retrieval solution (TissuePro, cat#: HIER01-32R) for 30min at 85°C. Tissues were washed again with 0.1M PBS-Triton (0.1%, pH 7.4) and then incubated in a blocking solution (5% normal donkey serum (NDS, GeneTex, cat#: GTX30972, Irvine, CA) in 0.1M PBS-Triton (0.1%), pH 7.4) at room temperature for 60 min. Tissue was incubated in primary antibody solution staining consisting of 5% NDS in 0.1M PBS-Triton (0.1%, pH 7.4) with anti-5-HT (1/2000, rabbit, Immunostar #20080) and anti-CTB (1/2500, goat, Millipore #227040) in 4°C overnight. The following day, tissues were washed with 0.1M PBS-Triton (0.1%, pH 7.4) then incubated in secondary antibody solution consisting of 5% NDS in 0.1M PBS-Triton (0.1%, pH 7.4) with secondary antibodies conjugated to Alexa Fluor® 594 (donkey anti-rabbit 594; 1:500, Invitrogen, Ref#A11055) and Alexa Fluor® 488 (donkey anti-goat 488; 1;1000, Invitrogen, Ref#A21207) in a dark box at room temperature for 2 hours. Sections were washed with 0.1M PBS-Triton (0.1%, pH 7.4) and mounted on positively charged slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. Slides were cover slipped with VectaShield Antifade Hard Set Mounting Medium (Cat#: H-1400). Fluorescently labeled sections were imaged via epifluorescent microscope with 20x magnification (Keyence BZ-X700, Keyence Corporation of America, Itasca, IL). Regions for imaging were determined by CtB-positive cell labeling within the C3-C5 ventral horn, signifying the phrenic motor nucleus. The sides of the spinal cord were marked prior to tissue sectioning to ensure accurate side determination during imaging and analyses.
**Determination of Serum Folate and Homocysteine**

Blood samples were taken from each rat prior to C2Hx and prior to terminal EMG recordings. Rats were anesthetized as described above. An I.V. catheter (Terumo Surflesh lot#180312A) was inserted into the tail vein and about 500µL of blood was withdrawn. The blood was inserted into a BD SST Microtainer® blood collection tube for serum (Ref#365967). The blood in the collection tube was left in 4ºC for 10 min and then was centrifuged at 2.5xg for 10min to separate the serum. The supernatant containing serum was extracted from the collection tube and placed into a sterile 1.5µL Eppendorf Tube®. The serum was stored at -80ºC. Serum folate levels were assed using a rat folic acid ELISA kit (LifeSpan Biosciences Catalog Number LS-F10084). Serum homocysteine levels were assed using a rat homocysteine ELISA kit (LifeSpan Biosciences Catalog Number LS-F32293). Both folate and homocysteine levels were determined according to the manufacturer’s protocols and were read at 450nm.

**Data Analysis and Statistics**

Raw RMS EMG amplitudes during spontaneous breathing were calculated, excluding spontaneous sighs, and analyzed for a 1-minute period. Separately, 4–8 spontaneous sighs were analyzed for each rat. During sustained tracheal occlusion, EMG bursts in the last 5-s of the 40s sustained tracheal occlusion were analyzed. Data during each experimental condition for each animal were averaged to represent the sample for that experimental condition per animal (Seven et al., 2018a).

Immunofluorescence was analyzed using a custom MATLAB (MathWorks, Natick, MA, USA) code. Thresholds were determined using a custom adaptive thresholding algorithm in MATLAB as previously described (Allen et al., 2019). This MATLAB code first identified CtB-labelled phrenic motor neurons. Then, the center of gravity of the
CtB-labelled phrenic motor neurons was calculated and the final region of interest was defined by a circular area with a 50 µm radius centered at the center of gravity of CtB-positive phrenic motor neurons (Seven et al., 2018b). Serotonergic structures were identified within this region of interest. We quantified the average number of immunolabeled structures (number of boutons), average immunolabeing area per structure (area per bouton), and average total area of serotonin-positive immunolabeling (number of boutons multiplied by the area of each bouton; total area). Despite known limitations in using immunofluorescence as an indicator of antigen concentration (Fritschy, 2008; Matos et al., 2010), we also quantified the average intensity of each structure (intensity per bouton). Folate manipulation remained blinded throughout analyses.

Statistical analyses were performed using JMP 11.0 (SAS Institute, Cary, NC). EMG and immunofluorescence data were analyzed using two-way repeated-measures ANOVA with independent variables being folate manipulation (deprived vs. control vs. supplemented) and spinal cord side relative to injury (ipsilateral vs. contralateral) as the repeated measure. Sub-acute and chronic time points were analyzed separately at each time point. Chi-squared analyses were performed on the number of animals with ipsilateral EMG recovery during spontaneous breathing in each group. Physiological variables, such as blood pressure and blood gas measurements, were analyzed with a one-way ANOVA. Serum folate and homocysteine levels were analyzed for the time points prior to C2Hx, 2 and 8 weeks post-C2Hx separately using a one-way ANOVA with the independent variables being folate manipulation (deprived vs. control vs. supplemented). We also analyzed serum folate and homocysteine levels using a two-
way repeated measures ANOVA with independent variables being folate manipulation (deprived vs. control vs. supplemented) and time (pre- vs. post-C2Hx) as the repeated measure. A significance level of 0.05 was set for all statistical comparisons. When significant differences were observed, Tukey-Kramer Honestly Significant Difference test was used for post hoc analyses. All data are displayed as mean ± standard error of the mean.

Results

Verification of C2Hx

Spinal segments from cervical level 1 to 3 consisting of the C2Hx site were visualized for anatomical completeness. The lesions were designated as complete if the left side of the 2nd cervical spinal segments was removed from the midline to the lateral edge leaving a visible gap. All C2Hx injuries were visually similar between animals (Figure 5-1B). Completeness of the injury was also confirmed during bilateral diaphragm EMG recordings with the reduction of ipsilateral diaphragm amplitude (Figure 5-1C).

Recovery of Ipsilateral Diaphragm EMG Activity is Not Folate Dependent

Pysiological measurements

Table 5-1 shows change in body weight in grams from pre-C2Hx to end point (ΔBW), respiratory frequency, body temperature (°C), heart rate, mean arterial pressure (mmHg; MAP), arterial oxygen (mmHg; PaO₂), arterial carbon dioxide (mmHg; PaCO₂), pH, and standard base excess (mEq/L; sBE) of each group represented as mean ± SE. There was an overall weight loss in rats after sub-acute C2Hx likely due to the short time of recovery after injury. After chronic C2Hx, rats have ample time to recover eating and drinking function and gain weight. There was no effect of folate manipulation on
change in body weight after sub-acute or chronic C2Hx. Rats in the control group after sub-acute injury had a significantly less MAP compared to deprived (p=0.0365) and supplemented (p=0.02) animals after sub-acute C2Hx.

**Diaphragm EMG amplitudes: sub-acute C2Hx**

Ipsilateral diaphragm EMG activity was present in only 20% of deprived, 20% of control and 33% of folate supplemented rats after sub-acute C2Hx during spontaneous breathing (Chi-squared likelihood ratio p=0.80, $X^2=0.435$; Pearsons p=0.08, $X^2=0.434$; $R^2=0.02$). In all rats, ipsilateral motor units were recruited during spontaneous sighs and sustained tracheal occlusion. There were no consistent effects of attempted folate manipulation on ipsilateral or contralateral diaphragm EMG function during spontaneous breathing at after sub-acute C2Hx (p=0.24; Figure 5-2A). Similarly, we did not see consistent folate effects on ipsilateral or contralateral diaphragm EMG function during spontaneous sighs (p=0.43; Figure 5-2B) or sustained tracheal occlusion (p=0.38; Figure 5-2C) at after sub-acute C2Hx. However, in all outcomes there was an effect of side in that the ipsilateral diaphragm EMG amplitude was significantly less than the contralateral EMG amplitude as expected (spontaneous breathing $p<0.0001$; spontaneous sighs $p=0.004$; sustained tracheal occlusion $p=0.007$).

**Diaphragm EMG amplitudes: chronic C2Hx**

EMG activity in the ipsilateral diaphragm was present in 71% of deprived, 50% of control, and 44% of supplemented rats after chronic C2Hx (Chi-squared likelihood ratio p=0.55, $X^2=1.24$; Pearsons p=0.55, $X^2=1.21$; $R^2=0.05$). In all rats, ipsilateral motor units were recruited during spontaneous sighs and sustained tracheal occlusion. There were no consistent effects of folate manipulation on ipsilateral or contralateral diaphragm EMG function during spontaneous breathing after chronic C2Hx (p=0.74; Figure 5-3A).
We also did not see any consistent folate effects on ipsilateral or contralateral diaphragm EMG function during spontaneous sighs (p=0.78; Figure 5-3B) or sustained tracheal occlusion (p=0.64; Figure 5-3C). In all outcomes there was an effect of side in that the ipsilateral diaphragm EMG amplitude was significantly less than the contralateral EMG amplitude (spontaneous breathing p<0.0001; spontaneous sighs p=0.001; sustained tracheal occlusion p=0.03).

**EMG reference point: intact rats**

Our focus was on folate manipulation of injured rats, but we also conducted a small number of studies on intact control rats to establish a reference point for diaphragm activity in uninjured rats. We measured bilateral diaphragm EMGs in 5 intact rats who received the control diet and sham I.P. injections. Two intact rats were run in parallel with the sub-acute group, and 3 intact rats were run in parallel with the chronic group. Bilateral diaphragm activity was recorded in urethane anesthetized intact rats during spontaneous breathing, spontaneous sighs and sustained tracheal occlusion. Since there were no differences in EMG amplitude across times or left versus right diaphragm, all intact EMG amplitudes were averaged to create the reference value. Data were not included in statistical analysis due to the low n. Average EMG amplitudes for the intact rats were 0.08 ± 0.04mV during spontaneous breathing, 0.13 ± 0.05mV during spontaneous sighs, and 0.27 ± 0.1mV during sustained tracheal occlusion and are represented as dotted lines of the graphs (Figure 5-2 and 5-3).

**Nomalized diaphragm EMG amplitudes**

Spontaneous breathing EMG amplitudes were normalized to spontaneous sighs amplitudes in each rat to understand the capacity of C2Hx rats to breathe relative to their maximum capacity. Since raw EMG data can be variable, we normalized RMS
EMG amplitudes to the near maximal behavior of spontaneous sighs (Mantilla and Sieck, 2011). When normalized to spontaneous sighs, folate manipulation did not have an effect after sub-acute and chronic C2Hx (Figure 5-4). There was no effect of folate manipulation after sub-acute injury (p=0.39), however, ipsilateral diaphragm EMG function was significantly less than contralateral (p<0.0001). After chronic C2Hx there was no effect of folate manipulation (p=0.65) or injury (p=0.15) on normalized ipsi- or contralateral diaphragm EMG function.

**Serotonergic Innervation of Phrenic Motoneurons is Not Folate Dependent**

Robust serotonin expression was around CtB labeled phrenic motor neurons in the ventral horn of the cervical spinal cord extending from cervical level 3 to 5 after sub-acute C2Hx (Figure 5-5 A-F) and chronic C2Hx (Figure 5-6 A-F). We quantified the average number of immunolabeled structures (number of boutons), average area of immunolabeling per structure (area per bouton), average total area of serotonin-positive immunolabeling (number of boutons multiplied by the area of each bouton; total area), and average intensity of each structure (intensity per bouton). Upon first observance, there were no obvious differences in serotonin immunofluorescence between folate manipulations in either sub-acute or chronic rats. In all rats, contralateral serotonin appeared thin and continuous, somewhat like “beads on a string”. Ipsilateral to C2Hx, serotonin structures appeared larger in all rats.

**Serotonin innervation after sub-acute C2Hx**

There was no folate treatment effect on the total area of serotonin-positive immunolabeling (p=0.64), number of boutons (p=0.41), area per bouton (p=0.09), or intensity per bouton (p=0.44) around the phrenic motor nucleus. However, there was a side effect on total area, area per bouton and intensity per bouton (Figure 5-5 G, I, J;
p<0.0001 for all), but not number of boutons (Figure 5-5H; p=0.89). There was more total area of serotonin-positive immunolabeling to the ipsilateral phrenic motor nuclei compared to contralateral to injury (Figure 5-5G). The area of each bouton was larger ipsilateral compared to contralateral to injury (Figure 5-5I). The intensity of each bouton was greater in boutons contralateral versus ipsilateral to injury (Figure 5-5J).

**Serotonin innervation after chronic C2Hx**

There was no folate treatment effect on total area of serotonin-positive immunolabeling (p=0.68), number of boutons (p=0.784), area per bouton (p= 0.12), or intensity per bouton (p=0.74) innervating the phrenic motor nucleus. However, there was a side effect on total area, area per bouton and intensity per bouton (Figure 5-6 G, I, J; p<0.0001). There was no effect of side on the number of serotonin boutons (Figure 5-6H; p=0.86). There was more serotonin immunopositive total area around ipsilateral phrenic motor neurons versus contralateral (Figure 5-6G, p<0.05). Bouton area was larger ipsilateral versus contralateral to injury (Figure 5-6I, p<0.0001). Bouton staining intensity was greater contralateral versus ipsilateral to injury (Figure 5-6J, p<0.05).

**Serotonin innervation reference point: intact rats**

Our focus was on folate manipulation of injured rats, but we conducted a small number of experiments on intact control rats to establish a reference point for serotonin immunolabeling around the phrenic motor nucleus. We measured serotonin immunofluorescence in 5 intact rats who received the control diet and sham I.P. injections; two intact rats were run in parallel with the sub-acute group and 3 in parallel with the chronic group. There were no differences in serotonin innervation across time points or across the left and right phrenic motor pools, so data were combined. Data were not included in the statistical analysis due to the low n. Averages and standard
error were 64.24 ± 16.99µm for total area, 49.15 ± 11.28 for bouton numbers, 1.28 ± 0.10µm for area per bouton, and 0.27 ± 0.08 A.U. for intensity per bouton are represented as dotted lines of the graphs (Figure 5-5 and 5-6).

**Folate and Homocysteine Levels in Rat Serum**

To verify that the folate deprived diet reduced folate levels, and that folate supplementation enhanced folate levels, relative levels of serum folate (Figure 5-7A) and homocysteine (Figure 5-7B) were determined. When folate levels are low, homocysteine levels are high, and visa-versa. We hypothesized that folate levels would be highest in the supplemented group, and lowest in the deprived group. We further hypothesized that homocysteine levels would be highest in the deprived and lowest in supplemented groups. Contrary to expectations, there were no effects of folate manipulations on folate levels in rat serum prior to C2Hx (p=0.47), 2 weeks post-C2Hx (p=0.15) or 8 weeks post-C2Hx (p=0.83). Similarly, there was no effect of folate manipulation on homocysteine levels in rat serum prior to C2Hx (p=0.39), 2 weeks post-C2Hx (p=0.52), or 8 weeks post-C2Hx (p=0.79). On the other hand, there was a significant C2Hx effect for both folate and homocysteine serum levels. Two and 8 weeks after C2Hx, serum folate levels were increased, and serum homocysteine levels were decreased 2 and 8 weeks post C2Hx (p<0.0001 for all). Thus, although we followed general guidelines presumed to enhance and/or diminish folate and homocysteine levels, our results suggest that we were not successful in doing so. All interpretations should be considered in this context.

**Discussion and Conclusion**

Although we intended to test the hypotheses that folate supplementation enhances, and deprivation hinders breathing recovery and serotonergic reinnervation of
phrenic motor nuclei post-cSCI, lack of evidence that our manipulations altered serum folate levels significantly limited interpretations based on our data. Thus, in retrospect, it is not surprising that none of the folate manipulations altered diaphragm function or serotonergic innervation of the phrenic motor nucleus after sub-acute or chronic C2Hx. Our findings, with respect to studies of folate and its effects, point strongly towards the need to verify plasma/serum levels of folate to assure the intended manipulation was successful. Reasons for our failure to successfully change serum folate levels are unclear.

On the other hand, our data do verify unique aspects of the C2Hx model of cSCI in spontaneously breathing rats. For example, C2Hx causes persistent reductions in ipsilateral diaphragm function during spontaneous breathing, spontaneous sighs and (maximal) tracheal occlusion. Further, after C2Hx, total serotonin immunolabeling area was greater ipsilateral versus contralateral to injury since ipsilateral (versus contralateral or control) serotonergic varicosities were larger. On the other hand, ipsilateral serotonin staining intensity per bouton was lower ipsilateral versus contralateral to injury. Finally, we report that C2Hx increases serum folate and decreases homocysteine levels at both times post-injury. The reasons for and functional significance of these folate changes are unclear.

**Folate Levels Post-C2Hx**

This is the first report to show that serum folate levels increase, and serum homocysteine levels decrease, post-C2Hx. Literature suggests that people with chronic SCI have reduced dietary folate intake (Groah et al., 2009; Perret and Stoffel-Kurt, 2011; Walters et al., 2009), although folate blood serum levels after SCI is not reported. It is possible that people with chronic SCI do not eat foods rich in folate because the
body already has increased folate levels. Folate maybe accumulating after SCI because is not being utilized by the body. In fact, global DNA methylation, CPG methylation, and SAM/SAH ratio is reduced in rats after SCI (Iskandar et al., 2010). In addition, the gut microbiota profile changes after spinal injury. Specifically, Lactobacillales, an order known to produce folate, changes in the gut of mice who received a T9 spinal contusion (Kigerl et al., 2016). The extent of changing folate synthesis and absorption in the gut after C2Hx is unknown but might contribute to the increased serum folate levels after C2Hx.

C2Hx Reduces Ipsilateral Diaphragm Function and Enhances Ipsilateral Serotonergic Innervation

After sub-acute and chronic C2Hx there was a significant reduction in ipsilateral diaphragm EMG amplitude, as expected (Fuller et al., 2006; Vinit et al., 2006). Although some rats never regained ipsilateral diaphragm activity after C2Hx during spontaneous breathing, we observed recruitment of ipsilateral motor units in all animals during sub-maximal behaviors of spontaneous sighs and sustained tracheal occlusion. We normalized spontaneous breathing to spontaneous sighs to assess the functional reserve. The 5 uninjured animals in this study had a normalized RMS EMG around 25%, consistent with literature that uninjured rats have a functional reserve around 20-30% (Mantilla et al., 2014). At both times post injury, functional reserve was less than 20%, both ipsilateral and contralateral to injury, possibly due to anesthetic suppression of breathing (Massey and Richerson, 2017).

It is well documented that serotonin immunofluorescence and the number of terminals below a spinal injury are reduced shortly after injury and spontaneously return to intact levels with time post injury (Camand et al., 2004; Golder and Mitchell, 2005;
Saruhashi et al., 1996). Contrary to previous reports that serotonin is reduced around the phrenic motor nucleus 2 weeks post-injury (Golder and Mitchell, 2005), we observed that the number of serotonin boutons around phrenic motor neurons was not different on the ipsi- and contralateral side of sub-acute (2weeks) or chronic (8weeks) C2Hx. Differences in data may be due to the size of the region of interest and the cervical spinal level quantified (C3-5 vs. C4), or specific sub-strain of Sprague Dawley rat used (colony 208A vs. 217). We observed reduced intensity of serotonin boutons ipsilateral compared to contralateral to injury. However, the total area of serotonin immunopositive labeling around ipsilateral phrenic motor neurons was increased due to the large size of each serotonin structure. Enlarged serotonin varicosities around the ipsilateral phrenic motor nucleus is also observed after 12 weeks post-C2Hx (Ciesla et al., in *ibid*).

**Attempted Dietary Folate Manipulations**

Unfortunately, our finding that the folate deprived group was not deficient of folate despite consuming a diet without folate for 1.5 (sub-acute) to 3 (chronic) months makes it impossible to make claims concerning the role of folate in deficits and/or recovery following spinal injury. It may be that the rats compensate for the lack of dietary folate by through gut folate production and/or preserving folate levels through coprophagy. Endogenous folate is synthesized by the gut microflora where small amounts are absorbed from the colon while a majority is released in feces (Rossi et al., 2011). One strategy to reduce folate absorption in the gut is to supplement animals with succinylsulfathiazole, a non-absorbed antibiotic drug that enhances folate depletion and inhibits folate synthesis by gut microflora (Challet et al., 2013). Although we did not supplement the animals with succinylsulfathiazole in this study, rat cages were changed 3 times per week to reduce the incidences of coprophagy.
Additionally, the folate supplemented group did not have higher folate levels than the deprived or control groups. Folate is a water-soluble vitamin and thus is not stored in the body for long periods of time. Based on the location of the supplemental folate I.P. injections, folate may have been readily taken up and metabolized by the liver. Indeed, there is variability in the length of time folate stays in the body based on the route of administration. Oral consumption of folate can last about 100 days (Gregory et al., 1998). Although this is dependent on the tissue being studied since folate is quickly removed from the blood serum. Whereas the half-life of folate after an intramuscular injection of folate is about 2 hours (Loew et al., 1987). Although correlated, the levels of folate are higher in cerebrospinal fluid than blood serum (Obeid et al., 2007; Reynolds et al., 1972). Thus, the manipulation may have a larger effect on cerebrospinal folate and homocysteine levels. In essence, it is essential to accurately report the route of administration, samples measured, and respective levels of attempted folate manipulations.

**Attempted Folate Manipulations Have No Effect on Diaphragm Activation or Serotonergic Innervation After C2Hx**

In the present study, we sought to explore the effect of folate supplementation and deprivation on breathing recovery after C2Hx. Contrary to our expectations, attempted folate manipulation did not affect breathing function after sub-acute or chronic C2Hx. This study also hypothesized that folate supplementation would increase serotonin innervation of phrenic motor neurons after C2Hx. Because folate has been shown to enhance axonal regeneration (Iskandar et al., 2004; Iskandar et al., 2010), is involved in the synthesis of serotonin (Serot et al., 2001), and folate deprivation is associated with low serotonin levels (Gospe et al., 1995; Moore et al., 2017), we utilized
the unique anatomy of serotonin within the spinal cord to study folate’s effects on serotonin innervation of phrenic motor nuclei. We did not observe an effect of attempted folate supplementation or deprivation on serotonin innervation of phrenic motor neurons after sub-acute or chronic C2Hx.

This was the first study attempting to investigate the effects of folate deprivation after cSCI, despite reports that folate intake is substantially reduced in people with chronic SCI (Groah et al., 2009; Perret and Stoffel-Kurt, 2011; Walters et al., 2009). Little is known concerning folate and its effects in the injured nervous system. Rats who received folate supplementation have a small but significant improvement in locomotor function 6 weeks after thoracic contusion injuries (Iskandar et al., 2004). In our study of C2Hx other elements did not recapitulate previous studies. Although serum folate levels were not ascertained in these studies, dietary/injection folate manipulations were similar (80µg/kg body weight) (Iskandar et al., 2004). The recommended amount of folate for a laboratory rat is 1mg/kg diet (Nutrition, 1995). Because the chow is already supplemented with 4 times the recommended value (4mg/kg folate), rats may have been past optimal folate levels, masking the potential benefits of additional folate. On the other hand, the failure of our protocols to increase plasma folate levels make interpretation difficult.

There is some suggestion that a peripheral nerve injury is needed in conjunction with a SCI for successful responses to folate supplementation to enhance regeneration (Iskandar et al., 2004; Iskandar et al., 2010; Miranpuri et al., 2017). It may also be that folate supplementation enhances functional improvements when combined with other therapies. For example, after combined folate with fetal stem cells after SCI, limb
function was enhanced versus folate or stem cells alone (Zhang and Shen, 2015). Folate supplementation may convey functional benefits only when combined with other treatments known to enhance breathing after C2Hx, such as acute intermittent hypoxia (Gonzalez-Rothi et al., 2015b).

This study was the first attempt to investigate the role of folate supplementation vs. deprivation on recovery of breathing function and serotonergic innervation after cSCI. Although we found no evidence that folate supplementation or deprivation impacts diaphragm function or serotonergic innervation of the phrenic motor pool following C2Hx, we found evidence that our folate manipulations were unsuccessful in changing plasma levels. This alone invalidates the first part of our study and points out the critical need to verify the success of our experimental manipulations. On the other hand, our findings do not discount the potential of folate to mediate functional recovery. Our data do make contributions in two areas: 1) we verify that ipsilateral diaphragm injury with a complete C2 hemisection remains suppressed for at least 8 weeks post injury; and 2) C2Hx increases folate levels and decreases homocysteine levels, which may alter outcomes post injury. Further studies are needed concerning the potential for folate to modulate functional recovery following cervical SCI.
Figure 5-1. Experimental timeline (A). Rats received respective diets 28 days prior to C2Hx. Intrapleural injections of CtB were administered 14 days prior to C2Hx. Intraperitoneal injections of supplemental folate or saline were administered beginning 3 days prior and continuing for 14 days post C2Hx. Diaphragm EMGs were recorded and tissue was harvested 14 (sub-acute) or 56 (chronic) days post injury. Representative image of left lateral C2Hx (B). Cervical segments 1 through 3 were labeled with Luxol Fast Blue to label white matter (blue) and cresyl violet (purple) to label nissel bodies. Completion of the injury, circled in red, was confirmed with the absence of white and gray matter from the lateral most side of the cord to the midline. Representative diaphragm EMG traces of three breaths during spontaneous breathing in a folate control rat after C2Hx (C). Ipsilateral diaphragm EMG (top) shows absence of spontaneous breaths in coordination with spontaneous breaths in the contralateral diaphragm (bottom). Both signals have synchronous heart rate artifacts.
Table 5-1. Physiological measurements in folate deprived, control and supplemented rats during spontaneous breathing and change in body weights from before C2Hx to end point represented as mean ± SE. There were no significant differences in physiological parameters. All data were analyzed with a one-way ANOVA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Acute</th>
<th>Chronic</th>
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<tbody>
<tr>
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<td>Control</td>
</tr>
<tr>
<td>ΔBW (g)</td>
<td>-18.8</td>
<td>-46.6</td>
</tr>
<tr>
<td>Frequency</td>
<td>138.7 ±8.8</td>
<td>124.1 ±5.9</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>37.2 ±0.2</td>
<td>37 ±0.1</td>
</tr>
<tr>
<td>HR</td>
<td>381 ±16</td>
<td>381 ±10</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>99 ±10</td>
<td>72 ±7</td>
</tr>
<tr>
<td>PaO2 (mmHg)</td>
<td>89.9 ±2.6</td>
<td>96.6 ±3.6</td>
</tr>
<tr>
<td>PaCO2 (mmHg)</td>
<td>44.3 ±1.2</td>
<td>42.7 ±2.1</td>
</tr>
<tr>
<td>pH</td>
<td>7.362 ±0.017</td>
<td>7.364 ±0.023</td>
</tr>
<tr>
<td>sBE (mEq/L)</td>
<td>0.7 ±1.3</td>
<td>0.2 ±1.2</td>
</tr>
</tbody>
</table>
Figure 5-2. Raw root-mean-squared EMG amplitudes during spontaneous breathing, spontaneous sighs, and sustained tracheal occlusion after sub-acute C2Hx. There was no effect of attempted folate manipulation in any of the respiratory behaviors assessed, spontaneous breathing ($p=0.24$; A), spontaneous sighs ($p=0.43$; B), and sustained tracheal occlusion ($p=0.38$; C). In each behavior assessed, there was a statistically significant effect of side, in that the amplitude of ipsilateral EMGs were less than the amplitude of contralateral EMGs ($p<0.001$ spontaneous breathing; $p=0.004$ spontaneous sighs; and $p=0.007$ sustained tracheal occlusion). Dashed lines correspond with EMG amplitudes in intact animals shown for reference. All data were analyzed with a two-way repeated measures ANOVA with independent factors being folate dose and side as the repeated measure. Bars denote means ± 1 SEM. * $p<0.01$
Figure 5-2. Continued
Figure 5-3. Raw root-mean-squared EMG amplitudes during spontaneous breathing, spontaneous sighs, and sustained tracheal occlusion after chronic C2Hx. There was no effect of attempted folate manipulation in any of the respiratory behaviors assessed, spontaneous breathing (p=0.74; A), spontaneous sighs (p=0.78; B), and sustained tracheal occlusion (p=0.64; C). In each behavior assessed, there was a statistically significant effect of side, in that the amplitude of ipsilateral EMGs were less than the amplitude of contralateral EMGs (p=0.0043 spontaneous breathing; p=0.001 spontaneous sighs; and p=0.03 sustained tracheal occlusion). Dashed lines correspond with EMG amplitudes in intact animals for reference. All data were analyzed with a two-way repeated measures ANOVA with independent factors being folate dose and side as the repeated measure. Differences were considered significant if p<0.05. Bars denote means ± 1 SEM. * p<0.01 and + p<0.05.
Figure 5-3. Continued
Figure 5-4. Raw root-mean-squared EMG amplitudes during spontaneous breathing normalized to spontaneous sighs. EMG amplitudes during spontaneous breathing were normalized as percent of spontaneous sighs. After sub-acute C2Hx (A), there was no effect of attempted folate manipulation on normalized EMG amplitude (p=0.39). However, there was a significant effect of side in that the amplitude of normalized ipsilateral EMGs were less than the amplitude of normalized contralateral EMGs after sub-acute C2Hx (p<0.0001). After chronic C2Hx (B), there was no effect of attempted folate manipulation (p=0.65) or injury (p=0.15) on normalized diaphragm EMG amplitudes (B). Dashed lines correspond with EMG amplitudes in intact animals for reference. All data were analyzed with a two-way repeated measures ANOVA with independent factors being folate dose and side as the repeated measure. Differences were considered significant if p<0.05. Bars denote means ± 1 SEM. * p<0.01
Figure 5-5. Serotonergic boutons within 50µm radius around the phrenic motor nucleus after sub-acute C2Hx. Representative images (20x) of serotonergic innervation of the ipsilateral (left) and contralateral (right) phrenic motor nucleus in a folate control rat after sub-acute C2Hx. Cholera toxin B fragment (green; A and D) expression in retrogradely labelled phrenic motor neurons, serotonin immunofluorescence (red; B and E), and merged imaged of CtB and serotonin (C and F). There was no effect of attempted folate manipulation on any of the immunohistochemistry parameters. However, there was an effect of side. The total area of serotonin immunolabeling was significantly greater around the ipsilateral phrenic motor nucleus compared to the contralateral phrenic motor nucleus in each group (p<0.0001; G). There was no side effect on the number of serotonergic boutons (p= 0.89; H). The area of each bouton was greater in the boutons ipsilateral compared to those contralateral to injury (p<0.0001; I). The intensity per bouton was significantly less on the ipsilateral boutons in the folate supplemented group compared to contralateral boutons (p<0.0001; J). Dashed lines correspond with serotonin in intact animals for reference. All data were analyzed with a two-way repeated measures ANOVA with independent factors being folate dose and side as the repeated measure. Differences were considered significant if p<0.05. Bars denote means ± 1 SEM. * p<0.01. Scale bar = 100µm.
Figure 5-5. Continued
Figure 5-6. Quantification of area per serotonergic boutons within 50um radius around phrenic motor neurons after chronic C2Hx. Representative images of serotoninergic innervation of the ipsilateral (left) and contralateral (right) phrenic motor nucleus in a folate control rat after chronic C2Hx. Cholera toxin B fragment (green; A and D) expression in retrogradely labelled phrenic motor neurons, serotonin immunofluorescence (red; B and E) and merged image of CtB and serotonin (C and F). There was no effect of attempted folate manipulation on any of the immunohistochemistry parameters. The total area of serotonin immunolabeling was significantly greater around the ipsilateral phrenic motor nucleus compared to the contralateral phrenic motor nucleus in each group (p<0.0001; G). There was no side effect on the number of serotonergic boutons (p=0.86; H). The area of each bouton was greater in the boutons ipsilateral compared to those contralateral to injury in each group (p<0.0001; I). The intensity per bouton was significantly less in ipsilateral boutons in each group compared to contralateral boutons (p<0.0001; J). Dashed lines correspond with serotonin in intact animals for reference. All data were analyzed with a two-way repeated measures ANOVA with independent factors being folate dose and side as the repeated measure. Differences were considered significant if p<0.05. Bars denote means ± 1 SEM. * p<0.01. Scale bar = 100µm.
Figure 5-6. Continued
Figure 5-7. Folate and homocysteine levels in rat serum. Folate (A) and homocysteine (B) serum levels were assessed in folate deprived, control, and supplemented rats prior to C2Hx and at the terminal time points of 2 and 8 weeks post-C2Hx. There was no effect of attempted folate manipulation on the levels of folate or homocysteine in rat serum prior to C2Hx (p=0.47; p=0.39 respectively), 2 weeks post-C2Hx (p=0.15; p=0.52 respectively), and 8 weeks post-C2Hx (p=0.83; p=0.79). There was a significant effect of C2Hx for both folate and homocysteine serum levels (p<0.0001). Dashed lines correspond with serum folate and homocysteine levels in intact rats for reference. All data were analyzed for the time points prior to C2Hx, 2 and 8 weeks post-C2Hx separately using a one-way ANOVA with the independent variables being folate manipulation (deprived vs. control vs. supplemented), and using a two-way repeated measures ANOVA with independent variables being folate manipulation (deprived vs. control vs. supplemented) and time (pre- vs. post-C2Hx) as the repeated measure. Differences were considered significant if p<0.05. Bars denote means ± 1 SEM. * p<0.01
Figure 5-7. Continued
 CHAPTER 6  
SUMMARY AND FUTURE DIRECTIONS

Cervical spinal cord injuries (cSCIs) cause profound respiratory impairment, often requiring mechanical ventilation (Winslow and Rozovsky, 2003). Along with reduced breathing ability, people with cSCIs loose upper limb function and have a high prevalence of sleep apnea (Anderson, 2004; Fuller et al., 2013). cSCI disrupts brain and brainstem inputs, including descending serotonergic projections to spinal motor nuclei. Serotonin modulates motor neuron excitability, and underlies the capacity for spinal motor plasticity (Baker-Herman and Mitchell, 2002). Thus, it is important to understand how serotonergic innervation of spinal motor nuclei is altered after cSCI to be able to maximize benefits from serotonin-linked therapeutic interventions. The aims of this dissertation were to understand serotonergic innervation of spinal motor nuclei involved in key motor functions, with emphasis on breathing, after chronic incomplete cSCI. I also investigated the potential of two novel therapeutic strategies: intermittent hypoxia and folate supplementation. These treatments were predicted enhance serotonergic reinnervation of motor nuclei, providing the necessary substrate for serotonin-dependent respiratory motor plasticity after cSCI. By harnessing such plasticity, we hoped to recover at least some lost breathing ability, preventing the need for ventilatory support with the attendant loss of independence.

In Chapter 2, I investigated the effects of cSCI on serotonergic innervation of phrenic and intercostal motor nuclei. Spontaneous serotonin re-innervation 12 weeks after C2Hx was enhanced in phrenic and nearly complete in intercostal motor nuclei, demonstrating the potential for serotonin-dependent motor plasticity. The number of ipsilateral serotonergic structures around motor neurons was reduced, but their size
was enlarged, leading to full recovery of total serotonergic innervation area within the phrenic motor nucleus. The nearly complete serotonergic innervation was presumed to be due to the spouting and hypertrophy of spared serotonin terminals since C2Hx did not affect serotonin crossing the cervical commissure. However, contrary to predictions, none of the IH protocols studied had any effect on serotonergic innervation of phrenic or intercostal motor nuclei 12 weeks post-C2Hx.

In Chapter 3 I focused on serotonergic innervation of brainstem hypoglossal motor nuclei rostral to injury since they play a key role in regulation of upper airway patency during breathing. Daily “low-dose” AIH enhanced serotonergic innervation of hypoglossal motor nuclei in both uninjured and C2Hx rats, but there was no C2Hx effect on serotonergic innervation of hypoglossal motor nuclei. This finding may suggest the potential for repetitive AIH to increase serotonin-dependent XII motor plasticity, as has been reported in prior studies (Wilkerson and Mitchell, 2009; Zabka et al., 2003).

In another analysis, recovery of serotonergic innervation in axial and forelimb cervical motor nuclei was investigated (Chapter 4). Overall serotonin innervation was reduced after C2Hx but IH had no effects.

Lastly, in Chapter 5, I investigated the effects of folate supplementation and deprivation on serotonergic innervation of phrenic motor nuclei and diaphragm function after sub-acute and chronic C2Hx. To our surprise, serum folate levels were unchanged by the manipulation methods used, despite the fact that these same doses and delivery routes were reported to have functional effects in other studies. However, serum folate levels are seldom reported in the literature, emphasizing the need to verify the efficacy of attempts to manipulate folate levels. On the other hand, folate levels were increased
after C2Hx. Accordingly, without effective folate changes, neither serotonergic innervation of phrenic motor neurons nor diaphragm EMG activity were altered by attempted folate manipulations. However, in this study, we do verify that ipsilateral serotonergic structures around phrenic motor neurons were larger 2 and 8 weeks post-C2Hx, presumably a direct effect of the injury and serotonergic neuron axotomy.

**Serootonin Innervation Around Motor Neurons After Cervical Spinal Injury**

**Phrenic Motor Nuclei**

Taken together, these studies demonstrated substantial serotonergic reinnervation of the phrenic motor nuclei at 2, 8, 12, and 20 weeks post-injury (Chapters 5, 2, and Appendix A respectively). At all times studied, there were significant increases in serotonergic structure size ipsilateral to C2Hx. Previous studies in our lab reported significant reduction in serotonergic innervation of ipsilateral phrenic motor neurons 2 weeks post-C2Hx, with partial spontaneous reinnervation 8 weeks post-C2Hx (Golder and Mitchell, 2005). In Chapter 5, I did not observe reductions in serotonergic innervation of phrenic motor nuclei 2 weeks post-injury; instead, I observed increased serotonin structure size. However, in the published images of this earlier study, serotonin terminals do indeed appear larger after C2Hx versus intact cervical rats (Golder and Mitchell, 2005), consistent with our results. Because of the larger but fewer structures post-injury, I report increased serotonergic immunolabeling near phrenic motor neurons 2 to 20 weeks post-C2Hx.

One possibility as to why this study did not have reductions in serotonin 2 weeks post-injury could be the size of the region of interest quantified. In the Golder study (Golder and Mitchell, 2005), the size of the region of interest surrounding phrenic motor neurons was not stated, and only some sections contained back-labeled for phrenic
motor neurons; some sections were described as “in the region where phrenic motor
neurons should be located” (Golder and Mitchell, 2005). Since the region of interest
used by Golder may have encompassed more than the phrenic motor nucleus, overall
innervation may have appeared reduced (Golder and Mitchell, 2005). Another difference
is that, Golder and Mitchell quantified serotonin in C4 (Golder and Mitchell, 2005),
whereas we quantified serotonin from C3 to C5. Although not well studied, it is possible
that serotonin exhibits differential innervation at different spinal levels.

It is also possible that differences in rat strain or substrain (Baker-Herman et al.,
2010; Fuller et al., 2001a; Golder and Mitchell, 2005; Golder et al., 2005), or other
factors such as normal diet or genetic variations, could account for the difference in
results. A commonality in these studies is the breed of rat used – Sprague Dawley.
Golder and Mitchell investigated the difference in serotonin innervation of phrenic motor
nuclei in both Sprague Dawley (inbred) and Lewis (outbred) rats. Although there was a
trend towards less serotonin in Lewis versus Sprague Dawley rats, the trend towards
reduced serotonin after C2Hx and partial spontaneous reinnervation was similar in both
(Golder and Mitchell, 2005). Thus, differences in serotonin innervation 2 weeks post-
C2Hx in this study versus previously published data is unlikely to be due to differences
among rat strains.

Overall, I conclude that increased in serotonin bouton size is characteristic of
serotonergic innervation in the phrenic motor nucleus ipsilateral to injury, likely due to
terminal sprouting from spared crossed-spinal serotonergic projections. In other words,
spared serotonergic terminals sprout quickly after injury, stabilizing serotonergic
innervation and providing the necessary substrate for serotonin-dependent plasticity.
The majority of serotonin in the spinal cord originates from raphe neurons in the brainstem and sends bilateral projections throughout the spinal cord (Azmitia, 1999; Skagerberg and Björklund, 1985). C2Hx itself severs these descending projections, removing spinal serotonin ipsilateral to injury. Because C2Hx is a lateral injury, contralateral spinal serotonergic projections remain intact and sprout to reinnervate the structures investigated.

To support this concept, I assessed crossing serotonergic projections in the cervical spinal cord. This analysis included serotonin around the commissure of central canal. Although little is known on the extent and exact locations of spinal serotonin decussations, they likely occur in the central commissure of the gray matter near the central canal. Serotonin projects down the spinal cord in ventrolateral and medial tracts that have regional neural innervation (Skagerberg and Björklund, 1985); however, since the spinal cord is not continuous on its ventral surface, this dissertation assumes crossing projections occur in the gray matter around the central canal (layer X). In Chapter 2, I found that there were no differences in crossing serotonin fibers of C2Hx rats versus spinally intact rats, supporting the notion that recovering serotonin innervation of ipsilateral phrenic motor nucleus results from sprouting of spared serotonin axons versus long distance regeneration.

**Intercostal Motor Nuclei**

After observing the large increase in serotonergic innervation of the phrenic motor nuclei, I was interested in another important respiratory motor pool, the intercostal motor nuclei, and whether C2Hx would have the same effects on serotonin structures. The intercostal motor nuclei are in the thoracic spinal cord, further caudal from the injury site, and express robust serotonin-dependent plasticity (Fregosi and Mitchell, 1994;
Navarrete-Opazo and Mitchell, 2014a). Serotonergic boutons that innervate intercostal motor nuclei are also enlarged 12 weeks post injury (Chapter 2), suggesting serotonergic fiber sprouting near intercostal motor nuclei, similar to those phrenic motor nucleus reinnervation. Collectively, these findings suggest the potential for robust serotonin-induced plasticity and functional recovery with time post-cSCI in Sprague Dawley rats.

**Significance of Enlarged Serotonergic Structures**

Although I did not test function of the enlarged serotonin structures, I hypothesize that they are functional and able to release serotonin with raphe neuron activity. Serotonin terminals are reported to increase in number and lengthen their synaptic active zones after C2Hx (Tai et al., 1997). In other circumstances swollen serotonin terminals have been observed, such as advancing age (Behan and Brownfield, 1999; Behan et al., 2002; Ko et al., 1997), injury (Mamounas et al., 2000; Mamounas et al., 1995; Tai et al., 1997; White et al., 1985), neurodegeneration, and neurotoxins (Azmitia, 1999; Azmitia et al., 1978; Azmitia and Nixon, 2008; Azmitia et al., 2011; van Luijtelaar et al., 1989). In addition, bouton swelling may result from innate changes in synthesis, reuptake, storage, receptor expression or cell death (Behan and Brownfield, 1999).

Since serotonin levels correlate with spontaneous functional recovery after SCI (Hashimoto and Fukuda, 1991), and the injured cord is capable of serotonin dependent phrenic motor plasticity (Gonzalez-Rothi et al., 2015b), I suspect these large serotonin terminals around phrenic and intercostal motor neurons to be functional.

**Axial and Forelimb Motor Nuclei**

Larger serotonin structures after injury were observed in serotonergic projections that innervated the phrenic and intercostal motor neurons, but not axial or forelimb
motor nuclei (Chapter 4). The axial motor nuclei are heavily innervated with serotonin, and are involved in both respiratory (i.e. accessory respiratory muscles such as scalenus) and non-respiratory functions (i.e. posture) (Pilarski et al., 2019; Shinozaki et al., 2019). Since I cut through the muscles on the back of the neck to perform C2Hx, axial muscles were cut, limiting our ability to make robust conclusions to some extent. Since C2Hx diminishes forelimb function (Gonzalez-Rothi et al., 2015c), and spinal serotonin levels correlate with improvements in locomotor function after SCI (Hashimoto and Fukuda, 1991) we also investigated motor nuclei associated with forelimb function. In both motor pools, there were reduced serotonin terminal numbers and total serotonin immunolabeling area after C2Hx since enlarged terminals/structures were not observed.

One possibility as to why serotonin terminals are enlarged in phrenic and intercostal but not axial or limb motor nuclei is that serotonin innervation is activity dependent. Breathing is a constantly active behavior, which requires the repeated activation of respiratory motor neurons, specifically the phrenic. Since serotonin modulates motor neuron activity, these constantly active neurons may be releasing factors, like neurotrophins, that increases serotonergic innervation near them. Since breathing is a necessary behavior for life, there may be innate protective factors in place to ensure that these necessary motor neurons function properly

**Hypoglossal Motor Nuclei**

The hypoglossal motor nuclei were investigated in Chapter 3 given their role in regulating upper airway tone on a breath to breath basis, and potential contributions to sleep apnea, a frequent condition in people with cSCI (Berlowitz et al., 2005; Dempsey et al., 2010; Fregosi, 2011; Sankari et al., 2014a; Sankari et al., 2019). This motor pool differs from the spinal motor pools investigated in this dissertation because it is located
in the medulla (rostral to injury) versus spinal cord (caudal to injury). Serotonergic projections that innervate the hypoglossal motor neurons arise from the caudal raphe nucleus (Barker et al., 2009), and are often the very same raphe serotonergic neurons innervating the phrenic motor nucleus (Holtman et al., 1984). Although XII serotonergic projections are rostral to the spinal injury and they are not directly severed by C2Hx, they may be profoundly affected by axotomy in their spinally projecting collaterals. In Chapter 3, I hypothesized that serotonergic innervation of the hypoglossal motor neurons would change after injury due to the compensation that occurs in the spinal cord. Previous studies have reported changes in the brainstem (Bezdudnaya et al., 2017), including hypoglossal activity (Golder et al., 2001), after SCI. In this dissertation I did not observe changes in serotonergic overall innervation, number, or size of structures around hypoglossal motor nuclei 12 weeks post-C2Hx. I speculate that previous reports of changes in the brainstem due to SCI may be a result of mechanisms independent of serotonin.

**Serotonin Contributions to Intermittent Hypoxia Induced Plasticity**

**Serotonin Dependent Phrenic Plasticity after Chronic C2Hx**

Important to this dissertation are two pathways of IH induced respiratory plasticity, the Q and S pathways. Typically, the Q pathway is referred to as the serotonin-dependent pathway and the S pathway is referred to as the adenosine-dependent pathway (Dale-Nagle et al., 2010a), although some serotonin receptors are Gs protein coupled (e.g. 5-HT7 (Hochman et al., 2001)) and activate the S pathway to phrenic motor facilitation. Acutely after SCI when serotonin is reduced, the S-pathway to plasticity dominates (Navarrete-Opazo et al., 2015). After chronic SCI when spinal serotonin is recovered, the Q pathway to plasticity dominates (Golder and Mitchell,
2005; Navarrete-Opazo et al., 2017b; Navarrete-Opazo et al., 2015). However, I found that serotonergic innervation is not reduced 2 weeks post-C2Hx. Contrasting to previous reports, this dissertation reports serotonergic innervation is increased at 2 weeks and remains enhanced up to 20 weeks post-injury. These findings suggest that the shift from the S to Q pathway after injury may be due to spinal inflammation. Inflammation, such as that resulting from SCI, undermines Q pathway plasticity (Huxtable et al., 2015). Thus, the S pathway may dominate acutely after SCI due to SCI inflammation, and with time post-injury, as inflammation is stable, the Q pathway dominates.

Although serotonergic innervation to ipsilateral phrenic motor neurons was recovered 2 weeks post-injury, there was no recovery of ipsilateral diaphragm function during baseline breathing (Chapter 5). Thus, serotonin reinnervation of phrenic motor nuclei is not sufficient to elicit baseline breathing recovery, however it may be involved in plasticity. After injury serotonin innervation is available for serotonin targeted therapies that enhance breathing function such as AIH induced serotonin-dependent plasticity (Q pathway). Though, having the monoamine available for use is only half of the equation; serotonin receptors need to be available for a response to occur.

In parallel studies, my lab investigated changes in receptors known to be necessary for the Q and S pathways, respectively. In these parallel studies using the same IH protocols in spinally intact rats and 12 weeks after C2Hx (unpublished data, manuscripts in preparation). These receptors include 5-HT2a, 5-HT2b (Q pathway), 5-HT7, A2a, and A1 (S pathway). In uninjured rats, none of the IH protocols studied in this dissertation affected any of the receptors of interest. However, there were IH protocol effects on 5-HT2a, 5-HT7, and A2a receptors after C2Hx. C2Hx, exposure to IH28-2/2
upregulated 5HT2a receptor expression compared to C2Hx rats exposed to Nx28. 5-HT7 receptor expression was upregulated in rats after C2Hx who were exposed to IH28-5/5 and IH28-2/2 compared to C2Hx rats exposed to Nx28. Lastly, there was a stepwise increase in A2a receptor expression (Nx28 < dAIH28 < IH28-5/5 < IH-28-2/2) with IH28-2/2 having the most significant effect after C2Hx. There were no effects of IH on 5-HTb or A1 receptors after injury; however, A1 receptor expression was reduced in rats with C2Hx compared to spinally intact rats. The unpublished data on serotonin and adenosine receptor expression suggests that the S pathway is dominating after chronic C2Hx and exposure to IH28-2/2. IH28-2/2 is thought to be neuroinflammatory and associated with sleep apnea. This pathway may be dominant after chronic SCI because of more adenosine accumulation at the phrenic motor nucleus. In addition, pericytes are known to increase capillary constriction after SCI (Li et al., 2017). Therefore, it is possible that the IH protocols in this dissertation resulted in greater tissue hypoxia than expected, presumably shifting the balance towards the adenosine dependent S pathway.

I investigated anatomical changes in serotonergic innervation of phrenic (and other) motor neurons after C2Hx but did not investigate the functional aspects of this enhanced innervation. The availability of serotonin at the ipsilateral phrenic motor nucleus lays the foundation for enhanced capacity for recovery of serotonin-dependent plasticity. In a parallel study investigating the same IH protocols, ipsilateral pLTF 12 weeks post-C2Hx was equal (or even larger) than in spinal intact rats when normalized to baseline values. This finding suggests that recovered/increased serotonergic innervation of the phrenic motor nucleus was sufficient to elicit serotonin-dependent
pLTF (Gonzalez-Rothi et al., unpublished). These data are consistent with the idea that larger serotonergic structures post-C2Hx are indeed functional. dAIH28 further enhanced ipsilateral pLTF, suggesting that the larger boutons lay the foundation for enhanced LTF with serotonin-dependent IH therapy.

**Intermittent Hypoxia Effects on Serotonin in Spinal and Brainstem Motor Nuclei**

This dissertation identified variations in serotonin innervation in response to various IH protocols and spinal injury. Most strikingly was the change in serotonin innervation of phrenic motor nuclei, caused by C2Hx (Chapter 2). Serotonergic innervation in spinal intact rats after CIH are consistent with previous reports of CIH induced, serotonin dependent phrenic motor plasticity in intact rats (Ling et al., 2001). dAIH28 did not enhance serotonergic innervation of phrenic motor nuclei, in apparent contrast with previous studies from our laboratory that reported rAIH enhanced serotonergic innervation of the phrenic motor nucleus in spinally intact rats (Satriotomo et al., 2012). In that earlier study, a larger region of interest was quantified: a square with lengths of 200 µm resulting in a total area of 40,000 µm². Our circular area of interest had a radius of 50 µm, with an area of about 7,850 µm². Satriotomo et al. quantified C4 and C5 (Satriotomo et al., 2012) whereas I quantified C3-5. Further, their rAIH protocol was 3 times per week for 10 weeks. Thus, there was a different pattern of presentation spread over a longer time frame (2.5 versus 1 month; 28 continuous days of AIH, mild and moderate CIH versus AIH 3x/week). It is important to understand differences in IH protocols and optimize specific protocols for spinally intact and injured applications.

Similar to phrenic motor nuclei, intercostal and hypoglossal nuclei elicit serotonin dependent plasticity (Bach and Mitchell, 1996; Fregosi and Mitchell, 1994; Navarrete-
Locomotor nuclei also exhibit putative serotonin dependent functional plasticity (Lovett-Barr et al., 2012; Prosser-Loose et al., 2015). Although not necessary to explain their findings, we not report changes in serotonergic innervation of intercostal, axial, or forelimb motor nuclei after exposure to any IH protocol. Rather our data demonstrate that sufficient reinnervation occurs to potentially support serotonin-based functional recovery after cSCI.

In the hypoglossal motor nuclei, I report enhanced serotonergic innervation after dAIH28 exposure in both intact and injured rats. This observation could explain a previous report that 7 consecutive days of dAIH elicits hypoglossal plasticity in a strain of rats with normally low LTF expression (Wilkerson and Mitchell, 2009). During sleep, raphe serotonin activity is reduced contributing to a collapsible airway (Veasey, 2003; Veasey et al., 1996). Increasing serotonin to hypoglossal motor neurons may help stabilize airway patency in people with sleep apnea by phasic and tonic activation of hypoglossal motor neurons during sleep (Cao et al., 2010). Importantly, this data supports AIH as a potential therapy for sleep apnea in people with chronic SCI, by harnessing serotonin-dependent mechanisms to elicit hypoglossal plasticity.

Optimization of Intermittent Hypoxia Protocol

The results of this dissertation show serotonergic reinnervation of phrenic motor nuclei with chronic SCI. Since serotonin is available after SCI, we can utilize serotonin dependent based therapies to increase breathing function after cSCI, such as AIH. However, there is a need to optimize AIH protocols. The studies in this dissertation were a first step towards AIH optimization because it compared four different IH protocols in spinal intact and injured groups: 1) dAIH28, 10 5 minute episodes of 10.5% O\textsubscript{2} interspersed with 21% O\textsubscript{2} every day for 4 weeks; 2) IH28-5/5, 5 minute episodes of
10.5% O2 interspersed with 21% O2, 8 hours a day, every day for 4 weeks; 3) IH28-2/2, 2 minute episodes of 10.5% O2 interspersed with 21% O2, 8 hours a day, every day for 4 weeks; and 4) rAIH, 10 5 minute episodes of 10.5% O2 interspersed with 21% O2, 4 times a week for 3 months.

Although the IH protocols studied did not have significant differences in serotonergic innervation of the phrenic motor nuclei, they may have elicited other effects not investigated in this dissertation. For example, I did not study inflammatory components or adenosine, factors involved in inhibiting the Q pathway. The ideal IH protocol to use for treatment after SCI would be one that elicits the largest amount of plasticity with the least amount of spinal inflammation. In other words, the optimal therapy would have the shortest exposure time to IH. Among many changes after SCI, spinal vasculature is altered after SCI resulting in more pericyte constriction of capillaries (Li et al., 2017). As mentioned in the section above, this increased constriction reduces the amount of oxygen delivered to the tissue. Thus, the hypoxia being delivered during AIH may be more hypoxic than expected. Since AIH elicits plasticity by stimulating the carotid body with low oxygen, shorter hypoxia episodes may be more beneficial after chronic SCI. In fact, in human studies of IH after SCI, the IH protocol used is 15 episode 1-2 minutes of 11% O2 for up to 5 days (Hayes et al., 2014; Trumbower et al., 2017; Trumbower et al., 2012). In rats, short exposures of just 45 seconds elicits profound serotonin dependent pLTF (Mahamed and Mitchell, 2008). Thus, we are currently investigating spinal tissue oxygen levels in intact and cSCI rats during IH exposures, and the effects of shorter hypoxic episodes on respiratory plasticity after chronic SCI.
Repeated AIH appears safe for rats and humans, with no evidence of pathology such as hippocampal death or reactive gliosis (Gonzalez-Rothi et al., 2015b; Lovett-Barr et al., 2012). Our lab tested the safety of rAIH after chronic cSCI and found no pathological consequences of rAIH on organs including the liver, bone, heart, etc. (Gonzalez-Rothi et al., unpublished). In addition, in a collaboration with the Foster laboratory in the Department of Neuroscience, I tested the hypothesis that repeated AIH (10 episodes of 2 minutes of 10.5% O₂ interspersed with 2 minutes of 21% O₂ for 5 consecutive days with 2 days off for 4 weeks) does not impact memory in aged rats with and without systemic inflammation. I found that AIH does not impair spatial memory in a water maze, novel location recognition, or inhibitory avoidance in old rats with and without systemic inflammation (Barter et al., unpublished). Collectively, these results verify that repetitive AIH is safe. Shorter hypoxic episodes may be the most beneficial to elicit serotonin dependent functional recovery and plasticity after chronic SCI.

**Folate Contributions to Functional Recovery and Serotonin Innervation after Spinal Injury**

Although previous studies report supplemental folate enhances neural regeneration and locomotor recovery after SCI (Iskandar et al., 2004; Iskandar et al., 2010; Miranpuri et al., 2017), we are unable to draw conclusions concerning the role of folate in Chapter 5. Neither folate supplementation via injections nor deprivation via diet had any documented impact on circulating (serum) folate levels. Thus, our findings that these manipulations have no impact on breathing ability or serotonergic innervation of phrenic motor nuclei were to be expected. Since folate is involved in serotonin synthesis, exploring the effects of more effective folate manipulations on the amount of serotonin within these sprouting terminals needs further investigation.
In this dissertation I report levels of folate and homocysteine in rat blood serum, which did not change due to folate manipulation. I did show that C2Hx alone increases serum folate levels and reduces plasma homocysteine, both 2 and 8 weeks post-injury. Understanding the effects of folate interventions is necessary to interpret results and understand the efficacy of treatment. However, many studies on folate's ability to regenerate axons and improve function after SCI do not report folate or homocysteine levels. Thus, there is a need for transparency in future publications investigating drug interventions.

Future Directions

Future studies should address the functional significance of fewer, enlarged serotonergic boutons after cSCI. Further, there is a need for greater understanding of factors that restore ipsilateral serotonergic innervation so quickly post-injury, such as detailed understanding of mechanisms leading to sprouting of crossed-spinal serotonergic fibers near phrenic motor neurons after cSCI.

Once we have greater understanding of serotonergic reinnervation below a spinal injury, future studies should address therapies that explore restored or even increased serotonergic innervation to enhance breathing and other somatic motor behaviors after cSCI. Understanding anatomical changes and functions of spinal serotonergic projections after cSCI may help guide therapeutic approaches to enhance function, and overall quality of life, for people living with SCI.

I propose experiments to address the significance of more distant but enlarged structures, including the timing of transient loss and spontaneous re-innervation. How quickly serotonin innervation is lost in the phrenic motor nucleus after C2Hx is unknown. These experiments would investigate serotonergic innervation of phrenic motor nuclei at
various time points after C2Hx from hours to months. Experiments are important to test the hypothesis that crossed spinal serotonin projections are sprouting, the molecules triggering that response, and their ability to restore normal serotonergic function.

Moving forward with anatomical examination of serotonin boutons after C2HX, it is important to use more reliable measurements. Immunofluorescence is a qualitative measurement with subjective interpretations at multiple steps such as antibody dilutions, exposure times during image acquisition, and thresholds during analysis to name a few. Future directions of this dissertation include utilizing electron microscopy techniques to investigate the number and size of serotonin terminals as well as the amount of synaptic and non-synaptic connections on motor neurons. This technique would allow precise identification of the anatomical changes of serotonin terminals after C2Hx. Identifying the amount and location of the serotonin transporter (SERT) on the presynaptic terminal will also provide information on the extent of volumetric transmission from enlarged terminals after chronic SCI. If SERT is farther from the synapse after injury, it would indicate that serotonin molecules are diffusing away from the synapse to bind to non-synaptic receptors (Murphy et al., 2004).

Another gap in this dissertation is that it does not address the functionality of enlarged serotonergic boutons. Thus, an important future direction of this study is to test the functional aspects of the serotonergic boutons that are innervating the phrenic motor nucleus after chronic C2Hx. One experiment to address this would be to measure serotonin levels in the ventral spinal cord, near the phrenic motor nucleus, using microdialysis assays (Gerin et al., 2010). Other methods such as high performance liquid chromatography measure 5-HT in the ventral spinal cord (Hashimoto and Fukuda,
1991; Mitchell et al., 2000). Using these techniques, I would determine the concentration of serotonin being released from the remaining boutons to understand their function in spinal intact rats and after C2Hx. In addition, investigating serotonin dependent phrenic activities, such as pLTF or diaphragm EMG recordings, would also provide functional information on enlarged serotonergic boutons after injury.

I would also like to test the hypothesis that the enhanced serotonergic innervation of respiratory motor neurons after SCI is due to sprouting of spared contralateral projections. To do this, I propose to use adeno-associated viruses (AAV) to trace serotonergic projections from the caudal raphe throughout the spinal cord after C2Hx (Skagerberg and Björklund, 1985). Methods for this experiment have been troubleshooting in our lab (Nair et al., unpublished). In short, AAVs conjugated to different fluorophores will be injected to either sides of the medulla targeting the caudal raphe nuclei. Once the virus has integrated into the cells (~4weeks) I will perform a C2Hx. Immediately after C2Hx I predict a significant loss of ipsilateral serotonergic projections caudal to C2Hx, determined by loss of respective ipsilateral AAV-conjugated fluorophore immunolabeling. In a series of experiments at multiple stages post-injury I would identify the time course of spontaneous serotonergic innervation of ipsilateral motor nuclei caudal to injury. With these experiments I would be able to determine if the spontaneous re-innervation was due to contralateral or ipsilateral raphe projections.

I am also interested in understanding the role of serotonergic sprouting in the spinal cord after other types of spinal injury and how this would affect breathing and plasticity. For example, serotonin in the spinal cord can be reduced through the use of neurotoxins such as p-chloroamphetamine or 5,7-dihydroxytryptamine. Other
interventions include p-chlorophenylalanine which reduces serotonin levels, or reserpine which blocks the vesicular monoamine transporter (Hashimoto and Fukuda, 1991; Mamounas et al., 2000; Mamounas et al., 1995). Previous studies using these drugs report enlarged projections of serotonergic neurons as they spontaneously recover. In addition, these interventions are known to reduce other monoamines. Interestingly, noradrenaline and dopamine are also important for functional recovery after spinal injury. Spinal levels of noradrenaline and dopamine correlate with function and recovery after SCI (Hashimoto and Fukuda, 1991; Mitchell et al., 2000). I am also interested in the innervation patterns of noradrenaline and dopamine to motor neurons, specifically the phrenic motor neurons, after chronic spinal injury.

The studies included in this dissertation are important for future work concerning serotonin targeted therapies to enhance respiratory and non-respiratory function and plasticity after cSCI. This thesis demonstrates increased spontaneous innervation and sprouting of serotonin projections to phrenic motor nuclei 2, 8, 12, and 20 weeks after C2Hx. Understanding serotonin innervation of motor nuclei after cSCI is important to refine therapeutic strategies to improve breathing and other functions after cSCI.
APPENDIX A
ENHANCED SEROTONERGIC RE-INNERRVATION OF IPSILATERAL PHRENIC MOTOR NUCLEI AFTER CHRONIC CERVICAL SPINAL CORD INJURY, BUT NOT LONG-TERM REPETITIVE ACUTE INTERMITTENT HYPOXIA

Overview
After a cervical spinal cord injury (cSCI), serotonin innervation to the ipsilateral phrenic motor pool is disrupted. Previous reports show that 8 weeks after injury serotonin partially re-innervates the phrenic motor nucleus (Golder and Mitchell, 2005). Serotonin is a critical modulator for acute intermittent hypoxia induced spinal respiratory motor plasticity (Bach and Mitchell, 1996). Repetitive acute intermittent hypoxia (rAIH) increases serotonergic innervation of the phrenic motor nucleus in spinally intact rats (Satriotomo et al., 2012). The impact of prolonged rAIH on serotonergic innervation below a cSCI has not been investigated. We tested the hypothesis that prolonged rAIH exposure, 3 months post-injury, stimulates sprouting of serotonergic neurons around phrenic motor neurons 20 weeks after cSCI.

Methods
All experiments were conducted with adult male (350-450 g) Sprague-Dawley rats (Colony 217, ENVIGO Laboratories). Rats were housed in pairs in a controlled environment (12 hour light/dark cycles) with food and water ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida. A total of 30 rats were studied (11-12 weeks of age). Spinally intact and C2 hemisection (C2Hx) rats were randomly assigned to intermittent hypoxia exposure or normoxia control groups at 8 weeks post-injury and exposed 4 times a week for 12 weeks. Experimental groups with rat numbers were: 1) spinal intact +
Nxormoxia (Nx; n=8); 2) spinal intact + rAIH (n=6); 3) C2Hx + Nx (n=7); 4) C2Hx + rAIH (n=9). Experimental timeline and rAIH protocols are displayed in Figure A-1.

Rats were randomly assigned to the C2Hx group. Anesthesia, C2Hx, and animal care were performed as described previously (Fuller et al., 2008; Gonzalez-Rothi et al., 2015c). Rats were anesthetized with 3.5% isoflurane (in 100% O2) and maintained at 2-2.5% isoflurane (in 100% O2) via nose cone throughout the surgery and confirmed by the absence of toe pinch and palpebral responses. Body temperature was maintained at 36.5–37.5°C with a heating pad. Artificial tears were applied to prevent eye damage. Nails were clipped from forelimbs and hind limbs, and the surgical site was shaved and cleaned (chlorhexidine scrub, 58829-140-01, First Priority, Inc., Elgin, IL). The C2Hx was performed by making a dorsal incision on the neck of the rat and dissecting the muscle to expose the C2 lamina. Following C2 laminectomy and durotomy, the left side of the C2 spinal cord was hemisected with a microknife, caudal to the C2 dorsal roots. The overlying dura was subsequently sutured with 9-0 ethilon nylon suture, the overlaying muscles were sutured with 3-0 Polysorb absorbable suture and the skin was closed with 9 mm stainless steel wound clips. Each rat received post-operative care following surgery which included pain management with an analgesic (buprenorphine, 0.03 mg/kg, s.q., Hospira< IL) and an anti-inflammatory drug (meloxicam, 2 mg/kg., s.q. Portland, ME) given at 12 and 24-hour intervals for 2 days post-surgery. Rats received lactated ringers solution (5 ml 2x/day, s.q.) and were manually fed with a nutritional supplement (Diet Gel Boost; Clear H2O; Westbrook, ME) until adequate volitional drinking and eating resumed.
Starting 8 weeks post-C2Hx, rats received either rAIH or Nx 4 times a week for 12 weeks. For the duration of the exposures, rats were placed in PlexiGlass gas exposure cylinders. Normoxia control rats received continuous gas flow of normoxia (21% O₂, 1.5 hours per day; Figure A-1B). Rats assigned to the rAIH groups received 10, 5 minute episodes of 10.5% O₂ alternating with 5 minute normoxic intervals for a total duration of 1.5 hours/day (10 hypoxic episodes per day; Figure A-1C).

At the end of the study, rats were perfused intracardially with cold 0.1M phosphate buffer saline (PBS), followed by paraformaldehyde (4% paraformaldehyde w/v in 0.1M PBS, pH 7.4). The spinal cord was harvested and post-fixed overnight in paraformaldehyde (4% paraformaldehyde w/v in 0.1M PBS, pH 7.4) and cryoprotected at 4°C in 20% sucrose solution in 0.1M PBS for 3 days, followed by 30% sucrose solution in 0.1M PBS for 3 days. Cervical (C3-C5) spinal segments were sectioned in transverse plane (40µm thickness) using a freezing microtome (Leica SM 200R, Buffalo Grove, IL). Tissues were stored in antifreeze solution at -20°C until processed (30% glycerol, 30% ethylene glycol, 40% 0.1 M PBS, pH 7.4). Every 12th section was selected and stained for serotonin using DAB (3,3’-Diaminobenzidine) immunohistochemistry protocol.

Free-floating sections were washed with 0.1M PBS (pH 7.4), then incubated in heat induced epitope retrieval (TissuePro, Cat#: HIER01-32R) for 30 minutes at 85°C. The sections were then quenched for 1 hour at room temperature. Quenching solution consisted of a mixture containing 30% methanol, 1.5% of 30% H₂O₂, and 68.5% 0.1M PBS (pH 7.4). Tissues were washed again with 0.1M PBS (pH 7.4) and then incubated in a blocking solution (10% normal goat serum (NGS, GeneTex) in 0.1M PBS-Triton.
(0.1%, pH 7.4) at room temperature for 60 minutes. Primary antibody staining was performed by incubating tissue sections in 3% NGS in 0.1M PBS-Triton (0.1%, pH 7.4) with anti-5-HT (1/10,000, rabbit serum, Immunostar #20080) for 60 minutes at room temperature and then 4°C overnight.

The following day, tissues were washed with 0.1M PBS-Triton (0.1%, pH 7.4). Secondary antibody staining was performed by incubating tissue in 3% NGS in 0.1M PBS-Triton (0.1%, pH 7.4) with secondary biotinylated goat anti-rabbit IgG (donkey anti-rabbit 594; 1:500, Jackson ImmunoResearch, Code# 711-036-152) at room temperature for 2 hours. Sections were washed with 0.1M PBS-Triton (0.1%, pH 7.4). Tissue were then incubated in ABC solution for 1 hour at room temperature (Vectastain, Cat# PK-6100). Sections were then washed with 0.1M PBS. Sections were placed in DAB solution (Vector Laboratoroes, Cat# SK-4100) for about 10 seconds. Sections were washed with 0.1M PBS. Tissue was mounted on positively charged slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. The next day tissue was rehydrated with distilled water for 5 minutes, dehydrated with a series of increasing ethanol solution for 5 minutes each (70%, 95%, 100%, 100%), and then dipped in histoclear three times for 5 minutes each. Slides were then coverslipped. Slides were cover slipped with mounting medium (Richard-Allan Scientific Ref# 4112). Serotonin labeled sections were captured using a bright field microscope with 10x and 20x magnification (Keyence BZ-X700, Keyence Corporation of America, Itasca, IL). The sides of the spinal cord were marked prior to tissue sectioning to ensure accurate side determination during imaging and analyses.
Serotonin was quantified using a custom MATLAB (MathWorks, Natick, MA, USA) code. Phrenic motor nuclei location was determined using the well-defined anatomical location within each cervical segment. A point at the center of the phrenic motor pool was selected and a circular region of interest was created with a radius of 50µm. Serotonergic structures were identified within this region of interest around putative phrenic motor nuclei. We quantified the average number of serotonergic structures (number of boutons), average area of immunolabeling per structure (area per bouton), average total area of serotonin-positive immunolabeling (number of boutons multiplied by the area of each bouton; total area), and average intensity of each structure (intensity per bouton). Independent serotonin structures were determined by being larger than 4 pixels and being at least 1 pixel apart. Thresholds were determined using a custom adaptive threshold algorithm in MATLAB as previously described (Allen et al., 2019). The adaptive threshold was calculated by constructing a pixel intensity histogram from the image. The pixel intensity corresponding to the 99th percentile was selected as the adaptive threshold. Groups remained blinded throughout analyses.

Statistical calculations were performed using JMP 11.0 (SAS Institute, Cary, NC). Serotonin immunofluorescence data were analyzed using a Mixed Model with repeated measures. The independent variables were injury (intact vs. C2Hx), protocol (Nx vs. rAIH), and spinal cord side relative to injury (left/ipsilateral vs. right/contralateral as the repeated measure). A significance level of 0.05 was set for all statistical comparisons. When significant differences were observed, individual comparisons were made based on the Fisher Least Significance Difference post hoc test. All data are displayed as mean ± standard error of the mean.
Results

Serpottin labeling was present in spinal sections of each group (Figure A-2). Using 20x images, we assessed serotonergic innervation within a 50µm radius of putative phrenic motor neurons. Upon first observance, there were no obvious differences in serotonin innervation between groups. However, in the C2Hx groups, the DAB positive structures innervating the ipsilateral motor pools appeared larger.

We first quantified the number of serotonergic boutons (Figure A-3). There were no significant effects of rAIH or C2Hx on the number of serotonergic structures innervating the putative phrenic motor nucleus. Next, we quantified the average area of each bouton innervating the putative phrenic motor nucleus (Figure A-4). There were no significant effects of rAIH on the area of each bouton, but there was a significant effect on the interaction of injury and side (p=0.0158). Regardless of rAIH or Nx, the area of each bouton was significantly larger ipsilateral to C2Hx compared to boutons in the left putative phrenic motor pool of spinally intact groups (p=0.007).

Next, we quantified the total area of serotonergic innervation within the region (Figure A-5). This was determined by multiplying the number of boutons by the size of each bouton. There were no significant effects of rAIH on the total area of serotonergic innervation, but there was a significant effect of the interaction of injury and side (p=0.0164). Regardless of rAIH or Nx, the total area of innervation was significantly greater ipsilateral to C2Hx compared to boutons in the left putative phrenic motor pool of spinally intact groups (p=0.0112). Lastly, we quantified the average intensity of each serotonergic bouton around putative phrenic motor nuclei (Figure A-6). There were no significant effects rAIH on the area of each bouton, but there was a significant effect on the interaction of injury and side (p=0.0067). The intensity per bouton was significantly
larger ipsilateral to C2Hx compared to boutons in left putative phrenic motor nuclei of the spinally intact groups (p=0.002) and compared to boutons contralateral to C2Hx (p=0.0234), irrespective of rAIH or Nx.

**Discussion and Conclusion**

Serotonergic innervation of ipsilateral phrenic motor nuclei is transiently reduced after injury, with reduced levels only 2 weeks post-C2Hx and spontaneously recovery by 8 weeks post-C2Hx (Golder and Mitchell, 2005). In addition, rAIH enhances serotonergic innervation of the phrenic motor nuclei in spinally intact rats (Satriotomo et al., 2012). Because of this transient reduction in serotonergic innervation after injury and the ability of rAIH to enhance serotonergic innervation of the phrenic motor nuclei, we hypothesized that exposure to rAIH 4 times a week for 12 weeks would increase serotonergic innervation of phrenic motor neurons in spinally intact rats and enhances serotonergic innervation of the ipsilateral phrenic motor nucleus in rats 20 weeks after C2Hx. We report that serotonergic innervation of ipsilateral phrenic motor nuclei is enhanced after C2Hx. In rats with chronic C2Hx we observed that the number of serotonergic boutons did not change, but the area and intensity of each bouton was significantly larger ipsilateral to injury resulting in greater area of innervation to ipsilateral phrenic motor nuclei.

Previous studies on serotonergic innervation of phrenic motor nuclei 30 days post-injury report an increase in the number of serotonergic terminals that synapse onto phrenic motor neurons. In addition, the active zones of serotonin synapses on phrenic motor neurons also increased 30 days after C2Hx (Tai et al., 1997). Because the rats in this study were 20 weeks post-C2Hx, a very chronic time post injury, we suspect that the increased bouton size observed is due to terminal sprouting. This is consistent with
the literature that suggests that serotonin neurons enlarge and the terminals swell
during sprouting (Mamounas et al., 2000; Mamounas et al., 1995). The present study
investigated the anatomical distribution of serotonin of serotonin 20 weeks after C2Hx.
Further studies are needed to determine the functionality of these larger structures.

Our lab has shown that serotonergic innervation of phrenic motor neurons is
enhanced with exposure to rAIH 3 times week for 10 weeks (Satriotomo et al., 2012).
This study exposed rats to rAIH 4x week for 12 weeks. Although not statistically
significant, we observed a trend towards rAIH enhancing total serotonergic innervation
of phrenic motor nuclei in spinally intact rats. Ongoing experiments in our lab are aiming
to optimize the intermittent hypoxia protocol that will provide most beneficial effects of
AIH therapy for spinally intact and spinally injured rats.

Overall, we report spontaneous sprouting of serotonergic boutons 20 weeks after
C2Hx. This increased sprouting was observed as increases in bouton size and overall
innervation of serotonin to ipsilateral phrenic motor nuclei. Experiments are underway to
further enhance this spontaneous serotonergic innervation after chronic C2Hx. In
addition, future studies include optimization of rAIH therapy to utilize serotonin
innervation of the phrenic motor nucleus to improve breathing recovery after cSCI.
Figure A-1. Experimental timeline and repetitive acute intermittent hypoxia protocol. A. Rats were randomly assigned to either spinally intact groups or they received a cervical spinal hemisection at C2 (C2Hx). The groups included: 1) spinal intact+Nx (n=8); 2) spinal intact+rAIH (n=6); 3) C2Hx+Nx (n=7); 4) C2Hx+rAIH (n=9). 8 weeks post-C2Hx (or 8 weeks after group assignment in the spinally intact groups) rats began either normoxia or repetitive acute intermittent hypoxia (rAIH) 4 times a week for 12 weeks (20 weeks in total). B. Normoxia protocol consisted of 21% O₂ for 1.5 hours. C. rAIH consisted of 10, 5 minute episodes of 10.5% O₂ alternating with 5 minute normoxic intervals for a total duration of 1.5 hours (10 hypoxic episodes).
Figure A-2. Representative images of serotonergic innervation of putative phrenic motor nuclei in spinally intact and C2Hx rats exposed to normoxia. Representative 2x (top) and 20x (bottom) images of the ipsilateral (left) and contralateral (right) ventral spinal cord of spinally intact (A) and C2Hx (B) rats exposed to normoxia. Serotonin labeling (brown) was present in all spinal sections. The black circle in the 20x images represents the region of interest around putative phrenic motor neurons with a radius of 50µ. 20x scale bar is 100µ.
Figure A-3. Number of serotonin boutons innervating 50µm radius around putative phrenic motor neurons. The number of serotonergic boutons innervating a 50µm radius around putative phrenic motor nuclei was assessed on the left/ipsilateral (left) and right/contralateral (right) side of the spinal cord in spinally intact rats exposed to normoxia (white n=8) and rAIH (white and black stripes n=6), and C2Hx rats exposed to normoxia (gray, n=7) and rAIH (gray and black stripes, n=9). Prolonged exposure to rAIH (4 times a week for 12 weeks) did not affect the number of serotonin boutons innervating a 50µm radius of putative phrenic motor nuclei in rats that were spinally intact or 20 weeks after left cervical hemisection at C2 (C2Hx). Data were analyzed using a repeated measures Mixed Model with injury (intact vs. C2Hx) and protocol (Nx vs. rAIH) as between subject variables, and side (left/ipsilateral vs. right/contralateral) as the within subject repeated measure. There were no statistically significant differences in the number of boutons. Differences were considered significant if p<0.05. Data are displayed as mean±SEM.
Figure A-4. Area per serotonergic bouton innervating the putative phrenic motor nucleus. The area of serotonergic boutons innervating a 50µm radius around putative phrenic motor neurons was assessed on the left/ipsilateral (left) and right/contralateral (right) side of the spinal cord in spinally intact rats exposed to normoxia (white n=8) and rAIH (white and black stripes n=6), and C2Hx rats exposed to normoxia (gray, n=7) and rAIH (gray and black stripes, n=9). Prolonged exposure to rAIH (4 times a week for 12 weeks) did not affect the size of serotonin boutons innervating a 50µm radius of putative phrenic motor neurons in rats that were spinally intact or 20 weeks after left cervical hemisection at C2 (C2Hx). Ipsilateral serotonergic boutons innervating the putative phrenic motor pool after C2Hx were significantly larger compared to spinally intact rats (p=0.007). Data were analyzed using a repeated measures Mixed Model with injury (intact vs. C2Hx) and protocol (Nx vs. rAIH) as between subject variables, and side (left/ipsilateral vs. right/contralateral) as the within subject repeated measure. Individual comparisons were made based on the Fisher Least Significance Difference post hoc test Differences were considered significant if p<0.05. Data are displayed as mean±SEM.
Figure A-5. Total Area of serotonergic innervation within a 50µm radius of putative phrenic motor neurons. Total serotonergic innervation within a 50µm radius around putative phrenic motor neurons was assessed on the left/ipsilateral (left) and right/contralateral (right) side of the spinal cord in spinally intact rats exposed to normoxia (white n=8) and rAIH (white and black stripes n=6), and C2Hx rats exposed to normoxia (gray, n=7) and rAIH (gray and black stripes, n=9). Prolonged exposure to rAIH (4 times a week for 12 weeks) did not affect the size of serotonin boutons innervating a 50µm radius of putative phrenic motor neurons in rats that were spinally intact or 20 weeks after left cervical hemisection at C2 (C2Hx). Ipsilateral serotonergic boutons innervating the putative phrenic motor pool after C2Hx were significantly larger compared to spinally intact rats (p=0.0112). Data were analyzed using a repeated measures Mixed Model with injury (intact vs. C2Hx) and protocol (Nx vs. rAIH) as between subject variables, and side (left/ipsilateral vs. right/contralateral) as the within subject repeated measure. Individual comparisons were made based on the Fisher Least Significance Difference post hoc test. Differences were considered significant if p<0.05. Data are displayed as mean±SEM.
Figure A-6. Average intensity per serotonin bouton within a 50µm radius of putative phrenic motor neurons. Average intensity of serotonergic boutons innervating a 50µm radius around putative phrenic motor neurons was assessed one the left/ipsilateral (left) and right/contralateral (right) side of the spinal cord in spinally intact rats exposed to normoxia (white n=8) and rAIH (white and black stripes n=6), and C2Hx rats exposed to normoxia (gray, n=7) and rAIH (gray and black stripes, n=9). Prolonged exposure to rAIH (4 times a week for 12 weeks) did not affect the size of serotonin boutons innervating a 50µm radius of putative phrenic motor neurons in rats that were spinally intact or 20 weeks after left cervical hemisection at C2 (C2Hx). Ipsilateral serotonergic boutons innervating the putative phrenic motor pool after C2Hx were significantly larger compared to spinally intact rats (p=0.002) and contralateral to C2Hx (p=0.0234). Data were analyzed using a repeated measures Mixed Model with injury (intact vs. C2Hx) and protocol (Nx vs. rAIH) as between subject variables, and side (left/ipsilateral vs. right/contralateral) as the within subject repeated measure. Individual comparisons were made based on the Fisher Least Significance Difference post hoc test. Differences were considered significant if p<0.05. Data are displayed as mean±SEM.
APPENDIX B
INTERMITTENT HYPOXIA EXERTS PROTOCOL SPECIFIC EFFECTS ON BDNF EXPRESSION IN PHRENIC MOTOR NEURONS OF SPINAL INTACT AND INJURED RATS

Abstract

Brain-derived neurotrophic factor (BDNF) is necessary and sufficient for acute intermittent hypoxia (AIH)-induced phrenic motor plasticity. Repetitive AIH exposure enhances BDNF expression within phrenic motor neurons of spinally intact rats, but its effects after cervical spinal injury (cSCI) are unknown. In contrast, chronic intermittent hypoxia (CIH), mimicking sleep apnea, elicits neuropathology that may undermine plasticity. We tested the hypothesis that BDNF expression in phrenic motor neurons is upregulated with therapeutic AIH, and down regulated with CIH, in spinally intact and injured rats. BDNF expression was assessed in rats with and without C2 hemisection (C2Hx; 12 wks post-injury) exposed to 28 days of: 1) normoxia; 2) daily AIH (dAIH28, 10, 5min 10.5% O\textsubscript{2} episodes per day, 5min normoxic intervals); 3) mild CIH (IH28-5/5, 5min 10.5% O\textsubscript{2} episodes, 5min intervals, 8 hrs/day); and 4) moderate CIH (IH28-2/2, 2min 10.5% O\textsubscript{2} episodes, 2min intervals, 8 hrs/day). Rats received intrapleural Cholera toxin B injections 14 days before C2Hx to label phrenic motor neurons. BDNF expression within and around identified phrenic motor neurons was assessed via immunofluorescence using a custom MATLAB algorithm. Spinally intact and injured rats have differential responses to AIH and CIH. dAIH28 increased BDNF expression within and around phrenic motor neurons of spinally intact rats. In contrast, after C2Hx, BDNF within and around phrenic motor neurons was unaffected by dAIH28 and was highest after IH28-2/2. Thus, whereas only dAIH28 enhances BDNF expression in spinally intact rats, similar effects were observed with IH28-2/2 after C2Hx. These data highlight
the potential of intermittent hypoxia preconditioning to enhance the capacity for BDNF-dependent phrenic motor plasticity and (possibly) functional recovery after cSCI.

Overview

Brain-derived neurotrophic factor (BDNF) is necessary and sufficient for acute intermittent hypoxia (AIH) induced spinal respiratory plasticity known as phrenic long-term facilitation (pLTF) (Baker-Herman et al., 2004). AIH is an emerging therapy that improves respiratory and non-respiratory motor function in rats and humans after cervical spinal cord injury (cSCI) (Hayes et al., 2014; Lovett-Barr et al., 2012; Trumbower et al., 2017; Trumbower et al., 2012). Mechanisms of rAIH on plasticity and functional recovery after cSCI include new synthesis of spinal BDNF (Baker-Herman et al., 2004; Gonzalez-Rothi et al., 2015b; Lovett-Barr et al., 2012). Previous reports show reduced spinal BDNF 1 – 7 days post injury, correlating with reduced motor function (Garraway et al., 2011; Strickland et al., 2014). With time post injury, increased BDNF levels correlate with functional recovery (Garraway and Huie, 2016; Jakeman et al., 1998). In uninjured rats, rAIH enhances pLTF (Ling et al., 2001; Wilkerson and Mitchell, 2009) and increases BDNF expression in phrenic motor neurons (Satriotomo et al., 2012). However, the effects of rAIH on BDNF expression in phrenic motor neurons after chronic cSCI has not been investigated. It is necessary to understand the availability of BDNF within phrenic motor neurons to guide therapeutic approaches to harness spinal plasticity and breathing recovery after chronic cSCI.

Conversely, chronic intermittent hypoxia (CIH) protocols simulating periods of low oxygen associated during sleep apnea, may elicit neuroinflammation and potentially undermine the benefits of AIH. However, some CIH protocols (12 hours of 5min hypoxia episodes interspersed with 5min normoxia episodes for 7 nights) have been reported to
elicit pLTF in spinal intact (Ling et al., 2001) and injured rats (Fuller et al., 2003). About 80% of people with cSCI also suffer from sleep apnea (Berlowitz et al., 2005; Fuller et al., 2013; Sankari et al., 2014a). Thus, it is important to understand the effect of various intermittent hypoxia protocols, from therapeutic rAIH to CIH, in order to optimize respiratory neuroplasticity and functional recovery after chronic cSCI. Indeed, the balance between the therapeutic vs pathogenic effects of intermittent hypoxia (IH) is a matter of “dose” (Navarrete-Opazo and Mitchell, 2014b). The differential BDNF expression in phrenic motoneurons following various “doses” of rAIH in intact rats is unknown. In addition, although rAIH elicits pLTF after cSCI, the effects of rAIH on phrenic motor neuron BDNF expression after chronic incomplete cSCI is unknown.

Here we investigate the effects of various protocols of IH on BDNF expression in phrenic motor neurons in spinally intact and injured rats. We hypothesize that BDNF expression in phrenic motoneurons is upregulated with therapeutic rAIH, and down regulated with CIH, in spinally intact and injured rats. BDNF expression was assessed in the phrenic motor nucleus of rats with and without C2 hemisection (C2Hx; 12 wks post-injury) exposed to 28 days of: 1) normoxia; 2) daily AIH (dAIH28, 10, 5min 10.5% O₂ episodes per day; 5min normoxic intervals); 3) mild CIH (IH28-5/5, 5min 10.5% O₂ episodes, 5min intervals; 8 hrs/day); and 4) moderate CIH (IH28-2/2, 2min 10.5% O₂ episodes, 2min intervals; 8 hrs/day). We report differential effects of IH on uninjured vs injured rats. Whereas dAIH28 enhances phrenic BDNF expression in spinally intact rats, IH28-2/2 enhances BDNF expression 12 weeks post-C2Hx. This study will guide better understanding of the mechanism behind various rAIH doses and will allow us to optimize the promising results already seen with rAIH after cSCI.
**Methods**

All experiments were conducted with adult male (350-450 g) Sprague-Dawley rats (Colony 217, ENVIGO Laboratories). Rats were housed in pairs in a controlled environment (12 hour light/dark cycles) with food and water *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida. A total of 79 rats were studied (11-12 weeks of age). Spinally intact and C2 hemisection (C2Hx) rats were randomly assigned to intermittent hypoxia exposure groups at 8 weeks post-injury and exposed for 28 consecutive days to: 1) normoxia (Nx28); 2) daily acute intermittent hypoxia (dAIH28), 3) moderate IH (IH28-5/5; and 3) more severe IH (IH28-2/2). Experimental groups with rat numbers were: 1) spinal intact+Nx28 (n=8); 2) spinal intact+dAIH-28 (n=10); 3) spinal intact+IH28-5/5 (n=10); 4) spinal intact+IH28-2/2 (n=10); 5) C2Hx+Nx28 (n=9); 6) C2Hx+dAIH28 (n=8); 7) C2Hx+IH28-5/5 (n=12); and 8) C2Hx+IH28-2/2 (n=12).

**Phrenic Motor Neuron Back Labeling**

Rats were anesthetized with 3.5% isoflurane (in 100% O2) and maintained at 2-2.5% isoflurane (in 100% O2) via nose cone throughout the surgery. Depth of anesthesia was confirmed by the absence of toe pinch and palpebral responses. All rats were injected intrapleurally with Cholera toxin B fragment (CtB) subunit bilaterally (0.2% w/v CtB; dissolved in sterile H2O; Calbiochem, Billerica, MA) to retrogradely label phrenic motor neurons 14 days prior to C2Hx (Dale-Nagle et al., 2011; Guenther et al., 2010; Mantilla et al., 2009). 25 μL of CtB was loaded into a 25 μL Hamilton syringe attached to a 9.52 mm sterile needle for bilateral injections (2 × 12.5 μL = 25μL total per animal) at the 5th intercostal space ~6 mm deep.
C2Hx Injury

Anesthesia, C2Hx, and animal care were performed as described previously (Fuller et al., 2008; Gonzalez-Rothi et al., 2015c). Rats were anesthetized with 3.5% isoflurane (in 100% O2) and maintained at 2-2.5% isoflurane (in 100% O2) via nose cone throughout the surgery and confirmed by the absence of toe pinch and palpebral responses. Body temperature was maintained at 36.5–37.5°C with a heating pad. Artificial tears (Rugby, NDC 0536-1086-91) were applied to prevent eye damage. Nails were clipped from forelimbs and hind limbs, and the surgical site was shaved and cleaned (chlorhexidine scrub, 58829-140-01, First Priority, Inc., Elgin, IL). The C2Hx was performed by making a dorsal incision on the neck of the rat and dissecting the muscle to expose the C2 lamina. Following C2 laminectomy and durotomy, the left side of the C2 spinal cord was hemisected with a microknife, caudal to the C2 dorsal roots. A gap (~1 mm) at the injury site was then created by gentle aspiration. The overlying dura was subsequently sutured with 9-0 ethilon nylon suture, the overlaying muscles were sutured with 3-0 Polysorb absorbable suture and the skin was closed with 9 mm stainless steel wound clips. Each rat received post-operative care following surgery which included pain management with an analgesic (buprenorphine, 0.03 mg/kg, s.q., Hospira< IL) and an anti-inflammatory drug (meloxicam, 2 mg/kg., s.q. Portland, ME) given at 12 and 24-hour intervals for 2 days post-surgery. Rats received lactated ringers solution (5 ml 2x/day, s.q.) and were manually fed with a nutritional supplement (Diet Gel Boost; Clear H2O; Westbrook, ME) until adequate volitional drinking and eating resumed.
Intermittent Hypoxia Protocols

Starting 8 weeks post-C2Hx (or 6 weeks after CtB injections in intact rats), rats were housed in a custom-designed PlexiGlass gas exposure cages with free access to food and water. Respective IH protocols were administered to the cages daily for 4 weeks. Normoxia control rats received continuous gas flow of normoxia (21% O\textsubscript{2}, 8 hours per day). Rats assigned to the dAIH groups received 10, 5min episodes of 10.5% O\textsubscript{2} alternating with 5min normoxic intervals for a total duration of 1.5 hours/day (10 hypoxic episodes per day). Rats assigned to the IH28-5/5 groups received 5min episodes of 10.5% O\textsubscript{2} alternating with 5 min normoxic intervals for a total duration of 8 hours/day (6 hypoxic episodes per hour for 8 hours = 48 hypoxic episodes per day). The alternating 5min hypoxia/normoxia protocols results in 6 hypoxic episodes per hour which is equivalent to the clinical criteria (apnea-hypopnea index, AHI) for diagnosing mild sleep apnea (American Academy of Sleep Medicine, 1999; Young et al., 2008). The rats assigned to the IH28-2/2 groups received 2min episodes of 10.5% O\textsubscript{2} alternating with 2min normoxic intervals for a total duration of 8 hours/day (15 hypoxic episodes per hour for 8 hours = 120 hypoxic episodes per day). This protocol results in 15 hypoxic episodes per hour which is equivalent to the clinical criteria (apnea-hypopnea index, AHI) for diagnosing moderate sleep apnea (American Academy of Sleep Medicine, 1999; Young et al., 2008).

Tissue Histology and Analysis

Rats were perfused intracardially with cold 0.1M phosphate buffer saline (PBS), followed by paraformaldehyde (4% paraformaldehyde w/v in 0.1M PBS, pH 7.4). The spinal cord was harvested and post-fixed overnight in paraformaldehyde (4% paraformaldehyde w/v in 0.1M PBS, pH 7.4) and cryoprotected at 4°C in 20% sucrose.
solution in 0.1M PBS for 3 days, followed by 30% sucrose solution in 0.1M PBS for 3
days. To verify completion of the injury, cervical spinal segments C1-C3 were
embedded in paraffin, cut at 7µm, directly mounted on positively charged slides
(Fisherbrand, Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. Sections
were stained with Luxol Fast Blue and cresyl violet. Sections were first deparaffinized
via heating at 60ºC for 20min, then submerged three times in xylene for 5min each.
Sections were rehydrated by graded alcohol (100%-70%) for 2min each. Sections were
stained overnight using Luxol Fast Blue (0.1g Luxol Fast Blue in 100mL of 95% ethanol
and 0.5mL 10% acetic acid). The following day, tissues were rinsed with 95% ethanol
for 2min and then distilled water for 5min. Differentiation was achieved by dipping the
tissue in 0.5% lithium carbonate and 70% ethanol, then rehydrated by distilled water for
5min. Tissues were placed in 0.1% cresyl violet for 15min. Cresyl violet was rinsed by
dipping tissue into distilled water twice. Tissue was then dehydrated through graded
alcohol (70–100%) for 1min each, cleared in Histoclear twice for 2min each (National
Diagnostics, Atlanta, GA) and cover-slipped (Eukitt, Electron Microscope Science, PA).
Injury sites were imaged using bright field at 10x zoom level (BZ-x710, Keyence Co.,
Osaka, Japan) to confirm lateral C2Hx.

Cervical (C3-C5) spinal segments were sectioned in transverse plane (40µm
thickness) using a freezing microtome (Leica SM 200R, Buffalo Grove, IL). Tissues
were stored in antifreeze solution at -20°C until processed (30% glycerol, 30% ethylene
glycol, 40% 0.1 M PBS, pH 7.4). Every 12th section was selected and stained for
serotonin and CtB-labeled phrenic and intercostal motor neurons. Free-floating sections
were washed with 0.1M PBS (pH 7.4), then incubated in heat induced epitope retrieval
(TissuePro, cat#: HIER01-32R) for 30min at 85°C. Tissues were washed again with 0.1M PBS-Triton (0.1%, pH 7.4) and then incubated in a blocking solution (5% normal donkey serum (NDS, GeneTex) in 0.1M PBS-Triton (0.1%), pH 7.4) at room temperature for 60 min. Primary antibody staining was performed by incubating tissue sections in 5% NDS in 0.1M PBS-Triton (0.1%, pH 7.4) with anti-5-HT (1/2000, rabbit serum, Immunostar #20080) and anti-CTB (1/2500, goat serum, Millo pore #227040) in 4°C overnight. The following day, tissues were washed with 0.1M PBS-Triton (0.1%, pH 7.4). Secondary antibody staining was performed by incubating tissue in 5% NDS in 0.1M PBS-Triton (0.1%, pH 7.4) with secondary antibodies conjugated to Alexa Fluor® 594 (donkey anti-rabbit 594; 1:500, Invitrogen, Ref#A11055) and Alexa Fluor® 488 (donkey anti-goat 488; 1:1000, Invitrogen, Ref#A21207) in a dark box at room temperature for 2 hours. Sections were washed with 0.1M PBS-Triton (0.1%, pH 7.4) and mounted on positively charged slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. Slides were cover slipped with VectaShield Antifade Hard Set Mounting Medium (Cat# H-1400). Fluorescently labeled sections were captured using an epifluorescent microscope with 20x magnification (Keyence BZ-X700, Keyence Corporation of America, Itasca, IL). The sides of the spinal cord were marked prior to tissue sectioning to ensure accurate side determination during imaging and analyses. Phrenic motor neurons were determined by CtB-positive cell labeling within the ventral horn.

**Data Analysis**

BDNF immunofluorescence was quantified using a custom MATLAB (MathWorks, Natick, MA, USA) code. BDNF was identified within and around regions CtB-labeled phrenic motor nuclei. Within the cervical C3-C5 spinal sections, the custom
MATLAB code first identified CtB-labelled phrenic motor neurons versus non-positive areas and assigned them binary values (CtB-positive=1, CtB-negative=0) to determine phrenic vs. non-phrenic areas. For the region of interest (ROI) around CtB-labelled phrenic motor neurons, the center of gravity of the CtB-labelled phrenic motor neurons was calculated. The final ROI was defined by a circular area with a 50 µm radius centered at the center of gravity of CtB-positive phrenic motor neurons (Seven et al., 2018b). The center of gravity equation was defined as:

\[
\vec{G}(x, y) = \frac{\sum (p_i \times \vec{r}_i(x, y))}{\sum p_i}
\]

Center of gravity was calculated based on the distance from a standardized reference point (image origin) and binary values.

Thresholds were determined using a custom adaptive threshold algorithm in MATLAB as previously described (Allen et al., 2019). The adaptive threshold was calculated by constructing a pixel intensity histogram from the image. The pixel intensity corresponding to the 99th percentile was selected as the adaptive threshold. Intact or C2Hx and intermittent hypoxia exposure protocols remained blinded throughout analyses.

**Statistics**

Statistical calculations were performed using JMP 11.0 (SAS Institute, Cary, NC). BDNF immunofluorescence data was analyzed using a mixed model with repeated measures. The independent variables were injury (intact vs. C2Hx), IH protocol (Nx28, dAIH28, IH28-5/5, and IH28-2/2), and spinal cord side relative to injury (ipsilateral vs. contralateral as the repeated measure). Additionally, we analyzed the intact and C2Hx groups separately. In the intact groups, we averaged the left and right sides of spinal
cord and ran a one-way ANOVA with the independent variable being IH protocol (Nx28, dAIH28, IH28-5/5, and IH28-2/2). In the C2Hx groups, we ran a two-way repeated measures ANOVA with the independent variables being IH protocol (Nx28, dAIH28, IH28-5/5, and IH28-2/2) and spinal cord side relative to injury (ipsilateral vs. contralateral as the repeated measure). A significance level of 0.05 was set for all statistical comparisons. When significant differences were observed, individual comparisons were made based on the Fisher Least Significance Difference post hoc test. All data are displayed as mean ± standard error of the mean.

Results

Immunohistochemistry was used to visualize BDNF expression within phrenic motor neurons via the colocalization of CtB labeled phrenic motor neurons and BDNF (Figure B1). Upon first observance there appeared to be protocol specific difference in spinally intact and injured rats. BDNF expression in spinally intact rats appeared brightest after dAIH28 exposure. In rats 12 weeks after C2Hx, BDNF expression appeared brightest after IH28-2/2 exposure protocol. In all groups, the colocalization of CtB and BDNF occurred mostly in the soma of phrenic motor neurons. Representative images were chosen based on the optical density values that were closest to the respective group averages.

Within phrenic motor neurons, there were IH dependent effects on BDNF expression in spinal intact vs C2Hx rats (repeated measures Mixed Model; p=0.0495). To avoid the risk of type II error due to the high number of interactions, we split the data into spinal intact and C2Hx groups. In the spinal intact groups, there were no statistically or physiologically significant differences between the left and right sides of the spinal cord (Fisher LSD post hoc analysis p>0.05), so the data was averaged. There was a
significant effect of IH protocol on the uninjured rats (one-way ANOVA; p=0.0367).

Spinally intact rats exposed to dAIH28 had greater BDNF expression within phrenic motor neurons (dAIH28 vs Nx28 p=0.0481; and dAIH28 vs IH28-5/5 p=0.0052; Figure B-2A). After C2Hx, there were no significant effects on IH protocol (two-way repeated measures ANOVA; p=0.4658; Figure B-2B).

The signal to background ratio is also used to identify the intensity of immunofluorescent signal in relation to the image background. Within phrenic motor neurons, there were significant effects of IH protocol, spinal injury, and spinal cord side (injury vs IH protocol p=0.0013; injury vs side p=0.0284; and protocol vs side p=0.0392) in BDNF signal to background ratio. The spinally intact and C2Hx groups were analyzed separately to further identify these effects. There no differences in regard to the side of the intact spinal cord, so the data was averaged. In the spinally intact groups, there was significant effects of IH protocol (one-way ANOVA; p=0.001). BDNF expression was reduced in with both mild and moderate CIH exposure compared to Nx28 and dAIH28 exposure (Nx vs IH28-5/5 p=0.0136; Nx vs IH28-2/2 p=0.0469; dAIH28 vs IH28-5/5 p=0.0004; dAIH28 vs IH28-2/2 p=0.0018; Figure B-3A). After C2Hx, there were side specific IH protocol effects (two-way repeated measures ANOVA; p=0.0096).

Contralateral to injury, BDNF signal to background ratio was greatest after IH28-2/2 exposure compared to all other IH protocols on either side of the cord (vs IH28-2/2 ipsilateral p=0.002; vs Nx28 contralateral p=0.0212, ipsilateral p=0.0298; vs dAIH28 contralateral p=0.0281, ipsilateral p=0.0025; vs IH28-5/5 contralateral p=0.0075, ipsilateral p=0.0056; Figure B-3B).
BDNF expression in a ROI (50µ radius) around CtB-labeled phrenic motor neurons was also investigated to include aspects of the motor neurons outside of the just the soma, such as distal dendrites, and other supportive cells surrounding the phrenic motor neurons. There were significant effects of IH protocol and injury (Mixed Model; p=0.0314 and p=0.0179 respectively) on ROI BDNF expression. To further investigate these effects, we separated the uninjured and C2Hx groups. There were no differences due to the side of the uninjured spinal cord, so the data was averaged. Although there were no significant effects of ROI BDNF expression in spinally intact rats (one-way ANOVA, p=0.0704), there was a trend towards more ROI BDNF expression uninjured rats exposed to dAIH28, although this was not significant (Figure B-4A). After C2Hx, there was a significant effect of IH protocol on ROI BDNF expression (two-way repeated measures ANOVA; p=0.0312). Exposure to IH28-2/2 resulted in greater ROI BDNF expression, regardless of spinal cord side, 12 weeks post C2Hx (IH28-2/2 vs Nx28 p=0.0093; IH28-2/2 vs dAIH28 p=0.0153; IH28-2/2 vs IH28-5/5 p=0.0308; Figure B-4B).

Lastly, the BDNF signal to background ratio of BDNF expression in a ROI (50µm radius) around CtB-labeled phrenic motor neurons was quantified. There was a significant effect in the interaction of injury vs IH protocol (Mixed Model, p=0.0015). We again separated the uninjured and C2Hx groups to further identify the effects of IH protocols. In the spinally intact groups, there was no effect of spinal cord side, so we averaged the data. There was a significant effect of IH protocol in the BDNF ROI signal to background ratio of spinally intact rats (one-way ANOVA; p=0.0033). BDNF ROI signal to background ratio was greatest after dAIH28 exposure compared to and mild
and moderate CIH exposure (dAIH28 vs IH28-5/5 p=0.0011; dAIH28 vs IH28-2/2 p=0.0021; Figure B-5A). After C2Hx there was also an effect of IH protocol on ROI BDNF signal to background ratio (two-way ANOVA with repeated measures; p=0.0441). Similar to the ROI BDNF expression, ROI BDNF signal to background ratio was significantly greater after IH28-2/2 exposure on both the ipsilateral and contralateral spinal cord (vs Nx28 p=0.0396; vs dAIH28 p=0.0178; vs IH28-5/5 p=0.0137; Figure B-5B).

**Discussion and Conclusion**

We conclude that IH exerts protocol specific effects on BDNF expression in the phrenic motor nucleus of spinally intact rats versus rats 12 weeks post-C2Hx. While therapeutic daily AIH (dAIH28) enhanced BDNF expression within and around phrenic motor neurons in spinally intact rats, only moderate CIH (IH28-2/2) enhanced BDNF expression 12 weeks after C2Hx. The effects of IH28-2/2 in rats pos-C2Hx was only significant in a ROI around the phrenic motor neurons, though there was a trend towards enhanced BDNF expression within phrenic motor neurons after IH28-2/2 exposure.

**Daily AIH Enhances BDNF Expression in Spinally Intact Rats**

Evidence suggests that 7 consecutive days of AIH is enough to enhance BDNF expression in a ROI around phrenic motor neurons in uninjured rats (Lovett-Barr et al., 2012). The effects of BDNF expression after prolonged IH durations have also been investigated. In fact, repetitive AIH, 10 cycles of 5min hypoxia/normoxia episodes 3 times per week for 10 weeks, increases BDNF expression in a ROI around the phrenic motor nucleus of spinally intact rats (Satriotomo et al., 2012). We report enhanced BDNF expression after 28 consecutive days of AIH in uninjured rats. The optimal
duration of and pattern of AIH that is needed to enhance substrates necessary for respiratory plasticity in spinally intact rats needs further investigation.

CIH elicits spinal inflammation that may undermine the potential for AIH to elicit spinal plasticity (Huxtable et al., 2013; Navarrete-Opazo and Mitchell, 2014b; Vinit et al., 2011) which could have negative implications on therapeutic AIH interventions. This study suggests that mild and moderate CIH exposure reduces phrenic BDNF expression in uninjured rats. One speculation is that the inflammation resulting from CIH undermines mechanisms upstream of BDNF protein translation (Agosto-Marlin et al., 2017; Huxtable et al., 2015).

**CIH Enhances BDNF Expression 12 weeks Post-C2Hx**

AIH effects on functional recovery after SCI are dependent on the time post-injury (Gonzalez-Rothi et al., 2015b; Navarrete-Opazo and Mitchell, 2014b). AIH benefits after acute cSCI is thought to be due to BDNF independent mechanisms (Navarrete-Opazo and Mitchell, 2014b; Navarrete-Opazo et al., 2015). Whereas AIH benefits after chronic cSCI are thought to be due to BDNF dependent mechanisms dependent (Navarrete-Opazo et al., 2017b). AIH exposure for 7 consecutive days enhances phrenic BDNF expression in rats 1 week post-C2Hx (Lovett-Barr et al., 2012). However, we do not report enhance BDNF expression in rats 12 weeks post-C2Hx exposed to dAIH28. Conversely, BDNF expression 12 weeks post-C2Hx was enhanced after moderate CIH (IH28-2/2) exposure. These data suggest different mechanisms for plasticity after acute vs chronic cSCI.

About 80% of people with chronic cSCI also experience sleep apnea (Berlowitz et al., 2005). The moderate CIH protocol used in this study mimicked the episodes of low oxygen experienced during sleep apnea. Our data suggest that CIH may be a
protective mechanism after cSCI by enhancing prolaticity molecules. The ability of IH28-2/2 to enhance BDNF expression within phrenic motor neurons sets the stage for the capacity for plasticity and functional recovery after cSCI. The metaplasticity effects of the interaction of sleep apnea and cSCI need further investigation.

Larger effects were seen in ROI BDNF expression compared to BDNF expression within phrenic motor neurons. Quantification of co-labeled BDNF expression within phrenic motor neurons only includes the cell soma, whereas ROI around phrenic motor neurons include the soma, distal dendrites, and other cells such as glia and interneurons. SCI is associated with spinal inflammation and microglia activation (Alexander and Popovich, 2009). In addition, CIH causes neuroinflammation via mechanisms related to microglia activation (Huxtable et al., 2015). Thus, the higher BDNF expression observed in rats with chronic cSCI after IH28-2/2 exposure could be due to increased BDNF expression in microglia.

Our lab extensively studies the ability of AIH to elicit BDNF dependent phrenic motor facilitation and enhance breathing function in rats and people with chronic SCI (Gonzalez-Rothi et al., 2015b). Understanding the extent and effects of spinal BDNF expression is necessary for translating IH therapy to people. BDNF is a neurotrophin important for neuroprotection, synaptic transmission, axon survival, and plasticity (Garraway and Huie, 2016; Weishaupt et al., 2012). However, too much BDNF can cause neuropathic pain and spasticity (Garraway and Huie, 2016; Kerr et al., 1999; Yajima et al., 2005). Thus, there is a need to optimize IH protocols to spinal enhance BDNF, thus enhancing function, without eliciting pain for people with SCI.
After cSCI, the spinal cord is a new cord (Edgerton et al., 2001) and responds differently to IH than the intact spinal cord. Therapeutic AIH (dAIH28) enhances phrenic BDNF expression in uninjured rats; whereas moderate CIH (IH28-2/2) enhances phrenic BDNF expression 12 weeks after C2Hx. Thus, AIH interventions must be optimized to individuals. These data highlight the potential of IH preconditioning to enhance the capacity for BDNF-dependent phrenic motor plasticity and possibly functional recovery after cSCI.
Figure B-1. Representative images of BDNF expression in phrenic motor neurons. Representative images from spinally intact rats (Left) and rats 12 weeks post-C2Hx (Right) exposed to 28 days of either to Normaxia (Nx28), Daily acute intermittent hypoxia (dAIH28), Mild acute intermittent hypoxia (IH28-5/5), Moderate acute intermittent hypoxia (IH28-2/2). Images from left to right illustrate Cholera toxin subunit B-labeled phrenic motor neurons (green channel), BDNF (red channel), and their overlays (merged channels). Images were taken at 20x magnification with the phrenic motor nucleus at the center. The white scale bars represent a distance of 100µm.
Figure B-2. BDNF expression in phrenic motor neurons of uninjured and injured rats. BDNF optical density within phrenic motor neurons was quantified in spinally intact rats (A) and rats 12 weeks after C2 hemisection (C2Hx; B) exposed to 28 days of intermittent hypoxia (IH) protocols (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). A. BDNF optical density in spinally intact rats. dAIH28 exposure enhanced BDNF optical density versus Nx28 (p=0.0481) and IH28-5/5 (p=0.0052) in uninjured rats. B. BDNF optical density in rats 12 weeks after C2Hx. There were no effects of IH protocol on BDNF optical density on the ipsilateral (left) or contralateral (right) side of the spinal cord in rats 12 weeks after C2Hx. All data were analyzed with a repeated measures Mixed Model ANOVA; injury (intact vs. C2Hx) and IH protocol (Nx28, dAIH28, IH28-5/5 and IH28-2/2) were between subject variables; side (left/ipsilateral vs. right/contralateral) was the repeated measure. Spinally intact groups were analyzed using a one way ANOVA by IH protocol. C2Hx groups were analyzed using a two-way repeated measures ANOVA with independent variables being IH protocol and spinal cord side relative to injury (ipsilateral vs. contralateral as the repeated measure). Differences were considered significant if p<0.05. Data are displayed in arbitrary units (A.U.) as mean±SEM. Bars denote means ± 1 SEM with the individual rats denoted by individual data points.
Figure B-2. Continued
BDNF signal to background ratio in uninjured and injured rats. BDNF signal to background ratio within phrenic motor neurons was quantified in spinally intact rats (A) and rats 12 weeks after C2 hemisection (C2Hx; B) exposed to 28 days of intermittent hypoxia (IH) protocols (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). A: BDNF signal to background ratio in spinally intact rats. CIH protocols had significantly lower signal to background ratio compared to Nx28 and dAIH28 (IH28-5/5 vs Nx28 p=0.0136; IH28-5/5 vs dAIH28 p=0.0004; IH28-2/2 vs Nx p=0.0469; and IH28-2/2 vs Nx28-2/2 p=0.0018). B: BDNF signal to background ratio 12 weeks after C2Hx. BDNF signal to background ration was enhanced contralateral to injury with IH28-2/2 exposure (vs IH28-2/2 ipsilateral p=0.002; vs Nx28 contralateral p=0.0212, ipsilateral p=0.0298; vs dAIH28 contralateral p=0.0281, ipsilateral p=0.0025; vs IH28-5/5 contralateral p=0.0075, ipsilateral p=0.0056). All data were analyzed with a repeated measures Mixed Model ANOVA; injury (intact vs. C2Hx) and IH protocol (Nx28, dAIH28, IH28-5/5 and IH28-2/2) were between subject variables; side (left/ipsilateral vs. right/contralateral) was the repeated measure. Spinally intact groups were analyzed using a one way ANOVA by IH protocol. C2Hx groups were analyzed using a two-way repeated measures ANOVA with independent variables being IH protocol and spinal cord side relative to injury (ipsilateral vs. contralateral as the repeated measure). Differences were considered significant if p<0.05. Data are displayed in arbitrary units (A.U.) as mean±SEM. Bars denote means ± 1 SEM with the individual rats denoted by individual data points.
Figure B-3. Continued
Figure B-4. BDNF expression in region of interest around phrenic motor neurons. BDNF optical density was quantified in 50µm region around phrenic motor neurons of spinally intact rats (A) and rats 12 weeks after C2 hemisection (C2Hx; B) exposed to 28 days of intermittent hypoxia (IH) protocols (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). A. BDNF expression in region of interest around phrenic motor neurons in spinally intact rats. Within the region of interest around phrenic motor neurons (50 µm) in intact rats, we did not find a significant difference between any of the protocols (p>0.05). B BDNF expression in region of interest around phrenic motor neurons 12 weeks after C2Hx. After C2Hx, on both ipsilateral (left) and contralateral (right) sides of the spinal cord, IH28-2/2 had the greatest optical density (IH28-2/2 vs Nx28 p=0.0093; IH28-2/2 vs dAIH28 p=0.0153; IH28-2/2 vs IH28-5/5 p=0.0308). All data were analyzed with a repeated measures Mixed Model ANOVA; injury (intact vs. C2Hx) and IH protocol (Nx28, dAIH28, IH28-5/5 and IH28-2/2) were between subject variables; side (left/ipsilateral vs. right/contralateral) was the repeated measure. Spinally intact groups were analyzed using a one way ANOVA by IH protocol. C2Hx groups were analyzed using a two-way repeated measures ANOVA with independent variables being IH protocol and spinal cord side relative to injury (ipsilateral vs. contralateral as the repeated measure). Differences were considered significant if p<0.05. Data are displayed in arbitrary units (A.U.) as mean±SEM. Bars denote means ± 1 SEM with the individual rats denoted by individual data points.
Figure B.4. Continued
Figure B-5. BDNF signal to back ratio in region of interest around phrenic motor neurons. BDNF signal to background ratio was quantified in 50µm region around phrenic motor neurons in spinally intact rats (A) and rats 12 weeks after C2 hemisection (C2Hx; B) exposed to 28 days of intermittent hypoxia (IH) protocols (Nx28, white; dAIH28, light gray; IH28-5/5, dark gray; IH28-2/2, black). A. BDNF signal to background ratio in region of interest around phrenic motor neurons of spinally intact rats: There were significant effects of IH protocol on the signal to background ratio in a region of interest around phrenic motor neurons of spinally intact rats. dAIH28 exposure has significantly higher signal to background ratio compared to IH28-5/5 (p=0.0011) and IH28-2/2 (p=0.0021) but not Nx28. B. BDNF signal to background ratio in region of interest around phrenic motor neurons 12 weeks post-C2Hx: After C2Hx, on both ipsilateral (left) and contralateral (right) sides of the spinal cord, IH28-2/2 had the greatest optical density (IH28-2/2 vs Nx28 p=0.0396; IH28-2/2 vs dAIH28 p=0.0178; IH28-2/2 vs IH28-5/5 p=0.0137). All data were analyzed with a repeated measures Mixed Model ANOVA; injury (intact vs. C2Hx) and IH protocol (Nx28, dAIH28, IH28-5/5 and IH28-2/2) were between subject variables; side (left/ipsilateral vs. right/contralateral) was the repeated measure. Spinally intact groups were analyzed using a one way ANOVA by IH protocol. C2Hx groups were analyzed using a two-way repeated measures ANOVA with independent variables being IH protocol and spinal cord side relative to injury (ipsilateral vs. contralateral as the repeated measure). Differences were considered significant if p<0.05. Data are displayed in arbitrary units (A.U.) as mean±SEM. Bars denote means ± 1 SEM with the individual rats denoted by individual data points.
Figure B-5. Continued
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BIographiesketch

Marissa Carmae Ciesla was born in Brandon, Florida in 1990 to parents Edward and Carmen Ciesla. She grew up in Brandon, Florida with her older sister Lindsey and older brother Eddie. She graduated in 2009 from Brandon Senior High School before attending the University of South Florida. In 2012 she graduated with a Bachelor of Science degree in biomedical sciences with minors in psychology and biomedical Physics. Marissa continued her education at the University of South Florida, obtaining a Master of Science degree in medical sciences with a concentration in neuroscience in 2014. Marissa was an adjunct professor at Hillsborough Community College before entering the College of Medicine’s Graduate Program in Biomedical Sciences at the University of Florida in 2015. During her PhD, Marissa studied therapies to enhance serotonergic reinnervation of motor nuclei after cervical spinal cord injury. During her PhD, Marissa received many awards including the McKnight Brain Institute Fellowship in 2016, Bryan Robinson Endowment in 2018, and Caroline tum Suden Award in 2020. In addition, she was the president of the North Central Florida Chapter for the Society of Neuroscience where she participated in Brain Awarness Week and helped host a local conference. Marissa graduated in August 2020 with her PhD in biomedical sciences with a focus on neuroscience from the University of Florida College of Medicine. After completing her PhD, she plans to continue a career in biomedical sciences and share her love for science with future generations.