INTERMITTENT HYPOXIA INDUCES SPINAL PLASTICITY IN RATS WITH CERVICAL SPINAL CORD INJURY

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ABSTRACT

Many experimental therapies have been used in the search for effective approaches to improve recovery after spinal cord injury (SCI). One of the most promising approaches is the augmentation of spontaneously occurring plasticity in uninjured neural pathways. Acute intermittent hypoxia (AIH-brief exposures to reduced O_2 levels alternating with normal O_2 levels) elicits plasticity in respiratory and non-respiratory spinal systems in experimental animals. AIH treatment has also been shown to improve walking abilities in persons with chronic incomplete SCI. In this thesis, I first examined the effect of AIH treatment, alone or in combination with motor training, on functional recovery in a rat model of incomplete cervical SCI. Second, I examined the effect of AIH on the expression of plasticity- and hypoxia-related proteins in the spinal cords of SCI rats. In a randomized, blinded, normoxia-controlled study, rats were trained to cross a horizontal ladder and footslip errors were measured before surgery for SCI, 4 wks post-surgery, each day of daily AIH treatment, and 1, 2, 4 and 8 weeks after treatment. dAIH treatment consisted of 10 episodes of AIH: (5 min 11% O_2: 5 min 21% O_2) for 7 days beginning at 4 wks post-SCI. AIH-treated rats made fewer footslips on the ladder task compared to normoxia-treated control rats after 4 days of treatment and this improvement was sustained for 8 wks post-treatment. Importantly, daily ladder training was required for AIH treatment to facilitate recovery. AIH treatment + motor training also increased the expression of Hypoxia-inducible factor-1α (HIF-1α), Vascular endothelial growth factor (VEGF), Brain-derived neurotrophic factor (BDNF), tyrosine kinase B receptors (trkB) and phospho-trkB in spinal motor neurons in SCI rats compared to normoxia-treated SCI rats. In particular these hypoxia- and plasticity-related proteins were differentially expressed both temporally and

spatially in the spinal cord during AIH treatment. These findings demonstrate that AIH + motor training can augment neural plasticity and improve motor recovery in an animal model of SCI. Taken together with the promising findings from human SCI studies, the results of this thesis suggest that AIH has potential as an effective therapy to restore motor function after nervous system injury.

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DEDICATION

I would like dedicate this research work to my dear parents and teachers.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

5HT7	Serotonin type 7
AIH	Acute intermittent hypoxia
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
bHLH-PAS	Basic helix-loop-helix-PAS
ChABC	Chondroitinase-ABC
CIH	Chronic intermittent hypoxia
CNS	Central nervous system
CSPGs	Chondroitin sulphate proteoglycans
CST	Corticospinal tract
dAIH	Daily acute intermittent hypoxia
DAPI	4’6-diamidino-2-phenylindole
EAAs	Excitatory amino acids
EGF	Epidermal growth factor
EPO	Erythropoietin
EPO-R	Erythropoietin receptor
ERK	Extracellular-related kinase
ESCs	Embryonic Stem Cells
FGF	Fibroblast growth factor
FLK-1	fms-like tyrosine kinase-1
FLT-1	Fetal liver kinase-1
GAP-43	Growth-associated protein-43
GDNF	Glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFR-α1	Glycosyl-phosphatidylinositol-linked family of receptors α1
GLUT-1	Glucose transporter-1
HIF-1α	Hypoxia-Inducible Factor-1α

HIF-1β	Hypoxia-Inducible Factor-1β
HRE	Hypoxia response elements
IDV	Integrated density value
IGF-1	Insulin-like growth factor-1
IH	Intermittent hypoxia
iNOS	Inducible nitric oxide synthase
IPS	Induced Pluripotent Stem Cells
LTF	Long-term facilitation
MAP	Mitogen-activated protein kinase
MASCIS	Multicentre Animal Spinal Cord Injury Study
MAG	Myelin-associated glycoprotein
NASCIS	National Acute Spinal Cord Injury studies
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
NRP-1	neuropilin-1
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
NYU Impactor	New York University Impactor
O2	Oxygen
OCT	Optimal cutting temperature compound
OECs	Olfactory ensheating cells
Omgp	Oligodendrocytes myelin glycoprotein
OSU Impactor	Ohio State University Impactor
p75	Pan-neurotrophin receptor
PBS	Phosphate buffered saline
PI3	Phosphoinositide 3
PlGF	Placental growth factor
pLTF	Phrenic long-term facilitation

PKA	Protein kinase A
PKC	Protein kinase C
PSNs	Propriospinal neurons
rAIH	repetitive acute intermittent hypoxia
RM-ANOVA	Repeated measure-Analysis of variance
ROS	Reactive oxygen species
RST	Rubrospinal tract
SP	Substance p
SCI	Spinal cord injury
SEM	Standard error of the mean
TCA	Tri carboxylic acid
TGF-β	transforming growth factor β
TMS	Trimethoprim and sulfadoxine
TNF-α	Tumor necrosis factor alpha
trkA	Tropomyosine-related kinase A
trkB	Tropomyosine-related kinase B
trkC	Tropomyosine-related kinase C
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A
VEGF-B	Vascular endothelial growth factor-B
VEGF-C	Vascular endothelial growth factor-C
VEGF-D	Vascular endothelial growth factor-D
VEGFR-1	Vascular endothelial growth factor receptor-1
VEGFR-2	Vascular endothelial growth factor receptor-2
VEGFR-3	Vascular endothelial growth factor receptor-3

CHAPTER 1

REVIEW OF LITERATURE

1.1 Introduction

Spinal cord injury (SCI) is a serious devastating global problem, which mostly affects young persons aged 16 to 30 (NSCISC 2013). It is estimated that more than 2.5 million people live with spinal cord injury, with more than 130,000 new injuries reported each year worldwide (http://www.rickhansen.com; NSCISC 2009). In North America, SCI affects over 41,000 Canadians and 259,000 persons in the U.S.A., with approximately 1,100 and 12,000 new injuries occurring each year in Canada and the US respectively (http://www.rickhansen.com; Sekhon and Fehlings 2001; NSCISC 2009). The most common cause of spinal cord injuries is motor vehicle accidents, which account for 42% of reported SCI. The second most common cause of SCI are falls, accounting for 27% of reported SCI. The remaining causes include gunshot wounds 15%, sports related injuries 8% and non-traumatic injuries including spinal stenosis and spinal tumors, 9% of total SCI. SCI damages axonal pathways and interrupts synaptic transmission between brain and spinal cord and subsequently alters the motor, sensory and autonomic functions below the level of injury. These alterations abruptly affect multiple body systems, including respiration, limb movement, muscles, sexual function, bowel and bladder movement. Persons living with SCI, thus experience devastating physical, psychological, emotional and social consequences.

Importantly, most SCIs are incomplete and leave some uninjured axonal pathways. The sparing of undamaged pathways contributes to spontaneous recovery of some limb and respiratory function following SCI (Raineteau and Schwab 2001; Fouad and Tse 2008). This recovery is thought to be a result of spontaneous, but limited plasticity in uninjured spinal synaptic pathways (Goshgarian 2003). This partial and limited recovery can be slow and is often inadequate to restore normal function, so methods designed to enhance spinal plasticity might further improve recovery (Raineteau and Schwab 2001; Baker-Herman, Fuller et al. 2004; Golder and Mitchell 2005). Therefore new strategies to enhance endogenous mechanisms of spinal plasticity and restore normal function following SCI are critically needed. Strategies to enhance plasticity in the spinal cord following SCI include:

1) neurorehabilitative or functional training, (Dietz and Fouad 2014)**.**
2) electrical stimulation (Carmel, Berrol et al. 2010; Onifer, Smith et al. 2011; Dietz and Fouad 2014).
3) pharmacological stimulation (Dietz and Fouad 2014).
4) acute intermittent hypoxia (AIH) (Dale-Nagle, Hoffman et al. 2010; Dale, Ben Mabrouk et al. 2014).

All these plasticity promoting strategies are being found to facilitate plasticity by enhancement of synaptic transmission of spared pathways, alone or in combination (Onifer, Smith et al. 2011).

This thesis examines acute intermittent hypoxia as a therapy to enhance spinal plasticity in a rat model of experimental spinal injury. Acute intermittent hypoxia (AIH) is repetitive exposure to reduced oxygen levels for brief periods. In this chapter, I will first review the progression of cellular events after traumatic SCI, and then I will discuss the different rat models and experimental treatments that have been used to investigate SCI. I will then outline the evidence for contribution of neural plasticity to recovery from SCI and finally focus on what is known about acute intermittent hypoxia, particularly the evidence that AIH might be a good candidate treatment for enhancing plasticity and improving functional recovery after SCI.

1.2 Spinal Cord Injury

The spinal cord contains neural circuitry and motoneurons in the central core of grey matter and axonal pathways in the surrounding white matter. Many of the consequences of spinal cord injury arise from the interruption of the white matter axonal connections between the brain and spinal cord, resulting in paresis or paralysis and loss of sensation to the different parts of the body controlled by the spinal cord segments below the injury. An injury at the cervical level may cause paralysis of both arms and legs resulting in quadriplegia, whereas lower injuries may affect only the lower part of the body causing paraplegia (Tetzlaff, Fouad et al. 2009). Spinal cord injuries also can lead to other complications, including respiratory insufficiency, the leading cause of death in patients with high-cervical spinal cord injuries, sexual impotence, muscle spasticity and loss of bladder and bowel control (Winslow and Rozovsky 2003; Sipski, Alexander et al. 2006; Francis 2007; Opperman, Buchholz et al. 2010). The physical disabilities

associated with SCI vary greatly depending on the type and severity of the injury, the level of the spinal cord at which injury occurs, and the nerve fibers pathways that are damaged due to injury.

Spinal cord injuries in general can be classified as either complete injuries or incomplete injuries. With complete SCI, there is total loss of sensation and voluntary movement below the level of injury. Incomplete SCI is more common and is characterized by some degree of sensation and movement below the level of injury. It is possible that the classification of the injury might change during recovery (Kirshblum, Millis et al. 2004). Each type of SCI occurs in two phases primary and secondary phase of SCI, discussed in detail in the following Section 1.3.

1.3 Phases of Spinal cord Injury

There are two mechanisms by which SCI damages the spinal cord, a primary or mechanical injury and a secondary injury process. Primary injury causes damage locally, namely in the area of the vertebral fracture, and it is characterized by acute hemorrhage and ischemia. Secondary damage is mediated through multiple processes, including inflammation, apoptotic cell death, excitotoxicity within the first week following SCI, causing further destruction of neuronal and non-neuronal cells (Liu, Zhang et al. 2006). Secondary mechanisms of injury exacerbate lesion size and severity, which ultimately increases the functional deficits (Tator and Fehlings 1991). The identification and understanding of mechanisms which initiate and sustain the inflammatory response, apoptosis, excitotoxicity, could help us to develop new treatment strategies which prevent or reduce this secondary damage and improve functional recovery (Zhang, Yin et al. 2012). These primary and secondary mechanisms of spinal cord damage are described in more detail below.

1.3.1 Primary injury

The causes of SCI are diverse in origin and can result from contusion, compression, penetrations or maceration of the spinal cord (Kwon, Tetzlaff et al. 2004; Onifer, Rabchevsky et al. 2007). Acute SCI is a bi-phasic process involving primary and secondary mechanisms. The primary injury to the spinal cord occurs at the instant of impact and is commonly due to mechanical damage. This damage can be contusion resulting in cavity formation, compression caused by

increased pressure to the spinal tissue, laceration from sharp bone fragments or foreign objects, and shearing caused by bullets (Blight 2000). The most common cause of primary SCI is vertebral fracture, which typically tears the spinal cord tissues and produces characteristic damage of the gray and white matter (Choo, Liu et al. 2007; Rowland, Hawryluk et al. 2008; Choo, Liu et al. 2009). In addition to disrupting axons, mechanical damage causes death of neurons, oligodendrocytes and astrocytes, and endothelial cells located at the site of the lesion. Disruption of blood vessels also causes hemorrhage (Rowland, Hawryluk et al. 2008).

1.3.2 Secondary Injury

The secondary injury is a progressive degeneration beginning immediately after the primary injury and may last for days, weeks or months. It is a highly complex process and involves numerous mechanisms including ischemia, inflammation, generation of free radical species, necrosis and apoptosis, and dysregulation of ionic hemostasis (Kwon, Tetzlaff et al. 2004).

1.3.2.1 Ischemia

SCI induces changes in spinal cord blood flow at the systemic and local level and a major reduction in blood flow (ischemia) occurs at the lesion site (Sekhon and Fehlings 2001). There are several mechanisms that are responsible for ischemia, including vasospasm due to release of vasoactive amines, hemorrhages, endothelial swelling and thrombosis through platelet aggregation (Dohrmann and Allen 1975; Nemecek 1978; de la Torre 1981; Wallace, Tator et al. 1986; Tator and Fehlings 1991). Neurons are critically dependent on oxygen and glucose. Damage to blood vessels causing oxygen and nutritional insufficiency can lead to neuronal apoptosis (Tator and Koyanagi 1997).

1.3.2.2 Inflammation

The blood brain barrier is highly selective barrier that is responsible for preventing blood cells from invading spinal tissue. Following SCI, this barrier is physically broken, causing an increase in permeability, allowing the cells from the blood to invade and initiate the inflammatory response. This contributes to secondary damage in the spinal cord (Popovich, Yu et al. 1997; Bareyre and Schwab 2003). Inflammation is a universal defense response to tissue injury and is

initiated after SCI (Kwon, Tetzlaff et al. 2004). The inflammatory response includes the invasion of inflammatory cells (neutrophils, T-lymphocytes), and macrophages, and release of noncellular inflammatory components such as cytokines, prostaglandins, interleukins. The cellular and noncellular components mediate the inflammatory response and contribute to further tissue damage (Rowland, Hawryluk et al. 2008).

1.3.2.3 Excitotoxicity

Ionic homeostasis is necessary to maintain the calcium gradient across the cell membrane. This homeostasis of calcium ion gradients can be disrupted due to mechanical changes in the microvasculature and hemorrhage, which can lead to increased intracellular calcium ion concentration, causing the depolarization of the cell membrane (Choi 1988; Wells, Hurlbert et al. 2003; Kusters, Dernison et al. 2005). This in turn can cause increased release of the most prevalent excitatory neurotransmitter, glutamate, into the synaptic cleft. Regulation of glutamate concentration is necessary to maintain the normal cellular function of neurons. Glutamate activates NMDA receptors, which allow massive influx of calcium ions into the cell. This triggers calcium-induced calcium release from the intracellular calcium store into the cytoplasm (Mody and MacDonald 1995; Kwon, Tetzlaff et al. 2004). This elevated concentration of calcium ions in cytosol can trigger many calcium-dependent intracellular pathways and activate the lytic enzymes such as proteases, caspases, caplains, phospholipases, endonucleases, and lipoxygenase that alter cellular metabolism and cause dysregulation of mitochondrial oxidative phosphorylation leading to apoptotic cell death of neurons (Choi 1988; Dusart and Schwab 1994; Mody and MacDonald 1995; Wells, Hurlbert et al. 2003).

1.3.2.4 Apoptotic cell death

Apoptosis is a programmed cell death and it occurs around the lesion epicenter as well as within the areas of Wallerian degeneration in both ascending and descending tracts of white matter in the spinal cord (Emery, Aldana et al. 1998). Apoptosis has been identified in the spinal cord of rats and humans after SCI (Crowe, Bresnahan et al. 1997; Emery, Aldana et al. 1998; Wada, Yone et al. 1999; Mattson 2000; Byrnes, Stoica et al. 2007) . It may occur as a result of adverse changes in the cellular environment as described above, resulting in axonal demyelination or as a

result of Wallerian degeneration or by a combination of both (Barres, Jacobson et al. 1993; Dusart and Schwab 1994; Liu, Zhang et al. 2006; Whalley, O'Neill et al. 2006; Titsworth, Cheng et al. 2009). After traumatic SCI in rats, apoptotic pathways are activated in neurons in the first hours after injury, and hours to days later in oligodendrocytes adjacent to and distant from the injury site (Springer, Azbill et al. 1999; Mattson 2000; Soini, Kahlos et al. 2005).

1.4 Experimental rodent models of spinal cord injury

A variety of animal models including dogs, cats, guinea pig, primates and rodents have been developed to examine the mechanisms, pathophysiology and functional deficits following SCI, and also to test intervention strategies to develop effective therapies for the treatment of SCI. Rodents, such as rats and mice, have emerged as the main animal used in spinal cord injury research, making up 90% of laboratory animals used in SCI research. The advantages of using rodents include low costs of purchasing and housing, as well as a short life span.

Rat models of SCI are the most widely used to study the mechanism and consequences of SCI because many of the morphological, biochemical, functional and behavioural changes that occur after SCI are similar to those seen in humans after SCI. The most commonly used rat models of spinal cord injuries are transection models, compression models, contusion models and chemically-induced models (Geissler, Schmidt et al. 2013). Compression and contusion injuries are most common in humans (Geissler, Schmidt et al. 2013). There is no single model that has dominated in the field of SCI research and each model has advantages and disadvantages.

1.4.1 Contusion

The production of an experimental spinal contusion injury is the most commonly used method because these injuries are clinically relevance to SCI occurring in humans (Blight 2000). This method relies on an impactor device that hits the spinal cord and produces either a defined force upon or a defined displacement of the cord. The contusion model of spinal cord injury was first developed by Allen, who used a weight drop technique on the spinal cord in dogs (Allen 1911). This weight drop technique was later developed in rats to deliver a blunt contusive force to the spinal cord (Wrathall, Pettegrew et al. 1985). This weight drop technique is now widely used to

produce contusion models of SCI, and several devices are designed to produce blunt contusive injury in animals. The New York University (NYU) Impactor is a sophisticated device introduced by Gruner in 1992 (Gruner 1992). The NYU Impactor drops a 10-gram weight from 6.25, 12.5, 25 or 50 mm directly onto the exposed spinal cord. This weight impacts the spinal cord with a defined force and induces contusion injury quickly. A modified version of weight drop impactor was introduced at the Ohio State University Spinal Cord Research Centre, where researchers developed the Ohio State University (OSU) electromechanical spinal cord impactor, a device which induces the injury by solenoid-controlled air cylinder (Noyes 1987). The severity of injury depends on the velocity and height of weight drop onto the exposed spinal cord. Both NYU and OSU impactors apply and remove force within one second and so induce contusion quickly. These impactor produce contusion injury in animals that allow to study the mechanism of secondary damage to spinal cord.

The majority of SCI in humans is contusive in nature and a contusion model of SCI is generally accepted as being clinically relevant to SCI (Norenberg, Smith et al. 2004; Kwon, Hillyer et al. 2010). Contusion SCI models are ideal for study of the pathologies and mechanisms of secondary damage to the spinal cord (Nobunaga, Go et al. 1999). Moreover, contusion models of SCI have been useful for the study of neuroprotective strategies, plasticity and demyelination after SCI (Blight 2000; Onifer, Rabchevsky et al. 2007). Nevertheless, contusion models do not completely mimic clinical occurrences of SCI in that the contusion models require pre-injury laminectomy, including surgical removal of muscles, ligaments and part of the vertebra (Fukuda, Nakamura et al. 2005). The technique used to produce contusion injury by impactor also induces some undesirable damage to soft tissue in surrounding areas (Fukuda, Nakamura et al. 2005). Finally, the contusive injury model is not a good model for investigation of axonal regeneration due to incomplete nature of the injury and the complexity of the tracts (Talac, Friedman et al. 2004; Lee and Lee 2013)

1.4.2 Compression

The compression model of SCI delivers a sustained and static force to the spinal cord for a specific duration of time, in contrast to the contusion models that deliver a single rapid blunt

force to the exposed spinal cord. Compression models of SCI are highly reproducible and useful to study the secondary mechanisms and pathophysiology following SCI. They are used to examine the effect of potential therapeutic agents to protect neuronal and non-neuronal cell loss due to secondary injury, with the aim to limit the severity of injury and minimize functional deficit following SCI. Compression models produce a glial scar similar to those seen in human SCI (Rivlin and Tator 1978). Compression can be produced by balloon compression clip compression or a forceps compression.

Tarlov and his colleagues developed the balloon catheter technique, the first compression model of SCI (Tarlov, Klinger et al. 1953). The balloon catheter is a catheter placed within the spinal canal, and when expanded, can induce a slowly developing compression such as seen in spinal tumour. The severity of injury produced by balloon catheter can be controlled by the pressure of inflated balloon and duration of application or by both (Martin, Schoenen et al. 1992; Vanicky, Urdzikova et al. 2001). This balloon catheter technique produce slow compression SCI, and allow to study the mechanism of SCI produce by spinal tumour.

Rivilin and Tator developed a calibration clip compression model of SCI (Rivlin and Tator 1978; Rivlin and Tator 1978). In this model, the spinal cord is exposed by performing a laminectomy and the blades of an aneurysm clip are placed on both dorsal and ventral surfaces of spinal cord. Force is applied by closing the clip to compressing the spinal cord in a dorso-ventral direction (Fehlings, Tator et al. 1989; Onifer, Rabchevsky et al. 2007). The severity of the injury produced by calibrated clip compression can be controlled by adjustment of closing forces of the clip, the application time for compression or by controlling both (Onifer, Rabchevsky et al. 2007).

A compression injury model of SCI has also been produced using modified surgical forceps. Blight developed this technique to produced lateral spinal compression injury in the guinea pig (Blight 1991). This calibrated forceps compression produced a larger volume of tissue compression and displacement of spinal column as compare to aneurysm clip compression. Taken together compression models of SCI are highly reproducible and useful to study the mechanism of pathophysiology following SCI, and are used to develop potential therapies to protect the spinal tissue from secondary damage after injury.

1.4.3 Transection

In animal models of transection injuries, the transection is generally performed manually to either completely or partially severe the spinal cord. With incomplete models, one can severe both ascending and descending axonal pathways in specific area of spinal cord white matter i.e. hemisection, dorsal hemisection, lateral hemisection, dorsal quadrant. Transection models of SCI are less clinically relevant to human SCI compared to contusion injuries, as transection injuries are rarely seen in clinics (Onifer, Rabchevsky et al. 2007). Transection models of SCI are not useful to study the complex mechanism of pathophysiology of spinal cord or to examine the effect of neuroprotective strategies. Transection models have become popular and useful to study the functional recovery of specific axonal pathways, axon regeneration, cell transplantation, multiple treatment in combination or alone, biomaterial, drugs and growth factors (Bregman, Coumans et al. 2002; Shumsky, Tobias et al. 2003; Tobias, Shumsky et al. 2003; Knudsen, Moxon et al. 2011). The injury in this model can be induced without use of any special device.

1.4.4 Chemical-mediated spinal cord injury

1.4.4.1 Photochemical Ischemia Model of SCI

An ischemia model of SCI was first developed in rabbits (DeGirolami and Zivin 1982). This model was produced by occlusion of the abdominal aorta just below the renal arteries. Unfortunately, the spinal arterial system of rabbits is segmental and unlike that of humans and rats. In the latter, it is not possible to produce local ischemia in the spinal cord with occlusion of the aorta (Fazio 1971; Kanellopoulos, Kato et al. 1997). Moreover this model was invasive and required abdominal surgery to ligate the abdominal aorta to interrupt the blood flow (Kanellopoulos, Kato et al. 1997). The model was refined to produce local ischemia by photochemically-produced blood clots in rats (Watson, Prado et al. 1986; Prado, Dietrich et al. 1987). In this model, the photosensitive dye rose Bengal or erythrosine B is injected intravenously and enters the systemic circulation (Watson, Prado et al. 1986; Cameron, Prado et al. 1990; Hao, Xu et al. 1991). The spinal cord is irradiated with a laser light, and this laser light interacts with rose Bengal or erythrosine B dye, which activates the dye, inducing endothelial damage with platelet activation and thrombosis, resulting in local blood flow interruption. The laser light-dye interactions induces primary microvascular occlusion and produces an ischemia

model of SCI (Watson, Prado et al. 1986; Prado, Dietrich et al. 1987; Cameron, Prado et al. 1990; Hao, Xu et al. 1991). This model of SCI is highly reproducible, minimally invasive and represents the ischemic component of SCI (Hao, Xu et al. 1991; Onifer, Rabchevsky et al. 2007). Moreover, unlike other models of SCI, i.e. contusion, compression and transection, the ischemia model of SCI does not require laminectomy, thus reducing collateral damage to the spinal cord (Prado, Dietrich et al. 1987; Onifer, Rabchevsky et al. 2007). The major disadvantage of photochemically induced ischemia is that this type of injury in not clinically relevant to SCI. In addition, the size and volume of the lesion are difficult to control in this injury model (Kundi, Bicknell et al. 2013). This model allows the study of the secondary spinal tissue damage as a result of ischemia.

1.4.4.2 Chemical Excitotoxicity Model of SCI

Traumatic SCI causes the release of excitatory amino acids (EAAs) from neurons and the concentration of these EAAs rapidly rises to produce excitotoxicity. This excitotoxicity causes neuronal cell death and plays a major role in gray and white matter pathology (Park, Velumian et al. 2004; Onifer, Rabchevsky et al. 2007). Animal models have been developed to study the contribution of excitotoxicity to the secondary injury phase in traumatic SCI. The excitotoxic model of SCI can be produced by the administration of EAAs such as glutamate, aspartate, N-methyl-D-aspartate (NMDA), or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor agonist kainate. Administration of these chemicals into the spinal cord causes the death of both oligodendrocytes and neurons. Application of kainate or quisqualic acid, both agonists of AMPA receptors, induces degeneration of gray matter (Yezierski, Santana et al. 1993; Onifer, Cannon et al. 1997; Magnuson, Trinder et al. 1999; Onifer, Rabchevsky et al. 2007).

Applications of chemicals other than EAAs have also been used to induce spinal injury. Administration of the free redical peroxynitrite, calpain, hydrogen peroxide or the microglia activator zymosan into spinal cord causes damage to lipids and proteins, cell death of oligodendrocytes and neurons, and produces inflammation that ultimately develops into the pathology similar to that seen in secondary phase of traumatic SCI (Liu 1993; Hall 2001;

Popovich, Guan et al. 2002). In addition, micro-injection of lysolecithin or ethidium bromide causes demyelination and death of oligodendrocytes (Onifer, Rabchevsky et al. 2007).

Chemical models of SCI are not physically invasive and are useful to study the pathophysiology of secondary mechanisms of spinal cord injury, because they produce inflammation, demyelination of axons, cell death of both oligodendrocytes and neurons, and degeneration of both gray and white matter in spinal cord, all events which occur in secondary phase of traumatic SCI. Chemical models of SCI allow to study the contribution of excitotoxicity to the secondary injury phase in traumatic SCI.

1.5 Current approaches to treat experimental spinal cord injury

A variety of approaches have been used to treat experimental spinal cord injuries. These approaches are listed below and explained in detail in the sections 1.5.1-1.5.

1) Neuroprotective treatments which are focussed on reducing secondary damage (Yang and Piao 2003; Hall and Springer 2004; Thuret, Moon et al. 2006).
2) Regenerative treatments which focus on promotion of axonal regrowth and neuronal replacement through the application of neurotrophic factors, blocking of inhibitory factors and cell transplantation (McTigue, Horner et al. 1998; Simonen, Pedersen et al. 2003; Fouad, Klusman et al. 2004; Kim and Jahng 2004; Kocsis, Akiyama et al. 2004; Li, Liu et al. 2004; Schwab, Conrad et al. 2005; Thuret, Moon et al. 2006; Rossignol, Schwab et al. 2007; Bull, Johnson et al. 2008; Cao, Onifer et al. 2008; Ying, Roy et al. 2008; Li, Li et al. 2009; Sieck and Mantilla 2009; Stavridis, Dehghani et al. 2009; Ronaghi, Erceg et al. 2010).
3) Plasticity promoting treatments that focus on enhancing endogenous mechanisms of recovery. Some degree of spontaneous functional improvement is generally observed within one year of injury in spinal cord-injured patients (Mansel and Norman 1990; Fouad, Krajacic et al. 2011; Dale, Ben Mabrouk et al. 2014; Dietz and Fouad 2014). It is thought that this recovery arises from cellular, molecular and synaptic changes in spared axonal pathways, collectively termed plasticity. Approaches which enhance these processes are under investigation for their potential to improve functional recovery.

All of these experimental approaches have demonstrated therapeutic potential alone or in combination with other treatments (Tohda and Kuboyama 2011; Wang, Zhai et al. 2011). Many have been applied and tested *in vitro* and as well in animal models and a few have been translated into human clinical trials (Onifer, Smith et al. 2011; Ruff, Wilcox et al. 2011; Tohda and Kuboyama 2011; Dietz and Fouad 2014).

1.5.1 Neuroprotective treatments

As described in Section 1.3.2, a cascade of secondary injury events is initiated following the primary phase of spinal cord injury, including ischemia, electrolyte imbalance, production of free radicals, Wallerian degeneration, and inflammation which causes the death of neuronal and non neuronal cells. Collectively, this leads to significant increases in the severity and volume of the injury. The main objective of neuroprotective treatments is to reduce cell death of neurons, oligodendocytes and astrocytes, prevent vascular damage, excitotoxicity, and inflammation, and eventually reduce the lesion volume after primary SCI.

1.5.1.1 Neuroprotective effects of anti-inflammatory agents

Numerous strategies have been reported to provide neuroprotection in animal models of SCI. The use of corticosteroids has been well researched. Methylprednisolone is one of the most commonly investigated steroid agents for its neuroprotective effects, which have been shown to reduce inflammation, edema, release of free radicals and the excitatory neurotransmitter glutamate (Tohda and Kuboyama 2011; Zhang, Fang et al. 2014; Yilmaz and Kaptanoglu 2015) Application of methylprednisolone within eight hours of acute SCI has shown significant improvement in motor and sensory function in patients with complete or incomplete spinal cord injury (Bracken 2012; Chikuda, Yasunaga et al. 2013). The beneficial effects of high-dose methylprednisolone reported in a series of studies conducted by National Acute Spinal Cord Injury studies (NASCIS) in last decade of 20th century (Bracken, Shepard et al. 1990; Bracken, Shepard et al. 1997; Bracken 2012). According to NASCIS three phase randomized trial research report, a high-dose of methylprednisolone steroid therapy is the only drug therapy used worldwide for acute SCI shown to have efficacy when administered within eight hours of acute

SCI. The patients who received high-dose (30 mg/kg as a bolus followed by infusion 5.4 mg/kg/hr) methylprednisolone within the first eight hours of injury have been shown to have greater neurologic improvement (Bracken, Shepard et al. 1990; Bracken, Shepard et al. 1997). Research reports also indicate additional benefits of methylprednisolone when the maintenance dose is extended from 24 to 48 hours (Bracken 2012). Methylprednisolone is one of the most effective drug therapy investigated for its neuroprotective effects, and used worldwide for the treatment of acute SCI.

The disadvantages of using high-dose methylprednisolone to treat SCI are the adverse effects which include gastrointestinal bleeding or ulcers, respiratory tract infection, urinary tract infection, high blood glucose level and altered immune response due to decreased helper T-cells responses (Galandiuk, Raque et al. 1993; Suberviola, Gonzalez-Castro et al. 2008; Chikuda, Yasunaga et al. 2013). For more than decade, there has been ongoing debate about the beneficial effects and clinical impact of methylprednisolone in neurological recovery from SCI (Breslin and Agrawal 2012). The high incidence of complications and adverse side effects in patients treated with high-dose methylprednisolone have made its use controversial and debatable (Pandya, Weant et al. 2010).

There has been some research using agents directed at specific molecules in the secondary injury cascade. Spinal cord injury induces the expression of several inflammatory molecules including tumor necrosis factor alpha (TNF-α), interleukin (IL-1, IL-6, IL-10) and chemokines MIP-1 alpha and MIP-1 beta (Bartholdi and Schwab 1997; Bethea, Nagashima et al. 1999; Hausmann 2003). These inflammatory molecules may reduce functional recovery by contributing to the formation of a glial scar in addition to causing necrosis or apoptosis of neurons and oligodendrocytes (Hausmann 2003). Administration of the anti-inflammatory cytokine interleukin-10 results in neuroprotective effects by reducing the production of TNF-α, resulting in improved functional recovery following SCI in rodents (Bethea, Nagashima et al. 1999; Brewer, Bethea et al. 1999; Abraham, McMillen et al. 2004; Jackson, Messinger et al. 2005; Thompson, Zurko et al. 2013).

Spinal cord injury also leads to release of the excitatory neurotransmitters glutamate and aspartate, resulting in hyperactivity of neurons which can lead to neuronal death. Studies have shown that treatment with the glutamate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) can minimize the excitotoxic damage and reduce functional deficits in an animal model following SCI (Wrathall, Teng et al. 1997).

1.5.1.2 Neuroprotective effects of growth factors

Administration of growth factors including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), basic fibroblast growth factor (bFGF), insulin-like growth factors (IGF), glial cell-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) have all demonstrated therapeutic potential in experimental SCI, (Houweling, van Asseldonk et al. 1998; Lee, Green et al. 1999; Rabchevsky, Fugaccia et al. 2000; Ding, Mao et al. 2005; Sharma 2005; Hung, Tsai et al. 2007). It is thought that the primary role of these growth factors in SCI is to promote survival of the neuronal cells and later they play a role in axonal regrowth and neuronal sprouting (Rosner, Avalos et al. 2012).

Neurotrophic and growth factors have been shown to promote survival and protection of neurons following SCI (Kelamangalath and Smith 2013). Exogenous application of either BDNF or NT-3 through infusion into the lumbar subarachnoid space exerts neuroprotective effects and prevents neuronal cell death of axotomized rubrospinal neurons in rats (Novikova, Novikov et al. 2000). Application of BDNF in close proximity to cell bodies of rubrospinal neurons promoted survival of rubrospinal neurons in SCI rats (Ruitenberg, Blits et al. 2004; Kwon, Liu et al. 2007). In addition, exogenous administration of BDNF and NT-3 in newborn rats promoted survival of axotomized neurons following mid-thoracic SCI (Diener and Bregman 1994). Moreover, exogenous infusion of BDNF through an implanted cannula at the site of injury showed neuroprotective effects following thorasic SCI (Namiki, Kojima et al. 2000). Exogenous intrathecal administration of bFGF following SCI significantly reduces tissue damage and enhances functional recovery in a contusion model of SCI in rats (Rabchevsky, Fugaccia et al. 1999). Intravenous injection of nerve growth factor (NGF) increased the survival of neurons by

reducing the apoptosis signal and improved motor function recovery in the contusion model of SCI in rats (Zhang, Wu et al. 2014).

In addition to its role as angiogenic factor, vascular endothelial growth factor (VEGF) appears to play neurotrophic and neuroprotective roles in spinal cord and brain injury (Krum and Rosenstein 1998; Facchiano, Fernandez et al. 2002; Svensson, Peters et al. 2002). Furthermore, application of VEGF in neuronal cell culture of embryonic spinal cord of rats has demonstrated a neuroprotective effect on rat spinal cord neurons. VEGF mediates this neuroprotective effects by activating its VEGFR-2 receptors (Ding, Mao et al. 2005).

1.5.2 Treatment strategies to promote regeneration

Traumatic SCI initiates a number of cellular and molecular changes in and around the injury site. These cellular and molecular changes contribute to axonal damage and neuronal and non-neuronal cell death that leads to functional deficits. Degeneration of axons occur both below and above the level of injury (Rosner, Avalos et al. 2012). Apoptotic cell death of oligodendrocytes causes demyelination and degeneration of axons. This demyelination ultimately affects the conduction properties of intact axons, and reduces the transmission of electrical impulses (Hunanyan, Garcia-Alias et al. 2010). Another major change that occurs following SCI is formation of a glial scar. This glial scar is formed by reactive astrocytes, which secrete a number of extracellular matrix growth inhibitory molecules. These create an inhibitory environment and play a critical role in the failure of axonal regeneration, the most important inhibitory molecules being semaphorin-3, keratin, tenacin and chondroitin sulphate proteoglycans (Silver and Miller 2004; Fitch and Silver 2008; Rosner, Avalos et al. 2012).

Regenerative therapeutic strategies focus on functional recovery by promoting regrowth of axons, sprouting and growth of new axons from the cell body of neurons and the re-myelination of axons to allow conduction of electrical impulses. Regenerative therapeutical strategies can be categorized into three classes:

1) Strategies to block or neutralize inhibitory molecules in order to change the non-permissive growth environment to a permissive growth environment.

2) Application of growth factors to promote regeneration of axons, and
3) Transplantation of various types of cells to replace lost neurons and/or oligodendrocytes.

1.5.2.1 Strategies to block or neutralize inhibitory molecules

Growth inhibitory molecules such as chondroitin sulphate proteoglycans (CSPGs) and myelin-associated inhibitors play a role to make impermissive conditions for axon growth following SCI (Meves and Zheng 2014; Gundimeda, McNeill et al. 2015). Numerous studies have shown that the blocking of growth inhibitory molecule, such as CSPG, Nogo, Myelin-associated glycoprotein (MAG), and oligodendrocytes myelin glycoprotein (OMgp) are able to promote axonal growth and functional recovery to some extent following SCI in animals (Bregman, Kunkel-Bagden et al. 1995; Chen, Huber et al. 2000; GrandPre, Nakamura et al. 2000; Bradbury, Moon et al. 2002; Meves and Zheng 2014). Blocking of inhibitory molecules neutralizes the effect of inhibitory glycoproteins and chondroitin sulphate proteoglycans (CSPG) in the extracellular matrix to allow the growth of axons (Yiu and He 2006; Hunanyan, Garcia-Alias et al. 2010; Lee, McKeon et al. 2010).

Chondroitin sulfate proteoglycans (CSPGs) are present in the extracellular matrix of the glial scar and are up-regulated after SCI, blocking the regeneration of axons (Davies, Goucher et al. 1999; Kottis, Thibault et al. 2002; Jones, Margolis et al. 2003; Jones, Sajed et al. 2003; Barritt, Davies et al. 2006; Massey, Amps et al. 2008). Chondroitinase-ABC (ChABC) is a bacterial enzyme which acts on chondroitin sulphate proteoglycans to neutralize its inhibitory effect, which in turn improves functional recovery and promotes regeneration in injured spinal cord in various animal models (Bradbury, Moon et al. 2002; Chau, Shum et al. 2004; Houle, Tom et al. 2006; Galtrey, Asher et al. 2007; Hunanyan, Garcia-Alias et al. 2010; Lee, McKeon et al. 2010). In addition to this, treatment with ChABC promoted the sprouting of intact and injured axons in a rat model of SCI (Barritt, Davies et al. 2006). Treatment with ChABC enhanced the sprouting of intact corticospinal tract axons and promoted recovery of forelimb function following unilateral pyramidotomy in a mouse model of SCI (Starkey, Bartus et al. 2012).

In addition to CSPGs, a number of inhibitory molecules are expressed on myelin, including Nogo, myelin-associated glycoprotein (MAG), oligodendrocytes myelin glycoprotein (OMgp) (McKerracher, David et al. 1994; Chen, Huber et al. 2000; GrandPre, Nakamura et al. 2000; Kottis, Thibault et al. 2002). All three myelin associated proteins, Nogo, MAG and OMgp mediate their inhibitory effects through common receptor known as NgR1 (Cafferty, Duffy et al. 2010)

Nogo is a transmembrane myelin associated growth inhibitory protein. The Nogo gene encodes three major proteins, Nogo-A, Nogo-B and Nogo-C. Nogo-A is the most studied inhibitory molecule expressed in oligodendrocytes and exerts inhibitory effects on axonal and neurite outgrowth by activating its receptor NgR1 (Spillmann, Bandtlow et al. 1998; Chen, Huber et al. 2000; Pernet and Schwab 2012). Monoclonal antibody IN-1 raised against myelin-associated Nogo proteins NI-35 and NI-250, neutralizes the inhibitory effect of Nogo protein, and promotes neurite growth of sensory and sympathetic neurons in culture (Caroni and Schwab 1988; Schwab and Caroni 2008). IN-1 also enhances the regeneration of axons and functional recovery of locomotion in skilled forelimb reaching task in rats and monkeys following SCI and stroke lesion (Pernet and Schwab 2012). IN-1 application in a rat model of SCI neutralized the inhibitory effect of Nogo protein and promoted neurite and axonal regeneration of corticospinal tract axons over distance of 5-11 mm in rats (Schnell and Schwab 1990; Schnell and Schwab 1993; Brosamle, Huber et al. 2000).

Myelin-associated glycoprotein (MAG) was the first identified myelin-associated growth inhibitory protein and is expressed in oligodendrocytes and Schwann cells of CNS and PNS respectively (Filbin 1995). Like Nogo, it is also a transmembrane protein that exerts its neurite and axon growth inhibiting effects by activating its receptor NgR (McKerracher, David et al. 1994).

Oligodendrocyte-myelin glycoprotein (OMgp) is a third member of myelin-associated inhibitory protein. It is expressed in both neurons and oligodendrocytes in the CNS (Vourc'h and Andres 2004; Lee, Case et al. 2009). OMgp is a growth inhibitory protein that inhibits the neurite and axon outgrowth by activating NgR receptors (McKerracher, David et al. 1994; Kottis, Thibault et

al. 2002). Triple knockout mice for Nogo, MAG and OMgp myelin-associated inhibitory proteins exhibits axon growth above and below a spinal injury and promote behavioural recovery in a mouse SCI model (Cafferty, Duffy et al. 2010).

Taken together, the previous studies suggest that a variety of growth inhibitory molecules including CSPGs, Nogo, MAG and OMgp are released following SCI. Strategies which aim to neutralize or attenuate the effects of growth inhibitory molecules have the potential to promote regeneration and growth of axons and promote functional recovery in animal models of SCI.

1.5.2.2 Application of growth factors to promote regeneration

The application of growth factors alone or in combination to promote axonal regeneration following SCI has been studied in various experimental models (Giger, Hollis et al. 2010). These growth factors include brain-derived neurotrophic factors (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and glial cell-derived neurotrophic factor (GDNF) and each has demonstrated some potential to promote axonal sprouting and re-growth of damaged axon *in vivo* and *in vitro*. These growth factors not only promoted axonal growth in motor axons but also in sensory axons as well (Liu, Kim et al. 1999; Namiki, Kojima et al. 2000; Blesch and Tuszynski 2003; Tobias, Shumsky et al. 2003; Brock, Rosenzweig et al. 2010).

Brain-derived neurotrophic factor (BDNF) is an important member of the neurotrophin family. In addition to its role in neuronal survival, neuroprotection, neuronal differentiation and plasticity, it also plays a crucial role in axonal sprouting and regeneration (Hiebert, Khodarahmi et al. 2002; Vavrek, Girgis et al. 2006; Waterhouse and Xu 2009; Ma, Wang et al. 2011; Nagahara and Tuszynski 2011; Park and Poo 2013; Liao, Bouyer et al. 2015). Numerous studies have shown that BDNF plays an important role in axonal sprouting and regeneration following SCI in animals (Liu, Kim et al. 1999; Namiki, Kojima et al. 2000; Hiebert, Khodarahmi et al. 2002; Jin, Fischer et al. 2002; Lu, Jones et al. 2005). Exogenous application of BDNF by osmotic pump to the corticospinal neurons in the motor cortex enhanced the sprouting of injured corticospinal axons in rats following SCI (Hiebert, Khodarahmi et al. 2002). Furthermore, transplantation of BDNF-secreting fibroblast cells to the site of injury in a rat model of SCI

promoted regeneration of rubrospinal tract (RST) axon through and around the graft and also improved the functional recovery of forelimb use in a rat model of SCI (Liu, Kim et al. 1999; Jin, Fischer et al. 2002). Continous administration of BDNF for 14 days at the injury site promoted regeneration of axons in a rat clip compression model of SCI (Namiki, Kojima et al. 2000). Moreover, transplantation of BDNF-secreting bone marrow stromal cells at the site of injury has been shown to promote growth of axons in SCI rats (Lu, Jones et al. 2005).

Application of neurotrophin-3 (NT-3) at the site of injury in injured spinal cord has been shown to promote axonal sprouting and regeneration of transected and spared CST axons following SCI in rats (Schnell, Schneider et al. 1994; Grill, Murai et al. 1997; Zhou, Baumgartner et al. 2003; Ramu, Bockhorst et al. 2007). Furthermore, transplantation of NT-3 secreting fibroblasts at the site of injury promoted axonal growth of corticospinal neurons and improved functional recovery following SCI in rats (Tuszynski, Grill et al. 2003)

NGF is an important regulator and is widely located throughout the PNS and CNS (Aloe, Rocco et al. 2012). NGF plays a critical and important role in neuronal development, survival, axonal regeneration and synaptic plasticity (Chen, Zhang et al. 2013; Zhang, Wu et al. 2014). Although NGF is expressed in injured tissues after SCI, several studies have shown exogenous application of NGF reduced secondary damage and promoted neural regeneration and functional recovery following SCI in rats (Chen, Zhang et al. 2013; Zhang, Wu et al. 2014). Administration of NGF by implantation of a graft of genetically modified NGF-secreting fibroblast cells at the site of injury induced axonal growth in SCI rats (Tuszynski, Gabriel et al. 1996; Grill, Blesch et al. 1997).

Glial cell-derived neurotrophic factor GDNF belongs to a family of growth factors, transforming growth factor β (TGF-β). GDNF mediates its biological effect by activating its receptors in the glycosyl-phosphatidylinositol-linked family of receptors (GFR) α1–4 (Lin, Doherty et al. 1993; Iannotti, Li et al. 2003; Zhang, Ma et al. 2009). Placement of GDNF saturated gel foam at the site of lesion right after the injury has been shown to promote axonal regeneration in rats with cervical SCI (Dolbeare and Houle 2003). Furthermore, co-administration of a GDNF and Schwann cell graft at the site of injury has been shown to promote propriospinal axonal

regeneration and remyelination following SCI in rats (Iannotti, Li et al. 2003; Zhang, Ma et al. 2009; Deng, Hu et al. 2011; Deng, Deng et al. 2013). Taken together, it is apparent that application of various growth factors to the site of injury in SCI animals has the potential to promote neurite and axonal regeneration and this regeneration can be associated with improved functional recovery following SCI.

1.5.2.3-Transplantation of various types of cells

Primary and secondary mechanisms of SCI cause the progressive tissue damage and loss of neurons and oligodendrocytes (Rosner, Avalos et al. 2012). Spared axons lose their myelin sheaths due to the death of oligodendrocytes which ultimately disrupts the conductance of axonal pathways (Totoiu and Keirstead 2005). The aim of cell transplantation therapy following SCI is to provide direct replacement of lost cells, provide guidance elements, create a growth permissive environment for axon regrowth and sprouting, and provide remyelination of axons and release of variety of growth factors. A variety of cells have been used for cell transplantation following SCI, including Schwann cells, induced pluripotent stem cells (IPS), embryonic stem cells (ESCs) and olfactory ensheathing cells (OECs) (Blakemore 1975; Li, Field et al. 1997; Ramon-Cueto, Plant et al. 1998; Thomson, Itskovitz-Eldor et al. 1998; McDonald, Liu et al. 1999; Ramon-Cueto, Cordero et al. 2000; Reubinoff, Pera et al. 2000; Pinzon, Calancie et al. 2001; Keirstead, Nistor et al. 2005; Oudega and Xu 2006; Johnson, Parker et al. 2010; Tsuji, Miura et al. 2010; Nori, Okada et al. 2011; Rosner, Avalos et al. 2012; Guest, Santamaria et al. 2013).

The Schwann cell which forms myelin sheaths in the peripheral nervous system is one of the most widely studied cell types for cellular transplant therapies to repair spinal cord. Schwann cells have the ability to differentiate, migrate, proliferate, express variety of growth promoting trophic factors including NGF, BDNF (Park, Lim et al. 2010), adhesion molecules and extracellular matrix proteins such as laminin, integrins, N-cadherin, N-CAM, L1, contactin, laminin, and collagens to support axonal growth (Pierucci, Duek et al. 2009; Ghosh, Tuesta et al. 2012) and remyelination of axons (Biernaskie, Sparling et al. 2007; Rosner, Avalos et al. 2012; Guest, Santamaria et al. 2013). Schwann cells have great potential for repair of the injured spinal

cord. In animal models of SCI, Schwann cell transplantation into the lesion site has been shown to promote axonal regeneration, remyelination and significant improvement in hindlimb function in rats (Takami, Oudega et al. 2002; Zaminy, Shokrgozar et al. 2013). Large numbers of transplanted Schwann cells can also serve to fill the cystic cavities in injured spinal cord (Zaminy, Shokrgozar et al. 2013).

Embryonic stem cells (ESCs) are pluripotent stem cells that can be harvested from the inner cell mass (blastocyst) of developing embryos (Li and Lepski 2013). ES cell - derived progenitors are one of the most important cell sources of cell transplantation therapies to treat SCI (Okada, Shimazaki et al. 2004; Kumagai, Okada et al. 2009). Previous studies have demonstrated that ESCs transplanted into injured rat spinal cord differentiated into neurons, astrocytes and oligodendrocytes (McDonald, Liu et al. 1999; Keirstead, Nistor et al. 2005). Transplantation of mouse ESCs and human ESCs in rodent models of spinal cord injury promote functional recovery (McDonald, Liu et al. 1999; Keirstead, Nistor et al. 2005; Kumagai, Okada et al. 2009). Human ESCs-based therapies have advanced from pre-clinical towards clinical treatment of SCI (Keirstead, Nistor et al. 2005; Kumagai, Okada et al. 2009) although the use of human ESCs-base therapies have some complications due to ethical concerns in certain countries of the world (Nori, Okada et al. 2011).

To avoid concerns associated with ESC-based therapies, induced pluripotent stem cells (IPS) were established by Yamanaka and colleagues (Takahashi and Yamanaka 2006; Okita, Ichisaka et al. 2007; Takahashi, Tanabe et al. 2007; Nori, Okada et al. 2011). IPS cells can be generated by introducing genes into mouse / human skin fibroblast and blood cells (Takahashi, Tanabe et al. 2007; Nakagawa, Koyanagi et al. 2008; Li and Lepski 2013; Nakamura and Okano 2013). IPS cells demonstrate a proliferative and differentiation capacity almost comparable to ESC (Takahashi and Yamanaka 2006; Nori, Okada et al. 2011; Li and Lepski 2013). IPS cells are pluripotent cells and have the potential to generate various cell types including neurons, astrocytes and oligodendrocytes when differentiated (Takahashi, Tanabe et al. 2007; Tsuji, Miura et al. 2010).

Transplantation of human and mouse IPS-derived neurospheres (a cluster of neural stem cells) into a mouse model of SCI has shown survival, migration and differentiation of transplanted IPS cells into neurons, astrocytes and oligodendrocytes (Tsuji, Miura et al. 2010; Rosner, Avalos et al. 2012). In addition to this, expression of neurotrophic factors, angiogenesis, axonal regrowth and remyelination and synapse formation between transplanted neurons and host mouse neurons was also observed in injured areas (Tsuji, Miura et al. 2010; Nori, Okada et al. 2011). Moreover, significant functional recovery of locomotion was also observed in a mouse model of SCI receiving IPS –derived neurospheres (Tsuji, Miura et al. 2010; Nori, Okada et al. 2011). IPS cells are expected to overcome the ethical issues and immunological rejection that were associated with ESC-based therapies (Nori, Okada et al. 2011). IPS cells are expected to open a new era in biomedical science and regenerative medicine (Nakamura and Okano 2013).

Olfactory ensheathing cells (OECs), also known as olfactory ensheathing glia, are one of the most promising candidates for cell transplantation-based therapies to treat spinal cord injury. OECs are specialized glia of the olfactory system and are found along the olfactory nerve (Tohda and Kuboyama 2011) (Rosner, Avalos et al. 2012). The most important property of olfactory neurons is that neurogenesis can occur throughout life (Barnett and Riddell 2004). Numerous studies from animal models of SCI have demonstrated that OECs from the olfactory bulbs of adult rats transplanted into spinal cord lesion appear to promote axonal regeneration, remyelination and functional recovery of locomotion in rat model of spinal cord injury (Lu, Feron et al. 2001; Nash, Borke et al. 2002; Li, Decherchi et al. 2003; Torres-Espin, Redondo-Castro et al. 2014). In addition to the beneficial effect of OECs transplantation in an animal model, recent OEC transplantation in a human patient with complete SCI has shown some neurological benefits without adverse effects (Tabakow, Jarmundowicz et al. 2013).

Contrary to this, some studies have shown, after implantation of propria OECs 1 mm above and below the lesion site at C4 in SCI rats, that OECs failed to exhibit migration to the lesion site and these cells were also unable to promote axonal growth into the SCI (Lu, Yang et al. 2006). Moreover, transplantation of mucosal OECs in cervical SCI rats with a unilateral CST lesion improved forepaw reaching in rats with SCI but failed to exhibit the regeneration of severed CST axonal fiber to form the bridge across the lesion site (Yamamoto, Raisman et al. 2009).

In summary, some controversy exists over whether implantation of OECs promotes axonal regeneration following SCI in animals, in that some studies demonstrated that OEC implantation did not promote axonal regeneration in SCI animal models. This controversy may be due to the use of different OEC cell populations (OECs from the olfactory bulbs or from mucosa), the different SCI model used in various studies, difference in preparation of grafts, time of transplantation and the procedure to transplantation of OECs at the site of lesion in SCI animals (Riddell, Enriquez-Denton et al. 2004; Richter, Fletcher et al. 2005; Steward, Sharp et al. 2006; Munoz-Quiles, Santos-Benito et al. 2009; Zhang, Huang et al. 2011; Zhang, Huang et al. 2011; Chhabra and Sarda 2015)

1.5.3 Plasticity

Neurons communicate with each other by highly specialized structures known as synapses. These synapses are not static but dynamic in nature and show a high degree of plasticity (Tsanov and Manahan-Vaughan 2007; Mehta, Luck et al. 2013). The ability of neurons to rearrange their anatomical and functional connectivity in response to environmental input or based on previous experience is known as plasticity and it is a key feature of the central nervous system (Dietz 2006). Plasticity is the mechanism by which the nervous system fine-tunes its structure and function to meet the demands of the body in its environment (Blight 2004). The circuits within the spinal cord are capable of significant reorganization, in the form of both activity-dependent and injury-induced plasticity (Muir and Steeves 1997). Previous studies have shown that spinal neurons exhibit changes in neuronal processes in response to various stimuli (Edgerton, Roy et al. 1992; Harkema, Hurley et al. 1997; Harkema 2001). These rearrangements of neuronal circuits within the brain and spinal cord are possibly involved in recovery in both humans and animal models (Fouad and Tse 2008). Plasticity encompasses axonal sprouting, synaptic rearrangements and changes in cellular properties of neurons in the brain, brainstem and in spared neuronal circuits rostral and caudal to a spinal cord lesion (Fouad and Tse 2008). Axonal sprouting is generally defined as the outgrowth of branches from an axon that can occur in unlesioned axons or in lesioned axons but proximal to the injury site. The occurrence of spontaneous sprouting of axons has been shown after spinal cord lesion for a number of axonal pathways, including the corticospinal tract (Weidner, Ner et al. 2001; Bareyre, Kerschensteiner

et al. 2004; Fenrich and Rose 2009). The corticospinal tract (CST) is a descending fiber system known to be involved in fine motor control and often is examined in rodent models of SCI. Following incomplete thoracic SCI in rats, transected CST axons sent collaterals sprouting into the cervical gray matter to make new synapses with long and short propriospinal neurons (Bareyre, Kerschensteiner et al. 2004). Twelve weeks after the lesion, hindlimb CST contacts with short propriospinal neurons had been lost whereas contacts with long propriospinal neurons were maintained. These new anatomical contacts formed a novel circuit. Electrophysiology and behavioral testing confirmed that recovery was mediated to some extent through collateral sprouting of the CST and most likely involved the newly formed spinal alternative circuit (Bareyre, Kerschensteiner et al. 2004). Axonal sprouting is part of a general rewiring of cortical, supra- and intraspinal connections that at least to some extent explains functional recovery after spinal cord injury and it is thought to be an important constituent of rehabilitation in humans (Bareyre, Kerschensteiner et al. 2004).

Regardless of level or severity of SCI, some degree of motor, sensory and autonomic functional recovery has been observed in humans and animals due to spontaneous plasticity (Onifer, Smith et al. 2011). Nevertheless, this spontaneous plasticity is frustratingly slow and takes days, weeks or even months to manifest and is inadequate to completely restore normal function following SCI. Many pharmacological and activity-based approaches are available to promote naturally occurring repair mechanisms in injured and spared synaptic circuitries of the spinal cord (Fouad, Krajacic et al. 2011). Plasticity-promoting approaches exploit and enhance endogenous repair mechanisms in the CNS and could open up new avenues to treat traumatic injuries and diseases.

The endogenous mechanism of plasticity can be facilitated by pharmacological means. Various neuromodulators or pharmacological agents have been delivered through intravenous, intrathecal, gelfoam, cell grafting modes in different animal models (Dietz and Fouad 2014). Neurotrophic factors such as BDNF, NGF, and NT-3 are promising candidates to induce plasticity and regeneration (Ye and Houle 1997; Onifer, Smith et al. 2011; Dietz and Fouad 2014). Other pharmacological agents are used to inhibit or block molecules which inhibit axonal sprouting. One example is chondroitinase ABC, a bacterial enzyme which digests the inhibitory chondroitin sulphate proteoglycans, and promoted neuronal sprouting, axonal regeneration and

functional recovery in an animal model of SCI (Onifer, Smith et al. 2011). Similarly, administration of antibodies against the myelin-associated protein Nogo-A, neutralized the effects of Nogo-A and facilitated axon and neuronal sprouting in rats (Zorner and Schwab 2010). Applications of various pharmacological agents alone or in combination have been shown to promote plasticity in animal models of SCI (Onifer, Smith et al. 2011).

Activity-based therapeutic approaches have been also used both clinically and in experimental models of SCI to further enhance the spontaneous recovery of sensory, motor and autonomic functional recovery following SCI. Rehabilitative training is the most current and well established approach to promote plasticity and functional recovery in both spinal-injured humans and animal models of SCI (Behrman and Harkema 2000; Ying, Roy et al. 2005; Ying, Roy et al. 2008; Dietz and Fouad 2014).

Similar to activity-based or rehabilitative training, electrical stimulation can also induce plasticity in spinal cord circuitry (Carmel, Berrol et al. 2010; Dietz and Fouad 2014). Electrical stimulation of the forelimb area of motor cortex in rats triggered the expression of BDNF and improved functional recovery of skilled paw placement over a horizontal ladder following corticospinal tract (CST) SCI in rats (Carmel, Berrol et al. 2010; Fritsch, Reis et al. 2010). In addition to this, electrical stimulation also promoted the outgrowth of CST axonal termination ipsilateral to the injury side of SCI (Carmel, Berrol et al. 2010). Electrical stimulation of CST axons in the medullary pyramid in a unilateral pyramidal lesion enhanced the axonal sprouting and strengthened connections with spinal motor circuits ipsilateral to injury in rats (Brus-Ramer, Carmel et al. 2007). Taken together, electrical stimulation promoted axonal outgrowth and improved skilled motor function in rats with pyramidal lesions (Brus-Ramer, Carmel et al. 2007; Carmel and Martin 2014). Therefore electrical stimulation of motor cortex could be an effective approach to promote sprouting of spared CST axons to promote functional motor recovery in rats (Carmel and Martin 2014)

1.6 Intermittent hypoxia

Another method to induce spinal plasticity is intermittent hypoxia, the focus of this thesis. Intermittent hypoxia (IH) is repetitive exposure to brief periods (i.e. minutes) of low oxygen alternating with periods of exposure to normal oxygen levels. Intermittent hypoxia has been used to train competitive athletes (Zhu, Yan et al. 2010), to enhance the ventilatory output in healthy humans (Serebrovskaya, Karaban et al. 1999; Serebrovskaya 2002) and to improve high altitude adaptation (Gorbachenkov, Tkachuk et al. 1994). Intermittent hypoxia has also been applied to the treatment and prevention of human diseases including to combat bronchial asthma (Serebrovskaya 2002), ischemic coronary artery disease (Zhu, Xie et al. 2006), Parkinson's disease (Lin, Chen et al. 2002), and leukemia (Liu, Guo et al. 2006). Importantly for this thesis, two recent reports demonstrate the effectiveness of IH to improve leg function after SCI (Trumbower, Jayaraman et al. 2012; Hayes, Jayaraman et al. 2014). In spinal injured persons, acute intermittent hypoxia (AIH) treatment for five days increased walking speed and endurance in a randomized, placebo controlled, blinded cross-over design study (Hayes, Jayaraman et al. 2014). Functional benefits lasted up to one week post-treatment (Hayes, Jayaraman et al. 2014).

1.6.1-Chronic intermittent hypoxia (CIH)

Chronic intermittent hypoxia (CIH) is an experimental protocol that aims to reproduce many of the pathophysiological consequences of obstructive sleep apnea. In sleep apnea, the obstruction of the airway occurs repeatedly throughout the sleep period, resulting in intermittent reductions in blood oxygen levels. Severe protocols of CIH in animal models have deleterious side effects including systemic hypertension (Fletcher, Lesske et al. 1992; Fava, Montagnana et al. 2011; Lurie 2011; Ramar and Caples 2011; Nanduri, Makarenko et al. 2012) impaired baroreflex control of heart (Gu, Lin et al. 2007), metabolic syndrome (Tasali and Ip 2008), cognitive impairment (Row 2007; Grigg-Damberger and Ralls 2012; Bucks, Olaithe et al. 2013), neuronal death in hippocampus and neurobehavioral dysfunction (Hambrecht, Vlisides et al. 2007), synaptic transmission in the nucleus of the solitary tract (Kline, Ramirez-Navarro et al. 2007), neurodegeneration, oxidative stress and inflammatory responses (Row, Kheirandish et al. 2007).

Alongside these pernicious side effects, CIH protocols induce robust plasticity in the spinal cord. Chronic intermittent hypoxia elicits plasticity at multiple sites of the respiratory control system including carotid body chemosensitivity (Peng, Kline et al. 2001; Peng and Prabhakar 2004), increased synaptic strength in the nucleus tractus solitarious (Kline, Ramirez-Navarro et al. 2007), and increased synaptic strength in spinal pathways to phrenic motor neurons (Fuller, Johnson et al. 2003; Dale-Nagle, Hoffman et al. 2011). CIH given for 7 days (alternating 11% O_2 and air; 5 min periods; 12 hr per night; 7 nights) following C2 hemisection has been demonstrated to enhance spontaneous plasticity and improve phrenic motor output (Fuller, Johnson et al. 2003). This CIH mediated enhancement in spontaneous plasticity has been demonstrated to be serotonin dependent (McGuire, Zhang et al. 2004; McGuire and Ling 2005). Pre-treatment with CIH increases the sensitivity and phrenic response during hypoxia and augments the effect of an acute intermittent hypoxia (Ling, Fuller et al. 2001). Nevertheless, the deleterious side effects of CIH make it inappropriate to use as a therapeutic regime for spinal cord injury. Recent studies have demonstrated that there are more acute IH protocols which can elicit plasticity in spinal cord without pernicious side effects (Dale-Nagle, Hoffman et al. 2010).

1.6.2 Acute intermittent hypoxia (AIH)

Acute intermittent hypoxia (AIH) protocols involve exposure to fewer hypoxic episodes compared with chronic protocols. Daily acute intermittent hypoxia (dAIH), for example, is a paradigm in which the animal is exposed to 10 hypoxic episodes per day for 7 days (total 70 episodes of hypoxia in one week) when in comparison, a CIH protocol might consist of 504 episodes of hypoxia during the same time period (Vinit, Lovett-Barr et al. 2009; Wilkerson and Mitchell 2009). A variety of acute intermittent hypoxia paradigms can induce spinal plasticity by augmenting spared synaptic pathways in intact and spinally injured animal models (Bach and Mitchell 1996; Ling, Fuller et al. 2001; Fuller, Johnson et al. 2003; Golder and Mitchell 2005; Wilkerson and Mitchell 2009; Dale-Nagle, Hoffman et al. 2010). A single acute protocol composed of 3, 5-min episodes of AIH (35–45 mmHg arterial PO_2 , 25–30 mmHg arterial PO_2) in rats can induce spinal plasticity in respiratory motoneurons in the spinal cord for a short period of time (30-90 minutes) (Dale-Nagle, Hoffman et al. 2011; Hoffman and Mitchell 2011; Nichols, Dale et al. 2012). The duration of AIH-induced plasticity can be prolonged and

enhanced by repetitive use of AIH, such as daily exposure (dAIH; 10 episodes per day, 7 d) for one week (dAIH) (Dale-Nagle, Hoffman et al.) or exposure to AIH for three times per week for 3-10 weeks (3xwAIH) (Satriotomo, Dale et al. 2012; Dale and Mitchell 2013). dAIH elicits comparable effects to CIH such as increases in the expression of BDNF within the phrenic motor nucleus (Satriotomo, Dale et al. 2007) without deleterious side effects such as systemic hypertension and hippocampal cell death (Ling, Fuller et al. 2001; McGuire, Zhang et al. 2002).

The most thoroughly studied model of AIH-induced plasticity is long-term facilitation (LTF), the strengthening of synapses onto respiratory motor neurons (Mitchell, Baker et al. 2001; MacFarlane and Mitchell 2008; Mahamed and Mitchell 2008). Long-term facilitation is manifest as a progressive increase in the output of phrenic or hypoglossal motoneurons in response to 10 alternating exposures to 5 minute episodes of moderate hypoxia (e.g. 11% inspired oxygen) alternating with 5 minutes of normoxic exposure (Bach and Mitchell 1996; Ling, Fuller et al. 2001; Fuller, Johnson et al. 2003; Golder and Mitchell 2005). This increase in motoneuron output is sustained for at least 2 hours following the end of AIH exposure. LTF can be evoked in both anaesthetized (Wilkerson and Mitchell 2009; Sandhu, Lee et al. 2010) and unanaesthetized (McGuire, Zhang et al. 2003; McGuire and Ling 2005; Nakamura, Olson et al. 2010) rats and in humans during sleep (Pierchala, Mohammed et al. 2008; Vinit, Lovett-Barr et al. 2009). It is important that sustained exposure to hypoxia for the same duration, i.e. without alternation with normoxia, cannot induce LTF (Pamenter and Powell 2013). The episodic exposure to hypoxia is therefore, necessary to evoke the response and it has been demonstrated that at least three episodes of alternating exposures to low oxygen are required to evoke LTF in the respiratory motor system (Dale-Nagle, Hoffman et al. 2011; Nichols, Dale et al. 2012). Physiologically, long-term facilitation may serve as a compensatory mechanism to stabilize the respiratory output following periods of hypoxia (Wilkerson and Mitchell 2009).

In addition to effects in intact animals, AIH elicits recovery of both respiratory and forelimb function in rodent models of incomplete cervical SCI (Lovett-Barr, Satriotomo et al. 2012). In separate experiments, rats exposed to 10 episodes of 5 min 11% oxygen alternating with 5 min normoxia for 7 days at 4 wks post-SCI, showed sustained improvement in respiratory output or

skilled forelimb function during a ladder walking task. The latter results are from our lab and the confirmation and expansion of these results form the basis for this thesis.

1.6.3 Cellular / synaptic mechanisms of AIH-induced Plasticity

Current studies in animal models have revealed the cellular and synaptic mechanisms of LTF. AIH activates multiple cellular pathways, which are named, the Q pathway, S pathway, V pathway, and E pathway. Several of these pathways require synthesis of BDNF and / or activation of tyrosine kinase receptor B (trkB) (Dale, Ben Mabrouk et al. 2014). These cellular pathways all ultimately induce a change of excitability in motor neurons via phosphorylation and insertion of glutamate receptors in the post-synaptic membrane at the premotor neuron : motor neuron synapse (Fuller, Bach et al. 2000; Mahamed and Mitchell 2007; McGuire, Liu et al. 2008; Dale-Nagle, Hoffman et al. 2011).

1.6.3.1 Q pathway

The term Q pathway refers to the involvement of G_q protein-coupled metabotropic 5-HT_{2a} receptors (Vinit, Lovett-Barr et al. 2009; Dale-Nagle, Hoffman et al. 2011; Dale, Ben Mabrouk et al. 2014). AIH treatment triggers the episodic release of serotonin in the vicinity of phrenic motor neurons in the spinal cord, thereby activating the serotonin receptor 5-HT_{2a}, which, via a PKC pathway, results in increased synthesis of new BDNF (Vinit, Lovett-Barr et al. 2009). This BDNF, through its high affinity receptor trkB on the same or adjacent neurons, initiates a cascade of signalling through ERK and MAP kinase pathways (Fig, 1.1) (Baker-Herman and Mitchell 2002; Hoffman and Mitchell 2011). The end result is a form of plasticity known as pLTF, the increase in the output of phrenic motoneurons (Baker-Herman and Mitchell 2002; Vinit, Lovett-Barr et al. 2009; Dale-Nagle, Hoffman et al. 2011; Dale, Ben Mabrouk et al. 2014). This pathway induces spinal plasticity and strengthening the synaptic output in spinal motor neurons.

1.6.3.2 S pathway

The S-pathway is a cellular pathway known to induce pLTF independent of BDNF synthesis following AIH (Vinit, Lovett-Barr et al. 2009; Dale, Ben Mabrouk et al. 2014). G_s protein-

coupled metabotropic receptors such as adenosine A_{2A} receptors or serotonin type 7 (5HT7) are involved in this pathway, which also requires the synthesis of an immature trkB receptor isoform and phosphoinositide 3 (PI3) kinase / protein kinase A signaling (Fig, 1.1) (Golder, Ranganathan et al. 2008; Dale-Nagle, Hoffman et al. 2011; Dale, Ben Mabrouk et al. 2014).

1.6.3.3 V pathway

AIH upregulates vascular endothelial growth factor (VEGF) and VEGF-R2 expression in phrenic motor neurons and nonrespiratory spinal motor neurons (Dale and Mitchell 2013). VEGF-R2 is a member of the receptor tyrosine kinase family which activates signaling cascades similar to trkB (Zachary 2005; Dale-Nagle, Satriotomo et al. 2011). VEGF-R2 mediates signaling cascade via ERK and Akt activation (Fig, 1.1) (Zachary 2003). This signaling cascade induces pLTF similar to BDNF administration (Dale-Nagle, Satriotomo et al. 2011).

1.7.3.4 E pathway

AIH increases the expression of erythropoietin (EPO) in spinal motor neurons and its expression is regulated by HIF-1 (Dale, Ben Mabrouk et al. 2014). EPO and its receptor EPO-R are present in phrenic motor neurons (Dale, Satriotomo et al. 2012; Dale and Mitchell 2013). Cervical spinal injection of EPO activates the EPO-R and initiates an intracellular signaling cascade via ERK and Akt activation, which induces pLTF similar to BDNF / trkB and VEGF administration (Fig, 1.1) (Dale, Satriotomo et al. 2012; Dale, Ben Mabrouk et al. 2014).

In summary, several distinct intracellular signalling pathways associated with different growth/trophic factors are involved the production of LTF (Dale-Nagle, Hoffman et al. 2011; Dale-Nagle, Satriotomo et al. 2011; Dale, Satriotomo et al. 2012; Dale and Mitchell 2013). The activation of one pathway versus another following AIH appears to depend on factors such as intensity and / or duration of hypoxia (Mitchell and Terada 2011). Moderate form of AIH (AIH; 3, 5-min episodes; 35–45 mmHg arterial PO_2) induced serotonin dependent pLTF through the Q pathway whereas a more intense and severe form of AIH (AIH; 3, 5-min episodes; 25–30 mmHg arterial PO_2) shifts the mechanism of pLTF from serotonin-dependent to an adenosine-dependent

pLTF mechanism through the S pathway (Nichols, Dale et al. 2012). It is possible that each of these pathways has the potential to induce functional recovery following SCI.

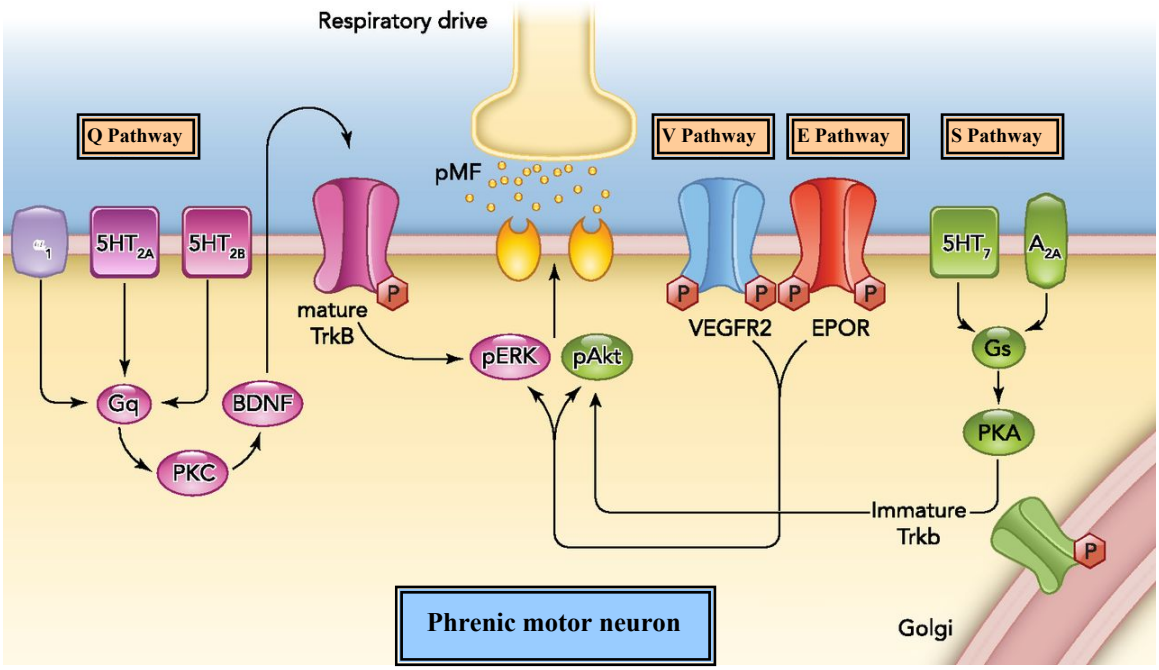

Figure 1.1: Working model of phrenic motor facilitation (pMF). The "Q" pathway is elicited by intermittent activation of Gq-coupled metabotropic receptors 5-HT2, which, via a PKC pathway, results in increased synthesis of new BDNF. This BDNF through its trkB, initiates a cascade of signalling through ERK and MAP kinase. The "S" pathway is elicited by Gs-coupled metabotropic receptors 5-HT7 and A2A, PKA activation, new synthesis of an immature trkB isoform, and downstream signaling via Akt phosphorylation/activation. The "V" and "E" pathways are activated by hypoxia-sensitive VEGF and EPO growth/trophic factors elicit pMF via ERK- and Akt-dependent mechanisms. Modified from E. A. Dale et al. Physiology 2014;29:39-48.

1.7 Neurotrophins

Changes in neurotrophin expression, particularly BNDF, play an important role in AIH-induced plasticity. The following section reviews the current knowledge on the contributions of neurotrophins to neural plasticity.

1.7.1 Importance of neurotrophins in plasticity

Neurotrophins are proteins that are secreted and synthesized by neurons and non-neuronal cells and subsequently bind to their appropriate receptors located on the target cell membrane. The neurotrophins are a family of neurotrophic factors that play a versatile role in the developing and mature nervous system including: development, survival, differentiation, migration, proliferation, maintenance, regeneration of neurite outgrowth and sprouting, and functional plasticity of CNS and PNS (Schinder and Poo 2000; Poo 2001; Thuret, Moon et al. 2006).

1.7.2 Neurotrophins and their receptors

The first member of the neurotrophin family to be described was nerve growth factor (NGF) and it was identified by Levi-Montalcini and Hamburger over 60 years ago (Levi-Montalcini and Hamburger 1951). Since then, NGF has become the prototypic neurotrophic factor (Levi-Montalcini 1987). Following its discovery, other members of the neurotrophin family have since been described, and the family now consists of NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (Huang and Reichardt 2001). Members of the neurotrophin family exert their effects through the interactions with two distinct classes of neuronal cell surface receptors, a high affinity tyrosine receptor kinase (trk) and a low affinity pan-neurotrophin receptor (p75). All neurotrophins are capable of binding to the low affinity p75 (p75RN) neurotrophin receptor (Chao 1992; Lu 2003) and each neurotrophin can also bind to a specific corresponding high affinity tyrosine kinase receptor, NGF with trkA, BDNF and NT-4/5 with trkB, and NT-3 with trkC (Barbacid 1993).

1.7.3 Nerve growth factor (NGF)

Nerve growth factor (NGF) is an important regulator of the central and peripheral nervous systems and it is widely located in the PNS and CNS (Aloe, Rocco et al. 2012). The largest

amount of NGF is produced in cortex, hippocampus, pituitary gland, thalamus, basal ganglia, retina and spinal cord (McAllister 2001). NGF is a polypeptide and exerts its biological effects through its specific tyrosine kinase receptor A (trkA). NGF also bind and activates the non-specific low affinity transmembrane glycoprotein pan-neurotrophin receptor (p75) (Huang and Reichardt 2001; Huang and Reichardt 2003; Schor 2005; Reichardt 2006; Aloe, Rocco et al. 2012). Subsequent to its binding with its receptors, NGF activates multiple cytosolic and / or endosomal signalling pathways. NGF controls and regulates the synthesis of neurotransmitters such as norepinephrine in sympathetic and sensory neurons (Otten, Schwab et al. 1977), neuropeptide expression such as Substance P (SP) in dorsal root ganglion, and calcitonin gene-related peptide from primary sensory neurons (Mearow and Kril 1995; Aloe, Rocco et al. 2012). NGF plays a critical and important role in neuronal development, survival, axonal regeneration and synaptic plasticity (Chen, Zhang et al. 2013; Zhang, Wu et al. 2014).

Exogenous administration of NGF through intracerebroventricular injection in postnatal day (PD) 7 rat pups exerted neuroprotective effects and reduced neurological deficits following hypoxic-ischemic brain injury (Holtzman, Sheldon et al. 1996). Although NGF is expressed in injured tissues after SCI, several studies have shown exogenous application of NGF reduced secondary damage and promoted neural regeneration and functional recovery following SCI in rats (Chen, Zhang et al. 2013; Zhang, Wu et al. 2014). This suggests that the normal expression level of NGF was not high enough to prevent or minimize the secondary damage to the tissues at the site of injury (Chen, Zhang et al. 2013). In addition to this, some deleterious effects are also linked with administration of NGF. Exogenous administration of NGF in rodents and humans can lead to a rapid activation and sensitization of cutaneous nociceptors causing hyperalgesia and pain (Andreev, Dimitrieva et al. 1995; Dyck, Peroutka et al. 1997). NGF increases the excitability of sensory neurons and induces peripheral sensitization that can lead to enhanced nocioception (Nicol and Vasko 2007). Moreover, exogenous administration of NGF causes extensive axonal sprouting of nociceptive primary sensory neurons in the spinal dorsal horn (Romero, Rangappa et al. 2000). Therefore, exogenous application of NGF to treat SCI is not a viable option for the reason that it causes hyperalgesia and pain.

1.7.4 Brain derived neurotrophic factor (BDNF)

BDNF is a second member of the neurotrophin family, discovered in 1982. BDNF has diverse roles as a neuronal modulator and plays a pivotal role in synaptic plasticity. BDNF regulates neuronal structure, function and connectivity through its high affinity receptor tyrosine kinase trkB and triggers the downstream signaling cascades that cause modifications in cellular function (Lu 2003; Massa, Yang et al. 2010). Increased excitatory activity induced by pharmacological agents, physical activity, and intermittent hypoxia will increase BDNF concentration in the hippocampus, cortex and spinal cord (Hartmann, Heumann et al. 2001; Baker-Herman, Fuller et al. 2004; Mattson, Duan et al. 2004). BDNF may also enhance synaptic input by increasing the strength of pre-existing synaptic connections and / or increasing sprouting and generation of new synaptic connection of spared neurons (Sieck and Mantilla 2009). BDNF plays an important role in neural plasticity and improves functional recovery following SCI through multiple mechanisms.

Earlier studies have demonstrated the essential role of BDNF in respiratory plasticity (Baker-Herman, Fuller et al. 2004). BDNF is required for synaptic plasticity associated with long-term facilitation in phrenic motoneurons. This effect is mediated through trkB (Baker-Herman, Fuller et al. 2004). Brief episodes of hypoxia in rats initiate the synthesis of new BDNF in the cervical cord, rather than release of existing BDNF (Baker-Herman, Fuller et al. 2004; Wilkerson and Mitchell 2009). It would be important to determine whether BDNF also mediates recovery of non-respiratory function after intermittent hypoxia treatment in rats with SCI.

1.7.5 Neurotrophin-3 (NT-3)

Neurotrophin-3 is the third member of the family of neurotrophins, discovered in 1990. NT-3 shares the properties of both BDNF and NGF. NT-3 plays an important role in survival, proliferation of neurons and neurite outgrowth (Arenas and Persson 1994; Barres, Raff et al. 1994; Beggs, Alvares et al. 2012). NT-3 is the first neurotrophin to be expressed in the PNS during embryogenesis and plays an important role in the survival and differentiation of PNS neurons during the perinatal development (Lessmann, Gottmann et al. 2003). During development, NT-3 shows the highest level of expression compared to BDNF and NGF, with

most prominent expression levels in the hippocampus, the neocortex, and the cerebellum (Zhou and Rush 1994). NT-3 is also produced by skin and muscle cells and is retrogradely transported by sensory neurons to the neuronal cell body (Helgren, Cliffer et al. 1997; Zhou, Deng et al. 1999; Jerregard, Akerud et al. 2000; Marconi, Terracina et al. 2003). NT-3 can bind with each member of the trk receptor family, but its primary biological role is mediated through the trkC receptors (Huang and Reichardt 2001). NT-3 also plays an important role in synaptic plasticity. Application of NT-3 on Xenopus neuromuscular junctions in culture results in an increase in amplitude of the excitatory post-synaptic current (Xie, Wang et al. 1997). In spinal neurons, NT-3 also promotes the maturation of synapses by increasing the levels of synaptic vesicle proteins, including synaptophysin, and synapsin 1 (Wang, Xie et al. 1995). After experimental SCI in rats, NT-3 appears to promote survival of neurons and axonal regeneration and improves locomotor performance (Dreyfus, Dai et al. 1999; Yang, Yang et al. 2009; Wang, Zhang et al. 2014).

1.7.6 Neurotrophin-4/5 (NT-4/5)

Neurotrophin-4/5 is the fourth member of the neurotrophin family to be discovered in 1991 (Hallbook, Ibanez et al. 1991). It is also known as NT-4, NT-5 and NT-4/5. Expression of NT-4/5 is prominent in the postnatal hippocampus, neocortex, cerebellum and thalamic nuclei and it continues to be prominent until adulthood (Friedman, Black et al. 1998). NT-4/5 exerts its biological effects through trkB receptors, and also binds with the low affinity receptor p75. NT-4/5 supports the survival of primary somatic and visceral sensory neurons and it also promotes the survival of axotomized developing or mature spinal motor neurons (Friedman, Kleinfeld et al. 1995; Erickson, Conover et al. 1996). Another study has demonstrated that grafted fibroblasts modified to secrete NT-4/5 promote axonal regeneration in rat model of SCI (Blesch, Yang et al. 2004).

1.8 Hypoxia-associated Proteins

To begin to understand the underlying mechanism of AIH-induced plasticity at the cellular level, we need to understand the cellular response to hypoxia. Cellular responses to changes in oxygen levels are essential for maintenance and survival of cells. Hypoxia is known to alter the

expression of many cellular proteins and their respective mRNAs, including Hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) (Nordal, Nagy et al. 2004; Ke and Costa 2006; Xiaowei, Ninghui et al. 2006; Dale-Nagle, Satriotomo et al. 2011; Dale and Mitchell 2013).

1.8.1 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is a 45 Da dimeric glycoprotein and a fundamental regulator of pathological and physiological angiogenesis (Rosenstein and Krum 2004). VEGF promotes endothelial cell formation and proliferation in several organ systems during embryonic development and after injury in many tissues, including the central nervous system (Skold, Cullheim et al. 2000). VEGF is critical for blood vessel growth in the developing and adult nervous system of vertebrates (Mackenzie and Ruhrberg 2012). In addition to its role as angiogenic factor, it also promotes neurogenesis, neuronal patterning, neuroprotection and neuronal migration in the embryonic brain (Rosenstein, Krum et al. 2010).

The VEGF family consists of six different members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and Placental growth factor (PlGF) (Ferrara, Gerber et al. 2003). VEGF-A is the most dominant member of VEGF family and key regulator of angiogenesis in the nervous system. The other growth factors in the VEGF family are found less frequently in the nervous system (Ferrara, Gerber et al. 2003; Rosenstein and Krum 2004). VEGF-A is composed of a collection of three isoforms. The human isoforms consist of 121, 165, 189 and 206 amino acids and are therefore termed VEGF121, VEGF165, and VEGF189. Mouse isoforms are VEGF120, VEGF164, VEGF188 (Ferrara and Alitalo 1999; Mackenzie and Ruhrberg 2012). $VEGF_{165}$ is the most predominant isoform produced in tissues including brain, although each isoform is able to support angiogenesis. Most biological effects of VEGF are mediated through an interaction with three VEGF receptor subtypes, VEGFR-1 or Flt-1 (Fetal liver kinase-1) and VEGFR-2 or FLK-1 (fms-like tyrosine kinase-1) and VEGFR-3 or neuropilin-1 NRP-1 (Millauer, Wizigmann-Voos et al. 1993; Mackenzie and Ruhrberg 2012). VEGF receptors are mainly expressed on endothelial cells of blood vessels and capillaries during embryogenesis. Animals with VEGF receptor defects die *in utero* (Fong, Rossant et al. 1995). Although VEGF isoforms display

differential affinities for VEGF receptors, all VEGF-A isoforms bind to the transmembrane tyrosine kinase receptors VEGFR-1 (FLT1) and VEGFR-2 (FLK1). The non-tyrosine kinase receptor VEGFR-3 or neuropilin-1, (NRP-1), preferentially binds with the VEGF164 isoform and activates several downstream pathways (Ruhrberg 2003; Rosenstein, Krum et al. 2010; Mackenzie and Ruhrberg 2012).

1.8.2 Role of VEGF in the CNS

VEGF was originally recognized for its role in angiogenesis, but it is no longer characterized solely as an endothelial mitogen. VEGF is known to play a versatile role in the central and peripheral nervous systems both *in vitro* and *in vivo* (Rosenstein and Krum 2004; Mackenzie and Ruhrberg 2012). These functions include neurogenesis, neuronal patterning, neuroprotection, axon guidance, neurotrophic, gliotrophic action, and anti-apoptotic action, (Mackenzie and Ruhrberg 2012). VEGF plays a significant role during development of the nervous tissue and directly influences Schwann cells, neuronal progenitor cells, astrocytes and microglia neurogenesis, as well as initiating endothelial differentiation and formation of vessels in the brain (Mackenzie and Ruhrberg 2012; Nowacka and Obuchowicz 2012). Current studies have shown that VEGF also improves cellular and behavioural function. *In vitro* studies have shown that application of VEGF increases survival and enhances neurite growth in dopaminergic neurons (Pitzer, Sortwell et al. 2003) and neocortical neurons (Khaibullina, Rosenstein et al. 2004) independent of its effect on blood vessels.

1.8.3-Role of VEGF in Spinal Cord Injury

Apart from its role in angiogenesis, VEGF appears to play neurotrophic and neuroprotective roles in spinal cord and brain injury (Krum and Rosenstein 1998; Facchiano, Fernandez et al. 2002; Svensson, Peters et al. 2002). Expression of VEGF is regulated by HIF-1α following SCI. VEGF, as a master regulator of angiogenesis, modulates the microcirculation and revascularization to restore blood flow, and revascularization can be seen the 3rd day after SCI. Revascularization reduces secondary damage after SCI and plays an important role in tissue repair and regeneration (Zhang, Magovern et al. 1997). Exogenous applications of VEGF through intrathecal injection at C4 in rats exerted neurotrophic and neuroprotective effects in the

CNS and elicited respiratory plasticity through ERK and Akt signalling pathways (Dale, Satriotomo et al. 2012; Dale and Mitchell 2013). In addition to this, exogenous application of VEGF exerted a neuroprotective effect and suppressed apoptosis in rat retinal neuron culture through ERK and Akt signalling pathways (Shen, Wu et al. 2010). Exogenous application of VEGF through intrathecal injection at C4 exerted neurotrophic and neuroprotective effects and induced spinal plasticity (pMF) in rats and enhanced the activity of the phrenic motor nerve (Dale, Satriotomo et al. 2012; Dale and Mitchell 2013)

1.8.4 VEGF after intermittent hypoxia

VEGF expression increases in spinal motoneurons in response to hypoxia in a manner thought to be neuroprotective in motoneurons (Sato, Morimoto et al. 2012). VEGF expression is regulated by hypoxia-Inducible Factor-1α (HIF-1α), a transcription factor activated in response to low oxygen (Rosenstein and Krum 2004; Xiaowei, Ninghui et al. 2006). AIH increases the expression of HIF-1α, which translocates to the nucleus and binds with HIF-1β, which in turn initiates the transcription of the VEGF gene to upregulate the expression of VEGF. After AIH, VEGF and its receptor VEGFR-2 were upregulated in both respiratory and non-respiratory motoneurons in the spinal cord and in neurons in the motor cortex (Sato, Morimoto et al. 2012; Dale, Ben Mabrouk et al. 2014). Recent studies have shown that VEGF and VEGFR-2 are both expressed in phrenic motoneurons where they induced phrenic motor facilitation (pMF) via ERK Akt intracellular pathways (Dale, Ben Mabrouk et al. 2014).

1.9 Hypoxia-inducible factor-1α (HIF-1α)

The cellular response to hypoxia is mediated through a transcription factor known as hypoxia inducible factor-1 (HIF-1). HIF-1 is a master transcriptional regulator of genes that control a number of adaptive responses to low oxygen tension in order to maintain oxygen homeostasis. HIF-1 regulates the expression of several dozen target genes including VEGF, erythropoietin (EPO), inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (Semenza, Nejfelt et al. 1991; Melillo, Musso et al. 1995; Stein, Neeman et al. 1995; Lee, Jiang et al. 1997; Kimura, Weisz et al. 2000; Ke and Costa 2006; Xiaowei, Ninghui et al. 2006; Xiong, Mahmood et al. 2010). The HIF-1 protein is a heterodimer and composed of two subunits: the HIF-1α subunit

and the constitutively expressed HIF-1β subunit (Wang, Jiang et al. 1995). Both subunits belong to the basic helix-loop-helix-PAS (bHLH-PAS) superfamily. HIF-α exists as multiple isoforms known as HIF-1α, HIF-2α, and HIF-3α (Ema, Taya et al. 1997; Flamme, Frohlich et al. 1997; Tian, Wu et al. 1997; Gu, Moran et al. 1998; Wenger 2002). HIF-1α is oxygen sensitive and it is activated under hypoxic conditions and is degraded in normoxic conditions by proteasomes. It has a very short half life (8 minutes) under normoxic conditions (Jewell, Kvietikova et al. 2001). In hypoxia conditions, HIF-1α translocates from the cytoplasm to the nucleus and dimerizes with HIF-1β subunit to form the active HIF heterodimer complex (Lando, Peet et al. 2002). This dimer complex binds with hypoxia response elements (HRE) in target genes to induce gene expression (Lando, Peet et al. 2002).

1.9.1 Importance of HIF-1α in SCI

Spinal cord injury increases the expression of both HIF-1α protein and HIF-1α mRNA (Ju, He et al. 2002; Xiaowei, Ninghui et al. 2006). HIF-1α is expressed in neuronal and non-neuronal cells in the spinal cord following SCI. HIF-1α mRNA also significantly increases following SCI (Xiaowei, Ninghui et al. 2006). The transcription of HIF-1α mRNA was initiated 6 hours post-SCI and reached a maximum on day 3 post-SCI, while HIF-1α protein levels increased 1-7 days following SCI and gradually reduced thereafter (Xiaowei, Ninghui et al. 2006). HIF-1α and its target genes could have an important impact on secondary damage after SCI because HIF-1α is responsible for activation of genes that facilitate the adaptation and survival of cells and tissue in low oxygen conditions. The most important of these genes is erythropoietin (EPO). Originally known to play a role in erythropoiesis (Ke and Costa 2006), EPO also has neuroprotective effects in the hippocampus and spinal motoneurons (Mennini, De Paola et al. 2006; Naganska, Taraszewska et al. 2010; Xiong, Mahmood et al. 2010; Dale, Ben Mabrouk et al. 2014). HIF-1α has the ability to activate the transcription of several glycolytic enzymes (Xiaowei, Ninghui et al. 2006). These enzymes further enhance the glycolysis pathway to supply energy to the cells to maintain their physiological functions for survival following SCI. The activity of these glycolytic enzymes lasts for several days.

Together, the overall effects of both hypoxia-associated proteins, VEGF and HIF-1α, appears to be beneficial for reducing secondary damage following SCI, and these hypoxia related proteins also appear to facilitate the process of repair. Therapies that augment the levels of these factors locally in the spinal cord might be beneficial for repair and recovery of SCI.

1.10 Summary and rationale for current studies

There are many animal models and experimental therapies that have been used in the search for effective approaches to improve recovery after spinal cord injury. One of the most promising approaches is the augmentation of spontaneously occurring plasticity in uninjured neural pathways. Current evidence strongly suggests that AIH is able to induce spinal plasticity by strengthening synapses onto respiratory motor neurons and enhancing motor output to the phrenic nerve. Moreover, AIH alters the expression of plasticity- and hypoxia-related proteins in respiratory motoneuron pools in the spinal cord. In animal SCI models, acute intermittent hypoxia improves respiratory and forelimb motor function (Lovett-Barr, Satriotomo et al. 2012) (Vinit, Lovett-Barr et al. 2009). Critically, AIH improves lower limb function and walking in spinal-injured humans (Trumbower, Jayaraman et al. 2012; Hayes, Jayaraman et al. 2014). Therefore, although there is still much to be investigated regarding the nature of AIH-induced motor recovery and the mechanisms underlying the effects of AIH, it is clear that AIH is a novel non-invasive experimental therapy for SCI that has strong potential for use in clinical SCI.

CHAPTER 2

AIMS AND OBJECTIVES

2.1 Aims and Objectives

The main aim of this research was to investigate whether acute intermittent hypoxia improves forelimb functional recovery in a rat model of spinal cord injury. AIH is a promising non-invasive therapy for spinal cord injury that has shown clinical benefits in persons with SCI. Preliminary work also showed that AIH improved forelimb function in rats with incomplete cervical spinal cord injury. This thesis extends this work to investigate the robustness of forelimb recovery in AIH-treated SCI rats, and the cellular changes which occur in spinal neurons in response to AIH treatment.

2.2 Rationale for current studies

There are many animal models and experimental therapies that have been used in the search for effective approaches to improve recovery after spinal cord injury. One of the most promising approaches is the augmentation of spontaneously occurring plasticity in uninjured neural pathways. Current evidence strongly suggests that AIH is able to induce spinal plasticity by strengthening synapses onto respiratory motor neurons and enhancing the motor output of the phrenic nerve. Moreover, AIH alters the expression of plasticity- and hypoxia-related proteins in respiratory motoneuron pools in the spinal cord. In animal SCI models, acute intermittent hypoxia improves respiratory and forelimb motor function (Lovett-Barr, Satriotomo et al. 2012) (Vinit, Lovett-Barr et al. 2009). Critically, AIH improves lower limb function and walking in spinal-injured humans (Trumbower, Jayaraman et al. 2012; Hayes, Jayaraman et al. 2014).

2.3 General Hypothesis

Acute intermittent hypoxia was initially investigated in the context of plasticity in spinal respiratory motor neuron pools. Although there is still much to be investigated regarding the nature of AIH-induced motor recovery and the mechanisms underlying the effects of AIH, it is

clear that AIH is a novel non-invasive experimental therapy for SCI that has strong potential for use in clinical SCI.

The purpose of this study is to investigate the effectiveness of a novel treatment, acute intermittent hypoxia (AIH), toward enhancing spinal plasticity after spinal injury.
Therefore, I proposed the general hypothesis that **acute intermittent hypoxia induces spinal plasticity and improves forelimb function in rats with cervical spinal cord injury.**

2.4 Specific Aims

My dissertation has two specific aims. The first specific aim focuses on investigating the effect of AIH on functional recovery of forelimb in a rat model of cervical spinal injury. The second aim focuses on determining the effect of AIH on expression of spinal cord proteins.

Specific Aim #1: To address the hypothesis that acute intermittent hypoxia improves recovery of limb function in rats with cervical dorsolateral funiculus lesions.

The following questions were addressed:

1. Does acute intermittent hypoxia (AIH) improve recovery of ladder walking in rats with SCI?
2. Is concomitant motor training required to facilitate the effect of AIH on recovery of ladder walking in rats?
3. Does AIH improve recovery of skilled paw use in rats with SCI?

Specific Aim #2: To address the hypothesis that AIH alters the expression of hypoxia- and plasticity-related proteins in a rat model of cervical spinal injury.

The following questions were addressed:

1. Does AIH alter the expression of hypoxia-related proteins, specifically hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) in the spinal cord of SCI rats?

2. Does AIH alter the expression of plasticity-related proteins, specifically brain-derived neurotrophic factor (BDNF) and trkB, in the spinal cord of SCI rats?

CHAPTER 3

Delayed intervention with intermittent hypoxia improves forelimb function in a rat model of cervical spinal injury

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Author Contributions

All the experiments were conceived and designed by myself, Dr. Gillian Muir, Dr. Erin Prosser-Loose and Dr. Gordon Mitchell. I performed the experiments, collected and analysed the data presented in this manuscript. This manuscript has been accepted for publication in the Journal of Neurotrauma in February 2015.

Transition Statement

This chapter focuses on the first of the two specific aims in this thesis, to address the hypothesis that acute intermittent hypoxia improves recovery of limb function in rats with cervical dorsolateral funiculus lesions.

3.1 Introduction

Spinal cord injury damages the axonal connections between the brain and the spinal cord, and the subsequent alteration of motor, sensory and autonomic function below the level of the injury has devastating consequences. More than 50% of injuries occur in the cervical region of the spinal cord, causing reduced motor control and sensory feedback from arms and hands as well as the legs (Devivo 2012). Most spinal injuries are anatomically incomplete, and spontaneous functional recovery does occur in the weeks or months after injury, attributed to plasticity in uninjured pathways in the spinal cord and throughout the central nervous system (Onifer, Smith et al. 2011; Cadotte and Fehlings 2013; Silva, Sousa et al. 2013). Nevertheless, rarely does motor and sensory function return completely and the focus of much of the contemporary research in this field, both pre-clinical and clinical, is directed toward enhancement of CNS plasticity after SCI in order to augment functional recovery.

Many different therapies and training regimes have been investigated (Kwon, Okon et al. 2011; Cadotte and Fehlings 2013; Dietz and Fouad 2014; Wilson, Forgione et al. 3013). Unfortunately, the majority of the pre-clinical therapies that have shown some success in a particular animal model have not proven robust enough to be replicated in other animal models, much less translated to the clinic (Heinemann, Steeves et al. 2012; Wilson, Forgione et al. 3013). A recent report has demonstrated the effective use of acute intermittent hypoxia (AIH) to improve motor function in persons with partial spinal injury (Hayes, Jayaraman et al. 2014). In an ongoing study, persons with incomplete SCI were able to walk faster and for longer distances after receiving AIH compared to normoxia treatment. This effect lasted for up to one week after treatment, the duration of the study so far (Hayes, Jayaraman et al. 2014). The current study focuses on a novel, non-invasive therapy AIH that has already shown some success in clinical investigation.

Acute intermittent hypoxia was initially investigated in the context of plasticity in spinal respiratory motor neuron pools (Dale, Ben Mabrouk et al. 2014). In a well-established animal model, brief (5 min) exposures to reduced oxygen levels (10.5% inspired O_2) in rats, alternating with exposures to normal levels (20% O_2), results in a sustained increase in phrenic motor

neuron output that outlasts the stimulus (Devinney, Huxtable et al. 2013). The mechanism of action is complex, but it is known to involve episodic increases in spinal serotonin, triggered by AIH-induced activation of carotid afferents. Spinal serotonin in turn stimulates new synthesis of brain-derived neurotrophic factor (BDNF) and activation of trkB receptors in spinal motor nuclei, resulting in strengthened synaptic input onto spinal motoneurons (Devinney, Huxtable et al. 2013).

Importantly, AIH has also been shown to promote plasticity after experimental SCI (Lovett-Barr, Satriotomo et al. 2012). Our laboratory has collaborated on a recent report demonstrating that repetitive treatment with AIH facilitates recovery of both respiratory function and forelimb function in rodent models of incomplete cervical SCI. This recovery is accompanied by long-term changes in neurotrophin expression in spinal motoneuron pools (Lovett-Barr, Satriotomo et al. 2012). In particular, we showed that AIH treatment initiated 4 wks after experimental SCI in laboratory rats produces a sustained improvement in skilled forelimb use.

Here we replicate these preclinical findings, in a randomized, blinded, normoxia-controlled study as outlined in the ARRIVE recommendations (Kilkenny, Browne et al. 2010) to show that animals receiving AIH treatment for 7 days at 4 wks post-injury made fewer footslip errors during ladder crossing for up to 4 wks post treatment compared to animals receiving normoxia-treatment. The current study differs from many pre-clinical studies of new therapies for SCI in that the onset of experimental treatment, in this case AIH, is delayed until 4 weeks after the injury, at a time when much spontaneous recovery has already occurred. This allowed us to assess the effect of AIH on residual functional deficits, in part to emulate the clinical situation more closely. This delay in the onset of treatment also revealed the variability in the amount of spontaneous recovery between animal subjects, requiring an extra methodological step in order to appropriately randomize treatment assignments (see methods).

Additionally, we investigated the role of motor training on AIH-induced recovery. In our original study, the assessment of footslip performance required that the rats voluntarily traverse a ladder multiple times for a food reward (Lovett-Barr, Satriotomo et al. 2012). Ladder performance was measured daily during the treatment week, which in itself constituted a form of

motor training. Task-specific training and other forms of exercise are well known to affect recovery after spinal injury (Kanagal and Muir 2009; Krajacic, Weishaupt et al. 2010; Alluin, Karimi-Abdolrezaee et al. 2011; Cote, Azzam et al. 2011; Fouad and Tetzlaff 2012). Therefore, in order to distinguish the effects of AIH from those of motor training, we examined the effect of AIH treatment on SCI rats with or without motor training. We also investigated the effect of AIH treatment on recovery of motor tasks other than ladder locomotion, tasks that required highly skilled use of the forepaw (two different reach-to-grasp tasks). For the ladder task, we hypothesized that rats with no motor training during the week of AIH treatment would not show improved performance, but that rats with concomitant ladder training (task specific) would show improvements in ladder performance. In brief, and consistent with Hayes et al (Hayes, Jayaraman et al. 2014), and others, our results indicate that task-specific training is required for AIH to improve ladder performance in this animal model of cervical SCI.

3.2 Materials and methods

3.2.1 Animals and Housing

Lewis male rats, 225-250g, were obtained from Charles River Laboratories (Quebec) and they were housed 3 rats/cage upon arrival to our facility and allowed to acclimate to the colony room for 5 days prior to handling. Temperature of the room was maintained at 20 °C and lights were on an automated cycle of 12hL: 12hD in a controlled room at the Animal Care Facility, Western College of Veterinary Medicine, University of Saskatchewan. Cages measured 51cm long x 28cm wide and contained wood chip bedding, PVC tubes for hiding and sleeping in, and wood blocks for chewing. Rats were fed rodent chow *ad libitum* until an approximate weight of 320g was reached, at which time they were restricted to 4 pellets/rat/day. Rats had *ad libitum* access to water throughout the study. Prior to initiation of behavioural training, rats were handled gently 10 min/day for approximately 3 days or until deemed comfortable with the handler. Individual animals were handled by the same person for all procedures. All animal procedures were approved by the University of Saskatchewan Committee on Animal Care and Supply and carried out in accordance with standards set out by the Canadian Council on Animal Care.

3.2.2 Experimental Design

Experiments were carried out in cohorts of 12 rats at a time. For the ladder task, each cohort was placed in two experimental groups (Gp): 1. ladder training during AIH / normoxia treatment week (task-specific), 2. No training during AIH / normoxia treatment week. For the other two different reach-to-grasp tasks, animals in Gp 3 were conditioned and assessed in the single-pellet reaching task, Gp 4 in the Montoya staircase task. The experimental timeline is illustrated in Fig 3.1A. At the start of each experiment, prior to SCI surgery, all animals were conditioned for the appropriate task as described below. Once the experiment began, animals in Gp 1 were exposed to the ladder task daily during the treatment week (at 4 wks post-SCI). For the two different reach-to-grasp tasks, animals in Gp 3 and 4 received daily training in either single pellet reaching task or Montoya staircase task during the AIH treatment week at 4 wks post-surgery.

3.2.3 Ladder-Walking Task

The horizontal ladder test was used for behavioral assessment of skilled locomotor ability of the rats. Rats were trained to cross a horizontal runway, the ladder apparatus was 120 cm in length, with 20 cm opaque plexiglass platforms at either end allowing the rats to turn around. The central ladder portion (80 cm in length) consisted of 2 mm diameter wire rungs spaced 2 cm apart. The ladder was positioned above the 45° angled mirror so both lateral and ventral aspects of the rat movements were visible in the digital video camera so movements of each rat were recorded by using a digital video camera.

Ladder conditioning and testing took place at the same time each day for each group of animals. Animals were initially conditioned by placing cage mates (3 rats) together in the apparatus with a food reward (several Cheerios) present on the platforms at either end for approximately 20 min per day. After 1-2 days, the food reward was only presented when animals had traversed the ladder from the opposite platform. After rats were consistently moving across the ladder, they were placed in the apparatus individually, and the food reward was similarly offered after the rat had traversed the length of the ladder. The ladder apparatus was wiped with 70% alcohol after each individual conditioning session. At the end of the 2 wk training period, rats would repeatedly cross the ladder consistently and quickly with very few long pauses or hesitations.

Performance on the ladder-task was assessed at the following time points: pre-surgery, pre-treatment, D 1 – 7 of Treatment (for experimental group #1 only), 1 day following treatment, and 1, 2, 4, and 8wk following treatment. These sessions were recorded at 60 frames/sec (EOS Rebel, T2i EOS 550D Canon). At each collection session, at least 12 complete crossings, without stops or hesitations by the subject, were recorded per rat.

All video analyses were performed by an assessor blinded to the treatment group. For each session, 10 crossings with no stops or hesitations were analyzed. Two non-consecutive complete stepping cycles were examined for each crossing, for a total of 20 samples per paw per animal per session. Paw placement of each paw was categorized as follows (Fig 3.1B): Grasp: contacts rung with both the digits and palmar/plantar surface of the paw; Digits: uses digit(s) only to contact rung, Wrist: rests palmar-carpal or plantar-tarsal area against rung rather than grasping; Error: paw does not bear weight but slips through rungs; Correction: paw is initially placed on a bar but immediately moved to a second bar within the same step. When an Error placement by any paw occurred, no further steps were analyzed until one complete stride cycle had taken place, so as to remove the confound of immediate subsequent paw placements being influenced by the foot slip. Once 20 full stride crossings were analyzed per rat, the occurrence of each of the 4 categories of paw placement was calculated as a percentage of total paw placements for each of the 4 paws. For each of the 4 experimental groups, repeated measures analysis of variance (RM-ANOVA, IBM SPSS Statistics 20 Software) was performed on each of the measures of Grasp, Digits, Wrist and Error steps for each paw, with treatment (AIH or normoxia) and time (pre-sx, pre-tx, post-tx, and 1wk, 2 wk, 4 wk and 8 wk post-tx timepoints) as variables.

3.2.4 Single pellet reaching task

Rats were conditioned to reach for a sugar pellet (45mg; Bioserve Inc., Frenchtown, NJ, USA) through a narrow opening in a reaching box as previously described (Whishaw, Pellis et al. 1993; Kanagal and Muir 2009). In brief, rats were placed individually into the reaching box with dimensions 45cm long x 13 cm wide, and a sugar pellet was placed in a small depression in the shelf positioned just outside the opening. The pellet was immediately replaced when the rat obtained the pellet by reaching through the opening with a forepaw. Each conditioning session

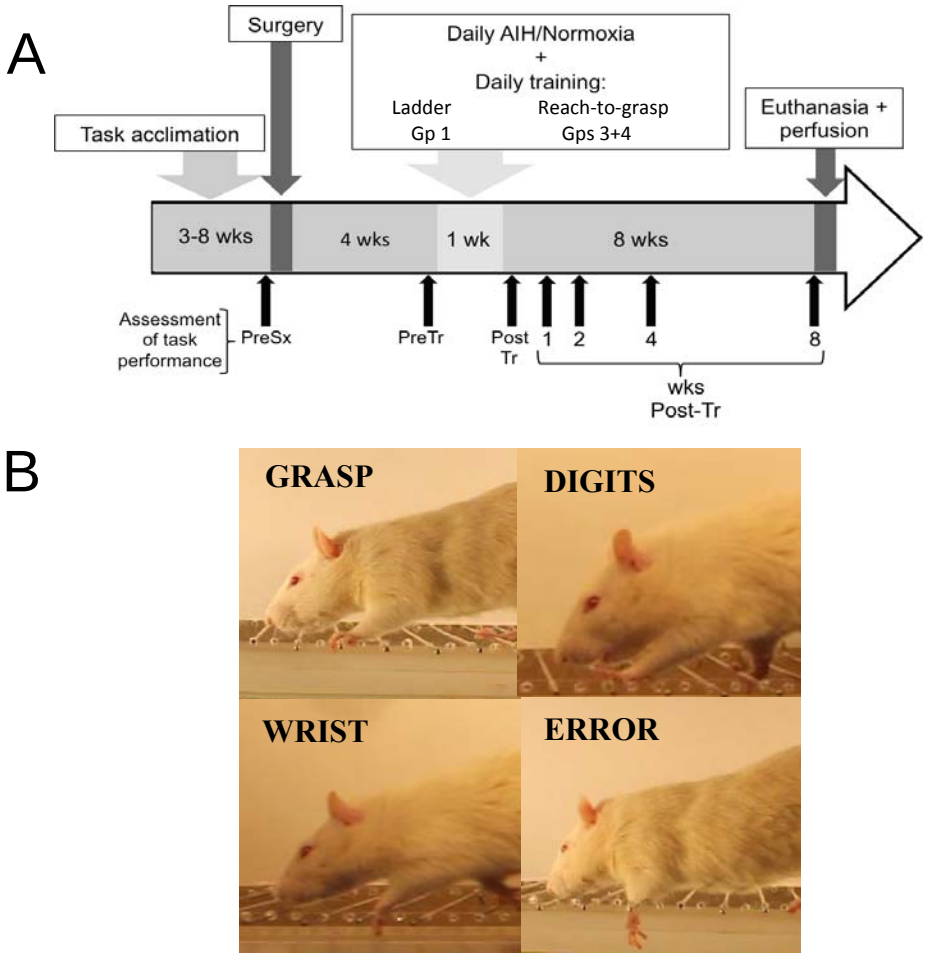

Figure 3.1: Summary of methodology used to investigate the contribution of motor training to AIH-induced recovery in SCI rats. Outcome measure for Groups 1 and 2 was ladder performance, so these animals were initially acclimated on the ladder task, and assessed at 7 different timepoints in the study (**A**). Group 1 was additionally trained /assessed on the ladder each day of the AIH/normoxia treatment week (not shown). Group 2 received no training during treatment week. The outcome measure for Groups 3 and 4 was reaching performance, so these animals were acclimated on the reaching task, and assessed on reaching performance at 7 different timepoints in the study (**A**). Illustration of paw placement classification for ladder performance assessment (**B**). Grasp: rat contacts rung with both the digits and palmar/plantar surface of the paw; Digits: uses digit(s) only to contact rung; Wrist: rests palmar region of the carpal area against rung rather than grasping; Error: slips through rungs without bearing weight on the paw. All assessors were blind to the experimental treatment.

consisted of 20 pellets placed successively on the shelf. When rats were reaching consistently, they were conditioned to retrieve a food reward at the back of the reaching box after each reach before approaching the reaching shelf at the front for the subsequent pellet. This ensured that each reach was independent of the previous attempt. For a reach to be considered successful, rats had to extend their forelimb through the slot, grasp the pellet in their paws, retract the pellet without dragging it along the shelf or dropping it, and then eat it, all in a single attempt. Multiple attempts at a single pellet were considered failed attempts. Rats could use a combination of different forelimb movements and all were considered successful if the above criteria were met. Conditioning was considered complete when each rat could obtain at least 10 of 20 pellets successfully, which required at least 4 – 6 weeks of conditioning at 5 sessions per week.

For each testing session, rats were acclimated by allowing them to reach for 5 pellets and then were digitally videotaped at 60 frames/sec (EOS Rebel, T2i EOS 550D Canon) as they reached for a further 20 pellets. Digital videos were examined by an assessor blinded to the treatment group. For each data timepoint, reaching performance was recorded and assessed over 3 testing sessions on each of 3 consecutive days and an average of the successful reaches over three days was used. This accounted for intra-individual variability in performance between days, as we have described (Kanagal and Muir 2009). Single pellet reaching performance was recorded pre-sx, pre-tx (pre-treatment), post-tx (post-treatment), and 1wk and 4 wk post-tx timepoints. Each post-treatment data timepoint was expressed as % successful reaches (= # successful reaches/20 pellets).

3.2.5 Montoya staircase task

The apparatus consists of a box (30 cm x 10 cm, 11 cm heights) with a staircase positioned along either side of the length of the box. Each staircase consisted of 7 steps, the highest steps positioned closest to the animal's nose. Three sugar pellets were placed on each step, each of which represented an increase in reaching difficulty. Conditioning sessions consisted of placing the animals inside the boxes daily for 15 min/day. The rats used their forelimbs to retrieve the pellets from the stairs. After 15 min, the number of pellets remaining was recorded. Rats were conditioned for 4 – 6 wks until each rat could achieve 70% successful reaches. Testing sessions

were carried out similarly to conditioning sessions and data expressed as the percentage of pellets eaten out of 42 total pellets provided. Animals were tested for the same timepoints as animals in the single-pellet reaching task, and data for each timepoint was expressed as the percentage of pellets eaten out of 42 total pellets provided.

3.2.6 Surgery

Rats were pre-medicated with glycopyrrolate (Sabex Inc., Boucherville, QC, Canada, 0.03mg/kg) to decrease oral secretions. Animals were placed under isoflurane anaesthesia, administered an antibiotic (trimethoprim and sulfadoxine (TMS), Trivetrin, Schering Canada Inc., QC, Canada, 30mg/kg SC) and pre-emptive analgesia (buprenorphine, Buprenex; Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA, USA, 0.05mg/kg SC). The surgical site over the dorsum of the neck was prepared by removing fur with clippers and cleaning the skin with stanhexidine and 70% isopropyl alcohol. Under an operating microscope, the spinal laminae of the 2nd and 3rd cervical vertebrae were exposed using sterile technique, and a laminectomy and durotomy performed to expose the 2nd cervical spinal segment. The dorsolateral funiculus was transected unilaterally on the left side using a modified 25-gauge bevel tipped needle. For rats in Gps 3 and 4, assessed on skilled reaching performance, the spinal lesion was performed on the side ipsilateral to the paw preferentially used for reaching. The muscle and skin were closed with a subcuticular suture technique. The entire time spent under anaesthetic was approximately 20 min. Following surgery, rats were administered 3 ml subcutaneous sterile saline and housed individually in cages equipped with wood chip bedding, a plastic tube and an extraneous heat source. Post-operative analgesia (buprenorphine 0.05mg/kg) and antibiotic (TMS, 30mg/kg) were administered for 48h post-surgery, and longer if necessary. Rats were monitored several times daily for 5 days post-surgery and assessed for change in weight, presence and severity of porphyrin, hydration, healing of the incision site, mobility, and general behavior. At day 3 post-surgery, rats were rehoused with their original cage mates as described earlier (*Animals and housing*).

3.2.7 Intermittent Hypoxia Treatment

3.2.7.1 Assignment of treatment groups

Four weeks post-surgery, rats were assessed for pre-treatment task performance as described earlier (ladder task, skilled reaching tasks). Due to the range in task performance after 4 weeks post-surgery, the following 2 steps were taken to ensure that the functional deficits between the treatment groups (AIH and normoxia) were equivalent. First, those rats that performed at 50% - 100 % of pre-surgery performance (e.g. that made fewer than 50% footslip errors on the ladder, or had less than a 50% decrease in reaching success) at this pretreatment time point were deemed not sufficiently injured and were removed from the study. Our previous experience with the ladder task shows that animals making less than 50% footslip errors at this timepoint continue to spontaneously improve over the subsequent weeks to make fewer and fewer errors over the following weeks. Therefore, only rats making between 50% and 100% footslip errors at 4 wks post-surgery were deemed sufficiently injured to be included in the study. Second, due to the range in task performance amongst those rats which successfully met the inclusion criteria, animals in each cohort were distributed between 2 different treatment groups, initially labelled A or B. The distribution was such that the ranges and average % footslip errors or successful reaches were equal between group A and B. One group in the cohort was then randomly assigned, using a coin toss, to receive AIH treatment and the other assigned to receive normoxia (normoxia) treatment.

3.2.7.2 Treatment

Rats were acclimated to the treatment apparatus by placing them for 30 min into custom-made Plexiglas chambers (1 rat per chamber; 30cm x 17cm x 12cm) under normoxia (21% inspired O_2), 1 d prior to the first treatment day as previously described (Lovett-Barr, Satriotomo et al. 2012). Subsequently, on each day of 7 days treatment, the rats were placed into the Plexiglas chamber and then exposed to AIH, consisting of ten 5 minutes hypoxic episodes (11% inspired O_2), alternating with 5 min normoxic intervals. Alteration in normoxic and hypoxic conditions were established by automatically switching the incoming air between premixed O_2 and N_2 gas (FlO_2 = 0.11) and medical air (FlO_2 = 0.21). Control animals were included in each set of experiments and they were simultaneously placed in adjacent chambers for the same total

duration of time under continuous normoxic conditions ($FIO_2 = 0.21$). The oxygen levels in the chambers were continuously monitored using an oxygen analyzer (AX300-1 Portable Oxygen Analyzer; Teledyne Analytical Instruments).

3.2.8 Histology

Rats were humanely euthanized following the 4wk or 8wk post-treatment time point. Animals were placed under deep isoflurane anaesthetic and trans-cardially perfused with saline and 4% paraformaldehyde. Spinal cords were removed, post-fixed overnight, processed and embedded in paraffin. Spinal cord was serially sectioned transversely at 8 μm throughout the injury site, sections stained with eriochrome cyanine and neutral red. Slides were examined under the light microscope (Zeiss Axioscop, Germany) to determine extent of damage at the lesion epicenter as previously described (Webb and Muir 2003; Webb and Muir 2004).

3.2.9 Statistical Analysis

For each of the 2 experimental groups AIH with and without ladder, repeated measures analysis of variance (RM-ANOVA, IBM SPSS Statistics 20 Software) was performed on footslip errors with treatment (AIH or normoxia) and time (pre-sx, pre-tx, post-tx, and 1wk, 2 wk, 4 wk and 8 wk post-tx timepoints) as variables. Bonferroni correction was used to compare measures at different time points. For each of the 2 reaching experimental groups, RM-ANOVA was carried out on the % successful reaches, with treatment (AIH or normoxia) and time (post-tx, and 1wk and 4 wk post-tx timepoints) as variables. All results are shown as mean $\pm$ SEM.

3.3 Results

In the first several days after surgery, most SCI rats had a detectable abnormality with the left forelimb when moving around their cages or on a flat surface. Signs included curled digits when non-weightbearing or weightbearing and moderate paralysis of the left forelimb segments. Some animals showed mild to moderate paralysis of the left hindlimb immediately post-surgery. After 4 wks post-surgery, however, animals did not show visible limb deficits except when tested on different motor tasks. For rats assessed on the ladder task, the only consistent change in limb usage was the increase in the number of footslip errors made by the left forelimb. For ladder Gp

2 (AIH without Ladder Training), there was no difference between treatment groups for the left forelimb performance. Histology revealed that all animals used in this study sustained damage to the left dorsolateral funiculus at the second cervical segment.

3.3.1 Experimental Group 1: AIH with Ladder Training

The result of RM-ANOVA indicate that there was a significant effect of AIH+ ladder training and time ($p < 0.05$). Result of RM-ANOVA revealed that rats treated with 7 days of AIH and daily ladder training initiated at 4 wks post-SCI made significant fewer footslip errors with the left forelimb after the treatment week when compared to control, normoxia-treated animals {F (1, 20) = 8.692, $p < 0.05$}, (Fig 3.2A). During the treatment week, AIH rats made fewer left forelimb errors than normoxia-control rats beginning at 4 days of treatment and this did not change throughout the week (Fig 3.2B). After treatment, AIH rats continued to make fewer footslips than control animals up to 4 wks post-treatment. At 8 wk post-tx, the range of footslip errors was large in the normoxia-treated group and there were no differences between AIH rats and normoxia rats. Analysis of the movements of the left forelimb in AIH-treated animals showed that there were more grasp steps, i.e. more normal steps compared with that of normoxia-treated controls following treatment {F (1, 20) = 9.123, $p < 0.05$, AIH n = 12; normoxia n = 10} (Fig 3.3).

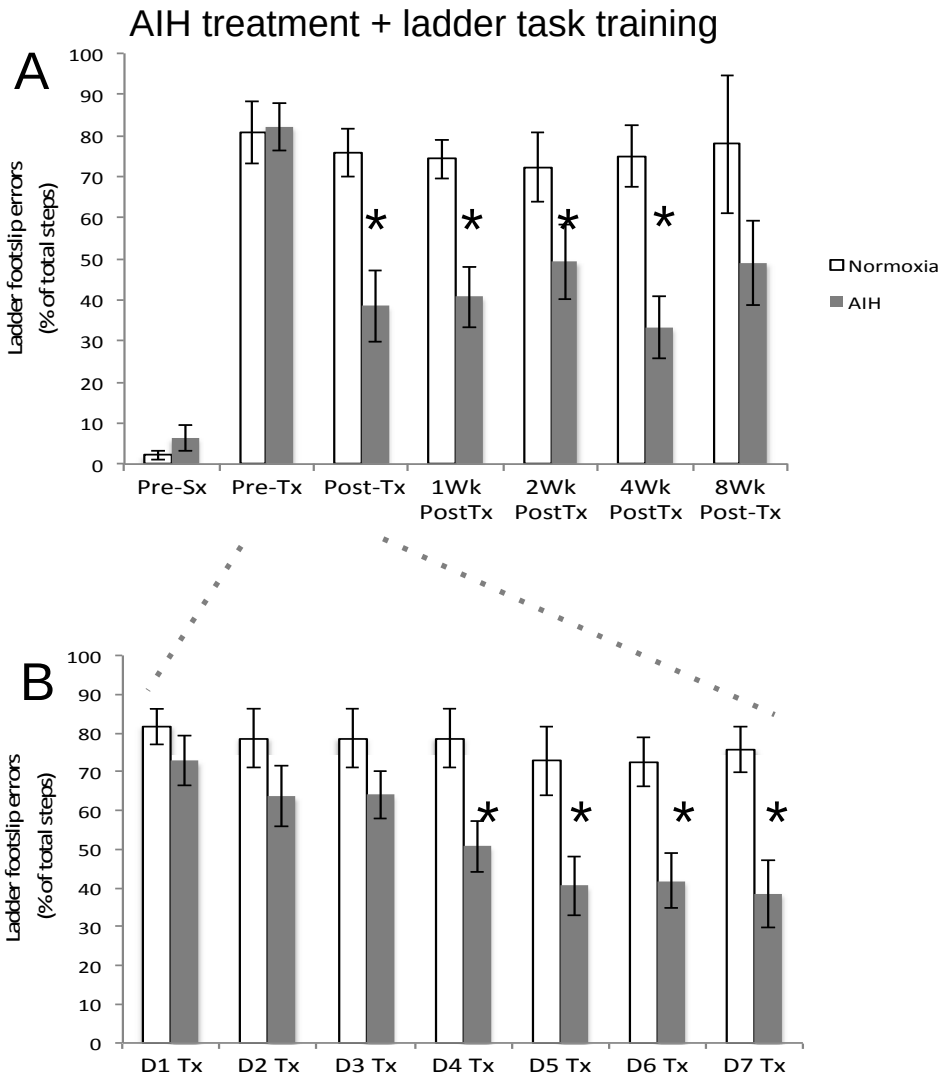

Figure 3.2: Delayed treatment with AIH improves skilled ladder locomotion in rats with incomplete cervical SCI. Rats receiving daily AIH treatment and concomitant ladder training for 7 days made fewer footslip errors on a ladder-walking task for up to 4 wks post-treatment compared to rats receiving normoxia (control) treatment (**A**). During the treatment week, AIH-treated rats made fewer footslip errors than normoxia-treated rats beginning at 4 days of treatment (**B**). Pre-sx: 1 day before surgery; Pre-tx: 4 wks after surgery and 1 day before the first treatment day; D = day of treatment; Post-tx: 1 day after the last treatment day; Wk Post-tx: number of weeks after last treatment day; * $p < 0.05$ difference between treatment groups, AIH n = 12; normoxia n = 10 (except at 8 Wk Post-tx: AIH n= 8, normoxia n = 7)

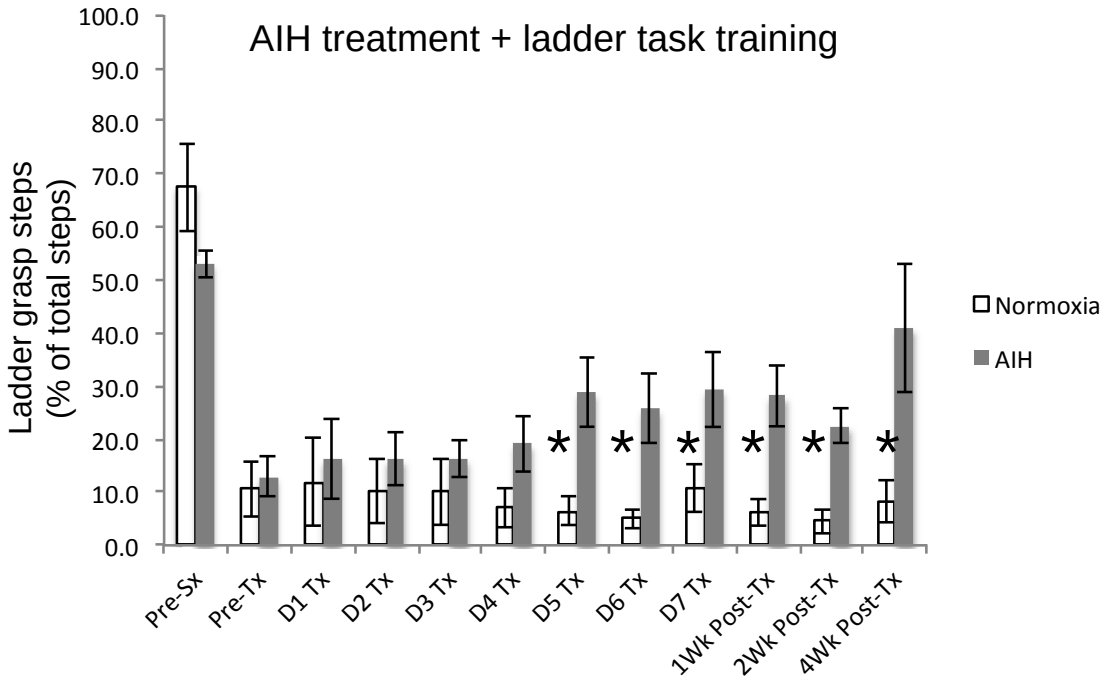

Figure 3.3: Rats treated with AIH and ladder training use more normal steps to cross the ladder compared to normoxia-treated controls. Rats receiving daily AIH treatment and concomitant ladder training for 7 days made more grasp steps on a ladder-walking task compared to rats receiving normoxia treatment. These differences first appeared during the treatment week and continued for up to 4 wks post-treatment. Pre-sx: 1 day before surgery; Pre-tx: 4 wks after surgery and 1 day before the first treatment day; D = day of treatment; Wk Post-tx: number of weeks after last treatment day; * $p < 0.05$ difference between treatment groups, AIH n = 12; normoxia n = 10.

3.3.2 Experimental Group 2: AIH without Ladder Training

Rats treated with 7 days of AIH without concomitant daily ladder training initiated at 4 wk post-SCI made the same number of footslip errors with the left forelimb after the treatment week as normoxia-treated animals (Fig 3.4). There were no significant differences in errors made by the left forelimb (the injured limb) between AIH and normoxia-treated animals at any timepoint {$F(1, 19) = 0.677$, $p > 0.05$, AIH n = 10; normoxia n = 11}.

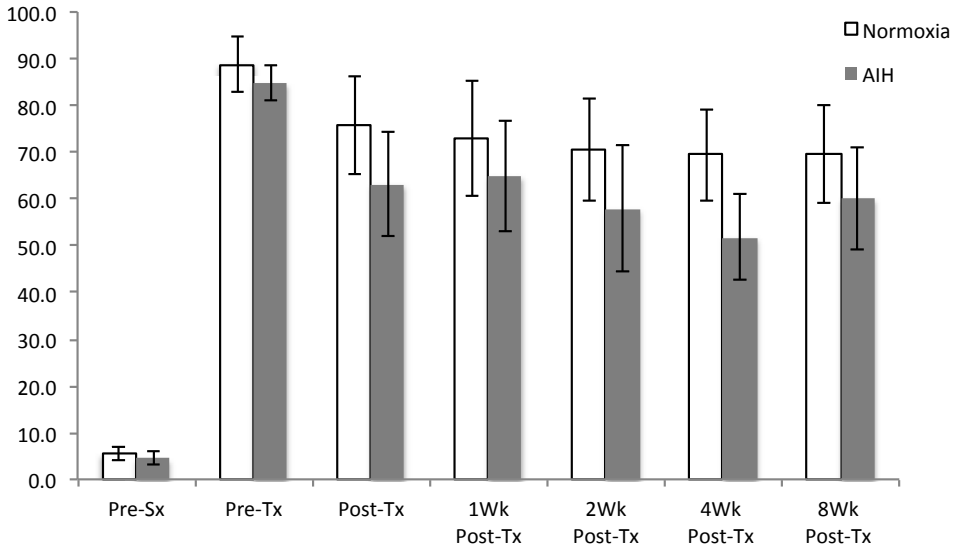

Figure 3.4: Delayed treatment with AIH without concomitant motor training does not improve skilled ladder locomotion in rats with incomplete cervical SCI. Rats receiving daily AIH treatment for 7 days without any form of daily motor training made the same number of footslip errors on a ladder-walking task compared to rats receiving normoxia (control) treatment. There were no differences in footslip errors between AIH and normoxia-treated animals at any timepoint. Pre-sx: 1 day before surgery; Pre-tx: 4 wks after surgery and 1 day before the first treatment day; Post-tx: 1 day after the last treatment day; Wk Post-tx: number of weeks after last treatment day ($p > 0.05$; AIH n = 10; normoxia n = 11).

3.3.3 Skilled reach-to-grasp tasks

Rats treated with daily AIH (7 days) plus daily task training at 4 wks post-SCI in either the single pellet reaching task (Figure 3.5A) or the Montoya staircase task (Figure 3.5B) did not show improved reach-to-grasp performance versus normoxia-treated, task-trained animals. Reaching success was not significantly different between AIH-treated and normoxia-treated rats at any time point. During the single-pellet reaching task (Figure 3.5A), rats used several different movement strategies, including shovelling or scooping movments, to successfully retrieve the food reward (see Methods). AIH-treated rats showed some improvement in reaching success versus normoxia-treated rats, but this did not reach statistical significance {F (1, 18) = 2.911, p > 0.05, n = 10/gp}. In contrast, the Montoya staircase task is designed to allow only one series of forelimb movements to retrieve the food reward and thus is a more demanding reaching task. AIH had no effect on reaching success in this task {F (1, 22) = 0.677, p > 0.05, n=12/gp}.

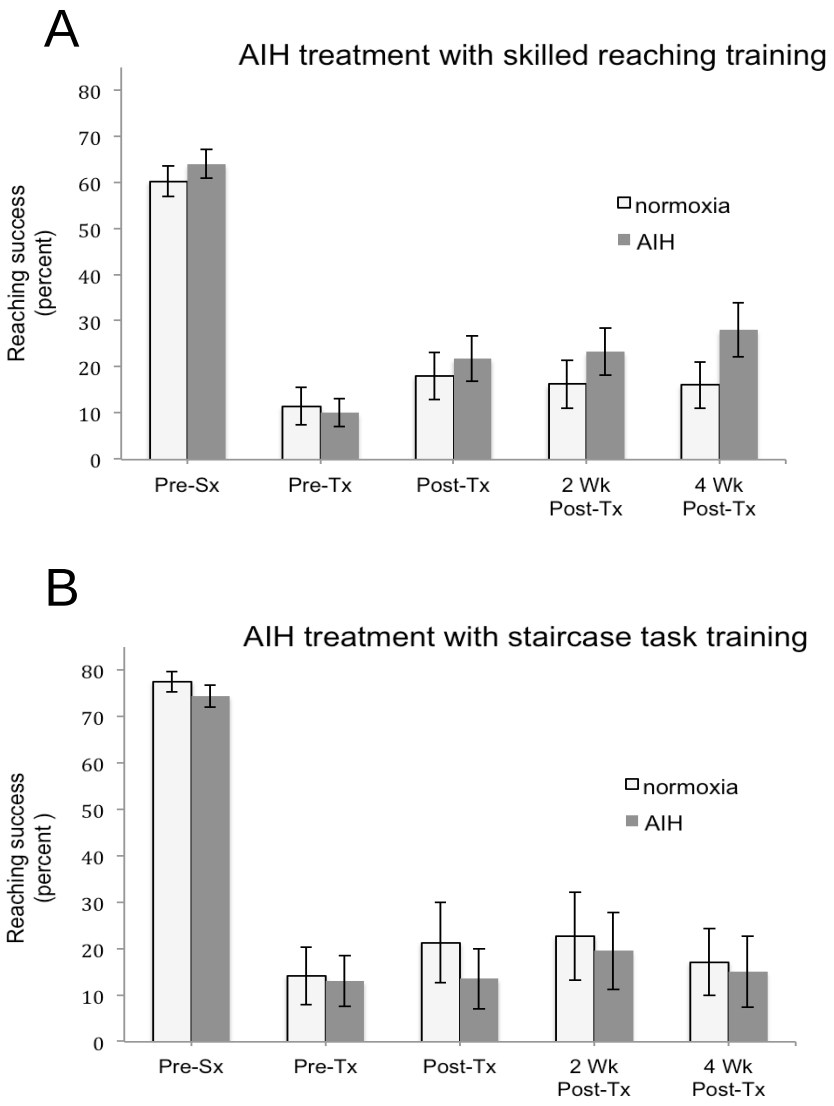

Figure 3.5: Delayed AIH treatment does not improve reach-to-grasp success in SCI rats receiving task specific motor training. (**A**) On the single-pellet reaching task, rats treated with AIH and daily reaching training showed a trend toward increased reaching success after the treatment week, but were not statistically different from normoxia-treated rats (p=0.10, n=10/gp). (**B**) On the staircase task, rats treated with AIH and daily staircase task training did not show improved reaching success, and there were no differences between AIH- and normoxia-treated rats (n=12/gp). Post-Tx: 1 day after the last treatment day; Wk Post-Tx: number of weeks after last treatment day.

3.4 Discussion

In summary, our results indicate that 7 days of AIH treatment initiated 4 wk after incomplete cervical spinal injury will improve footslip performance on a ladder task in SCI rats when combined with daily ladder task training. This improvement in performance was retained up to 4 wks after the end of treatment but was not present at 8 wks post-treatment. Rats that received AIH treatment without task training during the treatment week did not improve their footslip performance over that of normoxia-treated animals. One week of delayed AIH treatment also does not improve skilled reach-to-grasp performance in cervical SCI rats. Therefore, AIH treatment facilitates recovery of footslip performance on the ladder task only when treatment is accompanied by specific ladder-task training.

3.4.1 Daily AIH – possible mechanism of action

Our finding that ladder performance improves with daily AIH and daily ladder training in SCI rats is consistent with earlier findings that AIH improves both ladder performance and breathing in rats with cervical SCI (Lovett-Barr, Satriotomo et al. 2012). As described in the methods, the current experimental design included randomized treatment assignment and blinded assessments to strengthen the reliability of our findings. These results are also consistent with the recent report of AIH-induced improvements in human subjects with incomplete SCI (Hayes, Jayaraman et al. 2014). In a placebo-controlled, randomized double-blind study, treatment with AIH improved walking speed in SCI subjects. When combined with locomotor training, AIH also improved walking distance for up to one week post treatment, the latest time point reported. Importantly, the treatment paradigm in this clinical study differed only slightly from that our current study. Human SCI subjects received 5 days of AIH treatment, consisting of breathing 9% O_2 for 90s alternating with 21% O_2 for 60s, repeated 15 times. The paradigm in the current study, modelled after earlier animal studies, was 7 days of 11% O_2 for 5 min alternating with 5 min 21% O_2, repeated 10 times. Clinical locomotor training was carried out 1 hour after the end of AIH treatment, as was the ladder task training during the present study. The comparable results between the clinical and pre-clinical studies, in light of the shared methodology, suggest that similar mechanisms may be responsible for AIH-induced recovery in both situations.

Although the mechanisms for AIH-induced locomotor enhancements are not known, there is strong evidence that AIH-induced recovery of breathing after SCI is a result of enhancement of synaptic input onto spinal motoneurons (Devinney, Huxtable et al. 2013). It is well established in rats that AIH triggers episodic spinal serotonin release, resulting in increased expression of BDNF and phosphorylated trkB in spinal motor nuclei (Devinney, Huxtable et al. 2013). The activation of trkB elicits downstream intracellular cascades eventually leading to enhanced synaptic inputs on spinal motoneurons. Important for the present study, serotonin, BDNF and trkB show increased expression in motor nuclei throughout the spinal cord after AIH treatment (Lovett-Barr, Satriotomo et al. 2012; Satriotomo, Dale et al. 2012), suggesting that similar mechanisms could be involved in AIH-induced recovery of both respiratory and forelimb function. In addition, exposure to the specific regime of AIH used in the present study (5 min 11% O_2: 5 min 20% O_2) results in improved oxidative phosphorylation in rat liver, cardiac and skeletal muscle tissue, a change which lasts up to 3 months after treatment (Serebrovskaya, Nosar et al. 2013). Although not yet investigated, it is possible that similar effects occur in neural tissue, forming part of the underlying mechanisms for AIH-induced recovery.

3.4.2 Delayed treatment compared to immediate treatment

As described earlier, treatment with AIH did not commence until 4 wks after surgery, at a time point when much spontaneous recovery had already occurred in this rodent model of incomplete SCI. Essentially, this allowed us to examine the effect of AIH on residual deficits after recovery from SCI, a situation that differs from many experimental pre-clinical studies and is arguably more relevant to the clinical condition. For any particular lesion model, the amount of recovery and the time required for spontaneous recovery to occur will depend upon a number of factors, including the severity and type of lesion, rat strain, housing conditions and of course the particular outcome measures assessed. In our experience with the lesion model in this study, most spontaneous recovery has occurred by 4 wks post-injury, although recovery is not 100% complete. In particular, we know that animals making fewer than 50% footslip errors on the ladder at 4 wk post-surgery will continue to improve over the following weeks without any experimental intervention, whereas animals making more than 50% footslip errors do not improve consistently. Since we were interested whether AIH could facilitate recovery in subjects

with residual deficits, we focused on those animals which were less likely to recover spontaneously, i.e. those which met an inclusion criteria of >50% footslip errors at 4 wks post-injury. We also applied this inclusion criteria to the skilled reach-to-grasp motor tasks, in that only animals with a reaching performance at 4 wks post-injury of less than 50% compared to pre-surgery would be included in the experimental groups. Our decision to use inclusion criteria based on functional abilities, rather than anatomical lesion size, is strengthened by recent findings that behavioural recovery is not correlated with lesion size for incomplete cervical lesions in rats (Fouad, Hurd et al. 2013; Hurd, Weishaupt et al. 2013). In future studies, more can be done to examine the effect of treatment on sets of animals with more closely matched abilities, to determine whether AIH facilitates recovery better in animals with more, or less, severe deficits.

One major consequence of the delay in treatment onset was a variation in the amount of spontaneous recovery between individual animals that had received the same spinal lesion 4 wk earlier. Each cohort of animals had a range of functional abilities at the start of treatment, and this variability needed to be managed in order to effectively compare AIH and control treatments. The first step was, as described above, to include only those animals that, from our best information, would retain residual functional deficits. Even within this set of animals with greater than 50% errors, random assignment of individual animals to either AIH or control treatment would not necessarily result in a pair of treatment groups with equivalent abilities. Instead, for each experimental cohort, we first assigned animals to one of two groups based on their motor performance so that the groups were matched as closely as possible for the mean and range of errors. AIH or control treatment was then randomly assigned to the group, satisfying our requirements for random assignment of treatment while still maintaining valid comparisons of treatment effects within each cohort.

3.4.3 Task-specific exercise facilitates AIH-induced recovery of ladder performance

The results from experiments 1 and 2 and from earlier findings strongly suggest that concomitant training in the ladder task is necessary for AIH-induced recovery of ladder performance in SCI rats (Figs 3.2, 3.3). Footslip errors were reduced only when animals received both AIH

treatment and ladder training. We considered our method of recording ladder performance in SCI rats as a form of ladder task training largely because of some key characteristics of our ladder task methodology, which differ from the methods used in most other studies of footslip errors on a horizontal ladder or grid. For a detailed description, see (Metz and Whishaw 2009). In the present study, animals were conditioned to repeatedly cross the ladder voluntarily while recording occurred and as such, were not repeatedly handled during the recording sessions as is necessary with unconditioned animals. In the present study, animals would cross the ladder at least 10 – 12 times each recording/training session, and the data were averaged from at least 20 steps per session. Finally, the ladder used in the present study had regularly placed rungs rather than having irregularly spaced rungs (Metz and Whishaw 2009), as pilot studies in our lab showed the same number of footslip errors and forepaw grasping postures made by either uninjured rats or SCI rats regardless of the rung spacing. Rats in the present study voluntarily crossed the ladder many times during each recording session, which effectively also comprised the training sessions. Similarly, task training in the form of overground walking combined with AIH treatment also improved walking distance more than did AIH alone in human SCI subjects (Hayes, Jayaraman et al. 2014), although, unlike in the present study, AIH alone also improved walking speed in human SCI subjects.

3.4.4 AIH for 1 wk does not improve reach-to-grasp

The fact that AIH did not significantly improve skilled reach-to-grasp performance indicates as much about the nature of these tasks as it does the robustness of AIH effects. Reach-to-grasp movements with the forepaws require specific brainstem-spinal pathways, including the rubrospinal axons, which are directly affected by the dorsolateral funicular lesion used here. In contrast, ladder locomotion involves some grasping but also more general limb movements, subserved by pathways more widely distributed in spinal white matter. It is therefore, not surprising that reach-to-grasp tasks are affected more severely after SCI than is ladder locomotion. During recovery of ladder locomotion, rats would be able to access multiple neural pathways to achieve appropriate paw placements, and plasticity in these pathways, possibly facilitated by AIH, contributed to fewer errors.

Reach-to-grasp movements require plasticity in a more limited number of pathways and thus recover less well than ladder locomotion after dorsolateral cervical SCI. Nevertheless, we can see parallel comparisons between the two different reach-to-grasp tasks, in that more recovery occurs in the task that allows for more behavioral plasticity, the single pellet task, compared to the more restrictive Montoya staircase task (Fig 3.5). The single pellet task allows the animal to use different combinations of forelimb segment movements to accomplish a 'successful reach'. Whereas uninjured animals tend to use a similar series of movements to obtain the food pellet, motor deficits in SCI rats prompts them to compensate with a range of movement combinations. This behavioural plasticity presumably reflects underlying neural plasticity. Although our results are suggestive that AIH might enhance this plasticity, reaching success in AIH-treated rats did not differ from normoxia-treated rats at 4 wk post-tx (Figure 3.5A). Longer treatments with AIH might allow AIH-induced plasticity to improve performance in the single-pellet reaching task. In contrast, the Montoya staircase task is a less permissive task. The design of the apparatus restricts the action of the limbs to a particular series of movements in order to obtain a sugar pellet. Less behavioural plasticity is allowed and thus recovery on this task requires plasticity in a limited number of neural pathways.

3.5 Sources of potential systematic error

There is a growing acknowledgement in the field of preclinical biomedical research that many animal studies are not carried out with sufficient methodological rigour or at least with the reporting of such standards (Kilkenny, Browne et al. 2010; Landis, Amara et al. 2012). The lack of methodological standards, required in clinical research, has undoubtedly contributed to the lack of reproducibility for many pre-clinical animals' studies in the spinal cord injury field, one of several biomedical areas that have been investigated (Steward, Popovich et al. 2012). We have addressed these issues in the present study by employing and reporting recommended methods such as randomization of treatment assignment, use of placebo or normoxia treatments, and blinded assessments, as described in Methods. Nevertheless, there are still issues of potential error or systematic bias that might affect our findings. For example, animals were pre-selected for inclusion in each of the experiments based on their functional abilities at 4 wk post-SCI. Although we felt this was a justified inclusion criteria because our previous experience

demonstrated that animals with smaller deficits would continue to recover, there was still a range of individual functional abilities even within the groups of animals that met the inclusion criteria.

In summary, we have found that delayed treatment with AIH and task-specific training facilitates recovery on a ladder task in rats with cervical SCI. These findings, in conjunction with the successful use of AIH in a recent clinical study, point to the potential of AIH as an effective treatment to augment plasticity and improve functional recovery after SCI.

CHAPTER 4

Acute intermittent hypoxia alters plasticity-related and hypoxia-related proteins expression in a rat model of cervical spinal injury

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Author Contributions

All the experiments were conceived and designed by myself, Dr. Gillian Muir, Dr. Valerie Verge and Dr. Gordon Mitchell. I have performed all the experiments, collected and analysed the data presented in this manuscript. I have generated all the figures and wrote the first draft of the manuscript which was revised by Dr. Gillian Muir and Dr. Valerie Verge.

Transition Statement

This chapter focusses on the second specific aims in this thesis, to address the hypothesis that AIH alters the expression of plasticity- and hypoxia-related proteins in a rat model of cervical spinal injury.

4.1 Introduction

Spinal cord injury damages axonal pathways, interrupts synaptic transmission between the brain and spinal cord and subsequently alters the motor, sensory and autonomic functions below the level of injury. Most SCIs are incomplete and leave some uninjured synaptic axonal pathways. The sparing of undamaged pathways contributes to spontaneous recovery of some limb and respiratory function following SCI although this spontaneous recovery of function can be frustratingly slow and inadequate to restore normal function following SCI.

A variety of approaches have been used to enhance functional recovery in animal models of SCI, including methods to enhance plasticity in spared synaptic neural pathways (Dale, Ben Mabrouk et al. 2014). Plasticity is a fundamental property of the CNS, by which the nervous system fine tunes and rearranges synaptic connectivity in response to both changes in synaptic activity, injury or both.

Intermittent hypoxia (IH), is well known to induce plasticity in multiple physiological systems (Navarrete-Opazo and Mitchell 2014). Intermittent hypoxia is the exposure of persons or animals to periods of low oxygen levels. IH protocols vary greatly in terms of O_2 % in inspired air, number of episodes, and duration of episodes, but can generally be divided into chronic IH (CIH) and acute IH (AIH) protocols (Ling, Fuller et al. 2001; Dale-Nagle, Hoffman et al. 2011; Devinney, Huxtable et al. 2013). Chronic IH protocols are associated with both detrimental and beneficial effects in multiple physiological systems whereas AIH protocols are reported to elicit beneficial effects without showing detrimental effects (Navarrete-Opazo and Mitchell 2014). In particular, AIH has been shown to induce spinal plasticity in respiratory and non-respiratory motor systems and to improve functional recovery following SCI. AIH-induced plasticity in respiratory motor systems has been well-documented and thoroughly studied (Baker-Herman, Fuller et al. 2004; Golder and Mitchell 2005; Wilkerson and Mitchell 2009; Dale, Ben Mabrouk et al. 2014). The impact of AIH on non-respiratory motor systems has been recently reported in persons with chronic SCI and in animals with experimental SCI. A single AIH exposure increases ankle strength in patients with incomplete chronic spinal cord injuries, and daily exposure to AIH (dAIH) for 5 days has shown that dAIH enhances walking speed and endurance

in persons with chronic incomplete spinal cord injuries (Trumbower, Jayaraman et al. 2012) (Hayes, Jayaraman et al. 2014). Work from our lab has shown that delayed exposure to dAIH in rats with incomplete cervical spinal injuries will improve ladder performance when combined with ladder training (Lovett-Barr, Satriotomo et al. 2012); Chapter 3 of this thesis).

The mechanism associated with AIH-induced facilitation of non-respiratory motor function in SCI animals has not been previously investigated. More information is known about AIH-induced changes in respiratory motor systems. AIH induces a form of respiratory motor plasticity in intact animals known as long-term facilitation (LTF). LTF is a progressive increase in respiratory motor output that lasts for at least one hour, and may serve physiologically as a compensatory mechanism to stabilize the respiratory motor output following AIH (Fuller, Johnson et al. 2003; Golder and Mitchell 2005; Devinney, Huxtable et al. 2013; Dale, Ben Mabrouk et al. 2014). Multiple convergent intracellular pathways are able to induce LTF in spinal motoneurons, which involve signalling through serotonin-PKC-BDNF-trkB-pERK; adenosine-PKA-pAkt; VEGF-pAkt pathways, among others (for review, see (Dale, Ben Mabrouk et al. 2014).

Although it is not known whether the same pathways underlying LTF are involved in motor plasticity after AIH treatment in spinal-injured animals, the expression of several proteins associated with these pathways are increased after AIH treatment in rats. After repetitive acute intermittent hypoxia (rAIH) (10 weeks of 3 x weekly AIH consisting of 10, 5-min episodes of 10.5% inspired O_2), $5HT_{2A}$, BDNF and its high affinity receptor trkB among other proteins, are increased in spinal motoneurons (Satriotomo, Dale et al. 2012). Similarly, BDNF and trkB expression are increased after dAIH, (7 days of 10, 5-min episodes of 10.5% inspired O_2) in spinal motor neurons at C4 and C7 spinal segments.

I was interested to determine whether the protocol of training and AIH, which facilitated motor recovery in SCI rats, described in Chapter 3 of this thesis, also induced changes in expression of plasticity- and hypoxia- related proteins in the spinal cord of SCI rats. In order to compare findings with published studies, I chose to examine changes in expression of BDNF, and its high affinity receptor trkB, the phosphorylated form of trkB, and VEGF. I also chose to examine

changes in the expression levels of hypoxia-induced protein HIF-1α. HIF-1α is a transcription factor which is activated in response to low oxygen and regulates the expression of many target genes, including VEGF, to maintain oxygen homeostasis (Calvani, Comito et al. 2012). In addition, I examined the expression of cell specific markers for astrocytes and microglia (GFAP and ED-1, respectively) to assess the response of these non-neuronal cells to AIH and motor training in SCI rats.

The protocol used in Chapter 3 showed improvements in ladder performance beginning at 4 days of AIH + motor training. In the current study, I was interested to determine whether changes in expression of proteins were evident early versus late in the treatment week. For that reason, the current study examines protein expression after only one day of AIH + training and compares this to protein expression after 7 days of AIH + training. Furthermore, to assess whether the treatment affects motoneurons in the cervical spinal cord only (the only location examined in published studies) or at different segmental levels, I examined protein expression in cervical motoneurons as well as lumbar motoneurons. In short, the current study shows that hypoxia- and plasticity-related proteins are differentially expressed both temporally and spatially within the spinal cord after dAIH + motor training in a rat model of incomplete cervical SCI.

4.2 Materials and Methods

4.2.1 Animals and Housing

Animal were housed as described in Section 3.2.1. A total of 12 animals were used in this study. After ladder training and testing, SCI surgery, animals were randomly assigned to receive either AIH or normoxia treatment (n = 6 per treatment group), and within each treatment group, animals were randomly assigned to receive either 1 day or 7 days of AIH + ladder training (n = 6 per group). The experimental groups were therefore 1 day normoxia; 7 day normoxia; 1 day AIH; or 7 day AIH (n = 3 per experimental group). The experimental timeline is shown in Figure 4.1.

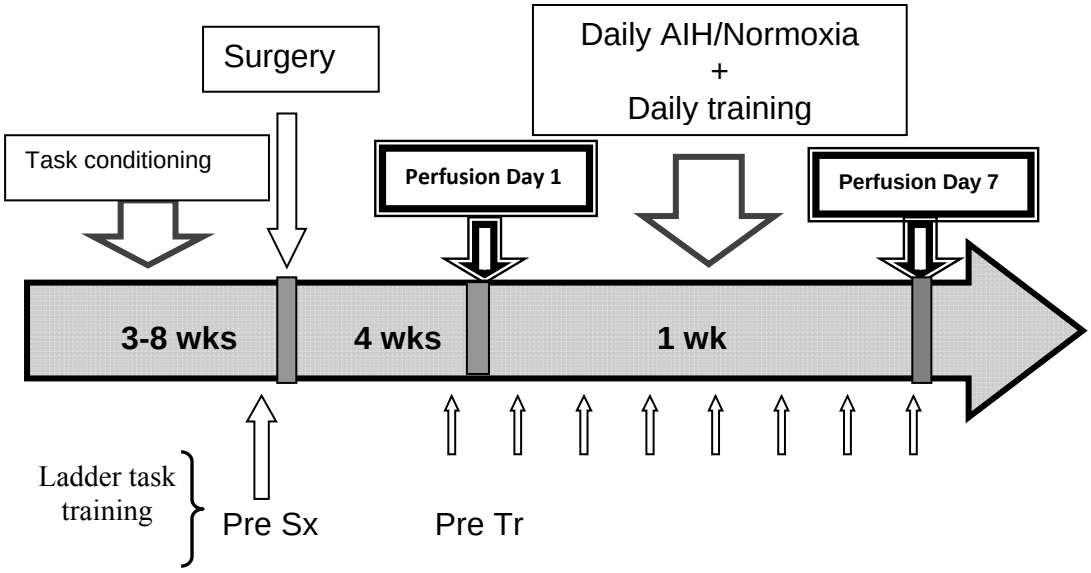

Figure 4.1: Summary of methodology used to investigate acute intermittent hypoxia-induced plasticity and hypoxia-related protein expression. All animals were initially conditioned on the ladder task, and were trained on the ladder task before spinal surgery (Pre Sx), 4 weeks after surgery (Pre-Tr), and every day of the 7 day AIH treatment. Spinal cord tissue was taken from a subset of animals (n = 6) euthanized and perfused after 1 day of treatment and training (Perfusion Day 1), and from the remaining animals (n = 6) after 7 days of treatment and training (Perfusion Day 7).

4.2.2 Ladder-Walking Task

Ladder acclimation, training, recording and analysis was carried out as described in Section 3.2.3.

4.2.3 Surgery

Surgery was carried out as described in Section 3.2.6.

4.2.4 Intermittent Hypoxia Treatment

Intermittent hypoxia treatment was carried out as described in Section 3.2.7.

4.2.5 Tissue Preparation

At day 1 and day 7 of AIH+training treatment, animals in the appropriate group were deeply anaesthetized with isoflurane and perfused by trans-cardial perfusion with heparinized phosphate-buffered saline followed by 4% paraformaldehyde. The vertebral column containing the spinal cord was removed and post-fixed overnight in 4% paraformaldehyde. The spinal cord was extracted from vertebral column and segments C6-C7 (containing motoneurons innervating the forelimb) and segments L4-L5 (containing motoneurons innervating the hindlimb) were postfixed for 1-1.5h in 4% paraformaldehyde and cryoprotected in 10% sucrose followed by 20% sucrose overnight at 4ºC. Subsequently, segments of spinal cord from AIH and control (normoxia) groups were embedded in a cryomold covered in OCT compound (Tissue Tek; Miles Laboratories, Elkhart, IN), then carefully frozen by immersing the mold in which they were frozen in isopentane cooled in a slurry of dry ice and acetone. The individual pieces of frozen spinal cord were subsequently released from the mold and annealed together with OCT compound, creating a larger block containing 8 pieces of spinal cord, representing each of the 4 experimental conditions (1 day normoxia; 7 day normoxia; 1 day AIH;7 day AIH) from 2 spinal cord regions (C6-7 and L4-5). With all 12 animals, this resulted in 3 blocks, each containing spinal cords from 4 experimental conditions x 2 spinal regions. Blocks were then stored at -80°C until sectioning. Tissues were sectioned at 10μm thickness with a Microm cryostat and thaw-mounted onto slides (VWR Superfrost Plus) and stored at -80°C until further processing for immunofluorescence. Thus, each slide contained eight sections. Each section represented each

experimental group**,** so that sections from the normoxia and AIH-treated (day 1 and day 7) animals, sampled from each of the forelimb and the hindlimb, were mounted on the same slides and processed under identical conditions to minimize processing variations.

4.2.6 Immunofluorescence

Slides were removed from -80°C refrigerator and allowed to air dry and reach room temperature for 30 minutes, then washed in 0.1M PBS, pH 7.4, for 30 minutes. PBS was changed every 10 minutes and excess liquid was removed from sections by suction. Prior to incubation with primary antibodies for BDNF or trkB, citrate antigen retrieval was performed. Briefly, slides were placed in 0.01M citrate buffer (10% 0.1M sodium citrate buffer in ddH2O, pH 6) at 50°C and then warmed to 90°C over 45min. Slides were then allowed to cool for 20min.

All slides were then incubated with blocking solution containing Sea Block buffer (Abcam) in primary diluent (0.1% Triton X-100 in 0.1M PBS) for 1hr at room temperature. Primary antibodies used in this study were diluted with 10 % Sea Block in primary diluent to the following concentrations: mouse anti-HIF-1α (NB 100-105, Novus Biological) 1:200, rabbit anti-VEGF (A-20, sc-152, Santa Cruz Biotech, Inc) 1:200, chicken anti-BDNF (Promega) 1:200, rabbit anti-trkB (794, sc-12, Santa Cruz Biotech, Inc) 1:100, rabbit anti ptrkB (phosphor Y705, abcam) 1:400, rabbit anti-GFAP (Dako Canada, Inc) 1:800, mouse anti-ED-1 (Cedarlane) 1:300. After 24hr incubation at 4°C in a humidified chamber, slides were washed for 30min in 0.1M PBS. Slides exposed to the various primary antibodies were then incubated with the appropriate secondary fluorescent antibodies. The following secondary antibodies were used in this study: goat-anti-rabbit Alexa Fluor 488, donkey-anti-mouse Alexa Fluor 488, DyLight 594 donkey-anti-mouse (Jackson Immuno Research Laboratories, Inc) at 1:2000, Cy3 Donkey-anti-rabbit (Jackson Immuno Research Laboratories, Inc) at 1:4000, goat-anti-chicken (F-1005, aves labs, Inc) 1:1000 diluted in 0.1M PBS. After 1hr incubation in the dark at room temperature, slides were washed for 30min in 0.1M PBS, and then coverslipped using ProLong Gold Antifade Reagent (P36931, Molecular Probes, Invitrogen).

4.2.7 Image Analysis / Quantification

Immunofluorescence-processed sections were examined using a Zeiss Axioskop with appropriate filters and photographs of all spinal cord regions of interest of sections mounted on the same slide were taken with the 20x objective under identical conditions. Six slides (two slides/animal) from each experimental group (representing different animals per group), were then used for quantification. Image analysis was performed in a blinded fashion in the region of interest (i.e. ventral grey matter) in all photographs of both C6-C7 and L4-L5 levels of spinal cords. To examine the degree of astrocyte reactivity or macrophage/microglia activation, GFAP and ED1 images respectively were analyzed using Image J software: 4 boxes (100μm X 100μm) each were placed at approximately equal intervals over ventral horn regions of spinal cord of each section and the mean gray value was obtained for each box. To examine expression of HIF-1α, VEGF, BDNF, trkB and ptrkB in motoneurons, images were analyzed using Northern eclipse software. Motor neurons in ventral grey matter on both sides of the spinal cord were circumscribed and the mean gray value for the marker being examined was obtained for each motor neuron. Background mean gray background values were determined and subtracted from the mean gray value for each motoneuron analyzed in that spinal cord section and then the net mean gray value determined for that grouping of motoneurons. For the 3 animals in each of 4 experimental groups (1 day normoxia; 7 days normoxia; 1 day AIH; 7 day AIH) mean gray values were averaged to obtain mean gray value for each group.

4.2.8 Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics v20 for windows Software. Differences between the mean gray values for each experimental group (1 day normoxia; 7 days normoxia; 1 day AIH; 7 days AIH) at C6-7 or L4-5 spinal segments for each of BDNF, trkB, phospho-trkB, HIF1-alpha, VEGF, GFAP and ED-1 were examined by using one-way analysis of variance (ANOVA) and Tukey HSD test was used for post hoc analysis. Differences were considered significant if $p<0.05$.

4.3 Results

For every marker examined in this thesis, controls without primary antibodies were performed to verify the authenticity of the positive immunofluorescence signal and to also ascertain that there was no nonspecific staining by the secondary antibodies used to visualize where the primary antibodies had bound antigen. For each marker examined, there was no detectable positive immunofluorescence signal detected when the primary antibody was omitted from the procedure. A representative image of what was observed can be seen in Fig 4.2.

4.3.1 AIH and motor training increases HIF-1α protein expression in spinal motor neurons at spinal segments C6-7 and L4-5.

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that regulates the expression of multiple genes in response to hypoxia. AIH treatment and motor training for 1 or 7 days increases HIF-1α protein expression in spinal motor neurons at C6-7 and L4-5 segments of spinal cord (Figs 4.3 – 4. 6). Photomicrographs of ventral grey matter of spinal cord processed for HIF-1α immunofluorescence show increased immunoreactivity in AIH-treated spinal-injured rats compared to normoxia-treated spinal-injured rats after 1 day (Day-1) and 7 days (Day-7) of treatment in both C6-7 (Fig 4.3) and L4-5 (Fig 4.4) spinal segments.

Quantitative analysis confirms that AIH treatment plus motor training for either 1 day or 7 days significantly increases HIF-1α protein levels in the motor neurons of ventral grey matter of C6-7 and L4-5 spinal segments in AIH-treated rats versus normoxia-treated rats ($p < 0.05$) (Fig 4.4, 4.6)

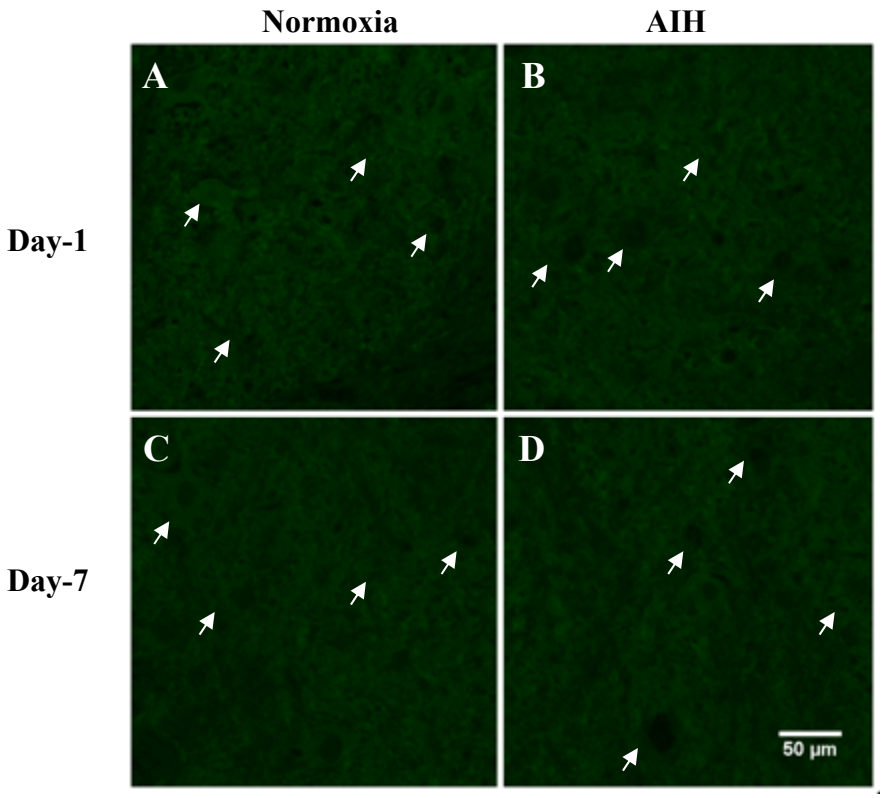

Figure 4.2: Representative Immunofluorescence control. Representative photomicrograph of spinal ventral horns at the C6-7 segment of spinal cord processed for HIF-1α protein expression without primary antibody (control). Note the lack of positive immunofluorescence signal in either 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus dAIH-treated (B,D) spinal-injured rats. Arrows identify representative motor neurons. Scale bar = 50 µm.

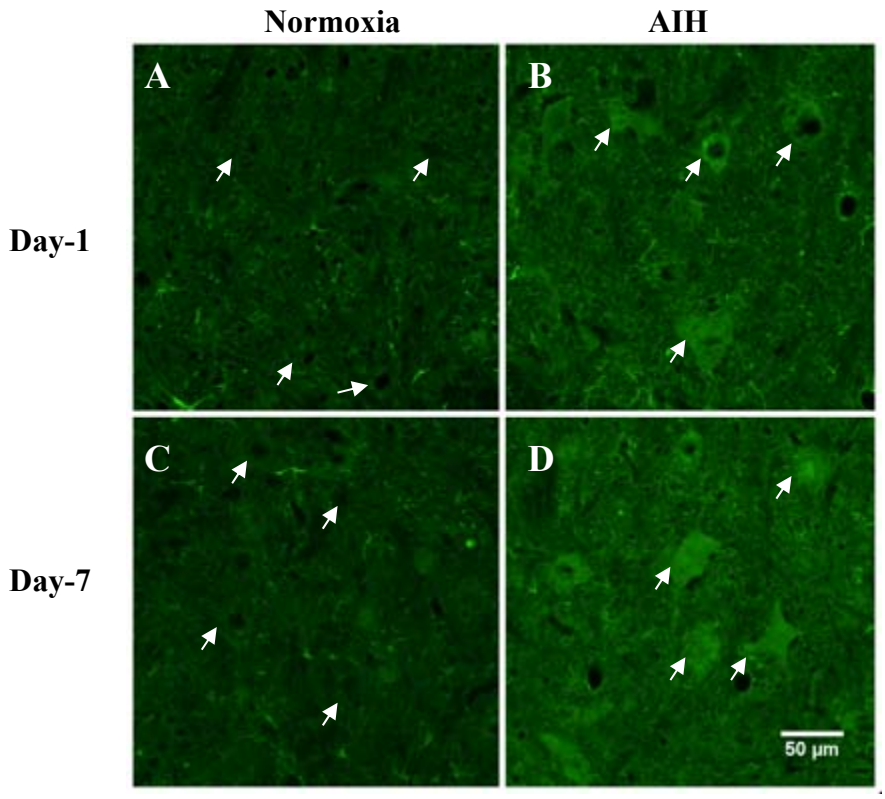

Figure 4.3: AIH treatment for either 1 or 7 days increases HIF-1α protein levels in the spinal segments C6 -7. Representative photomicrographs of the ventral grey matter in C6-7 spinal segments sections processed for HIF-1α immunofluorescence from 1 day (Day-1) or 7 day (Day-7) in normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Arrows indicate representative motor neurons. Scale bar = 50 µm.

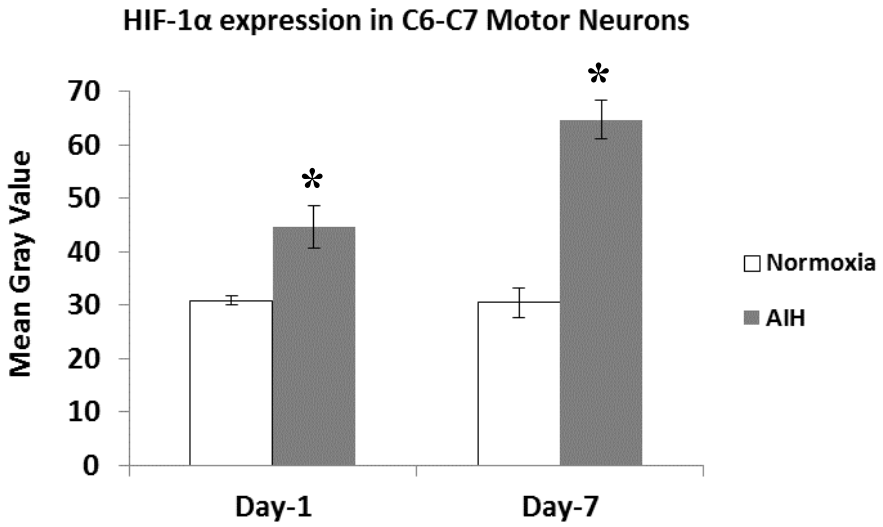

Figure 4.4: AIH treatment for either 1 or 7 days increases HIF-1α protein levels in motoneurons at spinal segments C6 -7. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values over individual motoneurons within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 60-65 neurons analysed/time point/experimental condition]. Asterisks indicate significant differences between experimental groups; * $p < 0.05$.

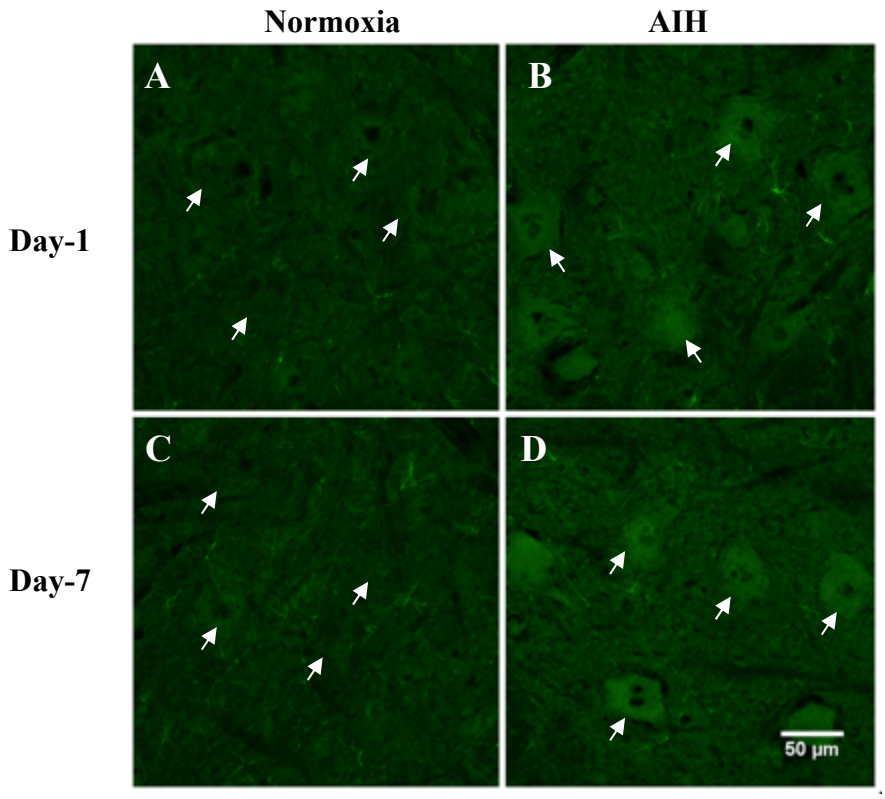

Figure 4.5: AIH treatment for either 1 or 7 days increases HIF-1α protein levels in the L4-5 spinal segments. Representative photomicrographs of the ventral grey matter in L4-5 spinal segments sections processed for HIF-1α immunofluorescence from 1 day (Day-1) or 7 day (Day-7) in normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Arrows indicate representative motor neurons. Scale bar = 50 µm.

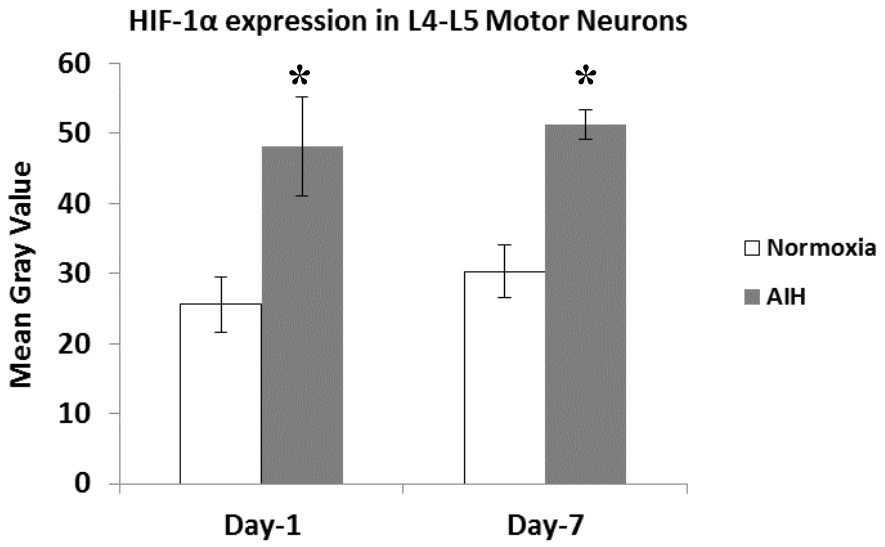

Figure 4.6: AIH treatment for either 1 or 7 days increases HIF-1α protein levels in motoneurons at L4-5 spinal segments. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values over individual motoneurons within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 80-85 neurons analysed/time point/experimental condition]. Asterisks indicate significant differences between experimental groups; * $p < 0.05$.

4.3.2 AIH and motor training increases VEGF protein expression in spinal motor neurons at spinal segments C6-7 and L4-5.

Vascular endothelial growth factor (VEGF) is a dimeric glycoprotein and a fundamental regulator of angiogenesis (Rosenstein and Krum 2004). Apart from its role in angiogenesis, VEGF appears to play neurotrophic and neuroprotective roles in spinal cord and brain injury (Krum and Rosenstein 1998; Facchiano, Fernandez et al. 2002; Svensson, Peters et al. 2002). Recent studies have shown that AIH induces the expression of VEGF and its receptors VEGFR-2 in phrenic motoneurons where they induced phrenic motor facilitation (pMF) via ERK Akt intracellular pathways (Dale, Ben Mabrouk et al. 2014).

To examine the possible effect of AIH treatment plus motor training for 1 day or 7 days on VEGF protein expression in spinal motor neurons, I processed the C6-7 and L4-5 segments of spinal cord for immunofluorescence for VEGF protein. AIH treatment and motor training for 7 days increased the expression of VEGF protein in spinal motor neurons at C6-7 and L4-5 segments of spinal cord but there was no marked change in VEGF immunoreactivity following 1 day of treatment and training. (Figs 4.7 - 4.10). Photomicrographs of spinal ventral grey matter processed for VEGF protein immunofluorescence show increased VEGF immunoreactivity in AIH-treated spinal-injured rats compared to normoxia-treated spinal-injured rats after Day-7 of treatment in C6-7 (Fig 4.7) and L4-5 (Fig 4.9).

Quantitative analysis confirms that AIH treatment plus motor training for 7 days significantly increases protein expression of VEGF levels in the motor neurons of ventral grey matter of spinal segments C6-7 and L4-5 in AIH-treated rats versus normoxia-treated rats ($p < 0.05$) (Fig 4.8, 4.10)

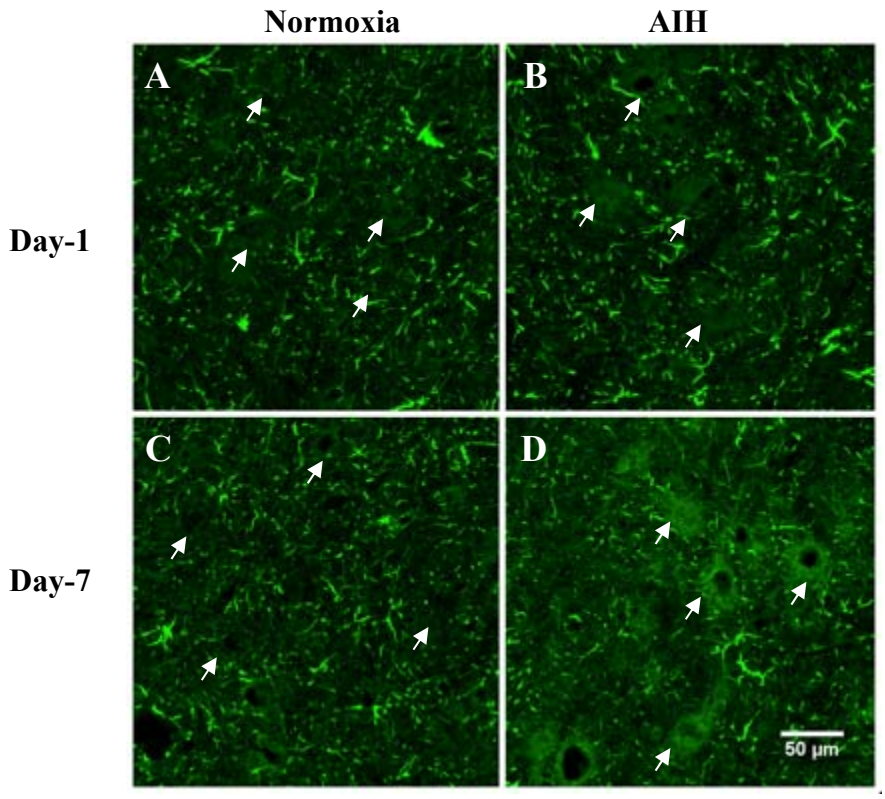

Figure 4.7: AIH treatment for 7 days increases VEGF expression in ventral grey matter of C6-7 spinal segments. Representative photomicrographs of the ventral grey matter in C6-7 spinal segments sections processed for VEGF immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Arrows indicate representative motor neurons. Scale bar = 50 µm.

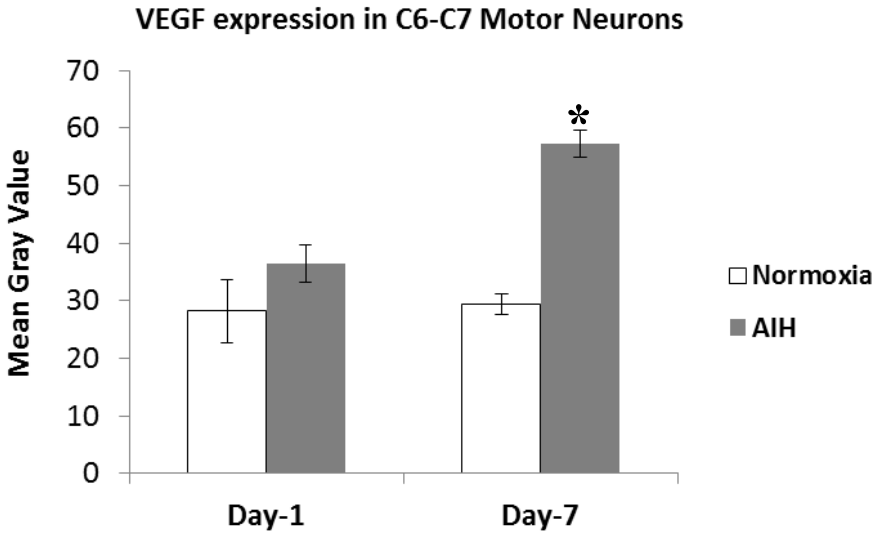

Figure 4.8: AIH treatment for 7 days increases VEGF protein levels in motoneurons at spinal segments C6-7. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values over individual motoneurons within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 60-65 neurons analysed/time point/experimental condition]. Asterisks indicate significant differences between experimental groups; * $p < 0.05$.

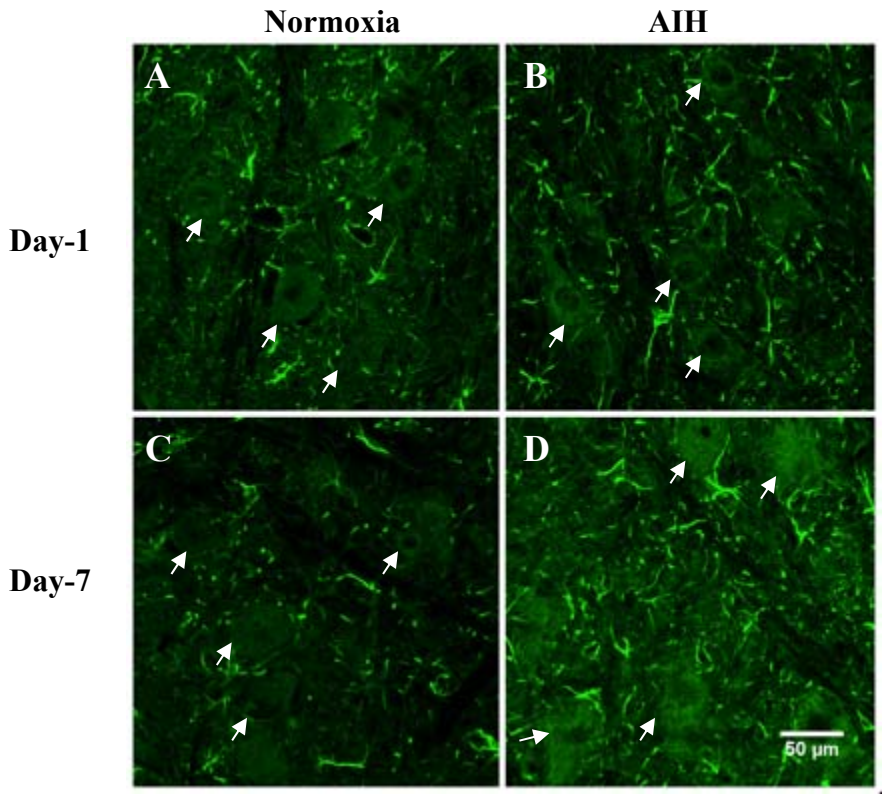

Figure 4.9: AIH treatment for 7 days increases VEGF expression in ventral grey matter of spinal segments L4-5. Representative photomicrographs of the ventral grey matter in L4-5 spinal segments sections processed for VEGF immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Arrows indicate representative motor neurons. Scale bar = 50 µm.

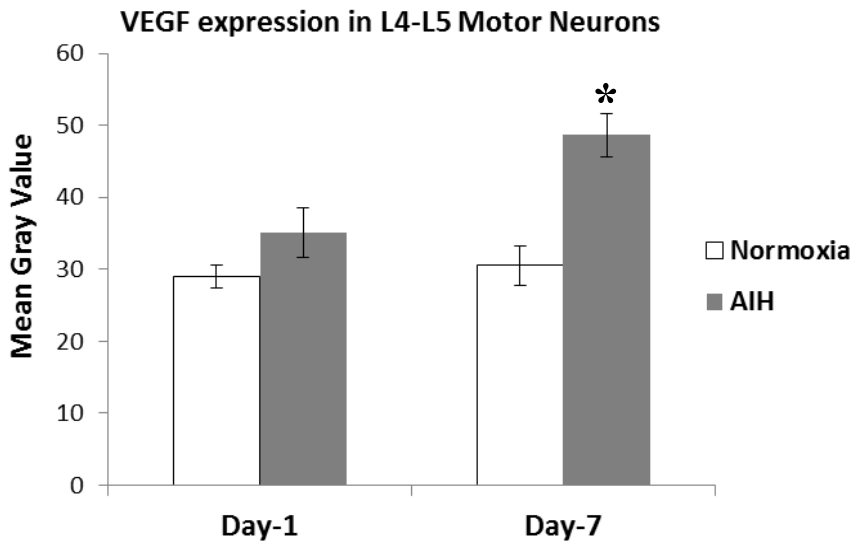

Figure 4.10: AIH treatment for 7 days increases VEGF protein levels in motoneurons at spinal segments L4-5. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values over individual motoneurons within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 80-85 neurons analysed/time point/experimental condition]. Asterisks indicate significant differences between experimental groups; * $p < 0.05$.

4.3.3 AIH treatment and motor training for 7 days increases BDNF protein expression in the ventral grey matter at spinal segments C6-7 and L4-5.

Brain-derived neurotrophic factor (BDNF) is an important member of the neurotrophin family and plays a diverse role in modulating neural plasticity and enhancing functional recovery following SCI (Hiebert, Khodarahmi et al. 2002; Vavrek, Girgis et al. 2006; Waterhouse and Xu 2009; Ma, Wang et al. 2011; Nagahara and Tuszynski 2011; Park and Poo 2013; Liao, Bouyer et al. 2015). BDNF mediates signaling through its high affinity receptor tyrosine kinase receptor B (trkB) (Weishaupt, Blesch et al. 2012; Mantilla, Gransee et al. 2013). Brief episodes of hypoxia in rats initiate the synthesis of new BDNF in the cervical cord, rather than release of existing BDNF (Baker-Herman, Fuller et al. 2004; Wilkerson and Mitchell 2009). It would be important to determine whether BDNF also mediates recovery of non-respiratory function after intermittent hypoxia treatment in rats with SCI.

To assess the possible effect of AIH treatment plus motor training for 1 day or 7 days on BDNF protein expression in spinal motor neurons, I processed sections from the C6-7 and L4-5 segments of spinal cord for immunofluorescence for BDNF protein. AIH treatment and motor training for 7 days increased the expression of BDNF protein in spinal motor neurons in the C6-7 and L4-5 segments of spinal cord but there was no change in BDNF immunoreactivity following one day of treatment and training (Figs 4.11 - 4.14). However, after 7 days of AIH treatment, BDNF immunoreactivity in AIH-treated spinal-injured rats was elevated compared to normoxia-treated spinal-injured rats at both C6-7 (Fig 4.11) and L4-5 (Fig 4.13) spinal levels.

Quantitative analysis confirms that AIH treatment plus motor training for 7 days significantly increases protein expression of BDNF levels in the motor neurons of ventral grey matter of spinal segments C6-7 and L4-5 in AIH-treated SCI rats versus normoxia-treated SCI rats ($p < 0.05$) (Fig 4.12, 4.14)

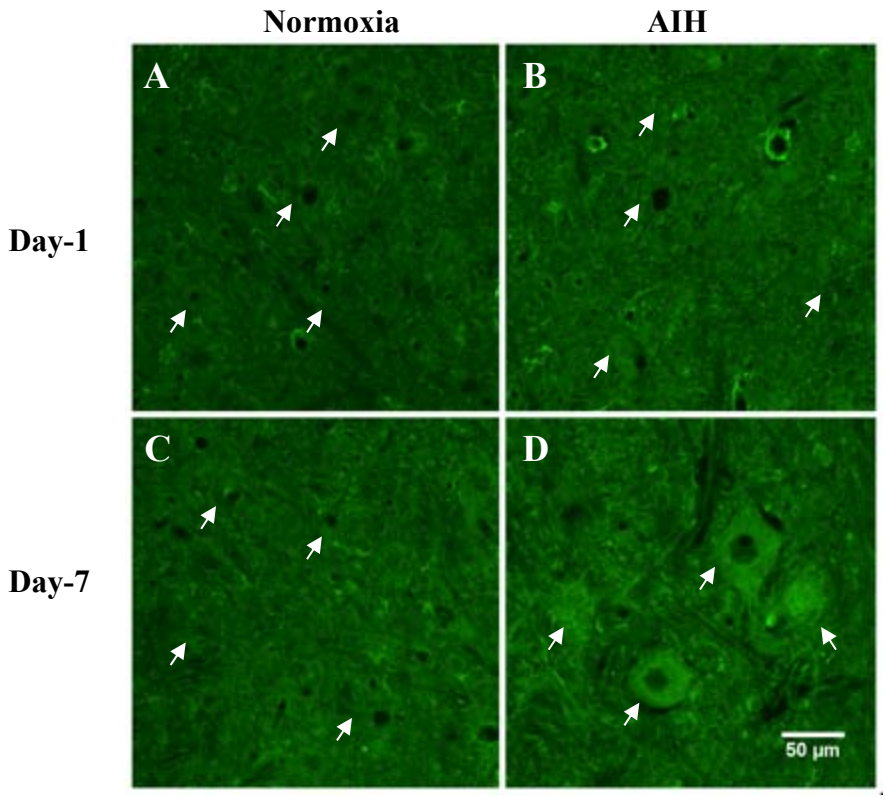

Figure 4.11: AIH treatment for 7 days increases BDNF expression in ventral grey matter of C6-7 spinal segments. Representative photomicrographs of the ventral grey matter in C6-7 spinal segments sections processed for BDNF immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Arrows indicate representative motor neurons. Scale bar = 50 µm.

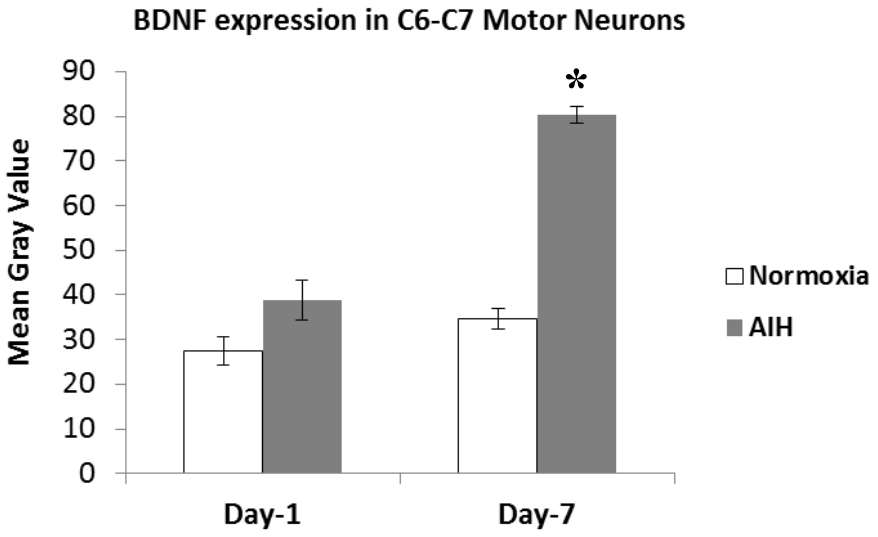

Figure 4.12: AIH treatment for 7 days increases BDNF protein levels in motoneurons at spinal segments C6-7. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values over individual motoneurons within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 60-65 neurons analysed/time point/experimental condition]. Asterisks indicate significant differences between experimental groups; * $p < 0.05$.

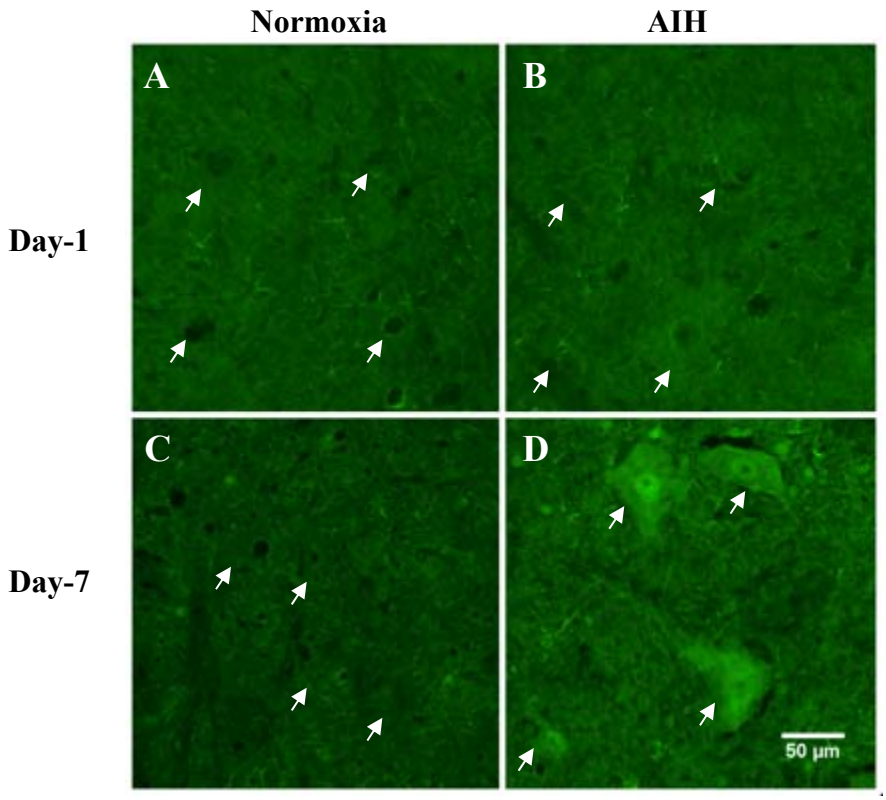

Figure 4.13: AIH treatment for 7 days increases BDNF expression in ventral grey matter of L4-5 spinal segments. Representative photomicrographs of the ventral grey matter in L4-5 spinal segments sections processed for BDNF immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Arrows indicate representative motor neurons. Scale bar = 50 µm.

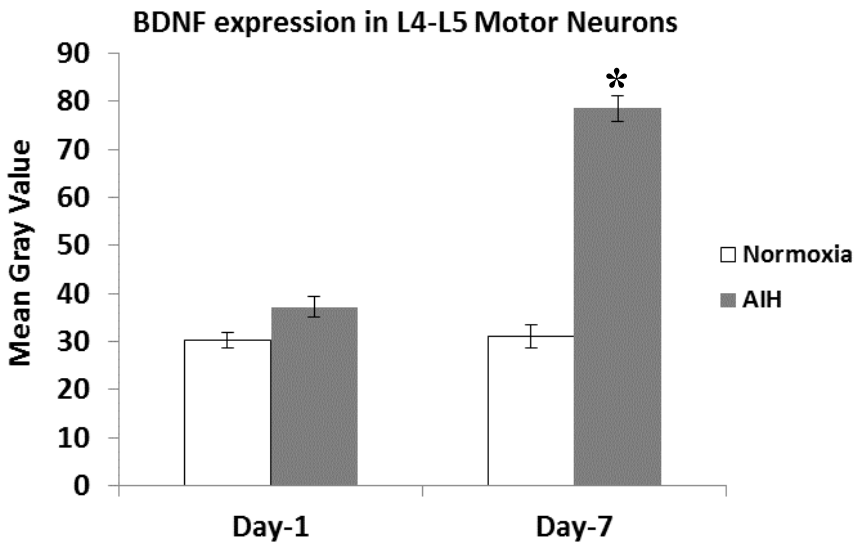

Figure 4.14: AIH treatment for 7 days increases BDNF protein levels in motoneurons at spinal segments L4-5. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values over individual motoneurons within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 80-85 neurons analysed/time point/experimental condition]. Asterisks indicate significant differences between experimental groups; * $p < 0.05$.

4.3.4 AIH treatment and motor training for 7 days increases trkB protein expression in ventral grey matter at spinal segments C6-7 and L4-5.

The tyrosine kinase B receptors (trkB) are transmembrane proteins that belong to the trk family of receptors for neurotrophins (Spencer-Segal, Waters et al. 2011). Similar to other tyrosine receptors, trkB receptors are activated by ligand-induced formation of receptor dimers (Schecterson and Bothwell 2010). BDNF-trkB signaling pathway is a key signaling pathway involved in activity dependent processes such as neural plasticity. Although it has been previously demonstrated that AIH exposure requires the activation of trkB receptors in spinal motor neurons to induce pLTF in phrenic nerve, it is also known that AIH increases both BDNF expression and extracellular adenosine levels in the spinal cord. (Baker-Herman, Fuller et al. 2004; Wilkerson and Mitchell 2009; Hoffman and Mitchell 2011; Lovett-Barr, Satriotomo et al. 2012; Satriotomo, Dale et al. 2012).

To assess the possible effect of AIH treatment plus motor training for 1 day or 7 days on trkB protein expression in spinal motor neurons, I processed the C6-7 and L4-5 segments of the spinal cord for immunofluorescence for trkB protein. AIH treatment and motor training for 7 days increased the expression of trkB protein in spinal motor neurons at C6-7 and L4-5 spinal segments but there was no change in trkB immunoreactivity following one day of treatment and training (Figs 4.15 - 4.18). Photomicrographs of spinal ventral grey matter processed for trkB protein immunofluorescence show increased trkB immunoreactivity in AIH-treated spinal-injured rats compared to normoxia-treated spinal-injured rats after Day-7 of treatment in C6-7 (Fig 4.15) and L4-5 (Fig 4.17).

Quantitative analysis confirms that AIH treatment plus motor training for 7 days significantly increases protein expression of trkB levels in the motor neurons of ventral grey matter of spinal segments C6-7 and L4-5 in AIH-treated rats versus normoxia-treated rats ($p < 0.05$) (Fig 4.16, 4.18)

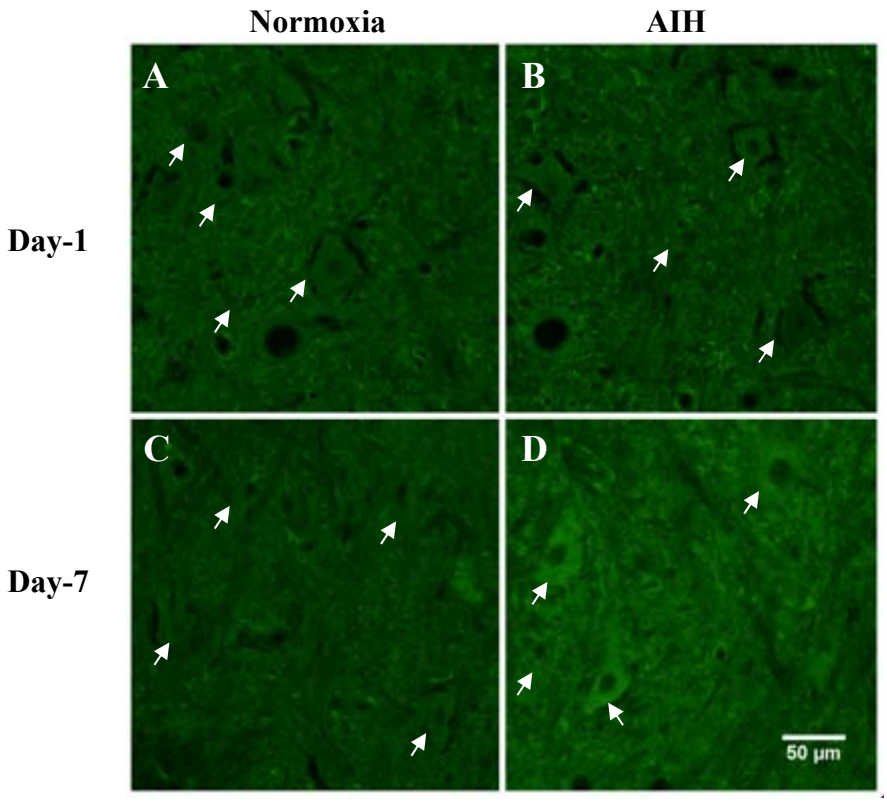

Figure 4.15: AIH treatment for 7 days increases trkB expression in ventral grey matter of C6-7 spinal segments. Representative photomicrographs of the ventral grey matter in C6-7 spinal segment sections processed for trkB immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Arrows indicate representative motor neurons. Scale bar = 50 µm.

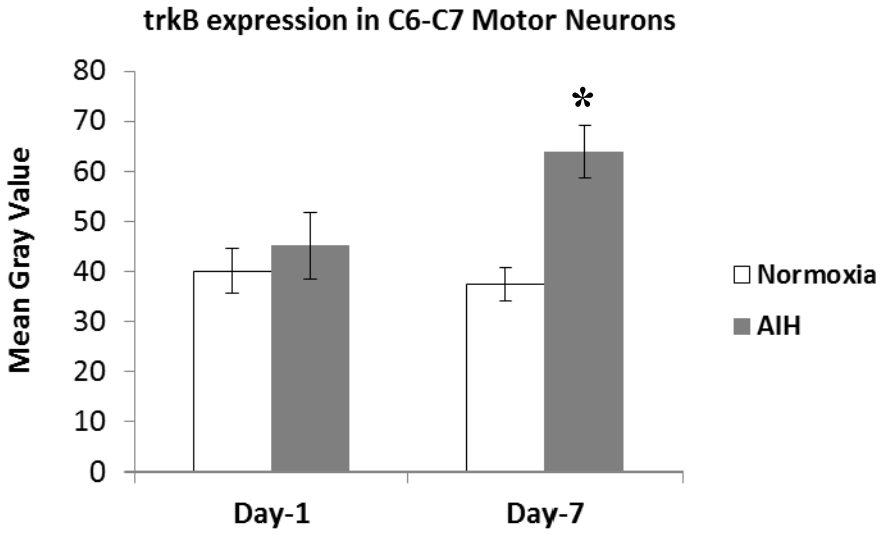

Figure 4.16: AIH treatment for 7 days increases trkB protein levels in motoneurons at spinal segments C6-7. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values over individual motoneurons within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 60-65 neurons analysed/time point/experimental condition]. Asterisks indicate significant differences between experimental groups; * $p < 0.05$.

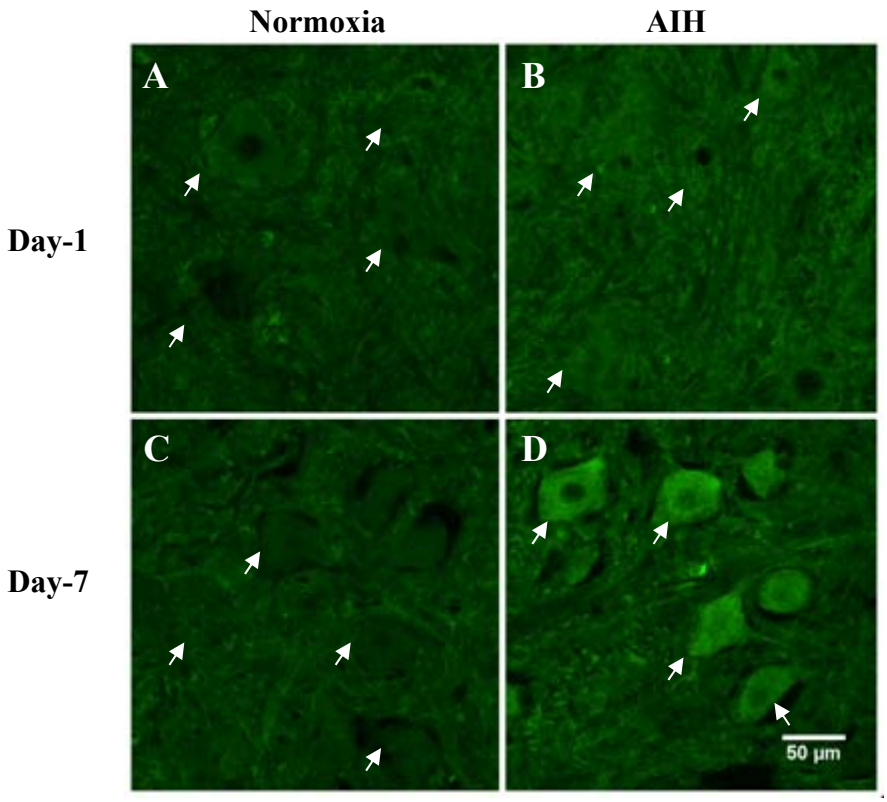

Figure 4.17: AIH treatment for 7 days increases trkB expression in ventral grey matter of L4-5 spinal segments. Representative photomicrographs of the ventral grey matter in L4-5 spinal segments sections processed for trkB immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Arrows indicate representative motor neurons. Scale bar = 50 µm.

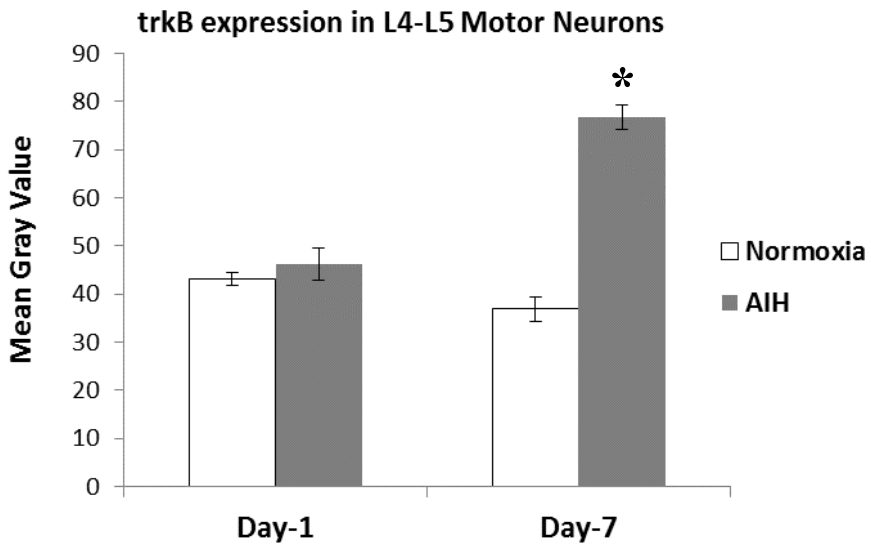

Figure 4.18: AIH treatment for 7 days increases trkB protein levels in motoneurons at L4-5 spinal segments. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values over individual motoneurons within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 80-85 neurons analysed/time point/experimental condition]. Asterisks indicate significant differences between experimental groups; * $p < 0.05$.

4.3.5 AIH treatment and motor training for 7 days increases phosphorylated trkB protein expression in ventral grey matter at spinal segments C6-7 and L4-5.

Since I observed increased expression of the BDNF receptor trkB and because the BDNF-trkB signaling pathway is a key signaling pathway involved in activity dependent processes such as neural plasticity, I decided to also examine the phosphorylation state of these BDNF receptors, as this would imply that the receptors had been activated. BDNF can induce rapid phosphorylation of trkB receptors (Klein, Nanduri et al. 1991; Jia, Chen et al. 2008; Guo, Ji et al. 2014). Phosphorylation of trkB is necessary for activation of many of the downstream pathways linked to BDNF's function (Babaei Bourojeni 2014). To assess the possible effect of AIH treatment plus motor training for 1 day or 7 days on phosphorylated trkB (ptrkB) protein expression in spinal motor neurons, I processed sections from the C6-7 and L4-5 segments of the spinal cord for immunofluorescence for ptrkB protein. AIH treatment and motor training for 7 days increased the expression of ptrkB protein in spinal motor neurons at C6-7 and L4-5 segments of spinal cord but there was no change in ptrkB immunoreactivity following one day of treatment and training and this may reflect the lack of notable increase in BDNF expression that I observed at this timepoint. (Figs 4.19 - 4.22). Photomicrographs of spinal ventral grey matter sections processed for ptrkB protein immunofluorescence show increased ptrkB immunoreactivity in the spinal motoneurons and surrounding neuropil in AIH-treated SCI rats compared to normoxia-treated SCI rats after 7 days of treatment at C6-7 (Fig 4.19) and L4-5 (Fig 4.21) spinal segment levels.

Quantitative analysis confirms that AIH treatment plus motor training for 7 days significantly increases the ptrkB levels in the motor neurons of C6-7 and L4-5 ventral grey matter of spinal segments when compared to normoxia-treated rats ($p < 0.05$) (Fig 4.20, 4.22)

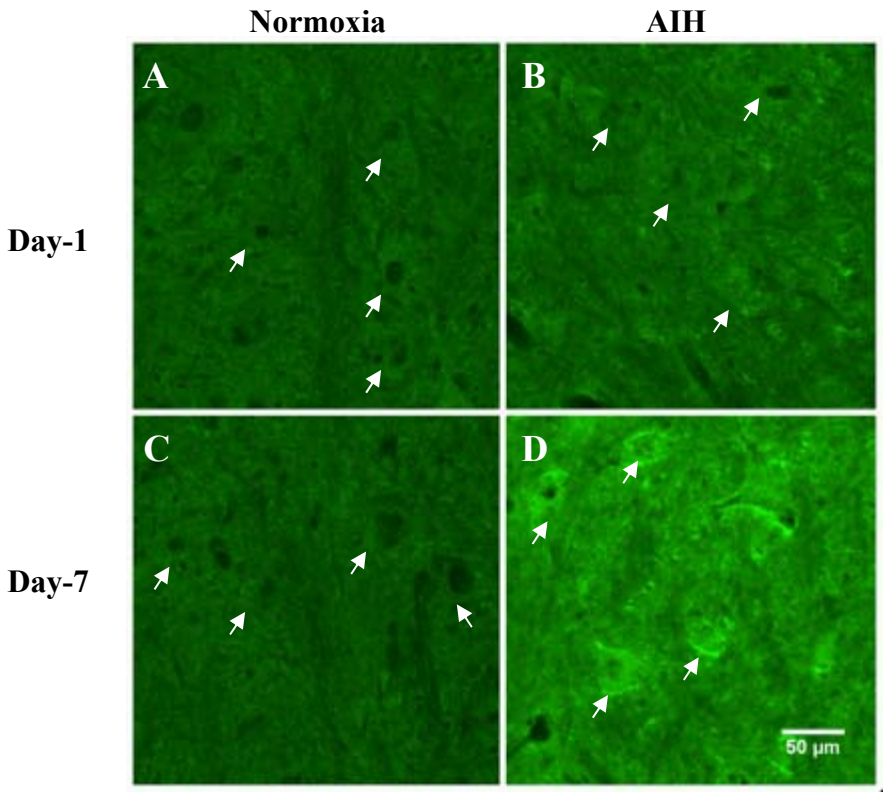

Figure 4.19: AIH treatment for 7 days increases ptrkB expression in ventral grey matter of C6-7 spinal segments. Representative photomicrographs of the ventral grey matter in C6-7 spinal segments sections processed for ptrkB immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Arrows indicate representative motor neurons. Scale bar = 50 µm.

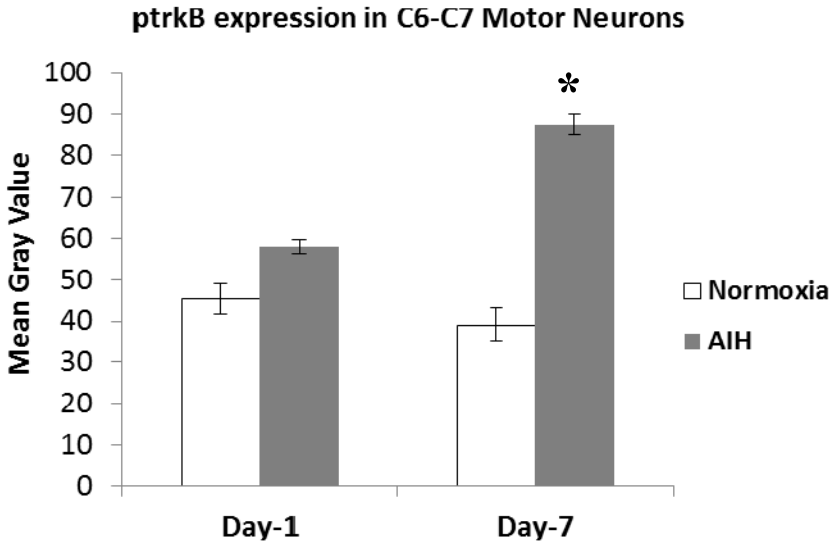

Figure 4.20: AIH treatment for 7 days increases ptrkB protein levels in motoneurons at spinal C6-7 segments. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values over individual motoneurons within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 60-65 neurons analysed/time point/experimental condition]. Asterisks indicate significant differences between experimental groups; * $p < 0.05$.

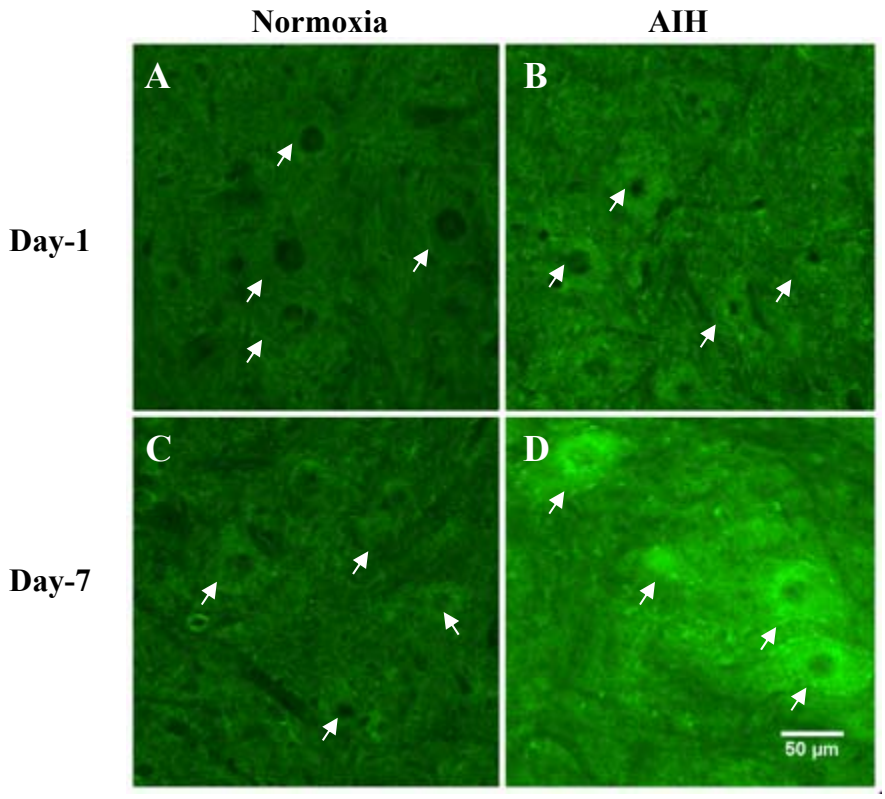

Figure 4.21: AIH treatment for 7 days increases ptrkB expression in ventral grey matter of L4-5 spinal segments. Representative photomicrographs of the ventral grey matter in L4-5 spinal segments sections processed for ptrkB immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Arrows indicate representative motor neurons. Scale bar = 50 µm.

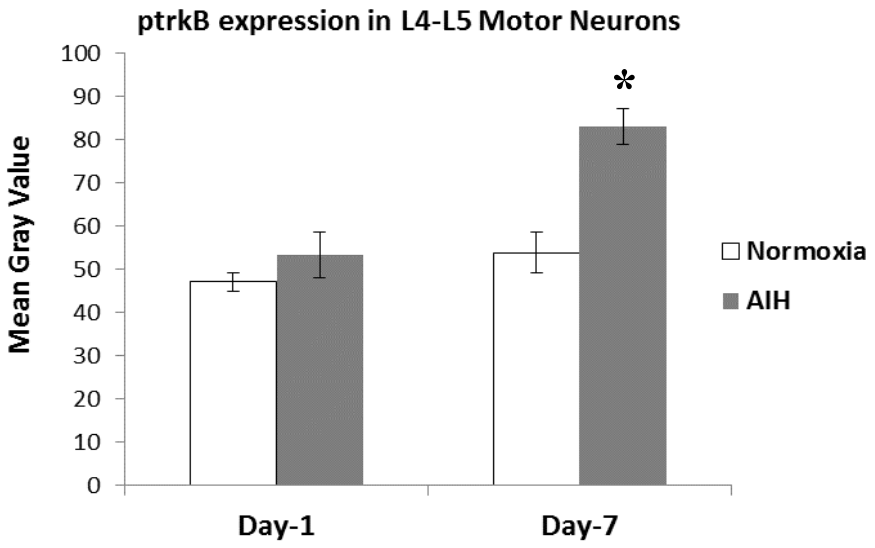

Figure 4.22: AIH treatment for 7 days increases ptrkB protein levels in motoneurons at L4-5 spinal segments. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values over individual motoneurons within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 80-85 neurons analysed/time point/experimental condition]. Asterisks indicate significant differences between experimental groups; * $p < 0.05$.

4.3.6 AIH treatment for 7 days attenuates GFAP protein expression in ventral grey matter at C6-7 segment of spinal cord.

Astrocytes play an important role in inflammatory process following SCI and treatments or factors that reduce the activation of astrocytes improve functional recovery following injury (Kang, Balordi et al. 2014). Astrocytes become activated following CNS lesions and express higher levels of the intermediate filament glial fibrillary acidic protein (GFAP), which is a marker for reactive astrocytes. AIH treatment and motor training for 7 days attenuated the expression of GFAP detected in ventral grey matter at the level of the C6-7 spinal segments, though there was no change in GFAP immunoreactivity following one day of treatment and training (Figs 4.23 – 4.26). Photomicrographs of ventral grey matter of spinal cord processed for GFAP immunofluorescence show decreased GFAP immunoreactivity in AIH-treated spinal-injured rats compared to normoxia-treated spinal-injured rats after 7 days of treatment (Day-7) in sections from the C6-7 spinal segments (Fig 4.23). Quantitative analysis confirmed that AIH treatment plus motor training for 7 days significantly decreased protein expression of GFAP in the ventral grey matter of spinal segments C6-7 in AIH-treated rats versus normoxia-treated rats ($p < 0.05$) (Fig 4.24).

Interestingly, AIH treatment and motor training for 1 day or 7 days did not alter the expression of GFAP in ventral grey matter at the L4-5 spinal segment level (Fig 4.25 – 4.26). Representative photomicrographs of ventral grey matter of L4-5 spinal cord processed for GFAP immunofluorescence reveal no difference in the levels of GFAP immunoreactivity detected in AIH-treated spinal-injured rats compared to normoxia-treated spinal-injured rats after either one day or 7 days of treatment (Fig 4.25). Quantitative analysis confirmed that AIH treatment and motor training for either 1 or 7 days had no quantifiable effect on GFAP protein expression in ventral grey L4-5 spinal segments (Fig 4.26).

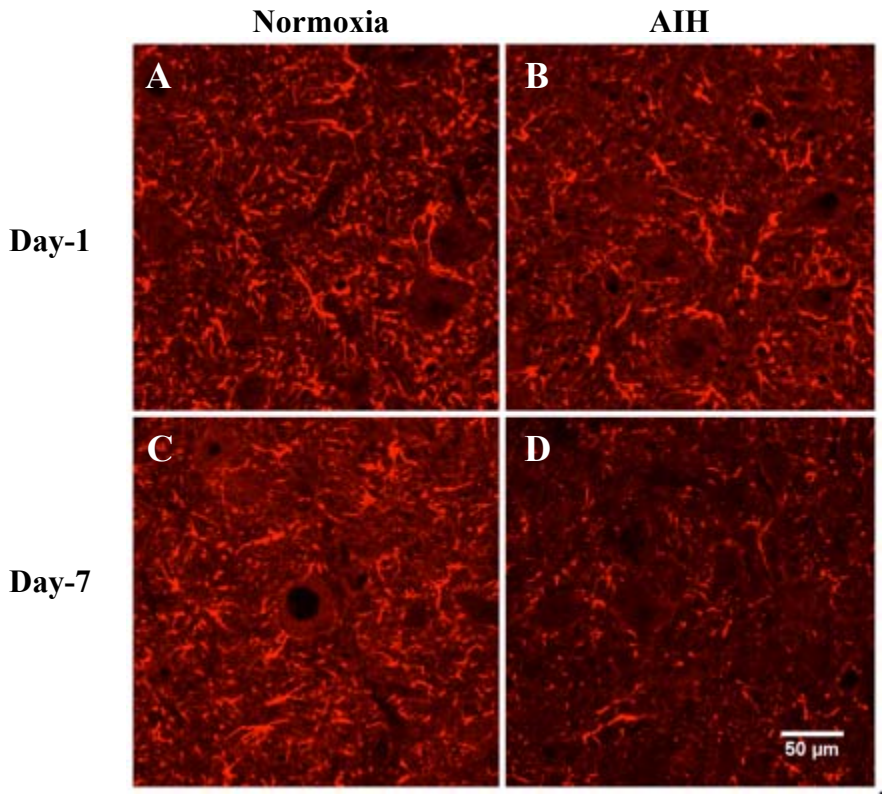

Figure 4.23: AIH treatment for 7 days decreases GFAP expression in ventral grey matter of C6-7 spinal segments. Representative photomicrographs of the ventral grey matter in C6-7 spinal segments sections processed for GFAP immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Scale bar = 50 µm.

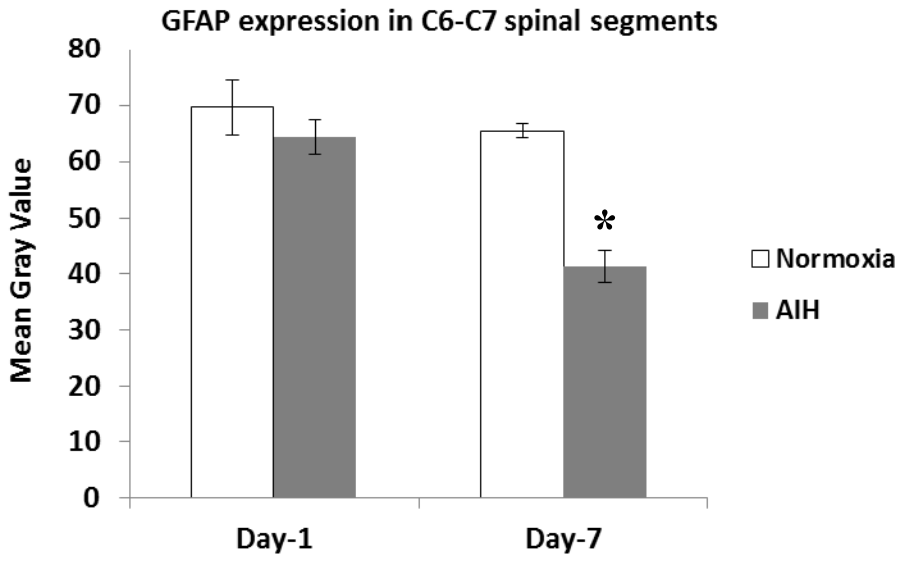

Figure 4.24: AIH treatment for 7 days decreases GFAP protein levels in ventral grey matter of C6-7 spinal segments. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 48 boxes (100μm X 100μm) analysed/time point/experimental condition]. Asterisks indicate significant differences between experimental groups; * $p < 0.05$.

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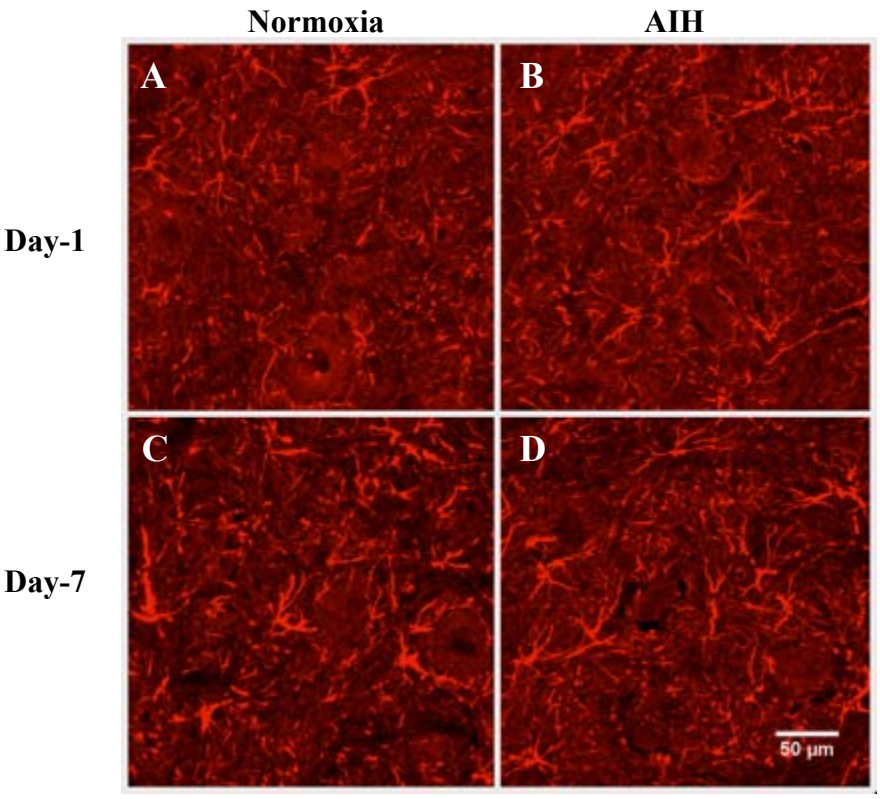

Figure 4.25: AIH treatment for either 1 or 7 days does not alter GFAP protein levels in the L4-5 spinal segments. Representative photomicrographs of the ventral grey matter in L4-5 spinal segments sections processed for GFAP immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Scale bar = 50 µm.

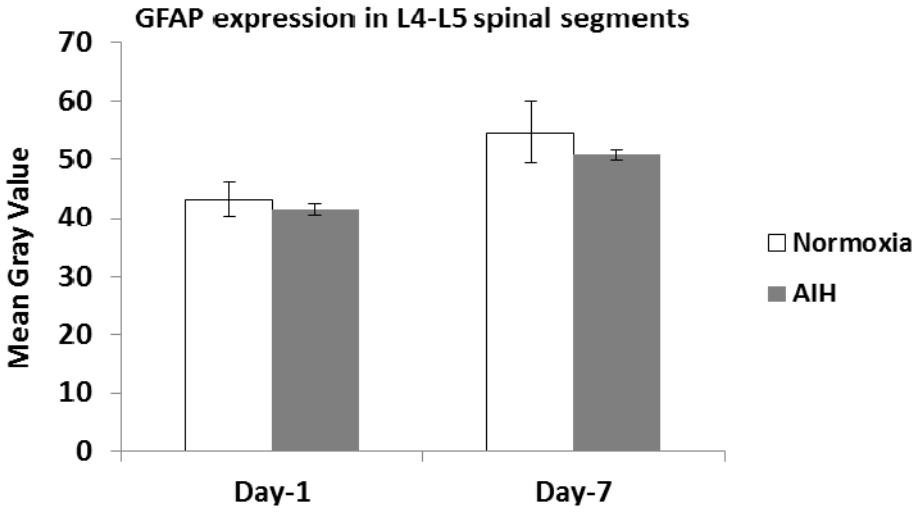

Figure 4.26: AIH treatment for either 1 or 7 days does not increase GFAP protein levels in ventral grey matter of L4-5 spinal segments. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 48 boxes (100μm X 100μm) analysed/time point/experimental condition].

4.3.7 AIH treatment for 1 or 7 days does not alter the ED-1 protein expression in the ventral grey matter of either C6-7 or L4-5 spinal segments.

Microglia cells are resident immune cells in central nervous system which play an important role in inflammation in the CNS. Microglia cells are present in resting states and become activated in response to brain or spinal cord injury. Reactive microglia cells accumulate at the site of injury, where they engulf damaged tissue or cell debris and can also play neuroprotective roles. ED-1 (aka CD-68) is a marker for activated microglia and can be used to detect activated microglia. There was no detectable ED-1 immunoreactivity in ventral grey matter of either AIH or normoxia-treated SCI rats after either 1 or 7 day of treatment at the C6-7 or L4-5 spinal segments, and thus AIH treatment and motor training did not appear to invoke this type of immune response in the spinal ventral grey matter at these levels (Figs 4.27 – 4.30). This can be visualized in photomicrographs of sections from the C6-7 and L4-5 ventral grey matter of spinal cord processed for ED-1 protein expression immunofluorescence which show no detectable ED-1 immunoreactivity in either the AIH-treated spinal-injured rats or the normoxia-treated spinal-injured rats following 1 day (Day-1) or 7 days (Day-7) of treatment.(Fig 4.27, 4.29).

Quantitative analysis confirms that there is indeed no discernible ED-1 detected in these animals at the levels analysed. (Fig 4.28, 4.30).

In contrast, ED-1 immunoreactivity is readily detectable in sections of the ventral grey matter from the C2-3 spinal segments, just proximal to the original spinal lesion. Representative photomicrographs of ventral grey matter sections of spinal cord processed for ED-1 protein expression immunofluorescence reveal that at all time points examined there are activated microglia/macrophages that can be discerned likely due to the proximity of this tissue to the original lesion site and also confirm that the ED-1 antibody employed was valid (Fig. 4.31).

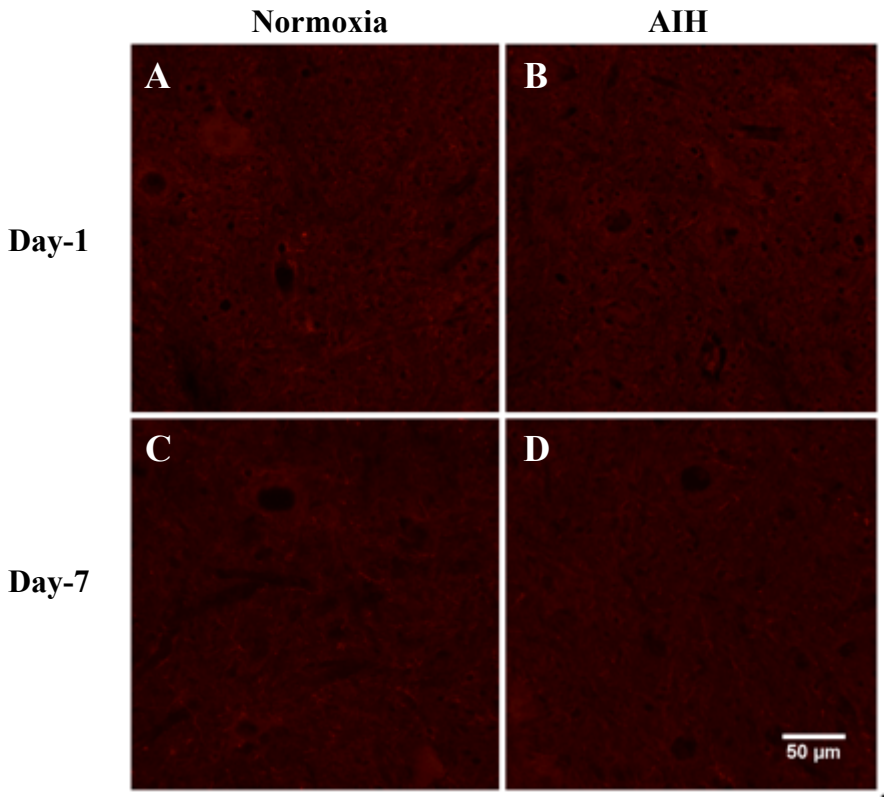

Figure 4.27: ED-1 protein expression was not detectable in the C6-7 spinal segments following AIH treatment for either 1 or 7 days. Representative photomicrographs of the ventral grey matter in C6-7 spinal segments sections processed for ED-1 immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Scale bar = 50 µm.

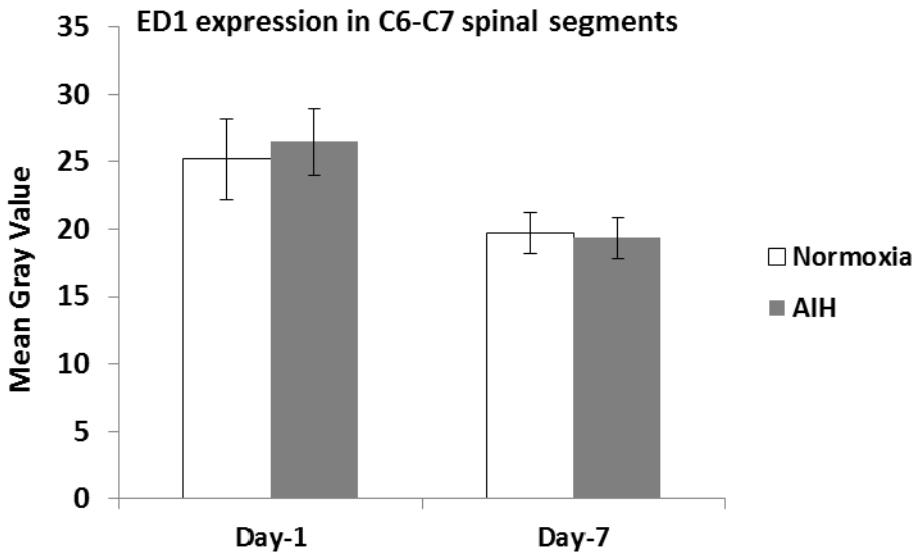

Figure 4.28: AIH treatment for either 1 or 7 days does not increase ED-1 protein levels in ventral grey matter of C6-7 spinal segments. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 48 boxes (100μm X 100μm) analysed/time point/experimental condition].

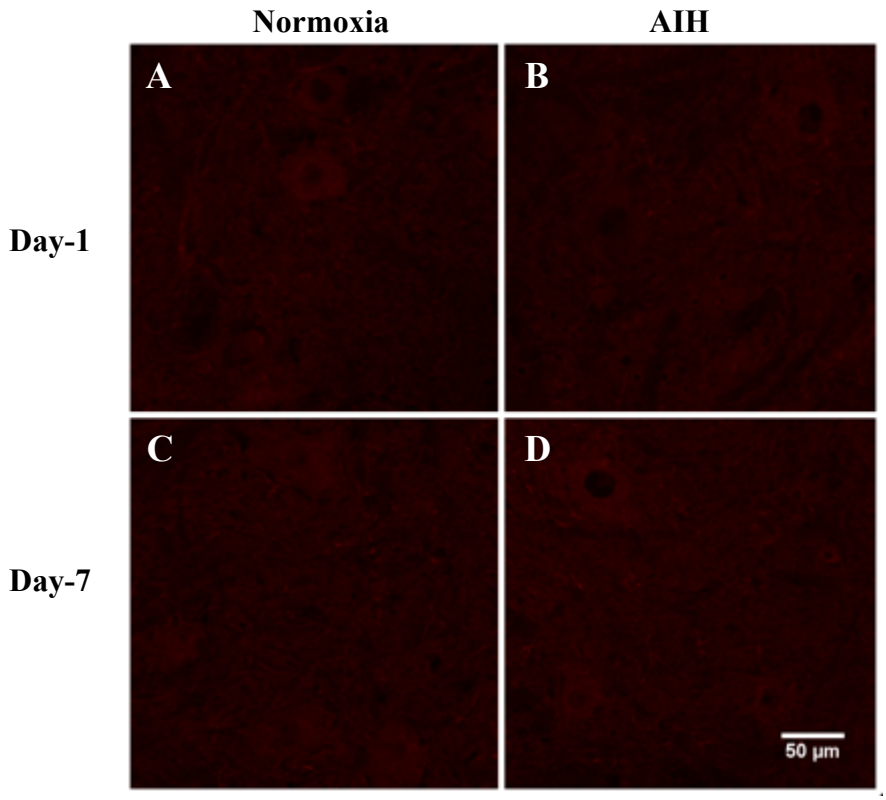

Figure 4.29: ED-1 protein expression was not detectable in the L4-5 spinal segments following AIH treatment for either 1 or 7 days. Representative photomicrographs of the ventral grey matter in L4-5 spinal segments sections processed for ED-1 immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Scale bar = 50 µm.

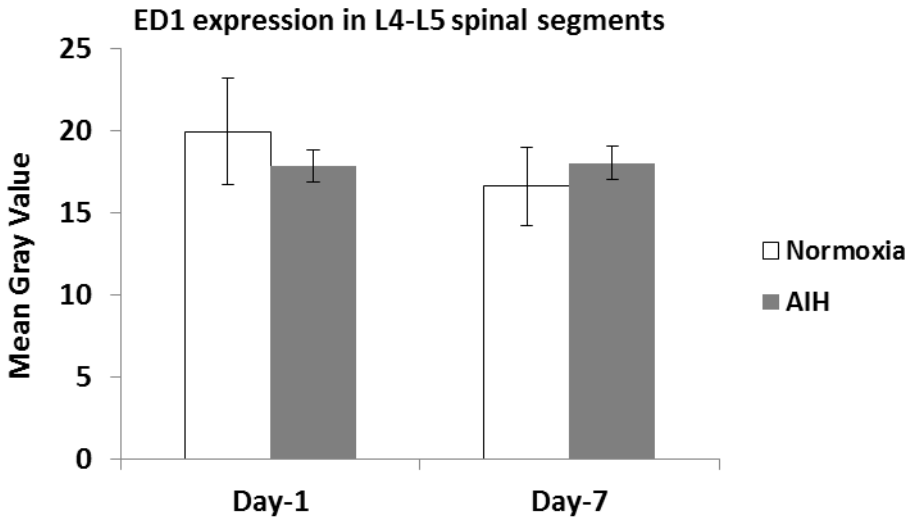

Figure 4.30: AIH treatment for either 1 or 7 days does not increase ED-1 protein levels in ventral grey matter of L4-5 spinal segments. Histograms summarize the mean immunofluorescence signal intensity detected ± SEM as measured in gray values within the ventral horn from normoxia- and AIH-treated spinal injured rats [n=3 rats per experimental group analysed; 48 boxes (100μm X 100μm) analysed/time point/experimental condition].

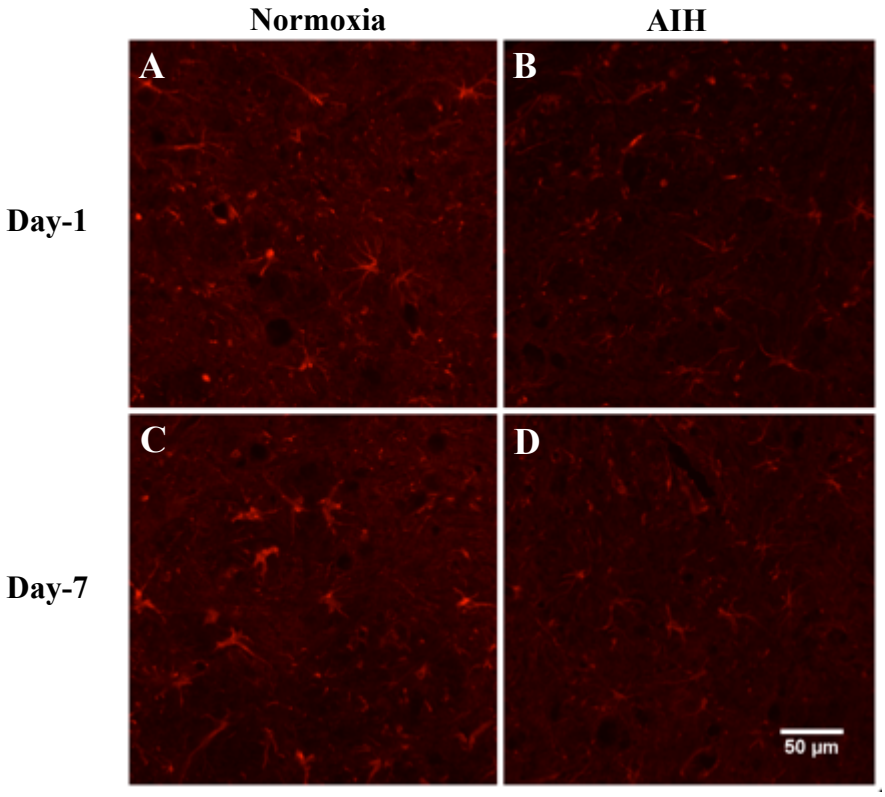

Figure 4.31: ED-1 protein expression was detectable in the C2-3 spinal segments proximal to lesion site. Representative photomicrographs of the ventral grey matter in C2-3 spinal segments sections processed for ED-1 immunofluorescence from 1 day (Day-1) or 7 day (Day-7) normoxia-treated (A,C) versus AIH-treated (B,D) spinal injured rats. Scale bar = 50 µm.

4.4 Discussion

In the present study, I assessed the combined effect of AIH and motor training on expression of hypoxia-associated (HIF-1α, VEGF) and plasticity-associated proteins (BDNF, trkB, ptrkB) in the spinal motor neurons of cervical SCI rats. I found that AIH (10 episodes of AIH, 5 min duration per day) plus motor training for 7 days significantly increased the expression of HIF-1α, VEGF, BDNF, trkB and ptrkB in spinal motor neurons of cervical SCI rats at spinal segments C6-7 and L4-5. The expression of the oxygen-sensitive transcription factor HIF-1α was also increased after only one day of AIH and motor training in C6-7 and L4-5. In contrast, AIH and motor training for 7 days reduced the expression of GFAP in the C6-7 spinal segments, although GFAP expression in L4-5 level spinal segments did not change. Similarly, there was no change in ED-1 expression, a marker for active microglia, as result of AIH treatment and motor training in SCI rats.

4.4.1 AIH and motor training increased hypoxia-associated protein expression

Hypoxia-Inducible Factor-1α (HIF-1α) is a master transcriptional regulator of genes controlling a number of adaptive responses to low oxygen tension in order to maintain oxygen homeostasis in mammalian cells. The HIF-1 protein is a heterodimer and composed of two subunits: the HIF-1α subunit and the constitutively expressed HIF-1β subunit (Wang, Jiang et al. 1995). HIF-1α is oxygen sensitive and it is stabilized and activated under hypoxia conditions and degraded in normoxia condition by proteasomes (Rosenstein and Krum 2004; Xiaowei, Ninghui et al. 2006). In hypoxic conditions, HIF-1α translocates from the cytoplasm to the nucleus and dimerizes with HIF-1β to form the active HIF heterodimer complex (Lando, Peet et al. 2002). This dimer complex binds with hypoxia response elements (HRE) in target genes to induce gene expression (Lando, Peet et al. 2002).

HIF-1 binds to promoter/enhancer elements and regulates the transcription of several dozen hypoxia-inducible target genes including VEGF, EPO, inducible nitric oxide synthase (iNOS), heme oxygenase-1, glucose transporter-1 and the glycolytic enzymes (Semenza, Nejfelt et al. 1991; Melillo, Musso et al. 1995; Stein, Neeman et al. 1995; Lee, Jiang et al. 1997; Kimura, Weisz et al. 2000; Ke and Costa 2006; Xiaowei, Ninghui et al. 2006; Xiong, Mahmood et al.

2010). Hypoxia induces the expression of HIF-1α in various cell types of the CNS including neurons, astrocytes, oligodendrocytes and microglia (Bergeron, Yu et al. 1999; Chavez and LaManna 2002). The present study demonstrates that the AIH treatment for either day 1 or day 7 enhances the expression of HIF-1α protein in spinal motor neurons at both C6-7 and L4-5 level of spinal segments. This enhancement of HIF-1α protein in spinal motor neurons supports the efficacy of the AIH protocol used in present study. Previous studies have shown that 1 hour of systemic hypoxia (6% O_2) is sufficient to increase HIF-1α protein expression in brain tissues of mice, especially in the neurons of cerebral cortex, granular layer of dentate gyrus and the hippocampus (Stroka, Burkhardt et al. 2001). Only a few minutes of hypoxia induces the expression of HIF-1α proteins in the human epithelial carcinoma cell line HeLaS3, and after one hour of anoxic hypoxia, HIF-1α protein expression reached its maximum level, and this maximum level was maintained for 4 hours (Jewell, Kvietikova et al. 2001). Moreover, exposure to low oxygen (6% to 7% O_2) for 30 minutes increased the expression of HIF-1α protein, but not its mRNA, 7 fold in rat retina, followed by a further increase after 3 hours of hypoxia to 15 fold compared to control animals (Crosson, Kroes et al. 2009). In short, exposure to low oxygen for only minutes to hours is sufficient to enhance HIF-1α protein levels. Therefore, the finding of the present study that HIF-1α protein expression increased in spinal motor neurons after only 1 day of AIH treatment is consistent with the published literature.

HIF-1α regulates the expression of VEGF, which is generally known for its role in angiogenesis and cell permeability but it is also known, through *in vitro* and *in vivo* studies, to play versatile roles in the central and peripheral nervous system (Senger, Perruzzi et al. 1986; Connolly, Heuvelman et al. 1989; Rosenstein and Krum 2004; Zachary 2005; Mackenzie and Ruhrberg 2012; Nowacka and Obuchowicz 2012). VEGF is a 45 Da dimeric glycoprotein and a fundamental regulator of pathological and physiological angiogenesis (Rosenstein and Krum 2004). VEGF promotes endothelial cell proliferation in several organ systems during embryonic development and after injury in various type of tissues, including the central nervous system (Skold, Cullheim et al. 2000). VEGF is critical for blood vessel growth in the developing and adult nervous system of vertebrates (Mackenzie and Ruhrberg 2012). Apart from its role in angiogenesis, VEGF appears to play neurotrophic and neuroprotective roles in spinal cord and

brain injury (Facchiano, Fernandez et al. 2002) (Krum and Rosenstein 1998; Svensson, Peters et al. 2002).

VEGF and its high affinity receptor VEGFR-2 are expressed in spinal motor neurons (Dale-Nagle, Satriotomo et al. 2011). Thrice-weekly AIH exposure (3x/week AIH: 10, 5-min episodes of 10.5% O(2); 5-min normoxic intervals) upregulated the expression of VEGF and its receptors in respiratory and non-respiratory motor neurons in the spinal cord (Dale-Nagle, Satriotomo et al. 2011; Satriotomo, Dale et al. 2012). Consistent with these previous findings, the current study shows that AIH treatment for 7 days also significantly enhanced the expression of HIF-1α and VEGF at both C6-7 and L4-5 levels in the spinal cord (Figs, 4.3-4.10). Interestingly, AIH exposure for 1 day altered the expression of HIF-1α but was not sufficient to alter the expression of VEGF in spinal motor neurons at C6-7 and L4-5. It is possible that more sustained elevation in HIF-1α levels beyond that produced by one day of AIH treatment is required to induce downstream increases in VEGF expression.

Whether the increased VEGF expression is related to improvements in ladder performance is not yet known. One series of studies has shown that exogenous application of VEGF elicits phrenic motor plasticity via the ERK/Akt intracellular pathways in respiratory motor systems (Dale-Nagle, Satriotomo et al. 2011; Satriotomo, Dale et al. 2012; Dale, Ben Mabrouk et al. 2014). These studies, in conjunction with the current finding of increased expression of VEGF protein at multiple levels of the spinal cord in response to AIH in the current study suggests that VEGF might have an important role in spinal plasticity and could contribute to facilitation of functional recovery in SCI animals (Sato, Morimoto et al. 2012; Dale, Ben Mabrouk et al. 2014).

4.4.2 AIH and motor training enhance the spinal expression of BDNF

Plasticity is a fundamental property of nervous system (Mitchell and Johnson 2003). Plasticity may involve alterations in circuitry within nervous system, such that neurons rearrange and fine tune their structural and/or functional connectivity based on previous experience. Circuitry within the spinal cord shows a high degree of plasticity, which can be induced by hypoxia, exercise, injury, stress, and pharmacological interventions or conditioning (Mitchell and Johnson

2003). Growth factors and trophic factors, including BDNF, play important roles in multiple forms of neural plasticity. BDNF mediates its effects through its high affinity receptor trkB. Exogenous application of BDNF promotes neuroprotection, axonal regeneration, survival of neurons and axonal sprouting at the site of injury in SCI animals (Bregman, McAtee et al. 1997; Novikova, Novikov et al. 2002; Weishaupt, Blesch et al. 2012; Mantilla, Gransee et al. 2013; Weishaupt, Li et al. 2013) and has been reported to improve functional recovery in SCI animals (Boyce, Park et al. 2012; Mantilla, Gransee et al. 2013). Nevertheless, there are recent reports of detrimental functional effects of exogenous BDNF, in that increases in spasticity were seen in SCI rats after BDNF administration (Fouad, Bennett et al. 2013). Additionally, exogenous administration of BDNF must overcome difficulties related to access across the blood brain barrier, down-regulation of BDNF receptors, and triggering of immune responses (Banks and Erickson 2010; Toth, Veszelka et al. 2011; Satriotomo, Dale et al. 2012). Whether the elevated levels of endogenous BDNF associated with AIH treatment are having a negative impact in these spinal injured animals is not evident as it is associated with improved behavioral outcomes.

My findings support treatments that increase BDNF levels endogenously might be more effective methods by which to manipulate BDNF levels to facilitate functional recovery. This is also observed in other studies. For example, treadmill training in rats is one method which has been shown to reduce spasticity through a BDNF-dependent mechanism (Tashiro, Shinozaki et al. 2014). AIH also increases the endogenous expression of BDNF and its high affinity receptors in spinal motor neurons and this BDNF is sufficient and necessary to induce respiratory plasticity (Baker-Herman, Fuller et al. 2004). Various protocols of AIH, i.e. dAIH and rAIH (10 episodes per day; 5 min of hypoxia with 5 min normoxia 3 times exposure per week for 10 weeks) increased the expression of BDNF, trkB and ptrkB in the phrenic motor nuclei (Wilkerson and Mitchell 2009; Lovett-Barr, Satriotomo et al. 2012; Satriotomo, Dale et al. 2012). The current study extends these previous findings by examining BDNF expression at multiple levels of the spinal cord and after 1 and 7 days of treatment after AIH treatment. In addition, animals were treated with both AIH and motor training.

Motor training alone has the potential to increase BDNF and promote plasticity in the brain and spinal cord (Vaynman and Gomez-Pinilla 2005; Skup, Ziemlinska et al. 2014). It is well

documented that motor training following SCI enhances BDNF protein expression and this increase in BDNF expression is associated with locomotor functional recovery following SCI in animals (Ying, Roy et al. 2005; de Leon, See et al. 2011). Various paradigms of locomotor training, including voluntary wheel running and forced treadmill training, enhanced the expression of BDNF protein, BDNF mRNA in spinal motor neurons and/or in their peripheral target skeletal muscles in spinal injured rats (Gomez-Pinilla, Ying et al. 2002; Dupont-Versteegden, Houle et al. 2004; Hutchinson, Gomez-Pinilla et al. 2004; Macias, Nowicka et al. 2009; de Leon, See et al. 2011; Joseph, Tillakaratne et al. 2012; Keeler, Liu et al. 2012). Furthermore, weight-supported treadmill training, either 100 or 1000 steps/training session daily for 4 weeks, has been shown to increase the BDNF expression in ventral grey matter of SCI rats (de Leon, See et al. 2011). Of particular interest to the current study, four weeks of treadmill training initiated one month after injury increased BDNF expression in the lumber spinal motor neurons in spinally transected rats (Joseph, Tillakaratne et al. 2012). Our current results show that one week of ladder training alone, initiated 4 weeks after injury, is not sufficient to increase BDNF expression in spinal motoneurons in SCI rats, though BDNF levels do increase when combined with concomitant AIH treatment (Figs 4.11 – 4.14).

In summary, our results suggest that AIH + motor training may be an effective method to endogenously increase BDNF levels to improve functional recovery after SCI and avoid the potential detrimental effects of exogenous BDNF administration.

4.4.3 AIH and motor training enhance the spinal expression of trkB and ptrkB

The tyrosine kinase B receptors (trkB) are transmembrane proteins which belong to trk family of receptors for neurotrophins (Spencer-Segal, Waters et al. 2011). These receptors are located in dendritic spines, axons and neuronal cell bodies (Gomes, Hampton et al. 2006; Samarajeewa, Goldemann et al. 2014). BDNF-trkB receptor signaling plays important roles in neurodevelopment, neuroprotection, differentiation, proliferation, activation of synaptic proteins, dendritic arborisation and neural plasticity (Kang and Schuman 1995; Chao 2003; Jia, Chen et al. 2008; Luikart, Zhang et al. 2008; Ohira and Hayashi 2009; Fenner 2012; Ma, Savas et al. 2012; Guo, Ji et al. 2014).

Similar to other tyrosine receptors, trkB receptors are activated by ligand-induced formation of receptor dimers, whereupon the dimerized trkB receptors rapidly phosphorylate each other and activate signaling pathways (Schecterson and Bothwell 2010). BDNF can induce rapid phosphorylation of trkB receptors, regulated by cyclic AMP signaling (Ji, Pang et al. 2005; Samarajeewa, Goldemann et al. 2014). Further, phosphorylation of trkB plays a significant role in regulating the functional properties of trkB receptors and its downstream pathways (Babaei Bourojeni 2014).

The increased spinal expression of both trkB and ptrkB after AIH and motor training in SCI rats found in the current study strongly suggests that this combined treatment increases both receptor expression and activation of trkB signalling pathways. While this is consistent with increased BDNF expression described in the previous section, it is important to note that trkB signaling pathways can also be activated in the absence of the ligand BDNF, through a G-protein coupled receptor (GPCR) mechanism such as that involving the A2a adenosine receptor (Lee and Chao 2001; Golder, Ranganathan et al. 2008). Activation of Gs protein-coupled A2a receptor increased trkB expression in the spinal cord and also increased the transactivation of trkB (Wiese, Jablonka et al. 2007; Golder, Ranganathan et al. 2008). Activation of A2a receptors increased the synthesis of immature trkB protein and phosphorylation of trkB in rat cervical spinal cord (Golder, Ranganathan et al. 2008). While BDNF-activated trkB receptors are present in the plasma membrane, GPCR activation of trkB occurs intracellular, particularly in the Golgi apparatus (Chao 2003; Rajagopal, Chen et al. 2004).

Whereas it has been previously demonstrated that AIH exposure requires the activation of trkB receptors in spinal motor neurons to induce pLTF in phrenic nerve, it is also known that AIH increases both BDNF expression and extracellular adenosine levels in the spinal cord. (Baker-Herman, Fuller et al. 2004; Wilkerson and Mitchell 2009; Hoffman and Mitchell 2011; Lovett-Barr, Satriotomo et al. 2012; Satriotomo, Dale et al. 2012). This suggests that there are two possible mechanisms for activation and phosphorylation of trkB receptors that may contribute to enhancement of neuronal activity and respiratory plasticity. Although not investigated in the current study, it is possible that AIH and motor training in SCI rats increased the spinal

expression of trkB and ptrkB through both BDNF-dependent and BDNF-independent mechanisms.

4.4.4 Effect of AIH and motor training on glial cells following SCI

Astrocytes are multifunctional cells that become active in response to brain and spinal cord injury (Kang, Balordi et al. 2014; Lee and MacLean 2015). Astrocyte activation, termed astrogliosis, plays an important role in neurological disorders including trauma, infections, stroke, and neurodegeneration (Sofroniew and Vinters 2010; Kang, Balordi et al. 2014; Lee and MacLean 2015). Activated astrocytes express high level of intermediate filament known as GFAP and it is thought that excessive or sustained activation of astrocytes contribute to chronic inflammation and neural dysfunction and can be deleterious to functional recovery.

The current results demonstrate that AIH and motor training in general has no effect on the activation of glial cells in SCI rats, with the exception that 7 days of AIH and motor training reduced the activation of astrocytes in the cervical spinal cord. This is consistent with previous studies that have demonstrated that AIH treatment does not alter the protein expression of astrocytes and microglia in the brain. (Lovett-Barr, Satriotomo et al. 2012; Satriotomo, Dale et al. 2012). Furthermore, chronic IH treatment (10 episodes per day, 3 x per week for 4 weeks) initiated 3 days after experimental SCI suppressed the inflammatory response by reducing the expression of inflammatory genes below and above the injury (Small, Nikodemova et al. 2014). It is not clear from the current results why 7 days of AIH plus motor training would reduce GFAP expression in the cervical cord but not in the lumbar cord, then this may have something to do with proximity to the injury site.

Microglia cells are resident immune cells in central nervous system which play an important role in inflammation in the CNS (Perry and Teeling 2013). Microglia exist in resting states but can become activated in response to brain or spinal cord injury (Watanabe, Yamamoto et al. 1999; Loane and Byrnes 2010). Reactive microglia cells accumulate at the site of injury, where they engulf damaged tissue or cell debris and play a neuroprotective role (Fu, Shen et al. 2014). At the same time, pro-inflammatory factors continuously released by activated microglia have

detrimental effects on neuronal function and survival (Iwama, Sugimura et al. 2011; Fu, Shen et al. 2014). A number of neurological disorders such as Alzheimer's disease, multiple sclerosis, Parkinson's disease, traumatic brain injury, ischemic and other brain diseases are linked with chronic activation of microglial cells (Fu, Shen et al. 2014). In the present research, AIH treatment and motor training did not alter ED-1 protein expression and thus did not increase microglial activation, in spinal ventral grey matter at segments C6-7 or L4-5 (Figs 4.27-4.30). The low expression levels of ED-1 in at C6-7 and L4-5 prompted the examination of ED-1 expression near the spinal lesion site, to serve as a positive control. ED-1 immunoreactivity was detectable in the ventral grey matter of spinal segments C2-3, adjacent to the spinal lesion, at 5 weeks post-injury (Fig 4.31). Thus, activated microglia are still present at the lesion site 5 weeks post-injury but AIH and motor training did not increase the levels of ED-1 immunoreactivity in any region of the spinal cord.

In summary, in the light of previous studies and the outcome of present research, it is likely that AIH treatment does not increase and may, in some instances, suppress activation of glial cells. This in turn might help to mitigate the detrimental effects of inflammation in the CNS by maintaining the normal non-reactive state of astrocytes and microglia. Further research is necessary in future to examine the effect of AIH on activation of astroglia and microglia.

4.4.5 Conclusion

In the present study, I have shown that a low-dose protocol of AIH treatment when combined with motor training in SCI rats, produced temporal and spatial differential expression of hypoxia- and plasticity-related proteins in spinal motor neurons. The pattern of expression of these proteins is consistent with the possibility that these changes underlie some of the functional improvements shown in Chapter 4 of this thesis. These findings, taken together with the capacity of AIH and motor training to improve walking abilities in persons with chronic incomplete SCI, suggests that AIH has great potential as a safe and effective therapy to restore motor function after nervous system injury.

CHAPTER 5

GENERAL DISCUSSION

5.1 Summary of findings

This thesis examined the use of acute intermittent hypoxia (repetitive exposure to reduced oxygen levels for brief periods, AIH) to enhance spinal plasticity at system and cellular levels and improve forelimb functional in a rat model of experimental cervical spinal injury. In the first part of the study, I examined the effect of AIH on behavioural recovery in cervical SCI rats. I then examined the effect of AIH on the expression of hypoxia- and plasticity-related proteins in spinal motor neurons of cervical SCI rats. This chapter of my dissertation summarizes the findings of my research and discusses the results of the behavioural study (chapter 3rd) and cellular study (chapter 4th) in general; I will also discuss the potential therapeutic role of acute intermittent hypoxia and possible future applications of AIH.

5.1.1 Summary of behavioural study

In the third chapter of this thesis, I tested the hypothesis that **acute intermittent hypoxia improves forelimb function in a rat model of cervical spinal injury.** This study, conducted in a randomized, blinded, normoxia-controlled manner, showed that animals receiving AIH treatment for 7 days at 4 wks post-injury made fewer footslip errors during ladder crossing for up to 4 wks post-treatment compared to animals receiving normoxia-treatment. In this case treatment is delayed until 4 weeks after the injury, at a time when much spontaneous recovery had already occurred. This allowed us to assess the effect of AIH on residual functional deficits, in part to emulate the clinical situation more closely. This delay in the onset of treatment also revealed the variability in the amount of spontaneous recovery between animal subjects, requiring an extra methodological step in order to appropriately randomize treatment assignments. The results of the behavioural the study addressed the hypothesis from section 2.4, Specific Aim # 1:

1. Delayed AIH treatment for 7 days combined with ladder training improved skilled ladder locomotion in rats with incomplete cervical SCI.

2. Delayed treatment with AIH for 7 days without concomitant motor training did not improve skilled ladder locomotion in rats with incomplete cervical SCI.
3. Delayed treatment with AIH for 7 days did not improve reach-to-grasp success in SCI rats receiving task-specific motor training.

In summary, the main finding of the third chapter of this thesis was that that delayed treatment with AIH and task-specific training facilitated recovery on a ladder task in rats with cervical SCI, though the same treatment did not improve recovery on reach-to-grasp tasks. These findings, in conjunction with the successful use of AIH in a recent clinical study (Hayes, Jayaraman et al. 2014), point to the potential of AIH as an effective treatment to augment plasticity and improve functional recovery after SCI.

5.1.2 Summary of cellular study

The fourth chapter of this thesis examined the changes in protein expression in response to AIH treatment and motor training, specifically changes in plasticity- and hypoxia-related proteins (HIF-1α, VEGF, BDNF, trkB and ptrkB) in motor neurons of the spinal cord at different segmental levels in spinal-injured animals. The purpose of this research was to determine whether AIH-induced plasticity in spinal motor neurons was limited to a specific segment or region of spinal cord or could be induced in entire spinal cord. In this chapter I have tested the hypothesis that AIH alters the plasticity-related and hypoxia-related protein expression in different regions of the spinal cord in a rat model of cervical spinal injury.

The results of the cellular study addressed the hypothesis from section 2.4, Specific Aim #2:

1. AIH treatment and motor training for either 1 or 7 days significantly increased hypoxia-associated HIF-1α protein expression in the spinal motor neurons of rats with incomplete cervical SCI. AIH treatment and motor training for 7 days significantly increased hypoxia-associated VEGF protein expression in the spinal motor neurons of rats with incomplete cervical SCI.

2. AIH treatment and motor training for 7 days significantly increased the expression of plasticity-related proteins BDNF, trkB and ptrkB in the spinal motor neurons in rats with incomplete cervical SCI.

In addition, I have found the:

1. AIH treatment and motor training for 7 days significantly decreased the expression of GFAP, a marker for activated astrocytes, in the ventral grey matter of C6-7 spinal segments in rats with incomplete cervical SCI.
2. AIH treatment and motor training for 1 or 7 days did not alter the expression of GFAP in ventral grey matter at L4-5 spinal segment in rats with incomplete cervical SCI.
3. AIH treatment and motor training for 1 or 7 days did not alter the expression of ED-1, a marker for microglia, at C6-7 and L4-5 spinal segments of rats with incomplete cervical SCI.

Taken together, these findings suggesting that AIH treatment and motor training for 7 days has the potential to induce plasticity at the cellular level by altering the expression of major plasticity- and hypoxia-related proteins at multiple spinal segmental levels in incomplete cervical SCI rats. In addition to this AIH treatment for 7 days does not alter the inflammatory response at C6-7 and L4-5 segments of spinal cord in rats with incomplete cervical SCI.

5.2 AIH induces plasticity and improves functional recovery in SCI rats

Intermittent hypoxia (IH) treatment has been a focus of research in the field of spinal plasticity for several decades (Navarrete-Opazo and Mitchell 2014). Various IH protocols from chronic IH to acute IH have been used to examine the effect of IH on physiological systems (Dale, Ben Mabrouk et al. 2014; Navarrete-Opazo and Mitchell 2014). AIH treatment has received considerable attention in recent years in the field of spinal plasticity (Navarrete-Opazo and Mitchell 2014). AIH is a novel non- invasive protocol which involves exposure to fewer hypoxic episodes compared with chronic protocols of IH. AIH can induce spinal plasticity by augmenting spared synaptic pathways in intact and spinal-injured animal models (Bach and Mitchell 1996;

Ling, Fuller et al. 2001; Fuller, Johnson et al. 2003; Golder and Mitchell 2005; Wilkerson and Mitchell 2009; Dale-Nagle, Hoffman et al. 2010). AIH induced spinal plasticity was initially investigated in the context of respiratory plasticity in spinal motor neurons. The most thoroughly studied model of AIH-induced plasticity is long-term facilitation (LTF), the strengthening of synapses onto respiratory motor neurons (Mitchell, Baker et al. 2001; MacFarlane and Mitchell 2008; Mahamed and Mitchell 2008). Physiologically, long-term facilitation may serve as a compensatory mechanism to stabilize the respiratory output following AIH (Wilkerson and Mitchell 2009).

In the current study, I have explored the effect of AIH on limb function in SCI rats. This is the first study to examine the effect of AIH on forelimb functional recovery in SCI rats by assessing performance on multiple behaviour tests in response to AIH alone or in combination with motor training i.e. ladder training and reaching task. I have demonstrated that AIH treatment for 7 days initiated 4 wks after incomplete cervical experimental SCI in laboratory rats, produce sustained improvement in forelimb performance on a ladder walking task when combined with daily ladder task training. These findings are consistent with a previous study from our laboratory that AIH elicits recovery of both respiratory and forelimb function in rodent models of incomplete cervical SCI (Lovett-Barr, Satriotomo et al. 2012). The outcome of the current study extended confidence in these earlier findings because in the current study I used randomized treatment assignments and blinded experimental design. Therefore the findings of both studies together strongly suggest that AIH, as a modest form of intermittent hypoxia can elicit beneficial effects when it combined with task-specific motor training.

Nevertheless, AIH treatment for 7 days did not significantly improve skilled reach-to-grasp task performance in cervical SCI rats. This may be due to the fact that multiple neural pathways are involved in the performance of ladder locomotion and AIH-induced plasticity in those multiple neural pathways likely contributed to improved ladder performance (Webb and Muir 2003; Muir, Webb et al. 2007). In contrast, the reach-to-grasp tasks are under the control of a limited number of spinal neural pathways and as such recovery requires plasticity in specific tracts (Whishaw, Gorny et al. 1998; Kanagal and Muir 2009; Hurd, Weishaupt et al. 2013). The smaller amount of

redundancy in the neural connections underlying reach-to-grasp movement might have contributed to lack of recovery on these tasks compared to ladder locomotion.

The lack of recovery of reach-to-grasp performance after 7 days of AIH and task training does not necessarily mean that AIH cannot facilitate recovery on these tasks. Results from Chapter 3 show a trend toward recovery on the single pellet reaching task, so it is possible that more than 7 days of AIH treatment might improve reach-to-grasp task performance in cervical SCI rats. Future experiments involving longer AIH treatments or a booster dose of AIH after 7 days of treatment i.e. every other week, might induce sufficient plasticity in neural pathways responsible for reaching movements to improve performance of reach-to-grasp task in SCI rats.

5.3 AIH improves motor functions in human patients with incomplete SCI

A variety of strategies have been used to treat experimental SCI, either alone or in combination (Kwon, Okon et al. 2011; Onifer, Smith et al. 2011; Tohda and Kuboyama 2011). Unfortunately, not many of these experimental strategies have been translated to the clinic. AIH is a novel non-invasive treatment strategy that is now advancing towards clinical application due to its therapeutic potential (Fields and Mitchell 2015). The first study to use AIH as a therapy for SCI reported that a single AIH exposure increased the ankle strength and plantar flexor torque in human patients with incomplete chronic spinal cord injury (Trumbower, Jayaraman et al. 2012). More recently, AIH treatment improved over-ground walking and endurance in persons with chronic incomplete SCI, and the impact of AIH treatment was enhanced when AIH treatment was combined with walking practice (Hayes, Jayaraman et al. 2014). Walking distance was increased more than 37% with combined AIH + walking practice (Hayes, Jayaraman et al. 2014).

The findings of the current study that footslip performance on a ladder walking task significantly improved with combined AIH treatment and ladder training in cervical SCI rats is consistent with the clinical study conducted with human subjects with chronic incomplete SCI (Hayes, Jayaraman et al. 2014). The treatment paradigm used in the current study on rats with cervical SCI is only slightly different from the clinical study. Human patients with SCI received AIH

treatment for 5 days, each day consisting of 15 AIH episodes of 9% O_2 for 90 seconds alternating with 60 seconds 21% O_2, and training occurred 1 hour after treatment. The paradigm used in present study consist of AIH treatment for 7 days, consisting of 10 episodes of 11% O_2 for 5 minutes alternating with 5 minutes of 21% O_2, with locomotor training in form of ladder task also carried out 1 hour following AIH treatment. All together, the evidence shows that AIH is a moderate form of intermittent hypoxia that has therapeutic potential to induce spinal plasticity and improve functional recovery, suggesting that AIH treatment with combinatorial therapies may promote greater functional recovery following SCI.

5.4 The role of BDNF and trkB signaling in spinal plasticity

Brain-derived neurotrophic factor (BDNF) plays an important role in modulating neural plasticity and enhancing functional recovery following SCI (Hiebert, Khodarahmi et al. 2002; Vavrek, Girgis et al. 2006; Waterhouse and Xu 2009; Ma, Wang et al. 2011; Nagahara and Tuszynski 2011; Park and Poo 2013; Liao, Bouyer et al. 2015). BDNF mediates signaling through its high affinity receptor tyrosine kinase receptor B (trkB) (Weishaupt, Blesch et al. 2012; Mantilla, Gransee et al. 2013). The findings of Chapter 4, that AIH treatment + training for 7 days enhances the expression of BDNF, trkB and ptrkB in spinal motor neurons at C6-7 and L4-5 spinal levels is consistent with published findings that AIH induces neurochemical changes in expression levels of. BDNF and trkB in spinal segments C4-5 (Lovett-Barr, Satriotomo et al. 2012; Satriotomo, Dale et al. 2012). These plasticity-related proteins may contribute to spinal plasticity following AIH. The fact that AIH elicits similar neurochemical changes within respiratory and non-respiratory motor nuclei within the spinal cord suggests that similar mechanisms might contribute to AIH-induced recovery of both respiratory and forelimb motor functions in SCI animals.

Previous studies have reported that SCI elevates the spinal expression of neurotrophins including BDNF, and that this increase in BDNF levels may contribute to spontaneous recovery in animal models of SCI (Dougherty, Dreyfus et al. 2000; King, Bradbury et al. 2000; Widenfalk, Lundstromer et al. 2001; Mantilla, Gransee et al. 2013). It is well documented that BDNF plays an important role in plasticity and improves functional recovery following SCI in experimental

animals (Joseph, Tillakaratne et al. 2012; Weishaupt, Blesch et al. 2012; Mantilla, Gransee et al. 2013; Dale, Ben Mabrouk et al. 2014; Gransee, Zhan et al. 2015). BDNF induces neuroplasticity through multiple mechanisms, including changes in excitability of spinal neurons, enhancing synaptic output, strengthening the existing synaptic connections, increasing new synaptic connections by axonal sprouting (Mitchell, Baker et al. 2001; Ying, Roy et al. 2005; Sasaki, Radtke et al. 2009; Boyce, Park et al. 2012; Weishaupt, Blesch et al. 2012; Gransee, Zhan et al. 2013; Mantilla, Gransee et al. 2013).

BDNF availability at the site of injury can be increased by two methods; 1) exogenous BDNF delivery to the site of injury, such as intrathecal injection, 2) endogenous mechanisms to increase local productivity or synthesis of BDNF, such as neural activity including AIH. Exogenous application of BDNF by intrathecal injection increases the BDNF and trkB signaling at the level of phrenic motor neuron and enhances functional recovery of rhythmic diaphragm activity in cervical SCI rats (Mantilla, Gransee et al. 2013). Exogenous application of BDNF to the lesion site promote hindlimb locomotor functional recovery in rats with complete thoracic spinal cord transection injuries (Boyce, Park et al. 2012). Furthermore transplantation of BDNF-secreting fibroblast cells to the site of injury in rat model of SCI promotes regeneration of rubrospinal tract (RST) axon through and around the graft and also improves the functional recovery of forelimb in rat model of SCI(Liu, Kim et al. 1999; Jin, Fischer et al. 2002). In addition to the beneficial effects of exogenous application of BDNF, there are several complications and difficulties linked with exogenous applications of BDNF, including inability of BDNF to cross the blood brain barrier, down-regulation of BDNF receptors, and immune responses triggered by delivery of foreign proteins (Banks and Erickson 2010; Toth, Veszelka et al. 2011; Satriotomo, Dale et al. 2012). In addition, adverse effects such as increased pain and muscle spasticity are also linked with exogenous BDNF application to treat SCI in experimental models (Groth and Aanonsen 2002; Coull, Beggs et al. 2005; Fouad, Bennett et al. 2013; Gransee, Zhan et al. 2015). Thus, exogenous BDNF application will not be a viable therapeutic strategy unless these adverse effects can be addressed.

In contrast to exogenous administration of BDNF, methods to enhance endogenous BDNF levels have shown more promise for promotion of beneficial effects after SCI. Exercise, electrical

stimulation and, as reported in this thesis, dAIH have all been demonstrated to increase endogenous levels of BDNF (Baker-Herman, Fuller et al. 2004; Wilkerson and Mitchell 2009; Carmel, Berrol et al. 2010; Fritsch, Reis et al. 2010; Joseph, Tillakaratne et al. 2012; Tashiro, Shinozaki et al. 2014). With respect to dAIH, it is important to distinguish acute intermittent hypoxia protocols from chronic intermittent protocols (CIH), the latter of which can produce deleterious side effects unrelated to endogenous BDNF. These deleterious effects include: systemic hypertension, impaired baroreflex control, cognitive impairments metabolic syndrome, neuronal death in the hippocampus, and neurobehavioural dysfunction, impaired synaptic transmission in the nucleus of the solitary tract, neurodegeneration, oxidative stress, and pro-inflammatory responses (Fletcher, Lesske et al. 1992; Gu, Lin et al. 2007; Hambrecht, Vlisides et al. 2007; Kline, Ramirez-Navarro et al. 2007; Row 2007; Row, Kheirandish et al. 2007; Tasali and Ip 2008; Fava, Montagnana et al. 2011; Lurie 2011; Ramar and Caples 2011; Grigg-Damberger and Ralls 2012; Nanduri, Makarenko et al. 2012; Bucks, Olaithe et al. 2013). In contrast, AIH is a moderate form of intermittent hypoxia that has the potential to induce spinal plasticity and improve functional recovery without showing detrimental effects (Trumbower, Jayaraman et al. 2012; Hayes, Jayaraman et al. 2014).

Taken together, the evidence from this thesis and others strongly suggests that AIH can enhance endogenous BDNF and trkB signaling at multiple levels of the spinal cord and that this signalling might play an important role in the recovery of forelimb motor function in cervical SCI rats (Lovett-Barr, Satriotomo et al. 2012; Satriotomo, Dale et al. 2012; Hayes, Jayaraman et al. 2014). The use of AIH presents an exciting therapeutic option to potentially harness neural plasticity and improve functional recovery in persons with chronic SCI.

5.5 Potential cellular and molecular mechanisms of AIH-induced plasticity

To understand the possible mechanisms of AIH-induced plasticity at the cellular level, it is necessary to understand the cellular response to hypoxia. Cellular adaptation to changes in oxygen level is essential for maintenance and survival of cells in physiological and pathological states. Hypoxia is known to change cellular functions by altering the expression of hypoxia-associated proteins and their mRNAs, including HIF-1α and VEGF (Nordal, Nagy et al. 2004;

Lovett-Barr, Satriotomo et al. 2012; Satriotomo, Dale et al. 2012; Dale and Mitchell 2013). The findings of this thesis demonstrated that AIH enhanced the spinal expression of key molecules known to play important role in spinal plasticity, including HIF-1α, VEGF, BDNF, trkB and ptrkB, consistent with earlier findings (Ke and Costa 2006; Xiaowei, Ninghui et al. 2006; Lovett-Barr, Satriotomo et al. 2012; Satriotomo, Dale et al. 2012; Dale and Mitchell 2013).

Cellular and molecular mechanisms of AIH-induced plasticity in respiratory motor neurons are well documented (Dale-Nagle, Hoffman et al. 2010; Dale, Ben Mabrouk et al. 2014). Ongoing studies have revealed that multiple converging cellular pathways, named the Q, S, V, and E pathways, are involved to induce spinal plasticity in response to AIH (Dale-Nagle, Hoffman et al. 2010; Dale-Nagle, Satriotomo et al. 2011; Dale, Satriotomo et al. 2012; Dale, Ben Mabrouk et al. 2014). Several of these pathways require BDNF synthesis and / or activation of its high affinity receptor trkB (Vinit, Lovett-Barr et al. 2009; Dale-Nagle, Hoffman et al. 2011; Dale, Ben Mabrouk et al. 2014).

The first and most thoroughly studied pathway, known as the Q pathway, is serotonin-dependent. The term Q pathway refers to the involvement of G_q protein-coupled metabotropic 5-HT_{2a} receptors (Vinit, Lovett-Barr et al. 2009; Dale-Nagle, Hoffman et al. 2011; Dale, Ben Mabrouk et al. 2014). AIH treatment triggers the episodic release of serotonin in the vicinity of phrenic motor neurons in the spinal cord, thereby activating the serotonin receptor 5-HT_{2a}, which, via a PKC pathway, results in increased synthesis of new BDNF. This BDNF through its high affinity receptor trkB on the same or adjacent neurons, initiates a cascade of signalling through ERK and MAP kinase pathways (Baker-Herman and Mitchell 2002; Hoffman and Mitchell 2011). The end result is a form of plasticity known as pLTF, the increase in the output of phrenic motoneurons (Vinit, Lovett-Barr et al. 2009; Wilkerson and Mitchell 2009; Dale-Nagle, Hoffman et al. 2010).

A second cellular pathway, known as the "S Pathway," induces serotonin-independent respiratory plasticity. This pathway does not require synthesis of new BDNF protein, but does require synthesis of new immature trkB receptor isoform, and phosphoinositide 3 (PI3) kinase / protein kinase B signaling (Golder, Ranganathan et al. 2008; Hoffman and Mitchell 2011).

Activation of either Q or S pathways is known to induce respiratory spinal plasticity resulting in LTF (Dale-Nagle, Hoffman et al. 2011; Dale-Nagle, Satriotomo et al. 2011; Dale, Satriotomo et al. 2012; Dale and Mitchell 2013). The BDNF and / or trkB signaling system is the centre of both pathways and also plays a critical role in multiple forms of spinal plasticity (Baker-Herman, Fuller et al. 2004; Dale-Nagle, Hoffman et al. 2010). In this thesis, I have found that AIH treatment and training enhances the expression of BDNF, trkB, ptrkB, in motor neurons at multiple spinal segments, and so it is possible that activation of the Q or S pathway might underlie improvements in motor performance in AIH-treated SCI rats.

This thesis also demonstrated that AIH treatment altered the expression of hypoxia-related proteins, HIF-1α and VEGF. Hypoxia-Inducible Factor-1α (HIF-1α) is a heterodimeric master transcriptional regulator of genes that controls a number of adaptive responses to low oxygen tension in order to maintain oxygen homeostasis in mammalian cells. The HIF-1 protein is a heterodimer and composed of two subunits: the HIF-1α subunit and the constitutively expressed HIF-1β subunit (Wang, Jiang et al. 1995). HIF-1α is oxygen sensitive and is stabilized and activated under hypoxia condition and degraded in normoxia condition by proteasomes (Rosenstein and Krum 2004; Xiaowei, Ninghui et al. 2006). In hypoxia conditions, HIF-1α translocates from the cytoplasm to the nucleus and dimerizes with HIF-1β subunit to form the active HIF heterodimer complex (Lando, Peet et al. 2002). This dimer complex binds with hypoxia response elements (HRE) in target genes to induce gene expression (Lando, Peet et al. 2002).

HIF-1 binds to promoter/enhancer elements and regulates the transcription of hypoxia-inducible target genes expression of several dozen target gene including VEGF, EPO, inducible nitric oxide synthase (iNOS), heme oxygenase-1 (Semenza, Nejfelt et al. 1991; Melillo, Musso et al. 1995; Stein, Neeman et al. 1995; Lee, Jiang et al. 1997; Kimura, Weisz et al. 2000; Ke and Costa 2006; Xiaowei, Ninghui et al. 2006; Xiong, Mahmood et al. 2010).

HIF-1α regulates the expression of vascular endothelial growth factor (VEGF). VEGF is a 45 Da dimeric glycoprotein and a fundamental regulator of pathological and physiological

angiogenesis (Rosenstein and Krum 2004). VEGF promotes endothelial cell formation and proliferation in several organ systems during embryonic development and after injury in various type of tissues, including the central nervous system (Skold, Cullheim et al. 2000). VEGF is critical for blood vessel growth in the developing and adult nervous system of vertebrates (Mackenzie and Ruhrberg 2012). Apart from its role in angiogenesis, VEGF appears to play neurotrophic and neuroprotective roles in spinal cord and brain injury (Facchiano, Fernandez et al. 2002) (Krum and Rosenstein 1998; Svensson, Peters et al. 2002).

VEGF and its high affinity receptors VEGFR-2 both are expressed in phrenic motor neurons (Dale-Nagle, Satriotomo et al. 2011; Satriotomo, Dale et al. 2012). AIH is also able to increase VEGF expression and mediate respiratory spinal plasticity through activation of the VEGF receptors VEGFR-2. The intracellular signalling pathways ERK and Akt are involved in this "V" pathway, activation of which will induces spinal plasticity (Dale-Nagle, Satriotomo et al. 2011; Sato, Morimoto et al. 2012; Satriotomo, Dale et al. 2012).

In addition to VEGF, HIF-1α also regulates the expression of erythropoietin factor (EPO) (Wang, Jiang et al. 1995; Wang and Semenza 1995). EPO and its receptors (EPO-R) are expressed in the brain but also in spinal motor neurons (Celik, Gokmen et al. 2002; Iwasaki, Ikeda et al. 2002; Mennini, De Paola et al. 2006; Dale, Ben Mabrouk et al. 2014). AIH increases the expression of EPO and its receptors EPO-R in phrenic motor neurons (Dale, Satriotomo et al. 2012). Through its receptor EPO-R, EPO initiates a signaling cascade via ERK and Akt activation and induces a form of respiratory plasticity similar to BDNF / trkB and VEGF (Dale, Satriotomo et al. 2012; Dale, Ben Mabrouk et al. 2014). This last pathway also known as the "E Pathway".

The final outcome of all of these cellular pathways, Q, S, V and E pathways is hypothesized to be the phosphorylation and/or insertion of glutamate receptors at the synaptic sites between pre-motor and motor neurons (Fuller, Bach et al. 2000; Mahamed and Mitchell 2007; McGuire, Liu et al. 2008; Dale-Nagle, Hoffman et al. 2011). AIH could thus change the excitability of motor neurons by increasing the strength of the synaptic connections between motoneurons and their premotor inputs. In this manner, all 4 cellular pathways which can produce pLTF have the

potential to induce functional recovery following SCI (Dale-Nagle, Hoffman et al. 2011; Dale-Nagle, Satriotomo et al. 2011; Dale, Satriotomo et al. 2012; Dale and Mitchell 2013).

While this thesis has not addressed in detail the cellular or molecular mechanisms that might underlie AIH-induced improvements in ladder locomotion in SCI rats, the results shown Chapter 4 suggest that at least the Q and V pathways and their downstream effectors might be involved to increase motoneuron output and improve recovery after SCI.

5.6 AIH and brain plasticity

Long term potentiation (LTP), the most widely studied form of synaptic plasticity, is a long lasting increase in synaptic efficacy between neurons that are activated simultaneously, and it forms the basis of learning and memory (Minichiello 2009). Repetitive activation of excitatory synapses in the hippocampus causes an increase in synaptic strength that could last for hours or even days (Bliss and Gardner-Medwin 1973; Bliss and Lomo 1973). Previous studies in rodents and higher primates, including humans, showed that the hippocampus is a critical component in various forms of long-term memory (Zola-Morgan and Squire 1993; Alvarez, Zola-Morgan et al. 1995; Nadel 1995). Furthermore, LTP provides an important insight into molecular mechanisms by which memories are formed (Eichenbaum 1995; Ajay and Bhalla 2006).

The hippocampus is the region in the brain where LTP has been most thoroughly studied. It is known that hippocampal BDNF plays an important role in both LTP and learning and memory formation (Minichiello, Korte et al. 1999). BDNF is expressed in many areas of CNS including the hippocampus where it is involved in both learning and memory formation (Lee, Everitt et al. 2004; Li, Peng et al. 2005; Minichiello 2009). Numerous studies have shown that endogenous BDNF, through its high affinity receptor trkB, plays a crucial role in the mediation and regulation of LTP in the hippocampus (Korte, Carroll et al. 1995; Chen, Kolbeck et al. 1999; Minichiello, Korte et al. 1999; Lu, Christian et al. 2008; Cowansage, LeDoux et al. 2010; Park and Poo 2013). Furthermore, blockade of endogenous BDNF-trkB signaling completely abolished LTP induction in *Xenopus laevis* retinotectal synapses (Du, Wei et al. 2009). Blockade of endogenous BDNF or its receptor trkB signaling by genetic mutation or by application of

specific monoclonal antibodies against BDNF or trkB–IgG protein, impaired LTP induction, demonstrating that secreted BDNF and trkB signaling is important for the induction of LTP (Korte, Carroll et al. 1995; Figurov, Pozzo-Miller et al. 1996; Kang, Welcher et al. 1997; Chen, Kolbeck et al. 1999). Exogenous application of BDNF or virus-mediated gene transfer of BDNF restored this impairment of LTP (Korte, Carroll et al. 1995; Patterson, Abel et al. 1996). Activity-based neural activities such as high-frequency stimulation (HFS) promotes synaptic activity and triggers the secretion of endogenous BDNF at synaptic hippocampal sites where it mediates LTP (Balkowiec and Katz 2000; Lu, Park et al. 2013).

An important avenue of research for the future would be an examination of the effect of AIH on hippocampal plasticity, including hippocampal LTP. The collective findings of the current thesis and previous work show that the expression of hypoxia and plasticity-related proteins, particularly VEGF, BDNF, trkB and ptrkB, are enhanced at multiple levels of the spinal cord in response to AIH treatment. AIH treatment exposes the whole body of the animal to low oxygen, including the brain, and so it is likely that AIH enhances the expression of hypoxia- and plasticity-associated proteins in the brain, including in the hippocampus. Multidisciplinary approaches could be used, including patch-clamp or intracellular recording in hippocampal cell culture or slice culture from animals treated either with AIH or normoxia. The relative expression of plasticity-associated proteins in hippocampus need to be examined in AIH and normoxia treated animals using immunofluorescence as described in Chapter 4 of this thesis for the spinal cord. The behavioural effect of AIH on learning and memory can be tested using running and swimming mazes. Studies such as these could open up new possibilities in the field of learning and memory research, including the examination of the effect of AIH as a novel therapy for learning and memory-related disorders.

5.7 Conclusion

AIH is a non-invasive treatment that can induce spinal plasticity by strengthening the spared synaptic pathways to respiratory and somatic motor neurons, and can also improve motor function following incomplete spinal cord injury in animals and in humans. AIH induction of plasticity and enhancement of recovery of respiratory and limb functions after SCI is associated

with up-regulation of hypoxia- and plasticity-related proteins, including BDNF and its high affinity trkB receptors, in motor neurons in ventral gray matter of spinal cord. These proteins also play important roles in the intra-cellular signalling pathways known to mediate increases in respiratory motoneuron output after AIH treatment in rats. In addition to altering protein expression at multiple levels of the spinal cord, it is likely that AIH affects plasticity-related protein expression in the brain and as such, could mediate changes in learning and memory processes.

5.8 Significance and Future Directions

The main aim of the proposed research was to investigate whether acute intermittent hypoxia can improve forelimb functional recovery in a rat model of spinal cord injury. The present research has used a multidisciplinary approach to begin to investigate the neural mechanisms that target spinal plasticity and offer a new basis for promoting significant recovery of forelimb function following cervical SCI. I have demonstrated the potential benefit of AIH training as a novel rehabilitation intervention for facilitating the restoration of forelimb function following cervical SCI in rats in safe and meaningful ways. The outcome of the present research will contribute to a growing body of work which aims to determine the optimum AIH dose to use in safe and significant ways to restore motor function in humans after incomplete spinal cord injury.

Future research in the area of AIH can follow three main directions. First, more research can be done on the clinical application of AIH in persons with SCI, such as determination of optimum doses for efficacy and safety and to combine AIH with other therapies. For example, AIH treatment can be used in combination with rehabilitation training or neuroprotective treatments, or with regenerative treatments which include application of neurotrophic factors, and cell transplantation to treat SCI. Second, more pre-clinical research needs to be carried out investigating the upstream and downstream effects of changes in BDNF-dependent and BDNF-independent mechanisms which underlie AIH-induced neural plasticity. Better understanding of the mechanisms by which AIH has its effects could lead to more refined or more efficacious treatment strategies. Finally, it is necessary to explore AIH-induced effects on brain plasticity,

including LTP, and the possible therapeutic applications which could be beneficial in a variety of nervous system disorders, including those related to learning and memory.

The above mentioned approaches to study the role and effects of this novel non-invasive treatment are yet to be explored and these possibilities open up promising potential therapies for a variety of disorders of the nervous system.

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