To my friends and family who provide endless support and affirmation as I work to achieve my goals
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<td>4xwAIH</td>
<td>10, 5 min episodes of 10.5% (O_2) per day; 5 min normoxic intervals; 4 x per week</td>
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<td>5-HT(_1)</td>
<td>Serotonin receptor 1</td>
</tr>
<tr>
<td>5-HT(_{2A})</td>
<td>Serotonin receptor 2A</td>
</tr>
<tr>
<td>5-HT(_{2B})</td>
<td>Serotonin receptor 2B</td>
</tr>
<tr>
<td>AADC</td>
<td>L-Amino acid decarboxylase</td>
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<td>AIH</td>
<td>Acute intermittent hypoxia</td>
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<td>AKT</td>
<td>Protein Kinase B</td>
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<td>AMPA</td>
<td>(\alpha)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
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<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
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<tr>
<td>C2Hx</td>
<td>C2 hemisection injury model</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CD11b</td>
<td>OX-42; cluster of differentiation marker 11b; Marker of macrophages</td>
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<td>CIH</td>
<td>Chronic intermittent hypoxia</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response binding element</td>
</tr>
<tr>
<td>CSPG</td>
<td>Chondroitin sulfate proteoglycan</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EPO</td>
<td>Erythropoetin</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein; marker of reactive astrocytes</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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IL-1β  Interleukin-1 beta
IL-6  Interleukin-6
iPMF  Inactivity-induced phrenic motor facilitation
Jak  Janus kinase
LIF  Leukemia inhibitory factor
M1  “Classically” activated microglia phenotype
MAG  Myelin-associated glycoprotein
mmHg  Millimeters of mercury
MRI  Magnetic resonance imaging
mTOR  Mammalian target of rapamycin
NG2  Neuroglial 2 proteoglycan
NgR1  Nogo receptor
NMDA  N-methyl-D-aspartate receptor
NT-4  Neurotrophin 4
Nx  Normoxia
OMgp  Oligodendrocyte/myelin glycoprotein
OPC  Oligodendrocyte precursor cell
OSA  Obstructive sleep apnea
PaCO₂  Partial pressure of carbon dioxide in arterial blood
PaO₂  Partial pressure of oxygen in arterial blood
PDE4  Phosphodiesterase 4
PFA  Paraformaldehyde
PI3K  Phosphoinositide 3-kinase
PKA  Protein kinase A
pLTF  Phrenic long-term facilitation
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<td>pMF</td>
<td>Phrenic motor facilitation</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PO₂</td>
<td>Partial pressure of oxygen</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<tr>
<td>Rheb1</td>
<td>Ras homolog enriched in brain</td>
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<td>ROCK</td>
<td>Rho-associated protein kinase</td>
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<tr>
<td>rAIH</td>
<td>Repetitive acute intermittent hypoxia</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SCI</td>
<td>Spinal cord injury</td>
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<td>SOCS3</td>
<td>Suppressor of cytokine signaling</td>
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<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
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<td>TAAR</td>
<td>Trace-amine-associated receptor</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>TrkB</td>
<td>Tropomyosin receptor kinase B</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>VEGF-R2</td>
<td>VEGF receptor-2</td>
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EFFECTS OF REPETITIVE ACUTE INTERMITTENT HYPOXIA ON LESION VOLUME FOLLOWING CERVICAL SPINAL CORD INJURY

By

Juan Gabriel Santiago-Moreno

May 2018

Chair: Gordon S. Mitchell
Major: Medical Sciences

Following spinal cord injury (SCI), the lesion progressively increases in size as inflammation and cell death causes axons withdraw from the site of injury. Strategies to promote growth through the injured spinal cord will require treatments to minimize or reverse such spinal degeneration. Repetitive exposure to acute intermittent hypoxia (AIH) has emerged as a promising new treatment to restore motor function after chronic SCI. AIH has been demonstrated to elicit spinal plasticity by upregulating growth/trophic factor expression, including BDNF and VEGF. The signaling mechanisms of AIH induced plasticity also share significant homology to established signaling mechanisms of axonal growth and regrowth following injury.

Aim 1 was designed to explore the potential for AIH diminish lesion volume following cervical SCI. Aim 2 was designed to assess the safety of AIH both in the central nervous system and in peripheral systems. Studies were initiated on rats with cervical spinal hemisections (a single slice injury); two months post-injury, the rats began three month exposures to repetitive AIH (10, 5 min episodes of 10.5% O₂ per day; 5 min normoxic intervals; 4 x per week) or sham normoxia. Rats were euthanized one day after treatment had ended, immediately following neurophysiological
assessment. Our results suggest that there is no significant difference in lesion volume between 4xwAIH treated rats and normoxic control. We also show no significant difference in gliosis in the hippocampus, suggesting long-term AIH is not associated with detectable CNS pathology. Regardless of long-term AIH’s effects, our results will be of major interest as we attempt to develop this promising new treatment for chronic spinal injuries, an injury for which there is currently no effective therapy.
In traumatic spinal cord injury (SCI), damage to the spinal cord generated by mechanical trauma causes deficits to motor, sensory and autonomic function. Excluding fatal injuries, approximately 17,500 individuals in the United States suffer spinal cord injuries annually. An estimated 285,000 individuals in the United States currently live with some kind of SCI (Silver et al., 2014; 2016). Clinically, the degree of impairment is highly variable and depends on both the injury severity and the region where trauma occurred. SCIs in the cervical region are among the most devastating because they disrupt downstream motor circuits including respiratory circuits. Indeed, the most common cause of death after cervical SCI is ventilatory failure (Frankel et al., 1998).

SCI results from trauma, disrupting both neural circuits and vascular architecture within the parenchyma of the spinal cord. The primary injury causes no gross damage to the spinal cord apart from cell death at the lesion site (Profyris et al., 2004). However, several mechanisms contribute to secondary injury and subsequent expansion of the lesion. Damaged vasculature leads to hemorrhage within the spinal cord and subsequent hematoma formation. Inflammation and oxidative stress trigger a wave of necrosis rostral and caudal from the lesion site which is irreversible. Axons retract away from the cytotoxic environment, further widening the lesion. A dense, fibrous layer of scar tissue envelopes the necrotic lesion. Within several weeks post-injury, activated macrophages in the lesion core consume necrotic tissue, forming progressive

CHAPTER 1
SPINAL CORD INJURY AND CNS REGENERATIVE FAILURE

Overview of Cervical Spinal Injury

In traumatic spinal cord injury (SCI), damage to the spinal cord generated by mechanical trauma causes deficits to motor, sensory and autonomic function. Excluding fatal injuries, approximately 17,500 individuals in the United States suffer spinal cord injuries annually. An estimated 285,000 individuals in the United States currently live with some kind of SCI (Silver et al., 2014; 2016). Clinically, the degree of impairment is highly variable and depends on both the injury severity and the region where trauma occurred. SCIs in the cervical region are among the most devastating because they disrupt downstream motor circuits including respiratory circuits. Indeed, the most common cause of death after cervical SCI is ventilatory failure (Frankel et al., 1998).

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cavitations which merge to yield a fluid-filled cyst contained within the scar tissue (Wallace et al., 1987; Profyris et al., 2004).

**Inflammation and Pathology**

This acute inflammatory response primarily causes BBB break-down, edema and recruitment of various immune cell types. Secondarily, inflammation is one of the major causes of widespread cell death following injury.

**Cytokines**

SCI triggers a rapid and transient elevation of pro-inflammatory cytokines in the lesion derived from upregulated expression in neurons and glia as well as extravasation from blood. The pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), leukemia inhibitory factor (LIF), and tumor necrosis factor (TNF) all peak expression at 24h post-injury. Expression quickly declines for all of these cytokines, but TNF demonstrates a second peak in the lesion epicenter at 28 days post-injury (Pineau and Lacroix, 2007). TNFα and IL-1β impair glutamate clearance, promoting excitotoxic cell death in neurons and oligodendrocytes (Chao et al., 1995; Takahashi et al., 2003). Increased TNFα and IL-1β expression also enhances vascular permeability in the lesion which contributes to BBB compromise and enhances leukocyte recruitment into the lesion (Schnell et al., 1999). Increased LIF and IL-6 expression have also been shown to enhance leukocyte infiltration (Lacroix et al., 2002; Kerr and Patterson, 2004).

**Blood-Brain Barrier Compromise**

While hemorrhage in SCI is primarily caused by mechanical injury to vascular architecture, BBB compromise allows for leakage of blood and serum elements from
otherwise intact blood vessels. After injury, perivascular stromal cells, fibroblast-like cells, pericytes, and other cells surrounding blood vessels rapidly proliferate and slough off of vessels to populate the lesion epicenter (Goritz et al., 2011; Fernandez-Klett et al., 2013; Soderblom et al., 2013). There is substantial erythrocyte and neutrophil infiltration within the first 24h post-injury with subsequent monocyte and lymphocyte infiltration in the following weeks.

**Microglial Activation**

Under homeostatic conditions, resident microglia are homogeneously distributed throughout the CNS. In response to ATP release from cell death however, respond by extending processes toward the lesion within seconds or minutes and migrating to the lesion site (Davalos et al., 2005; Dibaj et al., 2010). The primary role of these macrophages in the lesion is as phagocytes targeting necrotic tissue, but inflammatory cytokines in and around the lesion cause macrophages to take on distinct functional phenotypes. There is a wide spectrum of activated phenotypes in vivo, but the “classically” activated (M1) phenotype is canonically considered to be pro-inflammatory. Acute injury predominantly polarizes macrophages to M1 phenotype, but populations may shift to other phenotypes over the course of time with differential cytokine release. (Gordon and Taylor, 2005; Stout et al., 2005; David and Kroner, 2011).

**Cell Death and Axonal Retraction**

**Ischemia and Reperfusion**

Immediately following injury, ischemia resulting from vascular damage, vasospasm, thrombosis, and neurogenic shock deprives local tissues of oxygen and
compromises cellular metabolism. As a consequence of this local hypoxia, cells in the ischemic zone are driven to release glutamate while their reuptake mechanisms are impaired. High ambient glutamate levels activate AMPA receptors which depolarize the cell and allow for NMDA receptor activation. Over-activation of NMDA receptors pushes intracellular calcium levels past acceptable levels, triggering cell lysis or necrosis. This cascade triggered by ischemia is referred to as excitotoxicity. Neurons and oligodendrocytes are specifically vulnerable. (Profyris et al., 2004; Amar, 2008).

Ironically, reperfusion of ischemic tissues following injury only exacerbates tissue damage. Endothelial cells exposed to oxygen following a period of hypoxia perform enzymatic reactions which generate substantial reactive oxygen species (ROS) which can damage healthy cells (Guth et al., 1999). Along with the ROS emitted by neutrophils and necrotic cells, this adds to a potent cytotoxic stimulus (Profyris et al., 2004).

**Toxicity from Blood**

Within the lesion itself, the presence of blood products is one of the principal sources of secondary cell death following trauma (Aronowski and Zhao, 2011). Erythrocytes within the hematoma begin to lyse (hemolysis) quickly following hemorrhage, generating large quantities of toxic byproducts from the degradation of heme (Robinson et al., 2009). Apart from erythrocytes, blood-derived macrophages (i.e. neutrophils and monocytes) are significant sources of neuronal and glial toxicity. As byproducts of phagocytic activity, macrophages produce highly reactive and toxic free radicals. (Xiong et al., 2007). The same is true for classically activated (M1) microglia which release hydrolyzing enzymes, oxidative metabolites, and prostaglandins which damage neurons and glia (Silver et al., 2014). T and B lymphocytes mount immune
reactions against myelin proteins which exacerbates macrophage responses, further compromising BBB function and creating a toxic environment for both oligodendrocytes and neurons. (Hellings et al., 2002; O’Connor et al., 2005; Schroeter and Jander, 2005; Ankeny et al., 2006; Donnelly and Popovich, 2008).

**Axonal Retraction and Regeneration**

The cell damage most relevant to loss of function is axonal degeneration. Within 15min of injury, there is periaxonal swelling and disruption of myelin. Within 24h, axon contents can be observed in the extracellular space. Over time, injured axons exhibit other signs of pathology including widespread demyelination and abortive growth cones (Anthes et al., 1995). The sequela of this acute injury is a withering of severed axons called Wallerian degeneration in both rostral and caudal directions. This degeneration lasts for months in rat and years in humans (Gaudet et al., 2011).

Multiple factors are involved in the phenomenon of axonal retraction and degeneration. Attacks on the axonal tip by blood-derived macrophages have been deemed specifically responsible for axonal retraction both *in vitro* and *in vivo* following injury (Horn et al., 2008; Busch et al., 2009). As stated above, these attacks are mediated by reactions of T and B lymphocytes. Excitotoxicity resulting from ischemia and inflammation preferentially targets oligodendrocytes which form the myelin sheath around axons as well as the neurons those axons are connected to (Matute et al., 2001). Less acute mechanisms are at work as well. Programmed cell death (apoptosis) begins 6h after injury and continues for up to 3 weeks post-injury. This appears to be the primary mediator of delayed demyelination and axonal degeneration (Crowe et al.,
1997). Surviving oligodendrocytes are post-mitotic and cannot remyelinate axons (Keirstead and Blakemore, 1997).

**Scar Formation**

After direct injury and a period of secondary retraction, axon growth cones attempting to regenerate into the site of injury abruptly halt and form dystrophic end bulbs which rest indefinitely in the penumbra of the lesion (i.e. glial scar) and are considered to be the hallmark of regeneration failure (Ramon y Cajal, 1928; Tom et al., 2004). Much effort has been invested in the elucidation of which components of the lesion and glial scar are actually responsible for the hindrance of axonal growth and lack of functional network formation.

**Role of Astrocytes**

The inflammation and oxidative stress generated at the site of the lesion immediately drive astrocytes to migrate from the lesion epicenter to the very edges of the lesion (Fitch and Silver, 1997). The thin layer of reactive astrocytes at the lesion’s edge then begins to proliferate and thicken in a process called “astrogliosis” (Faulkner et al., 2004). Reactive astrocytes then accumulate intermediate filament proteins such as glial fibrillary acidic protein (GFAP), vimentin, and nestin, yielding a more hypertrophic morphology (This process also occurs with juxtavascular non-dividing astrocytes further from injury) (Wilhelmsson et al., 2004; Bardehle et al., 2013). Over time, the gliotic layer of astrocytes restructures to a mesh-like structure with projections perpendicular to the rostral-caudal axis of the spinal cord (Wanner et al., 2013).
Fibrotic Component of Scar

Growth-inhibitory extracellular matrix (ECM) molecules are produced by reactive astrocytes including a family of proteins referred to as chondroitin sulfate proteoglycans (CSPGs). This family includes aggrecan, brevican, neurocan, phosphacan, versican, and neuroglial 2 proteoglycan (NG2) (Margolis and Margolis, 1993). Phosphacan, neurocan, brevican, and NG2 produced within the glial scar have been demonstrated to as a major impediment to axonal growth both in vivo and in vitro (Cregg et al., 2014; Silver et al., 2014).

The production of ECM molecules is not unique to astrocyte, however. Type A pericytes, endothelial cells, and perivascular fibroblasts that proliferate as a result of vascular damage post-injury contribute to the deposition of type IV collagen, fibronectin, and laminin (Goritz et al., 2011; Kawano et al., 2012; Soderblom et al., 2013). Oligodendrocyte precursor cells (OPCs) within the lesion for example, are known to produce a mélange of both inhibitory and growth-promoting ECM molecules including NG2. NG2+ OPCs are among the most highly proliferating following injury and may even act as progenitors for different glial cell types. (Zai and Wrathall, 2005; Lytle et al., 2006; Busch et al., 2010). Their contributions to either the promotion or blockade of axonal regeneration are still highly debated. Because NG2 is one of the most highly upregulated CSPGs following injury, some have suggested that NG2+ cells are the major inhibitors of regeneration. In fact, the majority of axons severed after injury do not interact directly with astrocytes, but rather with NG2-producing OPCs (Busch et al., 2010; Filous et al., 2010). Following injury, NG2 glia form synapse-like connections with neurons, stabilizing their dystrophic tips and preventing further retraction. While
beneficial in the acute phases of injury, this interaction seems to prevent forward movement of axons through the scar as well (Silver et al., 2014)

All of these ECM-producing cells interact in the perimeter of the lesion to form a dense structure commonly referred to as the “glial scar”. This scar encapsulates the lesion, containing intra-lesion inflammatory and cytotoxic elements but, in effect, acts as the primary physical and chemical barrier to axonal regeneration post-injury (Silver et al., 2014).

**Lesion in Chronic Phase**

In acute phases of injury, axons are attacked and repelled by the inflammatory environment within the lesion. In chronic phases of injury, the inflammation is much more quiescent. Expression of the inflammatory cytokines IL1-β, TNFα, IL-6, and LIF peaks shortly after injury and rapidly downregulates within the first 24h of injury (Donnelly and Popovich, 2008). The cell types responsible for a good portion of cell death and axonal retraction during the acute phase of injury dwindle over time. Neutrophils accumulate rapidly in the injury, but are cleared within 7 days post-injury (Kigerl et al., 2006). In rats, lymphocyte infiltration peaks between 3 and 7 days post-injury and continually declines over the following 2 weeks. Populations of activated macrophages reach a plateau in the first week of injury and begin declining after 28 days (Sroga et al., 2003).

At 2-3 weeks post-injury, demyelination of axons near the lesion tapers off and the process of remyelination begins. This is in part due to the tapering of immune-mediated attacks on myelin proteins and inflammatory cytotoxicity toward
oligodendrocytes (Donnelly and Popovich, 2008). A much more prominent factor is the differentiation of OPCs into oligodendrocytes. While the oligodendrocytes left after injury are non-dividing, the progeny of OPCs are highly proliferating and apart from their role in glial scar formation, are largely responsible for the remyelination of axons (Lytle and Wrathall, 2007; Sellers et al., 2009).

While macrophages at the lesion clear away necrotic tissue, the glial scar maintains its integrity. Once all necrotic tissue is cleared, the former lesion site is no more than a fluid filled cyst surrounded by scar tissue (Profyris et al., 2004). Although the glial scar in the acute phase functions to shield healthy cells and axons from neuroinflammation within the lesion, the lack of inflammation in the chronic phase means that the glial scar only functions as a barrier which must be hurdled to grow axons and reestablish functional connections (Wanner et al., 2013).

Vascular Dysfunction

One often overlooked consequence of SCI is vascular dysfunction caudal to injury due to loss of supraspinal monoaminergic input. Axons projecting from the brainstem express the enzymes tryptophan hydroxylase and L-amino acid decarboxylase (AADC) which are involved in the synthesis of the neurotransmitters dopamine, norepinephrine, and serotonin. These neurotransmitters are responsible for maintaining myogenic tone and vasoconstriction in the CNS (Hardebo and Owman, 1980). When input from these axons is lost post-injury, the only remaining enzyme is AADC which sees substantial upregulation in pericytes surrounding spinal capillaries following SCI (Commissiong, 1985; Li et al., 2014). AADC alone is not sufficient for
neurotransmitter synthesis, but it does catalyze the synthesis of trace amines from amino acids. TAs activate serotonin 1 receptors (5-HT1), α2 adrenergic receptors, and specialized Trace-amine-associated receptors (TAARs), which all have effects on vasculature. Trace amines acting on pericytes constrict capillaries following SCI inducing severe tissue hypoxia (PO2 << 20mmHg) caudal to the injury. This tissue hypoxia is chronic and detrimental to motor function so long as monoaminergic input is not restored (Li et al., 2017).

Models of SCI
Animal models of SCI are an indispensable tool for studying the pathology of SCI as well as the efficacy of therapeutic interventions. When generating animal models of SCI, there are five different approaches which are modified and applied by researchers to achieve specific experimental conditions: contusion, compression, distraction, dislocation, chemical, or transection (Cheriyan et al., 2014). Each has unique advantages and disadvantages for different scientific aims.

The preferred models for studying pathophysiology of SCI in human involve contusion or compression (Nobunaga et al., 1999). Contusion models are the oldest and most commonly used models of SCI (Sharif-Alhoseini et al., 2017). In these models, an impactor device is used to inflict transient, acute injury to the exposed spinal cord. Compression models involve acute impact, like what is seen in contusion models, followed by persisting compression of the cord. These are useful to simulate the central canal occlusion that is often seen in human SCI and to study effects of compression and decompression (Sharif-Alhoseini et al., 2017). This compression is often inflicted by
clips, forceps, balloons, or purpose-built spinal cord strapping devices (Cheriyan et al., 2014).

Distraction models involve controlled stretching of the spinal cord to generate tension force. This model is generally used on larger animals. Dislocation models use lateral vertebral dislocation to replicate tension force like what is seen in distraction models, but without surgical exposure of the spinal cord. Chemical models use pharmacological agents to mimic specific features of injury such as excitotoxicity, ischemia, or demyelination (Cheriyan et al., 2014).

Transection models involve either a complete or incomplete dissociation of rostral and caudal segments. Translational potential for these models is limited because transected spinal cords are rarely seen in clinical practice and so cannot be said to accurately reflect the pathophysiology of SCI. This model is highly customizable and allows researchers to lesion specific tracts and neuronal populations in the spinal cord. Injuries are easily reproducible and provide an excellent model to evaluate tissue regeneration, plasticity, and functional recovery (Cheriyan et al., 2014; Sharif-Alhoseini et al., 2017).

In contusion, compression, and transection models, researchers have the option to either unilaterally, partially, or completely lesion the spinal cord. This decision is impacted by the region being lesioned and what is being evaluated in the study. In the cervical spinal cord, unilateral or partial lesions are most commonly used because complete lesions generate life-threatening pathology due to the disruption of respiratory pathways (Gensel et al., 2006). Complete lesions are more common in the thoracic and
lumbar spinal cord where life-threatening comorbidities are less likely to be life
threatening (Sharif-Alhoseini et al., 2017). This variability in approach may affect
experimental outcomes. For example, multiple studies have demonstrated differences in
plasticity between partial and complete transection models (Edgerton et al., 2004).
CHAPTER 2
AXONAL REGENERATION

Approaches to Axonal Regeneration

Following the acute phase of injury where cell death is actively ongoing, little can be done to salvage lost grey matter. Because of this, the strongest efforts should be directed at regeneration of axons and white matter which still maintain potential to grow and form functional connections despite damage. Over the years, a wide variety of approaches have been applied toward the goal of neural regeneration with varying degrees of success. Many approaches have involved bridging the physical gap formed by the lesion through cell transplantation, nerve grafting, or engineered biomaterials. Others involve the manipulation of intrinsic and environmental growth factors. For the sake of brevity, we will be discussing the latter approach as it reveals details more salient to the goals of this study.

Growth Inhibitors

In Chapter 1, we discussed the many facets SCI which create a cytotoxic or growth inhibitory environment in both the acute and chronic phases of injury. Acute inflammation, Wallerian degeneration, and the fibrotic component of the glial scar cause axons to retract away from the lesion site and remain in the lesion periphery, but we have not yet discussed the qualities of the CNS cells and environment which prevent long distance growth following injury.

Compared to the peripheral nervous system (PNS), axons in the CNS have relatively poor regenerative capacity following injury. This is partially due to intrinsic differences in cell physiology, but the predominant inhibitors of axonal growth seem to
be environmental (Silver et al., 2014). As early as the late 19th century, it was demonstrated that injured CNS axons will readily grow long distances through peripheral nerve grafts, implying that the PNS is much more permissive to growth than the CNS (Ramon y Cajal, 1928). It was later discovered that growth inhibitory factors were enriched in the CNS, specifically, in CNS myelin and oligodendrocytes. This presents challenges for researchers attempting to promote regeneration in chronic SCI when the process of remyelination is in full swing and myelin concentrations gradually increase.

Oligodendrocyte-derived myelin is one of the most potent inhibitors of neurite growth and plasticity in the adult CNS (Silver et al., 2014). In vitro, neurons derived from the dorsal root ganglion halt neurite outgrowth when they come in contact with any form of CNS myelin or isolated oligodendrocytes. Their growth cones collapse shortly after neurite outgrowth is halted like what is seen following SCI (Schwab and Thoenen, 1985; Schwab and Caroni, 1988; Fawcett et al., 1989). The repair capacity of the CNS is higher during development than at more mature stages. Experimental studies determined that this change from a growth-permissive to a non-growth-permissive environment coincide with myelin formation (Keirstead et al., 1992).

The proteins Nogo-A, myelin-associated glycoprotein (MAG), oligodendrocyte/myelin glycoprotein (OMgp), ephrins A3 and B3, semaphorins 4D, 5A and 3F, CSPGs, and myelin glycolipid sulfatide are all enriched in CNS myelin and act as potent growth inhibitors on multiple neuronal subtypes in vitro (Fawcett et al., 2012). Of these myelin-derived growth inhibitors, Nogo-A is the most-studied. Nogo-A-
mediated growth inhibition depends on the activation of Rho, a signaling molecule downstream of Rho-associated protein kinase (ROCK), and inhibition of cAMP response binding element (CREB). Nogo-A also downregulates mammalian target of rapamycin (mTOR), a key growth promoter (Hannila and Filbin, 2008; Joset et al., 2010). Genetic deletion of Nogo or Nogo receptor (NgR1) in mice have been demonstrated to promote axonal growth and even reestablish the degree of structural plasticity seen in development (Pernet and Schwab, 2012). Myelin's inhibition of neurite outgrowth and plasticity is thought to have evolved as a mechanism to stabilize the structure of CNS white matter and restrict developmental plasticity (Silver et al., 2014).

Intrinsic to the neuron, there are several key molecules and pathways that negatively regulate growth. Under homeostatic conditions, initiation of the phosphoinositide 3-kinase (PI3K)/mTOR cascade is antagonized by phosphatase and tensin homolog (PTEN). Downstream, upregulating TSC can antagonize this pathway at Rheb1 (Park et al., 2008). Suppressor of cytokine signaling 3 (SOCS3) negatively regulates the Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathway which is critical for cell growth and proliferation (Smith et al., 2009). Deletion of PTEN and SOCS3 together results in sustained, long-distance axonal regeneration of CNS axons (Sun et al., 2011).

**Growth promoters**

Some of the most well-known growth promoters are neurotrophins. Neurotrophins are a diverse family of small proteins that influence axonal growth, neuronal survival, synaptic plasticity, and neurotransmission. Their influences on growth
are highly dependent on where they are applied in relation to the injured neurons and the concentrations of their cognate receptors. For example, acutely following cervical SCI, delivery of exogenous brain-derived neurotrophic factor (BNDF) to the site of injury promotes robust regeneration of rubrospinal axons in vivo. However, the same response is not elicited if BDNF administration is delayed to 6-8 weeks post-injury (Shumsky et al., 2003; Tobias et al., 2003). This is largely attributed to the downregulation of BDNF’s high-affinity receptor, tropomyosin receptor kinase B (TrkB) in these axons after injury. Without TrkB receptors to sense high concentrations of BNDF at the injury site, the axons are not drawn to regenerate in that direction (Kwon et al., 2004). Interestingly, if either BDNF or another neurotrophic factor, neurotrophin-4 (NT-4), are applied directly to the soma of the injured neuron, the loss of TrkB receptors is diminished, axonal degeneration can be prevented acutely, and axonal growth can be stimulated up to a year post injury. (Kobayashi et al., 1997; Kwon et al., 2002).

A second messenger molecule downstream of neurotrophin receptor activation, cyclic adenosine monophosphate (cAMP) has been implicated as a major promoter of axonal regeneration following injury (Hannila and Filbin, 2008). Increased levels of intracellular cAMP activates protein kinase A (PKA), which phosphorylates cAMP response-element binding protein (CREB). CREB is a transcription factor that promotes the transcription of a suite of genes including interleukin-6 (IL-6), arginase I, secretory leukocyte protease inhibitor, and metallothionen which are important for axonal growth (Siddiq and Hannila, 2015).
Several other cell signaling molecules/pathways have also been identified as key elements in growth regulation. The kinase, mammalian target of rapamycin (mTOR) is an example of a growth-promoting molecule. In the neuron, mTOR activity is implicated in the process of new growth cone formation after injury in vitro and suggested as a critical determinant of regenerative ability within the CNS (Verma et al., 2005; Park et al., 2008). mTOR activates protein translation and ribosome biogenesis via phosphorylation of different substrates including ribosomal protein S6 and eukaryotic elongation factors. The end result is upregulated protein synthesis and cell growth (Guertin and Sabatini, 2007).

The activity of mTOR is mediated by the PI3K/protein kinase B (AKT) cell signaling pathway. AKT activation leads to phosphorylation and subsequent inhibition of tuberous sclerosis complex (TSC) 1 and 2 which in turn, results in subsequent engagement of Ras homolog enriched in brain (Rheb) to activate mTOR (Park et al., 2010). Another pathway implicated in axonal growth is the Ras/Raf/ERK pathway. This pathway works synergistically with the PI3K/AKT pathway when they are concurrently activated. Inhibiting Ras/Ras/ERK or PI3K pathway in retinal ganglion cells partially reduces neurotrophin-mediated outgrowth in vitro. Blocking both completely prevents any axonal outgrowth (Goldberg et al., 2002; Park et al., 2004).

The JAK/STAT pathway is a cell signaling pathway that is triggered by injury related cytokines and has been implicated in the promotion of growth in conditioning lesion models of axonal regeneration. Cytokines such as IL-6, ciliary neurotrophic factor, LIF are highly expressed after axonal injury and are known to activate the
JAK/STAT pathway. Initiation of this cascade phosphorylates STAT3 which translocates into the nucleus to initiate transcription of pro-growth proteins. Interestingly, one of the proteins regulated by STAT3 is its inhibitor, SOCS3, which thereby autoregulates the JAK/STAT pathway (Sun and He, 2010).

**Manipulating Growth Regulators**

Promoting or inhibiting axonal growth involves carefully adjusting the balance between endogenous growth promoters and growth inhibitors. For example, the inhibitory effects of Nogo-A, MAG, or CNS myelin can be counteracted with elevated levels of cAMP because their downregulation of growth depends on activation of Rho-A and CREB inhibition. Neurotrophic factors such as brain derived neurotrophic factor (BDNF), glial-derived neurotrophic factor, and nerve growth factor, which elevate levels of cAMP or P-CREB, can also do this (Cai et al., 1999; Hannila and Filbin, 2008). Concentrations of certain growth promoters such as BDNF and cAMP can be increased through direct exogenous administration (Kwon et al., 2002; Qiu et al., 2002). Changing the activity or environment of the animal using means such as exercise or hypoxia can also manipulate these pathways (Liu et al., 2012; Cho et al., 2015).

Growth regulators can also be manipulated via pharmacological means. Antibodies against Nogo-A have shown promise to promote structural plasticity and functional rehabilitation in rodent and clinical studies (Liebscher et al., 2005; Freund et al., 2007). The drug rolipram, a phosphodiesterase 4 (PDE4) inhibitor, elevates endogenous levels of cAMP in cells. After SCI, rolipram has shown to promote functional recovery and attenuate glial scar formation. Completely ablating the CSPG
component of the glial scar using the enzyme chondroitinase ABC (ChABC) has been shown in multiple studies to promote neurite outgrowth in chronic SCI (Massey et al., 2006). Alilain et al. (Alilain et al., 2011) even demonstrated functional regeneration of respiratory motor pathways with the use of ChABC.

To experimentally assess, the relationships between specific molecules and axonal growth, it is important to use drugs or treatments that are maximally selective for those molecules. With the expansion of bioinformatics however, it may be highly advantageous to seek promiscuous drugs which can syngergistically improve axonal regeneration by engaging multiple targets, thereby maximizing their therapeutic potential (Al-Ali et al., 2015).

**Assessment of Axonal Regeneration in Models of SCI**

Outcome assessments in models of SCI involve the evaluation of anatomy and function. Functional assessments are used to evaluate the extent of injury and efficacy of treatment either in the animal’s behavior or at the level of neurophysiology. For the purposes of axonal regeneration however, functional assessments are limited because they are readily influenced by mechanisms of plasticity which may strengthen spared pathways rather than regenerating severed ones. For this purpose, anatomical assessments are superior.

A powerful tool for assessing anatomical outcomes is magnetic resonance imaging (MRI). MRI can discriminate between white and grey matter, approximate the size of lesions, and discern the formation of scars and cavities in both living and dead animals (Sharif-Alhoseini et al., 2017). Functional MRI can even be used to distinguish...
recovery of sensory and motor function in living animals (Li et al., 2005). The major limitation of MRI is cost and access to MRI facilities, which is a significant barrier to studies with large sample sizes. There is also limited resolution with which to observe the structure and behavior of individual axons, although advances in diffusion tensor imaging have allowed researchers to resolve down to white matter fiber bundles (Li et al., 2015).

More traditional methods of anatomical assessment involve extraction of tissues from dead animals for histological analysis. Traditional histology involves staining of spinal cord tissues to assess tissue sparing, white matter loss, and cell counts. A well-known dye, specifically used to stain neurons and glia is cresyl violet. Cresyl violet dye has a deep purple color and binds to acidic structures in cells including ribosomes and nuclei as well as Nissl bodies which are specific to neural cells. Myelin can be stained for using one of many stains such as Luxol fast blue or osmium tetroxide (Sharif-Alhoseini et al., 2017). Because axonal retraction is such a large contributor to injury size, traditional histology can become a useful tool for evaluating widespread axonal growth, although it is highly difficult to differentiate specific cell types and fine structures such as axons and dendrites.

Like traditional histology, immunohistochemistry (IHC) is used to stain sections of tissue. However, rather than using chemical dyes, IHC labels proteins using antibodies specific to those proteins. The antigen antibody interaction is then visualized using an enzyme-conjugated antibody that can catalyze a color-producing reaction or to a fluorophore-labeled antibody which can be seen using fluorescence microscopy. The
latter is highly useful because multiple protein labels can be visualized on the same tissue using different fluorophores. For example, by staining for certain cytoskeletal proteins, researchers can see axonal projections in fine detail. They can simultaneously label growth-inhibitory/promoting cells or proteins to visualize their localization relative to each other.

Neuroanatomical tract tracing can be used to visualize specific neuronal projections and connections from regions injected with retrograde labels or viral vectors which cause cells to express proteins which can then be visualized (Gerfen and Sawchenko, 2016; Radtke et al., 2017). While a highly useful tool, this method only provides information limited to the regions that have been labeled. The majority of axonal tracts remain unlabeled and as such, cannot be visualized in the context of regeneration or structural plasticity.
CHAPTER 3
ACUTE INTERMITTENT HYPOXIA

Overview of Acute Intermittent Hypoxia

In an attempt to restore motor function following SCI, many treatment paradigms attempt to strengthen spared neural pathways by harnessing the innate plasticity of the nervous system. One well studied treatment paradigm is acute intermittent hypoxia (AIH). In its most studied applications, AIH induces spinal plasticity, strengthening synaptic connections to respiratory motor neurons, giving rise to long-lasting phrenic motor facilitation (pMF). These effects have also been demonstrated in nonrespiratory motor pathways following injury suggesting that this plasticity is global versus localized to the phrenic motor nucleus (Lovett-Barr et al., 2012; Trumbower et al., 2012).

Phrenic Long-Term Facilitation

AIH-induced phrenic long term facilitation (pLTF) was originally described as a persistent increase in phrenic motor output following repeated stimulation of the carotid sinus nerve (Millhorn et al., 1980a, b). This demonstrated that pLTF is a central, rather than peripheral mechanism. Later studies demonstrated similar pLTF through repeated chemoreceptor activation or hypoxic episodes. In each of these studies, pLTF was observed for at least 1h after stimulation (Bach and Mitchell, 1996; Mitchell et al., 2001).

The proposed mechanism is that episodic hypoxia elicits LTF via carotid chemoreceptor activation of serotonergic raphe neurons with excitatory bulbospinal and cranial projections to respiratory motor nuclei (McCrîmmon et al., 1995). The intermittent pattern is more important than the duration or severity of hypoxia. pLTF is elicited by Intermittent, but not sustained hypoxia (Baker and Mitchell, 1999). The actual details of
these hypoxic episodes are less stringent. Similar magnitudes of pLTF have been elicited with episodes ranging from 15 sec to 5 min and partial pressure of arterial oxygen (PaO$_2$) from 28 to 70 mmHg (Fuller et al., 2000; Peng and Prabhakar, 2004; Baker-Herman and Mitchell, 2008; Mahamed and Mitchell, 2008). pLTF is the most studied form of PMF and the most relevant to AIH as a treatment, but for the remainder of the thesis, we will use pMF as an umbrella term as the exact definition of pLTF can become limiting.

**Q pathway.** The “Q pathway” to pMF is mediated by metabotropic serotonin 2 receptor (5-HT$_2$) activation. It is named for the G$_q$ proteins to which 5-HT$_2$ receptors are coupled. Episodic serotonin release and 5-HT$_2$ activation have both been shown to be necessary and sufficient to elicit pMF without AIH. In fact, the pattern sensitivity of serotonin-induced pMF is very similar to AIH-induced pMF (MacFarlane and Mitchell, 2009). Therefore, AIH-induced LTF is considered to be predominantly driven by the Q pathway (Bockaert et al., 2006).

The current working model of the Q pathway is that repetitive, moderate hypoxia triggers serotonin release in the phrenic motor nucleus and in turn, activating 5HT$_2$ receptors and triggers G$_q$ signaling cascades (Baker-Herman and Mitchell, 2002). The signaling cascade relevant to Q pathway pMF includes extracellular signal regulated-mitogen-activated protein (ERK-MAP) kinase activity (Wilkerson and Mitchell, 2009), new synthesis of brain-derived neurotrophic factor (BDNF) (Baker-Herman et al., 2004), activation of its high-affinity receptor, TrkB (Baker-Herman et al., 2004) and downstream activation of protein kinase C-theta (PKCθ) (Agosto-Marlin and Mitchell, 2017). The
connection between PKCθ and pMF is still unclear, but it is predicted to involve phosphorylation of glutamate receptors on motor neurons as a blockade of spinal NMDA receptors effectively abolishes pMF (McGuire et al., 2008; Turner et al., 2017).

There also seems to be a differential mechanism between 5HT2A and 5HT2B receptors which is still being elucidated. 5HT2B receptor-mediated pMF is dependent on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity and nitric oxide (NO) generation by neuronal nitric oxide synthase (nNOS) whereas 5HT2A is not (MacFarlane et al., 2011; Macfarlane et al., 2014).

**S pathway.** The S Pathway is triggered by severe AIH (PaO2 25-35mmHg) as opposed to the more moderate AIH which triggers the Q pathway (Nichols et al., 2012). Severe AIH activates a pathway, independent of 5HT2 receptors, which is initiated by Gs protein-coupled receptors (Dale-Nagle et al., 2010a). The S pathway is initiated by either 5HT7 (Hoffman and Mitchell, 2011) or adenosine 2A (A2A) receptors (Nichols et al., 2012). The Gs signaling cascade initiated by these receptors includes cAMP signaling (Fields and Mitchell, 2017) via exchange protein activated by cAMP (EPAC) (Fields et al., 2015), activation of AKT (Golder et al., 2008), mTOR signaling (Dougherty et al., 2015), and 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., 2017).

**Cross-talk inhibition.** When the Q and S pathways are activated concurrently, pMF is attenuated or even abolished but, depending on severity of hypoxia, one pathway predominates. As a general rule, the Q pathway dominates pMF during mild to
moderate hypoxic episodes (PaO$_2$ ≥ 35mmHg) and the S pathway dominates during more severe hypoxia (PaO$_2$ ≤ 30mmHg) (Devinney et al., 2013). Reflecting this, low serotonin doses elicit robust pMF, whereas pMF is not observed with high serotonin doses unless 5-HT$_7$ receptors are blocked (MacFarlane and Mitchell, 2009). Activation of both 5-HT$_2A$ and 5-HT$_2B$ receptors inhibit 5-HT$_7$-derived pMF (Perim et al., 2018).

PKA activity, which is a consequence of cAMP signaling in the S pathway is necessary and sufficient to block 5-HT$_2$-induced pMF through inhibition of ERK (Fields and Mitchell, 2017). 5-HT$_2A$ and 5-HT$_2B$ have unique mechanisms that inhibit the 5-HT$_7$-induced S pathway. NOX activity from the 5-HT$_2B$-mediated Q Pathway generates ROS, particularly superoxide anions, which are quickly converted to hydrogen peroxide (Nauseef, 2014). Hydrogen peroxide depresses adenylyl cyclase (AC) activity and cAMP signaling, therefore inhibiting EPAC which is vital for the S pathway to occur (Perim et al., 2018). Currently unpublished by Perim et al. (2018) suggests that PKC generated by the 5HT2A-mediated Q pathway is responsible for suppression of the 5HT7 mediated S Pathway. Very little is known still about the A$_{2A}$ dependent S pathway and how it interacts with the Q pathway via cross-talk inhibition. A schematic of the Q pathway, S pathway and their cross-talk inhibition is shown in Figure 2-1.

**Other mechanisms of pMF.** There are three other known mechanisms of pMF which are far less studied than the Q and S pathways. Those are 1) VEGF-induced pMF, 2) EPO-induced pMF, and 3) inactivity-induced pMF (iPMF). Vascular endothelial growth factor (VEGF) signals via a tyrosine kinase coupled VEGF receptor-2 (VEGFR-2) and erythropoietin (EPO) signals through a JAK2-coupled receptor (EPO-R). Both
require ERK/MAPK and Akt as necessary intermediates for full pMF (Dale-Nagle et al., 2011; Dale et al., 2012). EPO, VEGF, and their receptors are upregulated in response to prolonged hypoxia (Forsythe et al., 1996). VEGF is known for its role in angiogenesis and cell permeability regulation. It has also been recognized as a neuroprotective and neurotrophic factor (Zachary, 2005). EPO has hematopoietic properties and is involved in ventilatory acclimatization (Dale et al., 2012). iPMF has been observed in models where respiratory neural activity has been reduced and then reestablished, leading to a robust facilitation. It is thought to be a major player in spontaneous recovery from SCI (Braegelmann et al., 2017).

**Metaplasticity**

Plasticity is often thought of as the nervous system’s active adaptation to changes in stimuli. Plasticity itself, however can change based on experience through a phenomenon called metaplasticity or “plastic plasticity”. In the case of AIH, cumulative benefit from repeated exposures would be an example of metaplasticity because the pMF (i.e plasticity) itself is exhibiting plasticity (Abraham and Bear, 1996; Fields and Mitchell, 2015).

**AIH as a Treatment for SCI**

AIH presents a promising treatment strategy to promote plasticity in both respiratory and somatic motor systems. As a treatment, it is non-invasive, non-pharmacological, and without any known negative side effects. Some evidence even suggests it may promote regrowth of axons to promote functional reconnection in the spinal cord following injury. Some spontaneous recovery of phrenic and somatic motor
function occurs in the weeks or months following injury as a result of the "crossed phrenic phenomenon" (Goshgarian, 2003). This spontaneous recovery is limited and therapeutic strategies that further strengthen these pathways would greatly benefit the recovery process.

**AIH Following Injury**

Following SCI, endogenous levels of serotonin decrease dramatically in the spinal cord (Hashimoto and Fukuda, 1991). In the phrenic motor nucleus, this loss of serotonergic input corresponds to a loss of ability to elicit AIH-induced pMF on the side of injury (ipsilateral). However, by 8 weeks post-injury, serotonergic innervation has recovered (albeit to significantly lower levels than pre-injury) and AIH-induced pMF can once again be elicited. Interestingly, on the non-injured (contralateral) side, capacity for pMF is limited even 8 weeks post-injury, suggesting compensatory responses to injury on the ipsilateral side prevent pMF expression on the contralateral side (Golder and Mitchell, 2005). This limited expression of contralateral pMF can be rescued through pretreatment with daily AIH 1 week post-injury (Vinit and Mitchell, 2009). These studies demonstrate that acutely post-injury, ability to elicit pMF and promote functional recovery is limited, but recovers in a manner dependent on serotonergic innervation below the injury. In fact, Hashimoto and Fukuda (1991) demonstrated that even in the absence of AIH, functional recovery was dependent on spinal concentrations of serotonin more than any other neurotransmitter.
**Chronic Intermittent Hypoxia**

Chronic intermittent hypoxia (CIH) type of intermittent hypoxia protocol which was originally developed to model the pathophysiological effects of obstructive sleep apnea (OSA). These protocols use frequent hypoxic episodes for an 8-12 hour period over the course of days or weeks. The end result of CIH is robustly increased plasticity in multiple sites of the respiratory system. This includes increased carotid body chemosensitivity, increased synaptic strength in the nucleus tractus solitarius (NTS), and increased strength in spinal respiratory motor pathways. Pretreatment with CIH also induces metaplasticity and can elicit pMF even 2 weeks post-injury (Fuller et al., 2003; Dale-Nagle et al., 2010b). This increased plasticity has been suggested to be a compensatory mechanism for stabilizing respiration in OSA (Mahamed and Mitchell, 2007). However, CIH cannot be applied as a therapeutic treatment as it has been associated with broad deleterious side effects. CIH can induce hypertension, impaired heart rate control, neurocognitive deficits, metabolic syndrome and tumor metastasis through mechanisms of inflammation, oxidative stress and transcriptional regulation. This is consistent with human OSA comorbidities (Almendros et al., 2014). More moderate AIH protocols would be appropriate in a clinical context to avoid pathology.

**Repetitive Acute Intermittent Hypoxia**

Repetitive acute intermittent hypoxia (rAIH) is an umbrella term for AIH paradigms that involve multiple treatments of AIH over days, weeks or months which are less severe than CIH protocols. Like CIH, rAIH protocols come in many forms. In general, however, these protocols involve 10, 5min episodes of moderate hypoxia (O₂: 10.5%) interspersed with 5 minute episodes of normoxia (O₂: 21%). The frequency and
duration of these protocols varies with each study. One well-studied variation of rAIH is daily AIH. In rats with SCI treated with daily AIH 1 week post injury, studies have observed functional recovery of diaphragm and intercostal activity (Navarrete-Opazo et al., 2015). Injured rats treated with daily AIH 4 weeks post-injury show restored breathing capacity and phrenic motor output as well as an increase in BDNF and phosphorylated TrkB expression in motor nuclei. Interestingly, non-injured rats treated with dAIH had no change in breathing capacity, but they displayed similar BDNF and pTrkB upregulation to the injured rats (Lovett-Barr et al., 2012). The potential benefits of rAIH are not limited to daily exposure. In non-injured rats exposed to rAIH 3 times per week for 10 weeks (3xwAIH), there is an upregulation of proteins and neuromodulators associated with multiple mechanisms of pMF in the phrenic motor nucleus (Satriotomo et al., 2012). Interestingly however, neither VEGF nor EPO-induced pMF were affected by 3xwAIH preconditioning (Dale and Mitchell, 2013). This suggests, the effects of rAIH may be strictly limited to Q and S pathway-mediated pMF. No pathophysiology has yet been associated with rAIH, although other factors may impose barriers to its implementation as a treatment in SCI.

**Inflammation and AIH Efficacy**

Both systemic and CNS inflammation present challenges as we attempt to apply AIH-induced plasticity in a translational context. Hypoxia itself induces CNS inflammation through the action of O$_2$-sensitistive prolyl hydroxylases (PHD). When IκB kinase (IKK-β) is disinhibited by PHD, hypoxia activates NF-κB to promote pro-inflammatory cytokine transcription (Cummins et al., 2006). The mechanism of inflammation via PHD is similar to the mechanism of HIF-1α which is a master switch for
gene expression in hypoxia (Rius et al., 2008; Prabhakar and Semenza, 2015). NF-κB works somewhat synergistically with HIF-1α. NF-κB is a transcriptional activator of HIF-1 and basal NF-κB activity is required for HIF-1 accumulation during hypoxia (Rius et al., 2008).

Q pathway-mediated pMF is blocked by even mild systemic inflammation (Huxtable et al., 2011; Huxtable et al., 2013). However, systemic inflammation has no effect on A2A-mediated pMF (i.e. S Pathway) (Agosto-Marlin et al., 2017). This inhibition of Q pathway pMF is mediated by a p38 MAPK-dependent mechanism (Huxtable et al., 2015; Huxtable et al., 2017). This means that in the acute stages of SCI, inflammation will undermine Q pathway pMF and the S pathway will dominate. In the chronic phases, the Q pathway comes back on top.

**Application to Non-Respiratory Motor Systems**

While the effects of AIH and its many variants have primarily been studied on the respiratory motor system, a growing body of literature suggests that these effects also extend to other somatic motor nuclei. Non-injured rats exposed to 3xwAIH demonstrate significant increase in plasticity-related neuromodulators and proteins in non-respiratory motor neurons like what has been seen in the phrenic motor nucleus (Satriotomo et al., 2016). Rats with cervical spinal injury exposed to daily AIH show increased forelimb function (Lovett-Barr et al., 2012). The majority of observations relating to non-respiratory motor rehabilitation following injury have been made in human clinical studies.
Evidence from Human Trials

Human therapeutic AIH trials are still in their infancy, but evidence continues to suggest that the same therapeutic effects elicited in rodent models can be demonstrated in humans. A study by Trumbower et al. (2012) measured voluntary ankle strength in 13 individuals with chronic, incomplete SCI before and after receiving AIH (15, 1min episodes; 9% O₂). Subjects demonstrated and improvement between 59-115% above baseline immediately post-AIH and function above baseline was maintained for 90min after the treatment had ceased. Tester et al. (2014) was able to demonstrate that rAIH can elicit pMF by measuring ventilatory function after 10 days of AIH treatment (8, 2min episodes; 8% O₂). Hayes et al. (2014) used daily AIH (15, 90sec episodes; 9% O₂) to improve both walking speed and endurance in patients with chronic incomplete SCI.
Figure 3-1. Q and S Pathway
CHAPTER 4
APPLYING LONG-TERM REPETITIVE AIH AS A POTENTIAL TOOL FOR
REGENERATION

Potential of AIH to Promote Axonal Regeneration

Mechanisms of AIH-dependent plasticity and axonal regeneration are closely related. One is associated with strengthening existing neural circuits, whereas the other establishes new circuits, their signaling pathways share enough homology for one to suggest that they may influence each other. Based on known cell signaling pathways, the Q pathway should promote axonal growth through synthesis of BDNF, activation of TrkB and phosphorylation of ERK which inhibits PDE4 (an inhibitor of cAMP activity). The S pathway should promote growth through upregulation of mTOR and cAMP. Because these pathways undermine each other through cross-talk inhibition, treatment paradigms should be designed to minimize concurrent activation.

During acute injury, S pathway will dominate due to its resistance to inflammation and abundant adenosine concentrations resulting from cell death. Protocols involving severe hypoxia would also allow the S pathway to dominate. Treatment in the acute phase however, provides a small therapeutic window and severe hypoxia is associated with deleterious side-effects. For these reasons, the Q pathway emerges as a preferred therapeutic target. Q pathway-mediated plasticity mechanisms can be elicited with modest doses of hypoxia, minimizing risk of deleterious effects. Additionally, optimizing treatment paradigms for the chronic phase of injury promises easier clinical translation for the millions living with SCI and not only the recently injured.
There is little precedent in the literature to suggest AIH will promote neural regeneration in the CNS. One study by Cho et al. (2015) demonstrated that axonal regeneration and neuromuscular junction innervation were enhanced following 3 days of daily AIH in a mouse model of sciatic nerve injury. However, they presented no data to suggest the same effect occurs in the CNS. Promising preliminary data from our lab has led us to believe that long-term, repetitive AIH may reduce lesion size and drive at least serotonergic innervation through the injury site (Santiago-Moreno et al., 2017). The study in question however suffered from small sample sizes and poor tissue quality. Serotonin is important not only because it is the monoamine neurotransmitter most closely linked to AIH-induced plasticity and functional motor recovery, but also because serotonergic projections into the spinal cord are almost exclusively supraspinal. This makes serotonin a convenient label to examine changes in axonal dynamics after injury at a single time point.

Methods

Experimental Groups

Experiments were conducted on male Sprague-Dawley rats (12-14 weeks of age) obtained from Envigo (Colony 208A). Rats were housed in pairs with free access to food and water. All procedures were approved by the University of Florida, Institutional Animal Care and Use Committee. Each rat received one of four treatments:

- C2 hemisection with AIH 4x per week (C2Hx + 4xwAIH; n= 12)
- 4xwAIH without C2Hx (Shelf + 4xwAIH; n = 10)
- C2Hx without AIH (C2Hx + Nx; n = 12)
• Neither C2Hx nor AIH (Shelf + Nx; n = 9)

The AIH protocol cycled between normoxia (5 min, 21% O₂) and hypoxia (5 min, 10.5% O₂) over the space of 1.5h. Rats received exposure treatments for a total of 12 weeks regardless of experimental group.

**Surgical Protocols**

**Recovery surgery.** Immediately before C2Hx, rats were anesthetized with isoflurane in a closed chamber and isoflurane was maintained (2-3%) using a nose cone as described above. The cervical spinal cord was exposed with a dorsal approach (C2 laminectomy and durotomy) and the cervical spinal cord was hemisected caudal to the C2 dorsal roots using a microscalpel. All rats received an analgesic (buprenorphine, 0.03 mg/kg sq) and lactated Ringer’s solution (5 mL sq) before the termination of isoflurane anesthesia. Wounds were sutured and skin was closed with wound clips.

**Repetitive Acute Intermittent Hypoxia**

For 4xwAIH and dAIH protocols, normoxic (21% O₂ and hypoxic (10.5% O₂) conditions were established in Plexiglas cylinder chambers (Therapeutiq Research) by mixing O₂ and N₂ gas with a computer-controlled mass-flow controller system (Flow Commander, Therapeutiq Research) to obtain the desired O₂ concentrations. Within the chambers, CO₂ and O₂ levels were continuously monitored throughout the entire protocol. Gas flowed through the chamber at a rate of 16 L/min, keeping chamber CO₂ concentrations below 0.5% at all times. Humidity (30-50%) and temperature (21-22°C) were maintained using a custom-made humidification system (A.K. Simon, University of Florida). On the experiment day, rats were placed in the chamber for a 30min
acclimation period. Once all rats were in chambers, experimental groups were administered the AIH protocol (10, 5-min 10.5% O₂ interspersed with 5-min 21% O₂, for a total of 90min). Control rats were administered continuous normoxia. For rats that received the IH7 protocol, conditions were established using a modified cage-top mounted on their normal housing enclosures (Therapeutiq Research). Because the rats also lived in the cages when not being treated, no acclimation period was established prior to each exposure. Once all cage tops were placed, the IH7 protocol was administered (120, 2-min 10.5% O₂ interspersed with 2-min 21% O₂, for a total of 8h). Rats had free access to food and water for the entirety of the exposure. All other factors were the same as the cylinder-treated rats.

**Tissue Harvesting and Processing**

Rats were euthanized immediately after neurophysiological measurements were completed while still urethane anesthetized. The thoracic cavity was opened and rats were perfused transcardially with 300-500mL of cold phosphate buffered saline (PBS, pH 7.4) followed by 300-500mL 4% paraformaldehyde (PFA; in PBS) solution. The brain and spinal cord (to upper lumbar) were collected. Tissues were post-fixed for 24h in PFA and then cryoprotected in a sucrose solution (30% in PBS) for dehydration at 4°C until they sank. Tissues that were not to be cut within 30 days were stored in antifreeze solution (30% Glycerol, 30% Ethylene Glycol, 0.01% Sodium Azide in PBS) at -20°C.

**Histology and Stereology**

**Hippocampus.** The brain was separated in two with a cut just rostral to the hippocampus. The hippocampal region of the brain was sectioned on a freezing
microtome (Leica SM2010R) at 20µm. Sections were stored in antifreeze solution at -20°C until stained.

**C1-C3 segment.** After paraffin embedding, tissues were sectioned longitudinally using a paraffin microtome (Microm HM340E) at 7µm. Sectioning began near the initiation of the ventral horn and proceeded dorsally. Sections were mounted to positively charged slides using a warm water bath with each new section being mounted to the next slide. A total of 100 sections were collected over 25 slides (4 sections/slide; 700µm) for each animal. Each slide was representative of the entire sectioned portion of spinal cord.

**Staining**

**Cresyl violet.** Slide-mounted sections were heated at 60°C to melt paraffin wax and deparaffinized with xylene. Tissues were then rehydrated through an ethanol gradient. Free-floating sections were mounted to slides and dried overnight before rehydrating in deionized H₂O. Sections were then stained with cresyl violet solution (...), rinsed, and dehydrated through an ethanol gradient before mounting with a toluene-based mounting medium (Richard-Allan Scientific™ Mounting Medium, Thermo Fisher Scientific).

**Immunohistochemistry.** Free-floating sections were washed with PBS with 0.1% Triton-X100 (PBS–Tx; 3 × 5 min) and incubated in PBS containing 1% H₂O₂ for 30min to block endogenous peroxidase activity. After washing (3 × 5 min) in PBS–Tx, tissues were blocked with 5% normal goat serum at room temperature (RT) for 60 min. Staining was performed by incubating tissue sections with anti-GFAP (1:1000, mouse
monoclonal, Millipore) or anti-CD11b (1:1000, mouse monoclonal, BioRad) overnight at 4°C. The following day, sections were washed with PBS-Tx (3 x 5 min) and incubated in biotinylated secondary goat anti-rabbit or goat anti-mouse antibody (1:1000, Vector Laboratories). Antibody detection was done using avidin-biotin complex method (Vectastain Elite ABC Kit, Vector Laboratories) followed by visualization with 3,3'-diaminobenzene peroxidase substrate (DAB Peroxidase Substrate Kit, Vector Laboratories) according to manufacturers’ instructions. Sections were then washed with PBS, placed on slides, dried at RT, dehydrated through an ethanol gradient, cleared with xylenes, and mounted using a toluene-based mounting medium (Richard-Allan Scientific™ Mounting Medium, Thermo Fisher Scientific).

All images of tissues processed in our lab were captured using the Keyence BZ-X710 microscope and its requisite software (BZ-X Viewer, Keyence). Analysis was performed using the Fiji open-source image processing package (Schindelin et al., 2012). Final photomicrographs were created with Adobe Photoshop software (Adobe Systems) and all images received equivalent adjustments to tone, scale, gamma and sharpness. Negative controls were performed by omitting the primary or secondary antibodies

Quantification and Statistical Analyses

Lesion volume analysis. Two slides of the C1-C3 tissue, 12 slides (84µm) apart, were stained with cresyl violet for lesion characterization and area analysis. Lesions were surprisingly inconsistent with complete lateral destruction only observed in a minority of tissues. To account for inherent variability between tissues, the area of
tissue spared on the ipsilateral side was measured and compared to the area of the contralateral side as an internal control. Ipsilateral area was divided by contralateral area and the median ratio was calculated from each animal. The ratio was then subtracted from 1 and multiplied by 100 to generate a “percent lesioned area” from each animal. Five animals were excluded from this analysis. Four were cryosectioned prior to the decision to paraffin embed the tissues and as such could not be used for comparison. Data were compared between C2Hx + Nx (n = 8) and C2Hx + 4xwAIH (n = 8) using a T-test. Differences were considered significant if \( p < 0.05 \). All values are expressed as mean ± 1 SEM

**Immunohistochemical analysis.** Expression of CD11b and GFAP in the hippocampus were quantified as previously described (Satriotomo et al., 2016). Four sections from each animal that included the CA1 region of the hippocampus were selected for immunohistochemistry using the DAB method. The investigator was blinded to the experimental treatment in all analyses.

Analyses of CD11b and GFAP in the hippocampus were performed on images taken at 20\( \times \) magnification using the Fiji open-source image processing package (Schindelin et al., 2012). All images were converted to eight-bit resolution, and threshold was set between 120 and 160 during all analyses; a threshold was chosen for each group in which all glial cells were visible but not saturated (i.e. images from both groups were treated identically). A cell count algorithm was run using the Analyze Particles function with Count and Average Size selected as measurements.
**Statistical analyses.** For CD11b and GFAP cell count and average cell size, a two-way ANOVA was run (factors: injury x treatment). ANOVA was followed by a Tukey *post hoc* test for multiple comparisons. Data are presented as mean ± SEM and significance was accepted at p ≤ 0.05
CHAPTER 5  
RESULTS, DISCUSSION, AND FUTURE DIRECTIONS

Results and Discussion

Long-Term AIH Does Not Affect Lesion Volume

Injury sites of 4xwAIH treated and normoxia control animals are shown below in Figure 5-1. A paired t-test \( (t_{(15)} = 0.183, \ p = 0.857, \ \text{paired t-test}) \) confirmed no significant difference between C2Hx + Nx (n = 9) and C2Hx + 4xwAIH (n = 8).

Long-Term AIH is Not Associated with Signs of Pathology in the Hippocampus

CD11b staining is shown below in the CA1 region of the hippocampus. Figure 5-2 shows equal CD11b immunoreactivity between all groups. A two-way ANOVA confirmed there were no significant effects of injury or treatment on the number of CD11b+ cells in the hippocampus \( (F_{(1,1)} = 0.239, \ p = 0.628) \). However, a two-way ANOVA for cell size suggested a significant main effect of Injury \( (F_{(1,1)} = 0.385, \ p = 0.03) \) which justified pairwise comparison with Tukey’s post hoc test which was significant when comparing C2Hx rats to shelf rats \( (p = 0.03) \) as well as the same groups within normoxia treatment \( (p = 0.050) \).

GFAP staining is shown below in the CA1 region of the hippocampus. Figure 5-3 shows equal GFAP immunoreactivity between all groups. A two-way ANOVA confirmed there were no significant effects of injury or treatment on the number of GFAP cells in the hippocampus \( (F_{(1,1)} = 0.0313, \ p = 0.861) \). However, a two-way ANOVA for cell size suggested a significant main effect of Injury \( (F_{(1,1)} = 6.113, \ p = 0.018) \) which justified pairwise comparison with Tukey’s \textit{post hoc} test which was significant when comparing
C2Hx rats to shelf rats ($p = 0.002$) as well as the same groups within normoxia treatment ($p = 0.003$).

**Conclusions**

**Specific Aim 1 Summary**

Long-term 4xwAIH does not appear to have significant effects on the size of injury. We have multiple hypotheses which may explain this. One hypothesis is simply that axonal regeneration does not necessarily equal gross changes in lesion morphology. During the analysis of spinal hemisections, the completeness of lateral destruction in the lesion was highly variable among both groups. This could be a result of investigator error or a byproduct of the microslice injury used in the C2Hx model. Microslice transections sever rostral and caudal portions of the spinal cord, but trauma is minimal and there is little empty space between freshly dissociated portions. Over a long period of time, this may increase the likelihood of spontaneous regeneration.

The dose and timing of AIH post-injury may also be factors in a lack of regeneration. Because moderate AIH emphasizes activation of the Q pathway over the S pathway, we may have been directly undermining the S-pathway mediated upregulation of cAMP to promote axonal growth via CREB phosphorylation in favor of promoting BDNF translation and TrkB activation.

**Specific Aim 2 Summary**

No significant differences were found in the size or number of microglia and astrocytes in the hippocampus between treated and control groups. Neuroinflammation is a well-documented pathology associated with more severe intermittent hypoxia
protocols. Lack of inflammation in any distant region of the CNS is indicative of a lack of global neuroinflammation. Injured groups were found to have significantly smaller microglia and significantly larger astrocytes than non-injured animals, but this appears to be a result of the injury itself and has no relevance on the safety of the treatment. Preliminary analyses of peripheral organ histology and physiological function done with these same rats also do not suggest any deleterious effect from long-term AIH treatment.

**Future Directions**

Currently, more experiments are planned with the tissues obtained through this study. At the injury, we plan to stain for both serotonin and Tuj1 to compare growth of serotonergic nonspecific axon networks in and around the glial scar. Differences in the composition of the fibrotic component of the glial scar between treated and non-treated groups are also of interest. Below injury, studies are underway to characterize the pattern of serotonin innervation to see how it correlates with the pattern of serotonergic innervation at the injury site.

In future studies, we would like to look at the three-dimensional nature of the changes we see in axonal network following injury. Using either traditional immunohistochemistry or neuroanatomical tracing of raphe projections with state of the art microscopy techniques such as CLARITY, we could visualize and model the cellular and molecular changes we are attempting to describe in this study in three-dimensions. Additionally, we would like to determine how best to apply AIH to promote neural regeneration and if this would be possible without undermining plasticity or causing significant pathology.
One potential experiment may be to use an AADC antagonist to relieve the vascular dysfunction and severe tissue hypoxia caudal to injury caused by pericytes, and create an environment that is more permissive to Q pathway activation. Another proposed experiment would attempt to maximize S pathway activation and evaluate if it indeed would promote more robust axonal regeneration. This could involve not only a more severe hypoxia protocol, as well as other experimental groups in which we would pharmacologically enhance the S pathway’s effects. This could be done with pertussis toxin, an inhibitor of G_{i/o} proteins that inhibit Adenylate Cyclase or with rolipram, a PDE4 inhibitor. Regardless of the experiments we choose for future studies, it is vital that we develop a protocol to evaluate changes in axonal growth and functional networks in real time. This could potentially be done using high resolution MRI on the same rat over time or by extracting tissues at multiple time-points after initiation of treatment.
Figure 5-1. Comparison of Lesion Size

A) A visual comparison of lesion morphology between the C2Hx + Nx group (Left) and the C2Hx + 4xwAIH group (Right). B) The graph below demonstrates their relationships to each other.
Figure 5-2. Comparison of Astrocytes in the Hippocampus

**Above:** A visual comparison of astrocytes in the hippocampus of Shelf +Nx (A), Shelf + 4xwAIH (B), C2Hx + Nx (C), and C2Hx + 4xwAIH (D). **Below:** Graphical comparisons of astrocyte number (E) and size (F).
Figure 5-3. Comparison of Microglia in the Hippocampus

**Above:** A visual comparison of microglia in hippocampus of Shelf +Nx (A), Shelf + 4xwAlH (B), C2Hx + Nx (C), and C2Hx + 4xwAlH (D). **Below:** Graphical comparisons of microglia number (E) and size (F)
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BIOGRAPHICAL SKETCH

Juan Gabriel Santiago Moreno was born in San Juan, Puerto Rico in 1995 to parents Vivian Moreno and Jorge Santiago, and is one of three children. He graduated from Titusville High School in 2013 and obtained his Bachelor of Science degree in Behavioral and Cognitive Neuroscience from the University of Florida in 2016. He then entered the Master of Science in Medical Science program at the University of Florida College of Medicine. He completed his MS degree and graduated in May 2018. His research focus was investigating the potential use of acute intermittent hypoxia to promote axonal regeneration following spinal cord injury.