BEHAVIORAL AND ANATOMICAL PLASTICITY FOLLOWING LOW THORACIC HEMISECTION AND CHONDROITINASE ABC TREATMENT IN THE ADULT CAT: ASSESSMENTS OF LOCOMOTION AND THE COUGH REFLEX

By

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To everyone who supported me over the years including all of my family and friends.

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BEHAVIORAL AND ANATOMICAL PLASTICITY FOLLOWING LOW THORACIC HEMISECTION AND CHONDROITINASE ABC TREATMENT IN THE ADULT CAT: ASSESSMENTS OF LOCOMOTION AND THE COUGH REFLEX

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Chondroitin sulfate proteoglycans (CSPGs) are potent inhibitors of neuronal growth following spinal cord injury (SCI). Digestion of CSPGs with Chondroitinase ABC (Ch'ase ABC) has been shown to significantly decrease their inhibitory properties *in vitro* and enhance axonal growth and promote recovery of locomotion in rodent models and our feline model of SCI. This study assessed the effects of intraspinal Ch'ase ABC delivery following low thoracic hemisection in the adult cat across motor tasks that are mediated by diverse levels of the neural axis. Adult cats received a left spinal T10 hemisection (hx) alone, hx+vehicle control or hx+Ch'ase ABC repeatedly delivered to the lesion site via a port system. Motor performance was assessed pre and post-injury over the course of 20 weeks during basic as well as skilled locomotion and during coughing. Two weeks following the last behavioral data collection point, fluorogold (FG) was injected bilaterally into the spinal cord caudal to the T10 hx. Specific components of the gait cycle such as the step cycle duration, swing and stance duration, knee flexion during swing, and paw drag were differentially affected during bipedal treadmill and overground locomotion following injury. Ch'ase ABC treatment did not significally affect these characteristics assessed during bipedal treadmill or overground locomotion. Ch'ase ABC

treatment significantly improved skilled pegboard locomotion. Ch'ase ABC treated cats were able to place their affected hindlimbs on the pegboard earlier and more frequently than SCI-only cats and developed a unique hindlimb motor strategy that differed from the one used prior to injury. To determine if Ch'ase ABC has an effect across diverse motor systems, the cough reflex also was assessed. The general characteristics of cough were not affected by our lesion paradigm as no changes in the parameters evaluated were seen post-hemisection. However, Ch'ase ABC treated cats showed a significant increase in esophageal pressure amplitudes above pre-injury values. In addition to behavioral improvements and changes, Ch'ase ABC also enhanced axonal plasticity. pNF-H immunoreactivity below the injury site, as well as retrogradely labeled neurons in the contralateral red nucleus, were significantly greater in cats treated with Ch'ase ABC than in controls. The findings presented are the first to demonstrate that Ch'ase ABC treatment can enhance skilled locomotor behavior, axonal growth, and cough esophageal pressures within the same animals. They also are the first to show that Ch'ase ABC can promote plasticity of anatomical substrates likely to underlie improvements observed in recovery of locomotor behavior following SCI in a large translational animal model.

CHAPTER 1
BACKGROUND

Spinal Cord Injury

Demographics, Etiology, and Functional Outcomes

In the blink of an eye, an entire life can be changed. Precarious behavior or simply the normal, daily events of life such as driving a car, walking to work, or riding a horse can unpredictably result in a life altering injury that will affect every facet of daily living. Individuals in the United States that survive trauma to the spinal cord, approximately 12,000 new cases each year, endure both physical and psychological impairments. The approximate 250,000 individuals currently living in the United States with SCI also require rehabilitative care for the duration of their lives causing great personal and societal expense.

All demographics are at risk of sustaining a spinal cord injury (SCI), but primarily these injuries transpire among Caucasian, young adult, males. The majority of SCI cases are attributable to motor vehicle accidents (42%), falls (27.1%), acts of violence (15.3%), and recreational sporting activities (7.4%) (NSCISC, 2008). For centuries, the prognosis for survival or recovery was dismal. During World War I, it was estimated that 90% of individuals sustaining a SCI died within one year of the injury and only one percent survived more than 20 years (Grundy et al. 2002). Since then, improvements in medical and surgical care, rehabilitative sciences, and technological advances have resulted in improved survival rates, care, and general life expectancy.

The physical impact of a traumatic spinal cord injury is vast and multifaceted. The extent of sensorimotor loss and dysfunction is dependent upon the lesion site and breadth of damage. The injury may involve physical transection of the cord, blunt contusion or compression of the cord, or a stretch injury (Schwab and Bartholdi 1996). Therefore, the functional outcome for

each individual is unique and the extent of paralysis, pain, spasticity, and necessary rehabilitation and therapeutic intervention is highly specialized. Injuries to one of the eight cervical segments of the spinal cord can result in quadriplegia, also termed tetraplegia. Individuals with this type of injury would have motor and sensory deficits in both upper and lower extremities, as well as diaphragm, bowel, and bladder dysfunction. Injuries occurring in the thoracic, lumbar, or sacral regions of the spinal cord result in paraplegia. This injury type causes loss of motor function and sensation in the lower extremities and loss of other specific specialized functions corresponding to dermatome level.

The completeness of the injury is a strong indication of the severity of the injury, and has served as the basis of categorization of functional severity for clinicians using the American Spinal Injury Association (ASIA) impairment Scale. Conventionally, a 'complete' spinal cord injury entails having no voluntary motor or conscious sensory function below the level of the lesion. This definition is difficult in application since the majority of 'complete' injuries are functionally 'complete' but anatomically 'incomplete' leaving a slight rehabilitative substrate intact (Bunge et al. 1997). An individual with an 'incomplete' injury maintains some sensory or motor function below the injury site. Data from the Model Spinal Cord Injury System since 2000 indicates that the most frequent neurologic category at discharge is incomplete tetraplegia (34.1%), followed by complete paraplegia (23%), complete tetraplegia (18.3%) and incomplete paraplegia (18.5 %) (NSCISC 2008). Basic and clinical scientists are actively pursuing therapeutic and rehabilitative strategies that target anatomical, biological, and pathophysiological features of injury in order to facilitate functional recovery.

Acute, Secondary, and Chronic Damage

Trauma to the spinal cord is described as occurring in a three phase process consisting of acute, secondary, and chronic damage (Schwab and Bartholdi 1996; Tator 1998), which may

overlap in their temporal progression. The initial traumatic mechanical injury resulting from compression, contusion, laceration, or stretch to the nervous tissue acutely affects axons, neurons, and blood vessels in the spinal cord. Within seconds to minutes, the acute process causes hypotension, hemorrhage, ischemic cell death, disrupted blood supply and flow, edema, changes in electrolytes , and neurotransmitter accumulation (Sekhon and Fehlings 2001). Spinal shock is used to describe an individual directly following the acute phase of injury. They experience muscle paralysis, reduced tone, and loss of reflexes below the level of the injury (Hiersemenzel et al. 2000). The severity of these effects increases with greater damage (Ditunno et al. 2004). Spinal shock may cause the prognosis to appear initially as a complete injury, and it is not until this shock subsides that the true extent of damage can be assessed.

The secondary process encompasses a continued cascade of cellular and biochemical processes from the primary phase, such as edema, electrolyte changes, and necrosis. Novel processes also occur during the secondary phase such as formation of free radicals (Demopoulos et al. 1980), altered calcium, sodium, and potassium permeability (Young and Koreh 1986), lipid peroxidation and hydrolysis (Anderson et al. 1985; Hall and Springer 2004), and inflammation that cause continuing cellular damage and death for weeks after injury.

The chronic phase of injury sets in within a few months to years. The initial lesion is filled with a fluid filled cyst, filled with a glial scar, or the formation of both occurs. These lesion features may act as physical and chemical barriers to axonal regeneration. Oligodendrocyte death, and subsequent de-myelination of axons decrease axonal conduction capacity. De-myelination in combination with molecular changes in the surviving neurons and aberrant axonal sprouting can result in chronic pain, which has been examined in animal models of spinal cord injury (Hulsebosch 2002).

Limited Regenerative Capacity of the Mammalian Central Nervous System

Plasticity and Regeneration after CNS Damage

The minimal intrinsic capability within the spinal cord to repair itself following traumatic injury has been a compelling quandary for scientists and physicians dating back as early as the 16th century BC. Ancient Egyptian hieroglyphic medical papyri described fractures and dislocations of the neck vertebrae followed by paralysis and sensory deficits as "an ailment not to be treated" (reviewed by (Hughes 1988)). This dismal diagnosis exemplifies the long-standing ideological dogma that the spinal cord has a limited regenerative capacity following insult or injury. Nearly three centuries later, the Italian medical doctor Felice Fontana made numerous innovative observations regarding the structure and regeneration of nerves. He observed that following sectioning of the rabbit hypoglossal nerve, the nerve underwent an apparent repair process that he interpreted as a result of injured tissue reproduction (Fontana 1787). The study of the regenerative processes in the nervous system was heightened by the middle of the twentieth century with the emergence of highly specialized neuronal fixation and morphological staining techniques (reviewed by (Gorio 1993)).

Santiago Ramon y Cajal, highly regarded as the father of modern neuroscience and well known for his groundbreaking work on the structure of the nervous system and the introduction of the neuron theory, diligently investigated the degenerative and regenerative capabilities of the central nervous system (CNS). In his earlier work on degeneration and regeneration of the nervous system, Ramon y Cajal aligned with the pessimistic dogma that the mature CNS was embodied by an absolute architecture. He wrote that "In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated" (Cajal 1991). In somewhat juxtaposed opinion, he also observed and commented on the presence of vigorous but abortive attempts at regeneration due to a postulated lack of trophic support.

Ramon y Cajal noted that in order for damaged axons to recapitulate growth following damage, the scientific community "must give to the sprouts, by means of adequate alimentation, a vigorous capacity for growth; and, place in front of the disoriented nerve cones…specific orienting substances" (Cajal 1991). During subsequent decades, the viewpoint that the CNS had the capacity for sprouting of undamaged axons as well as regeneration of damaged ones began to be more universally accepted based on new discoveries in the field.

The proof that undamaged axons in the central nervous system had the capacity to generate sprouts and collateral projections was a ray of hope that the CNS was a plastic, malleable substrate capable of re-wiring in response to neuronal damage. Following partial denervation of the feline spinal cord, intraspinal axons formed new processes termed collaterals and the quantity of sprouting was proportional to the area of damage, the more damage the more sprouting (Liu and Chambers 1958). This research proved that undamaged axons may play an important role following CNS damage by altering their morphology in order to compensate for the environmental substrate surrounding them. Collateral sprouting of intact axons within the spinal cord may allow for adaptability and functional recovery following spinal damage.

Re-organization following CNS damage may occur by regeneration of originally cut fibers or by collateral sprouting by neighboring intact fibers. With the advent of the electron microscope came the ability to assess plastic or regenerative changes at the synapse level. Following selective lesions of one pathway of the septal nuclei, a region receiving afferent input from two distinct sources, the distribution of synaptic terminals of the surviving fiber pathway was rearranged (Raisman 1969). In the normal, uninjured adult rat brain, afferents from the medial forebrain bundle (MFB) form synapses on soma and dendrites, whereas the fimbrial fibers terminate solely onto dendrites. Following fimbrial lesions, the MFB synaptic terminals

maintain their existing synapses, as well as form additional synapses to occupy vacated sites. Following lesions of the MFB, the fibrial fibers made synaptic connections on the soma, sometimes positioning on a previously vacated synapse. These experiments further show that de-afferentation mediates the sprouting of intact fibers and that they make functional synaptic connection in response to the injury environment. This finding suggested that the synaptic connections in the adult mammalian system are far more plastic and malleable than previously believed. Interestingly, Raisman postulated that this rapid re-wiring of existing intact synaptic connections may halt stimulation of anatomical regeneration following injury in the CNS (Raisman 1969).

Many subsequent studies across multiple neuronal systems have demonstrated that collateral sprouting in the adult mammalian CNS can act as a compensatory response to spinal cord injury. Tract-tracing studies in adult monkeys after unilateral pyramidotomy or spinal cord hemisections suggested that there is collateral sprouting of corticospinal axons distal to the injury (Kucera and Wiesendanger 1985; Aoki et al. 1986). A sprouting response of the ventral corticospinal pathway following transection of the dorsal corticospinal tract at C3 in adult rats also was seen and correlated with improved forelimb function (Weidner et al. 2001). It has also been found that following thoracic lesion in adult rats, corticospinal axons can sprout at the cervical level to form a new relay circuitry onto propriospinal neurons, which seemed related to improved hindlimb function (Fouad et al. 2001b; Bareyre et al. 2004; Courtine et al. 2008).

Inhibitory Environment

CNS myelin and oligodendrocytes (Schwab and Caroni 1988), as well as elements of the lesion scar (Fitch and Silver 2008), are recognized as the primary growth inhibitory substrates to CNS axonal regeneration after injury. Research has focused on the identification of specific inhibitory components of CNS myelin and the scar that inhibit axonal growth. These

components may serve as potential therapeutic targets to increase the axonal growth capacity following injury.

Until the early 1980s, a prominent theory dominated the field of CNS regeneration. This theory, formulated by Ramon y Cajal, postulated that the limited regenerative capacity was due to a lack of growth stimulating factors existing in the adult CNS. Pioneering work in the laboratory of Martin Schwab tested this trophic factor hypothesis. Researchers co-cultured adult rat optic nerve and sciatic nerve explants with dissociated peripheral neurons in the presence of increased nerve growth factor (NGF) (Schwab and Thoenen 1985). Axon outgrowth was immense into the sciatic nerve cultures, whereas the optic nerve explants were non-permissive growth substrates despite the presence of the growth promoting NGF. Therefore, they deduced that the CNS tissue, not the PNS tissue, contained potent neurite growth inhibitory substrates (Schwab and Thoenen 1985). Oligodendrocytes and myelin were later proven to be two non-permissive substrates for neurite growth (Schwab and Caroni 1988). Confirming these findings, it was demonstated that axonal regenerative failure in the chick spinal cord corresponds with the onset of spinal myelination and that with experimental delay of myelination the permissive period for axonal re-growth was extended (Keirstead et al. 1992).

Researchers also theorized that the lack of regeneration in the CNS was due to the intrinsic inability of adult neurons to reactivate their growth program. This assumption was disproven by transplant studies of autologous peripheral nerve grafts into the adult rat brainstem and spinal cord (David and Aguayo 1981; Richardson et al. 1984), and into the hamster retina (Keirstead et al. 1989). These transplants induced in-growth of axons from different populations of CNS neurons and stimulated elongation over centimeters. These studies proved that the CNS

is capable of recapitulating a developmental state, and it is the inhibitory environmental surroundings following damage, not the neurons themselves, that halts the regenerative process.

Myelin Inhibitors of Axonal Regeneration

Axon growth inhibitors associated with myelin play an important role in the failure of regenerative axonal growth in the mammalian CNS following injury. Nogo is debatably viewed as the most potent myelin-derived neurite growth inhibitor of CNS regeneration. Nogo activity was originally characterized by Schwab and colleagues through myelin fractionation experiments in the adult CNS (Caroni and Schwab 1988b). The Nogo gene and receptor have since been cloned and this has aided in the understanding of how this molecule mediates neurite growth inhibition (Fournier et al. 2002). The monoclonal antibody IN-1, directed against Nogo A and recognizing both Nogo A and B, overcame oligodendrocyte-mediated inhibition of axonal growth *in vitro* (Caroni and Schwab 1988a). *In vivo*, IN-1 producing hybridoma cells transplanted into young rat cortex increased sprouting and long distance corticospinal tract axon regeneration in rats (Schnell and Schwab 1990). Behavioral tests in adult rats revealed that chronic exposure to IN-1 improved locomotor function as well as sensorimotor reflexes following bilateral transection of the dorsal columns and dorsal corticospinal tracts (Bregman et al. 1995; Fouad et al. 2001a). Similarly, in adult mice immunized with CNS myelin or spinal cord homogenate, transected corticospinal tract axons were capable of long distance axonal regeneration (Huang et al. 1999).

Nogo A, B, and C transcripts exist through alternative promoter usage and alternative splicing (Fournier et al. 2002). They all share a universal carboxy terminal domain with two transmembrane regions separated by a 66 amino acid segment (Nogo-66) (Fournier et al. 2002). Nogo-A has been shown to have more than one potent inhibitory domain. The amino terminal domain (Amino-Nogo) as well as the 66 amino acid stretch, Nogo-66, inhibit neurite extension

and may act in a synergistic fashion to inhibit neurite outgrowth in the injured CNS (Fournier et al. 2001). Human Amino-Nogo of Nogo-A inhibits cerebellar nerve growth in a dose-dependent fashion (Prinjha et al. 2000), and Nogo-66 demonstrated neurite inhibitory activity by causing the collapse of growth cones and decreased neurite extension on assays of E12 chick dorsal root ganglion (DRG) neurons (GrandPre et al. 2000). The receptor for the Nogo-66 protein, (NgR), also has been proven to function as a growth inhibitor through studies that show that there is a positive correlation between NgR expression level in chick DRG neurons and sensitivity to myelin inhibition (Fournier et al. 2001). Blockage of Nogo, NgR, as well as Nogo-66 interaction with NgR may prove to be strategies to improve axonal regeneration in the injured adult CNS.

Many other elements inhabit CNS myelin and inhibit neuronal regeneration, one such component is myelin-associated glycoprotein (MAG). MAG is solely found in myelin sheaths of oligodendrocytes and Schwann cells (Filbin 1995), and inhibits neurite outgrowth of adult CNS neurons in vitro (McKerracher et al. 1994; Mukhopadhyay et al. 1994). NgR may function as a receptor for MAG (Domeniconi et al. 2002; Liu et al. 2002a) as well as for another myelin inhibitory protein oligodendrocyte-myelin glycoprotein (OMgp), demonstrating that NgR may mediate the inhibitory effect of Nogo, MAG, and OMgp, the three main myelin proteins that inhibit axonal growth. Signaling pathways through these receptor interactions may provide a molecular target for therapeutic interventions to overcome the lack of CNS regeneration.

Glial Response to Injury

Traditionally, the scar formed at the lesion epicenter following spinal trauma is referred to as the ‘glial scar’. Infiltration of other cell types primarily fibroblasts also can occur, especially with disruption of the blood-brain-barrier and dura. In order to stay consistent with previous literature, this dissertation will also use the nomenclature term ‘glial scar’ to refer to the scar environment, but it should be noted that there are other cells that comprise this scar milieu.

Inhibitory cellular components

Central nervous system injures lead to a barrage of molecular and cellular interactions in a futile attempt to repair damaged tissue. The formation of the glial scar composed of astrocytes and connective tissue elements functions immediately to reestablish the cellular and chemical integrity of the damaged CNS tissue. Juxtaposed to the role of the glial scar preventing infection and further tissue damage, its formation also generates a physical barrier for axonal growth (Windle and Chambers 1950) and produces inhibitory molecules that create impediments to axonal regeneration through the injured CNS. The most compelling evidence that the glial scar environment was the major growth impediment to axonal regeneration following mammalian CNS injury was clearly demonstrated by Silver and colleagues. Their microinjects of adult DRG cells into young adult rat spinal cords showed that these cells were capable of extending axons over long distances in myelinated tracts until they reached the glial scar (Davies et al. 1999). The primary cell types involved with the inhibitory scar environment are astrocytes, microglia, and oligodendrocyte precursors, with some enlistment of meningeal and stem cells (Fawcett and Asher 1999). Each cell type is responsible for the up-regulation of particular molecules that inhibit axonal regeneration (Fawcett and Asher 1999). These cells are recruited in a highly spatial and temporal fashion corresponding to their particular function.

Microglia from the surrounding tissue and macrophages are the first responders following CNS damage. Microglia activate, divide, and migrate to the injury milieu following CNS damage (Kreutzberg 1996). Macrophages are recruited to the injury site if the blood-brain-barrier (BBB) is compromised (Kreutzberg 1996). Microglia have the full capacity, when stimulated in the CNS injury environment, to release inhibitory molecules such as free radicals, nitric oxide and arachidonic acid derivatives (Fawcett and Asher 1999), although conflicting experimental evidence has shown that they can produce neurotoxic or neuroprotective effects in

vivo (Streit 1996). Experiments that transplanted microglial cells into the injured spinal cord, including or not including astrocytes, demonstrated that microglia were capable of stimulating CNS regeneration (Rabchevsky and Streit 1997). Overall, the evidence is conflicting regarding the effects of microglia *in vitro* and in *vivo*. It is theorized that the microglial presence in the glial scar environment following CNS injury may facilitate growth as opposed to inhibiting axonal regeneration.

Oligodendrocytes myelinate and, therefore, insulate central nervous system axons. Following CNS injury, axons undergo a degradation process which results in myelin debris release into the injury environment. The specific inhibitory properties of the myelin-derived group of molecules, was elucidated earlier in this manuscript. Mature oligodendrocytes *in vitro* inhibit neurite outgrowth in a contact-dependent manner and cause filopodial growth cone collapse (Bandtlow et al. 1990). Oligodendrocytes in the adult CNS possess the previously mentioned molecules Nogo and MAG, which have been proven to inhibit neurite growth. Oligodendrocytes also express the extracellular matrix glycoprotein tenascin-R, a member of the bi-functional inhibitory and growth promoting tenascin family. This glycoprotein is multifunctional and can induce cellular adhesion, differentiation and enhancement of neurite outgrowth as well as inhibition and repulsion of axonal growth (Pesheva and Probstmeier 2000).

Oligodendrocyte precursor cells (OPCs) are another cell type that is recruited during the post-traumatic glial response in the CNS. Their identification is marked by staining with an antibody to the inhibitory proteoglycan NG2 and the presence of receptors for platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (Grimpe and Silver 2002). These cells have also been shown to express other inhibitory proteoglycans such as neurocan, phosphacan,

and versican (Fawcett and Asher 1999; Asher et al. 2000), and therefore contribute to the inhibitory CNS environment after injury

Reactive astrocytes are the major constituents of the glial scar. The glial scar predominantly consists of an intermeshed network of astrocyte cells that have become hypertrophic, are densely packed with minimal extracellular space , and are surrounded by extracellular matrix (Fawcett and Asher 1999). Hypertrophic astrocytes are marked by an increased expression of the glial fibrillary acidic protein (GFAP) (Eng et al. 1987). Numerous *in vitro* and *in vivo* experiments have proven that this astroglial environment is exceptionally inhibitory to axonal regeneration (Reier and Houle 1988; McKeon et al. 1991).

Tissue culture models of the glial scar have shown that astrocytes are inhibitory to regenerating axons from both peripheral and central neuronal cell types (Fawcett et al. 1989; McKeon et al. 1995). This inhibitory nature is most likely due to the direct injury altering the astrocyte function from permissive to inhibitory. *In vivo* micro-lesion studies in the adult rat brain that axotomized small numbers of axons but caused minimal damage to the astroglial framework showed that regeneration occurred at far distances to the original lesion, whereas the immediate vicinity of the injury environment became inhibitory to axonal growth (Davies et al. 1996). This experiment extablished that the CNS injury caused astrocytes to release chemorepulsive molecules that was inhibitory to axonal regeneration. Many studies have examined the secreted, cell surface, and extracellular matrix (ECM) molecules produced by reactive astrocytes that might play an inhibitory role in axonal regeneration (Eddleston and Mucke 1993). In particular, the ECM has been shown to contain a conglomerate of axonal growth-promoting and growth-inhibitory molecules whose presence determines the success or failure of CNS axonal regeneration.

Extracellular matrix (ECM) molecules

The failure to support axonal regeneration in the mammalian spinal cord has been highly correlated to the presence and up-regulation of inhibitory proteoglycans associated with glial architecture and the ECM. The field of extracellular matrix molecules has recently expanded, including families of highly conserved attractant and repulsive guidance molecules such as netrins (Yu and Bargmann 2001), semaphorins (Pasterkamp and Verhaagen 2001), ephrins (Klein 2001), tenascins (Joester and Faissner 2001), integrins, and a variety of matrix proteoglycans, (Yamaguchi 2001). Proteoglycans are found in the matrices of all tissues, including the brain. They are a complex of polysaccharides and protein with immensely rich and broad functions in the body (Rhodes and Fawcett 2004). Glycosaminoglycans (GAGs) are the polysaccharide side chain units on proteoglycans. Proteoglycans are formed from the covalent bonding of these GAG side chains to the core protein. There are more than 30 primary structures of proteoglycan core proteins, and their GAG side chain make-up has been characterized (Bandtlow and Zimmermann 2000). This elucidates the remarkable diversity of proteoglycans within the body's tissues. Chondroitin sulfate proteoglycans (CSPGs) are the main axon growth inhibitory molecule in the glial scar and have been shown to be the primary cause of failed axonal regeneration in the injured adult CNS (Silver and Miller 2004).

Chondroitin Sulfate Proteoglycans (CSPGs)

CSPGs consist of a diverse variety of core proteins that are covalently bonded to chondroitin sulfate glycosaminoglycan (CS-GAG) side chains (Hartmann and Maurer 2001). CSPGs can be grouped into four major categories: the family of lecticans including aggrecan, neurocan, versican, and brevican (Yamaguchi 2000); phosphacan and receptor protein-tyrosine phosphatase (Maurel et al. 1994); the small leucine-rich proteoglycans including decorin and biglycan

(Galtrey and Fawcett 2007); and other CSPGs including NG2 (Galtrey and Fawcett 2007). There is large variation in the number and position of GAG chains attached to the core proteins.

CS-GAGs are composed of disaccharide chains of glucuronic acid (GlcA), which are linked to the core protein by the enzyme xylosytransferase (Galtrey and Fawcett 2007). Along the sugar side chains, the presence of different disaccharide units results in the formation of specific structural motifs known as CS-A (alternative name for chondroitin-4-sulfate, 4S), CS-C (alternative name for Chondroin-6-sulfate, 6S), CS-D, and CS-E. The sulfation patterns, created by the chondroitin sulfotransferases, of each of these units affects the binding properties of the GAGs and thus the overall function of the CSPG (Properzi et al. 2003).

CSPGs are important in the development, preservation, and ageing of the normal CNS and are the most abundant proteoglycan found within the CNS (Bandtlow and Zimmermann 2000), and there is strong evidence that in the adult CNS they may be involved in the control of plasticity (Rhodes and Fawcett 2004). CSPGs can function during development as axonal guidance molecules. Areas rich in CSPGs are inhibitory to developing axons within the dorsal root entry zone (Pindzola et al. 1993), and the roof plate of the developing spinal cord (Snow et al. 1990b). Studies utilizing isolated embryonic chick dorsal root ganglia showed that CSPGs are inhibitory to neurite outgrowth *in vitro* (Snow et al. 1990a). It has been demonstrated *in vitro* that this CSPG mediated developmental growth inhibition is eliminated with the enzymatic removal of chondroin sulphate (Brittis et al. 1992). Many *in vitro* studies have established the inhibitory nature, during development and in the adult, of the CSPG glycosaminoglycan (GAG) side chains or of the core protein itself (Dou and Levine 1994; Yamada et al. 1997; Niederost et al. 1999; Schmalfeldt et al. 2000).

CSPGs are the primary axon growth inhibitory molecule within the glial scar following CNS injury and play a pivotal role in regenerative axon failure after injury (Silver and Miller 2004). An *ex vivo* model of the glial scar provided further evidence of the inhibitory nature of CSPGs following CNS injury. Nitrocellulose filters were implanted into the adult rat cerebral cortex and subsequently removed and used as a substrate for neurite growth in explant cultures. The astrogliotic scar present on the filters contained CSPGs and were not permissive substrates for neurite extension (McKeon et al. 1991). Expression of several CSPGs, including versican, neurocan, brevican, and NG2 have been shown to drastically increase after injury to the rat brain (Moon et al. 2002).

Degradation of CSPGs with Chondroitinase ABC (Ch'ase ABC)

The axonal growth inhibitory properties of CS-GAG chains led to the isolation of chondroitin sulphate-degrading enzymes purified from several species of bacteria (Makarem and Berk 1968; Yamagata et al. 1968; Michelacci and Dietrich 1975; Ke et al. 2005). The bacterial enzyme isolated from *Proteus vulgaris* degrades CS-A (4-S), CS-B (dermatan sulfate), and CS-C (6-S) and therefore is termed Chondroitinase ABC (Ch'ase ABC). Commercially available Ch'ase ABC has been important in studying the effects of CS-GAG degradation *in vitro* and *in vivo*. Ch'ase ABC has been the bacterial enzyme of choice for many studies investigating enhancement of axonal regeneration and plasticity following central nervous system (CNS) damage.

CSPGs in the normal developing embryo have been shown to limit axonal growth within typical boundary regions *in vitro*. Snow and colleagues grew isolated E9 chick DRGs on nitrocellulose-coated petri dishes containing stripes of alternating lanes of the inhibitory molecules KS/CS-PG and the growth promoting molecule laminin. DRGs extended neurites on laminin stripes but when they encountered the KS/CS-PG lanes, the neurites either aborted

growth or traveled along the perimeter of the KS/CS-PG lanes. Enzymatic removal of the CS-GAG chains with Ch'ase ABC *in vitro* significantly decreased neurite inhibition, and when the cultures were treated with Ch'ase ABC and keratanase neurite inhibition was completely abolished. Therefore it is the CS-GAG side chains, not the core proteins, which possess the neurite inhibitory activity (Snow et al. 1990b).

CSPGs also have been shown to be expressed in the mature CNS following injury (McKeon et al. 1991). McKeon and colleagues implanted nitrocellulose filters into the adult rat cortex and subsequently the gliotic scar tissue was explanted *in vitro* and used as a substrate for embryonic retinal ganglion cell growth. The injury induced CS-PGs inhibited neurite extension, consistent with the prior literature that these molecules contribute to the minimal regenerative ability following trauma. These explants were then treated with Ch'ase ABC, which lead to a significant increase in mean neurite length of embryonic retinal neurons over the explanted scar surface (McKeon et al. 1995)

The first *in vivo* evidence that enzymatic degradation of CS-GAGs with Ch'ase ABC alone could diminish the inhibitory milieu of the injured nervous system was achieved by Moon and colleagues in the rat. Following unilateral nigrostriatal tract axotomy and treatment with Ch'ase ABC, significantly more dopaminergic nigral axons grew through the injury site and back to their original target (ipsilateral striatum) as compared to control treated animals (Moon et al. 2001). These results demonstrated that Ch'ase ABC treatment can enhance CNS axonal regeneration in the adult rat nigrostriatal tract and that this means of degrading CS-GAGs may be equally as effective at stimulating growth following CNS damage in other models.

Bradbury and colleagues assessed the effects of Ch'ase ABC on corticospinal tract (CST) axons following a cervical dorsal crush spinal injury in the rat using anatomical,

electrophysiological and behavioral measurements (Bradbury et al. 2002). In their model, Ch'ase ABC promoted regeneration of CST axons at and below the level of the crush injury, and electrophysiological recordings showed that these regenerated axons established functional re-connectivity. Behavioral assessments during skilled grid and beam walking tests showed that Ch'ase ABC treatment improved forelimb placement accuracy, and footprint analyses showed that these animals had walking patterns near normative values unlike vehicle controls. Overall, Ch'ase ABC treatment promoted regeneration of CST axons and enhanced functional behavioral recovery. In the same lesion model, Barrit et al. showed that Ch'ase ABC treatment facilitated injured CST axons to sprout around the lesion as well as grow through the lesion environment. Intact serotonergic axons were also found to have sprouted ventral and caudal to the spinal injury and intact spinal afferents were also found to sprout caudal to the spinal lesion (Barritt et al. 2006). These studies showed that Ch'ase ABC can influence spinal plasticity following spinal cord injury and that compensatory sprouting of intact axonal tracts may be partly responsible for functional recovery after Ch'ase ABC treatment. Massey and colleagues also demonstrated a sprouting phenomena following cervical dorsal column transection. Following application of Ch'ase ABC into the cuneate nucleus of adult rats following partial denervation of forepaw dorsal column afferents, the remaining primary afferent terminals were able to collaterally sprout into denervated areas of the cuneate nucleus and this was directly linked to functional recovery (Massey et al. 2006).

Following low thoracic lateral hemisection, Yick and colleagues assessed the ability of Ch'ase ABC to promote axonal regeneration of Clarke's nucleus (CN) neurons into a peripheral nerve (PN) graft at the lesion site. Implantation of PN grafts alone or in combination with BDNF infusion did not stimulate regeneration of CN neurons, whereas Ch'ase ABC administration

promoted axonal growth of CN neurons into the PN grafts (Yick et al. 2000). Similarly, the same laboratory illustrated that application of Ch'ase ABC promotes the growth of CN neurons into the rostral spinal cord through the lesion scar environment without PN transplantation in both neonatal and adult rats (Yick et al. 2003).

Ch'ase ABC treatment has also been assessed following contusive spinal cord injury. Caggiano et al. demonstrated that following moderate or severe low thoracic contusion, both somatic and autonomic motor recovery was promoted with Ch'ase ABC administration as assessed by open field locomotor and bladder function tests but with no correlative anatomical assessments (Caggiano et al. 2005). More recently, Iseda and colleagues demonstrated that following a single high-dose treatment with Ch'ase ABC, CST axons grew around and caudal to the lesion site following low thoracic hemisection but not contusion injury (Iseda et al. 2008).

Cafferty et al. engineered transgenic mice that expressed Ch'ase ABC via the *gfap* promoter following rhizotomy, and these transgenic mice had significant sensory axon regeneration and functional behavioral recovery (Cafferty et al. 2007). Similarly, they also found that following partial deafferentation of the spinal cord, Ch'ase ABC treatment mediates a functional plasticity of spinal circuitry, and a correlated recovery of function via undamaged afferent contributions (Cafferty et al. 2008). These studies therefore provide evidence that Ch'ase ABC treatment can also restore sensory function following nervous system damage.

Overcoming the impediments to axonal growth following central nervous system is multifactorial, and therefore many studies have assessed combinatorial treatment with Ch'ase ABC and other therapies to promote axonal growth. Combined treatment with BDNF and Ch'ase ABC was found to synergistically promote retinal fiber sprouting after denervation of the adult rat superior colliculus (Tropea et al. 2003). Chau et al. grafted Schwann cell-seeded

channels into low thoracic spinal hemisections in adult rats and delivered Ch'aes ABC close to the graft-host interface. Previous research had shown that due to the CS-PG deposition at the caudal graft-host interface, axons are unable to exit the caudal graft and enter into the caudal host spinal cord (Xu et al. 1995). Chau and colleagues found that with Ch'ase ABC treatment, axons from the rostral cord were able to traverse through the graft and into the host spinal cord (Chau et al. 2004). In a low thoracic transection model in adult rats, Fouad and colleagues combined Ch'ase ABC infusion to diminish inhibitory CS-PGs, Schwann cell bridges to provide an axonal growth promoting substrate, and olfactory-ensheathing glia (OEG) to allow axons to exit the Schwann cell bridge and re-enter the spinal cord. Animals receiving this combinatorial strategy showed significant improvements in locomotor recovery as well as serotonergic fiber growth through the cellular bridge and into the caudal spinal cord tissue (Fouad et al. 2005). Following thoracic spinal contusion, Ikegami et al. combined Ch'ase ABC treatment with neural stem/progenitor cell transplantation, which enhanced transplant cell migration and increased growth of growth-associated protein-43 (GAP-43) positive fibers (Ikegami et al. 2005). Houle and colleagues used a peripheral nerve graft (PNG) to bypass a cervical hemisection and direct regrowing axons into the distal spinal cord. Ch'ase ABC treatment promoted significant axonal growth past the distal end of the PNG back into the spinal cord and a correlated with functional behavioral recovery of the affected limb. Transection of the PNG caused complete loss of behavioral gain, suggesting that regenerating axons aided in the behavioral recovery (Houle et al. 2006). All of these combinatorial strategies utilizing Ch'ase ABC application in concert with other regenerative strategies prove promising as possible therapies following CNS, and especial spinal cord injury.

Our laboratory has recently demonstrated, for the first time in a species other than the rodent, that degradation of CS-GAGs with the bacterial enzyme Ch'ase ABC enhances spinal plasticity as well as locomotor recovery in the adult cat model (Tester and Howland 2008). The basic and skilled locomotor recovery seen in these Ch'ase ABC treated cats was most likely mediated by plasticity of local spinal circuitry including the central pattern generator (CPG) and/or plasticity of descending spinal tracts involved with the control of locomotion such as the rubrospinal tract.

Motor Systems Affected by Spinal Cord Injury

Locomotion

The locomotor repertoire of vertebrates is intricate and precisely orchestrated to perform a broad range of functions; therefore its control systems are multifaceted. The general design of the locomotor control system has been conserved among vertebrates, from the lamprey to mammals including humans. Vertebrate locomotion is controlled by a hierarchical tripartite neural system comprised of a central pattern generator (CPG), afferent feedback, and supraspinal input. The CPG is at the core and is responsible for the basic locomotor pattern, which can be modulated by input from supraspinal centers and peripheral input (Goldberger 1988). There are functionally distinct CPGs for each limb and in combination they can produce different motor patterns requiring differing levels of interlimb coordination. The locomotor CPG provides the basic motor signals common to all forms of locomotion. Unique details necessary for different forms of locomotion, in contrast, depend on interactions among the tripartite system (Buford et al. 1990). Physiological and anatomical studies also have demonstrated the presence of propriospinal connections throughout the rostro-caudal extent of the mammalian spinal cord with ascending or descending fibers that are important for mediating reflex control and coordination during locomotion. The propriospinal system has varied projection patterns that can span

between multiple segments such as between cervical and lumbar levels, as well as projecting only a few segments (Miller et al. 1973; Conta and Stelzner 2004).

The caudal spinal cord of numerous vertebrate species contains the necessary pattern generating circuitry to create locomotor patterns (Grillner et al. 1981; Rossignol and Dubuc 1994; Rossignol et al. 1996). T13 spinalized cats (Barbeau and Rossignol 1987) and kittens (Forssberg et al. 1980a; Forssberg et al. 1980b; Howland et al. 1995a) can walk on a treadmill, with plantigrade stepping of the hindlimbs. Therefore, the isolated spinal cord without receiving descending control can still produce complex motor outputs.

In many vertebrates, even decerebrate animals, the CPG for locomotion can be activated by stimulation of particular brainstem regions such as the subthalamic nucleus, nucleus cuneiform, pons, and the pyramids (Selionov and Shik 1984). There are at least four locomotor regions located in the brainstem that when stimulated either electrically or chemically induce locomotion. The first is the mesencephalic locomotor region (MLR), an area in the caudal cuneiform nucleus of mammals that when stimulated initiates locomotion in decerebrate cats placed on a treadmill (Shik and Orlovsky 1976). Its neurons project to the second locomotor region, the medullary reticular formation (MRF) and subsequently to interneurons in the spinal cord descending via the ventrolateral funiculus (VLF) (Jordan 1998). The MRF region regulates and aids in initiation of stepping pattern as well as interlimb coordination (Whelan 1996) and electrical stimulation of the MRF can produce locomotion (Mori et al. 1978). The third region, just medial to the MLR, referred to as the medial MLR (mMLR) also has shown to initiates locomotion when electrically stimulated (Garcia-Rill et al. 1983). Axons of the mMLR pass through the fourth region, the pontomedullary locomotor strip (PLS), and continue on to the MRF. The PLS runs through the lateral tegmentum of the brainstem and continues in the spinal

cord in the dorsolateral funiculus (DLF) (Whelan 1996). Stimulation of the PLS, as well as the MRF, elicits locomotor bouts, but the behavior appears fragmented and spastic (Whelan 1996). These pathways descending to the spinal cord via the VLF and DLF contain the capacity to control spinal motor circuitry

Neurons originating from several areas of the brainstem have been found to contribute to the initiation and maintainance of locomotion (for review see (Fouad and Pearson 2004)). Descending pathways that influence motor activity can be grouped into two principle systems according to their medial or lateral position in the spinal cord (Drew et al. 2002). The medial system includes the reticulospinal and vestibulospinal tracts while the lateral system includes the corticospinal and rubrospinal pathways. Although these systems are all important in locomotor control, this section will focus on the contribution of the rubrospinal tract.

In the cat, the course of the rubrospinal tract has been well described to immediately decussate in the mesencephalon and send its crossed bundle of fibers into the lateral funiculus of the spinal cord where it lies just anterior to the lateral corticospinal tract (Verhaart 1953; Verhaart 1955; Pompeiano and Brodal 1957; Hinman and Carpenter 1959; Kuypers 1964; Nyberg-Hansen and Brodal 1964; Schoen 1964). The mammalian red nucleus (RN) can be divided into a caudal magnocellular region (RNm) and a rostral parvocellular region (RNp). The hindlimb region of the RNm is located ventral and ventrolateral and projects to the lumbar enlargement. Research by Orlovsky and colleagues undertaken in cats walking on a treadmill, elucidated that cells in the RNm display spike bursts during active limb movements (Orlovsky 1972a). In the majority of mammals, including cats and primates, the rubrospinal tract influences flexor activity of the forelimb and hindlimbs (Kuypers 1964) during the swing phase (Orlovsky 1972b) and helps coordinate the spatiotemporal muscle activity pattern of the limbs

(Lavoie and Drew 2002). Several studies suggest that the red nucleus plays a role during coordinated, multi-articular whole limb movements such as reaching (Gibson et al. 1985; Miller et al. 1993; Sinkjaer et al. 1995; van Kan and McCurdy 2001). Red nucleus neurons have also been shown to increase their discharge frequency during voluntary gait modifications such as negotiation of an obstacle (Drew 1993). They are also involved in the control of coordination during locomotion of intralimb as well interlimb activity, and regulate muscle activity during the transport and placement phases of the step cycle (Lavoie and Drew 2002). This descending system is essential for regulation of the locomotor cycle and damage to this tract following spinal cord injury causes behavioral deficits. It has been shown previously that following a cervical hemisection in the adult rat, RST axons approach the rostral lesion edge but do not have the capacity to spontaneously re-grow through or caudal to the lesion (Houle and Jin 2001), therefore numerous experimental strategies to specifically promote RST axonal growth have been conducted in animal models of spinal cord injury.

Cough Reflex

Spinal cord injured individuals sustain weakening or paralysis of musculature vital to respiratory functions including the cough reflex. Cough is a ballistic behavior that can be elicited experimentally with mechanical or chemical stimulation. A cough is elicited via stimulation of central airway receptors that project via vagal afferents to second order neurons (Bolser et al. 2000), which then project to populations in the brainstem involved with respiratory control (Ezure et al. 1991). Pre-motor excitatory input to spinal motoneurons originates from bulbospinal expiratory neurons in the nucleus retroambigualis in the medulla (Bolser et al. 2002).

Cough is produced by a complex neural network within the brainstem. This network controls motoneurons supplying laryngeal, phrenic, intercostal, and abdominal motor pools. These motoneuron pools are activated in a precise and sequential manner to produce ballistic-

like expiratory pressures that peak within 200 ms from the end of the inspiratory phase in the cat and are characterized by intrathoracic pressures that can exceed 100 cmH_20 (Bolser 2002). The expulsive component of this behavior is generated by a coordinated activation of chest wall and abdominal musculature to elicit a cough. It is this spatiotemporal coordination that makes cough particularly sensitive to functional impairment by insults such as spinal injury. The principal expiratory muscles responsible for expiratory cough pressures are the anterolateral abdominals (rectus abdominis, transversus abdominis, external oblique and internal oblique). The motoneuron pools for these four expiratory abdominal muscles terminate at L3, but they have varying rostral extents with the rectus abdominis extending most rostrally (T4); the external obliques to T6; the transverses abdominis to T9; and and the internal oblique to T13 (Miller et al. 1987) in the cat.

A spinal pattern generator has not been identified for the cough reflex, but much like certain forms of locomotion, there is descending input for this behavior. Premotor drive to these expiratory motoneuron pools arises primarily from bulbospinal expiratory neurons in the caudal part of the ventral respiratory column (cVRC), corresponding to the caudal portion of the nucleus retroambigualis (NRA). It has been shown in the cat, (Merrill 1970) that the axons of the expiratory cVRC neurons immediately decussate in the medulla (Monteau and Hilaire 1991) between C1 and the obex (Arita et al. 1987; Miller et al. 1987; Miller et al. 1989) and descend into the contralateral ventral column of the lateral spinal cord (Merrill 1970; Merrill 1974; Richter et al. 1975; Merrill and Lipski 1987; Jiang and Lipski 1990; Kirkwood 1995). Antidromic mapping studies of the descending respiratory pathways have shown that expiratory axons, at the cervical level, form a very discrete tract that courses through the ventral white matter immediately below the base of the ventral horn (Davis and Plum 1972; Merrill 1974).

These descending expiratory axons become more dispersed throughout the ventrolateral white matter with caudal progression through the thoracic cord (Merrill 1974). From T1-L3 expiratory axons arborize expansively throughout the contralateral side of the spinal cord, with respect to the cell body, creating an extensive network spanning several spinal segments (Merrill 1974).

This network of expiratory axons contributes to both monosynaptic and multisynaptic drive to thoracic and lumbar expiratory motoneuron pools. Kirkwood and coworkers (Kirkwood et al. 1988) proposed that spinal interneurons have an important role in mediating this descending drive. Many of the expiratory-associated interneurons they identified had crossed axons and some descended at least five segments. Kirkwood suggested that these crossed interneuronal connections might mediate heterogeneous functions including inhibition of inspiratory thoracic motoneurons during the expiratory phase of breathing and excitation of chest wall motoneurons. It is reasonable that these interneurons provide a mechanism by which drive from each cVRC is bilaterally represented in the spinal cord. Recently similar propriospinal connections in the thoracic spinal cord have been implicated as an indirect pathway likely to mediate spontaneous recovery of basic locomotor function following spinal hemisection (Courtine et al. 2008). However, whether or not the cough motor system shows similar spontaneous recovery or retained function following partial lesions of the thoracic spinal cord has not been assessed.

CHAPTER 2
COUGH FOLLOWING LOW THORACIC SPINAL HEMISECTION IN THE CAT

Introduction

The expulsive component of cough is generated primarily by the coordinated activity of the anterolateral abdominal muscles (rectus abdominis, transversus abdominis, external oblique, and internal oblique). Input from the brainstem cough neural networks (Merrill 1970; Merrill 1974; Richter et al. 1975; Arita et al. 1987; Merrill and Lipski 1987; Miller et al. 1987; Miller et al. 1989; Jiang and Lipski 1990; Monteau and Hilaire 1991; Davis 1993; Kirkwood 1995) to the motoneuron pools for these primary expiratory muscles is disrupted following cervical and thoracic injuries involving the ventral and ventrolateral spinal cord (Davis and Plum 1972; Merrill 1974). In the cat, the motoneuron pools for these four muscles all terminate at L3, but they have varying rostral extents with the rectus abdominis extending most rostrally (T4); the external oblique to T6; the transverses abdominis to T9; and the internal oblique to T13 (Miller 1987). Further, from T1-L3 expiratory axons originating in the brainstem arborize expansively throughout the contralateral side of the spinal cord, creating an extensive network spanning several spinal segments (Merrill 1974). Due to the extensive arborization of descending motor axons we hypothesized that T9/10 lateral hemisection would not cause significant disruption of rectus abdominis EMG activity bilaterally or cough pressure generation.

In the present study lateral T9/10 hemisections were made in adult cats. These lesions transect the descending brainstem expiratory pathways on one side of the spinal cord, disrupting pre-motor drive to the caudal, ipsilateral expiratory motoneuron pools. Cough pressure generation and rectus abdominis muscle activity were characterized pre- and post-injury. Expiratory muscle recordings were made from the rectus abdominis because it contributes to the generation of cough expulsive forces in the cat (Tomori and Widdicombe 1969), plays a

significant role in increasing abdominal cavity pressure during cough (Bolser et al. 2000), and is easily accessed for repeated assessments. Our findings show that, despite considerable disruption of descending pre-motor drive from the brainstem to motoneuron pools of the primary expiratory muscles, the cough motor system shows substantial function following thoracic spinal cord injury (SCI).

Methods

Cough production was assessed pre- and post-spinal T9/10 left hemisection in six specific-pathogen-free adult, spayed female cats (6-8 lbs, Liberty Laboratories, NY). Hemisections and post-op care were performed as described previously (Tester and Howland 2008). Surgeries were performed under isoflurane anesthesia. Buprenorphine (0.02mg/kg) was given TID for 48h, and bladders expressed manually for 1-5 days, post-SCI. Cats were housed on thick cushions in the AALAC accredited animal facility and trained on a variety of locomotor tasks (5x/week) for a parallel study. Animal procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the University of Florida's IACUC.

Cough was assessed in isoflurane anesthetized, spontaneously breathing cats pre-injury and at 4, 13 and 21 wks post-SCI. Atropine sulfate (0.1 mg/kg, SQ) was given to block salivation and tracheal secretions. End-tidal CO_2 was monitored and isoflurane levels adjusted to maintain this parameter within 4-5%. A sterile abdominal field was prepared and paired bipolar Teflon-coated stainless steel wire electrodes placed 2-3 mm apart in the left and right rectus abdominis muscles approximately 2 cm caudal to the iliac crest and 1 cm lateral to midline. A ground electrode was placed in the left hamstring. An esophageal balloon catheter was placed at the midthoracic level and cough elicited by mechanical stimulation of the vocal folds and

epiglottis using an oral approach and a small length of flexible plastic tubing (Bolser 1991; Bolser et al. 1993).

Esophageal pressure (Pes) and left and right rectus abdominis (LRA and RRA) electromyograms (EMGs) were recorded. Pes (cmH_2O) was used as a measure of intrathoracic pressure generation. EMGs were amplified, rectified, band-pass filtered (200-5000 Hz), and integrated (time constant 50 ms). Coughs were identified by behavioral observation of the animal and the presence of Pes amplitude larger than 5 cm H_2O in response to the mechanical stimulus. Six parameters were calculated: Pes, percentage of LRA and RRA normalized cough amplitudes, esophageal rise times, and LRA and RRA rise times. To obtain RA EMG normalized amplitudes, amplitudes were obtained from moving averages, normalized to the largest burst at a given time point for each side in each cat, and expressed as a percentage of the largest burst. These normalized percentages were averaged for each cat at each time point. Rise times were determined as the elapsed time between 10% and 90% of the total rise time of the moving average. Individual cough rise times for each cat were averaged at each time-point.

Using SPSS software 14.0 (Chicago, IL), separate repeated measures, within-subjects ANOVAs were conducted to determine if esophageal parameters (pressure and rise time) differed across time points. Mixed (time x side) two-factor ANOVAs were conducted to determine if there was an effect of time or side on rectus abdominis EMG rise times and normalized percent amplitudes. Post-hoc Fishers LSD tests were used to isolate any differences identified with ANOVAs. An α level of 0.05 was used for all analyses.

Between 4 and 6 months post-SCI, cats were deeply anesthetized (sodium pentobarbital, >35 mg/kg I.P.) and transcardially perfused with 0.9% saline (200-400 mls) followed by 4% paraformaldehyde in 0.1M phosphate buffer (3.5L, pH 7.4). Tissue was blocked, cryoprotected

in 30% sucrose-paraformaldehyde, and sectioned at 25um on a cryostat. Four lesion segments were cut coronally and two longitudinally. One section of every ten was mounted onto chrom alum and poly-L-lysine-coated slides (chromium potassium sulfate and poly-L-lysine, Sigma-Aldrich, St. Louis, MO; gelatin, Fisher Scientific, Hampton, NH) and processed with cresyl violet (cresyl violet with acetate, Sigma Aldrich, St. Louis MO) and myelin (Eriochrome Cyanine R, Fluka, New York, NY) stains for basic histology to determine the extent of injury following procedures detailed previously (Tester and Howland 2008).

Results

A total of 256 coughs from six cats were analyzed across four time points: pre-injury (n=37); 4 weeks post-hemisection (wphx, n=87); 13 wphx (n=75); and 21 wphx (n=57). Cresyl violet and myelin stained serial sections of each lesion were assessed to determine the extent of SCIs. The lesions ranged from an under-hemisection with ipsilateral medial-ventral white matter sparing to a complete hemisection to an over-hemisection with disruption of contralateral gray and white matter (Figure 2-1). The expiratory pressures and bilateral RA EMGs features assessed in this study were not influenced by these injury magnitude differences.

All cats generated coughs pre- and post-injury under anesthesia in response to mechanical stimulation of the epiglottis and vocal folds. As assessed by EMGs, the RA muscles were normally silent during eupneic breathing. RA EMGs and Pes increased during mechanically-elicited cough. Individual coughs, as well as repetitive cough bouts, were frequently generated after injury (Figure 2-2). Pes and RA EMGs during coughing were similar to pre-injury recordings. The EMG patterns at all post-injury time points were typical of ballistic-like bursting observed in uninjured animals (Bolser et al. 2000) (Figure 2-2). Moreover, they were present bilaterally. Finally, the peak EMG activities of the LRA and RRA during cough

occurred simultaneously and were correlated with increases in Pes. Thus, qualitatively, these general cough characteristics appeared similar to those observed prior to injury.

Increases in Pes with cough averaged between 40 and 69 cm H_2O at all time points and no significant change in the average Pes was seen across time points (p=0.410). In addition, during some individual coughs post-operatively, Pes reached or exceeded 100cm H_2O indicating that the injured system was capable of generating the substantial pressures sometimes seen pre-injury, as well as in other reports of normal cats (Bolser et al. 2000). A significant effect of time on Pes rise time was found, F (3, 12) = 4.29, p = 0.028. However, post-hoc Fisher's LSD tests did not reveal significant differences between any time points. Despite this, a notable prolonged Pes rise time was present at 13 weeks post-injury during some coughs. When this prolonged rise time occurred, it was manifested without a change in the magnitude of mechanical Pes (Figure 2-2). Average rise times were 0.089 (pre-SCI), 0.087 (4 wphx), 0.12 (13 wphx) and 0.082 seconds (21 wphx).

Normalized EMG amplitudes during cough showed that both the LRA and RRA averaged between 60-72 and 61-79 percent of maximum respectively across time points. A two-factor ANOVA revealed no significant change in the average percent of maximum amplitude over time (p=0.67) or between the left and right RA muscles (p=0.587). As with LRA and RRA normalized amplitudes, no significant effects of time (p=0.183), side (0.136) or the interaction of time by side (0.690) were seen for EMG rise times.

Rectus abdominis EMG activity was observed during the inspiratory phase of coughing in most animals at all time points (Figure 2-2). This muscle activity was termed pre-expulsive because it occurred during the inspiratory phase and before the expulsive cough phase. There was no apparent change in pre-expulsive activity in the RA EMG at any post-injury time point

compared to pre-injury values and the duration of this pre-expulsive activity was similar to the 600-700 ms range reported by Bolser and colleagues (Bolser et al. 2000).

Discussion

The regenerative capacity of the central nervous system is limited (Steward et al. 2008). However, following incomplete spinal lesions in humans (Dietz et al. 1998; Dobkin et al. 2007; Fawcett et al. 2007) and animals (Rossignol et al. 1999; Weidner et al. 2001; Bareyre et al. 2004; Courtine et al. 2005; Courtine et al. 2008) some locomotor recovery can occur without apparent intervention. Our studies suggest that the cough motor system shows similar endogenous recovery or preservation of function following a range of incomplete thoracic lesions.

Following injury, function may be mediated through indirect or bypass pathways. Premotor drive to the motoneuron pools of the four primary expiratory muscles arises primarily from bulbospinal neurons in the caudal part of the ventral respiratory column (cVRC), corresponding to the caudal portion of the nucleus retroambigualis (Merrill 1970; Merrill 1974; Richter et al. 1975; Arita et al. 1987; Merrill and Lipski 1987; Miller et al. 1987; Miller et al. 1989; Jiang and Lipski 1990; Monteau and Hilaire 1991; Davis 1993; Kirkwood 1995). Kirkwood and colleagues (Kirkwood et al. 1988; Schmid et al. 1993) have proposed that spinal interneurons play an important role in mediating this descending drive. Many of the expiratory-associated thoracic interneurons they identified had crossed axons and spanned multiple segments. They suggested that these crossed interneuronal connections might mediate heterogeneous functions including inhibition of inspiratory thoracic motoneurons during the expiratory phase of breathing and excitation of chest wall motoneurons. It is reasonable that these interneurons may provide a mechanism by which drive from each cVRC is bilaterally represented in the spinal cord. This would enable expiratory cough muscles with motoneurons caudal and ipsilateral to a hemisection to receive cVRC input via multisynaptic connections.

Recently, contralaterally projecting cervical interneurons associated with phrenic (respiratory) motoneurons in the rat also have been identified (Lane et al. 2008) suggesting that respiratory-associated interneurons are present at multiple spinal levels in the normal and injured spinal cord. The potential importance of interneurons for recovery also has been reported recently with respect to locomotor recovery (Courtine et al. 2008). Following SCI in the mouse, thoracic interneurons were reported to mediate recovery of basic stepping in the relative absence of direct descending supraspinal connections to the spinal segments containing the hindlimb motoneurons.

Although reports are mixed, the clinical literature indicates that individuals with thoracic spinal injuries generally maintain inspiratory capabilities but experience expiratory dysfunction due to partial paralysis (Hemingway et al. 1958) and atrophy (Davis 1993; Kern et al. 2008) of the abdominal musculature. A variety of expiratory muscle conditioning or training techniques have been reported to improve some expiratory functions following SCI in humans (for review see (Van Houtte et al. 2006)). These include high frequency magnetic stimulation, surface muscle stimulation, and electrical spinal cord stimulation (Jaeger et al. 1993; Linder 1993; Lin et al. 2001; Lim et al. 2007; Lee et al. 2008). Significant atrophy of the expiratory muscles along with decreased functional capacity also has been shown in the cat following a T6 complete spinal transection (Kowalski et al. 2007). Further, electrical stimulation of the spinal cord at T10 for 6 months following T6 transection appears to prevent the muscle atrophy and support greater expiratory function (DiMarco and Kowalski 2008). Although specific conditioning of the abdominal muscles with respect to cough or other respiratory functions was not done in the current study, all cats were extensively trained on a variety of locomotor tasks 5x/week. Training involved treadmill walking as well as crossing of simple and challenging runways (for examples see (Tester and Howland 2008)). Substantial evidence suggests that locomotor

training can improve hindlimb/lower extremity motor functions post-SCI in animals and humans respectively (Edgerton et al. 2004; Thomas and Gorassini 2005; Behrman et al. 2006; Frigon and Rossignol 2006). Other studies indicate that it improves abdominal expiratory muscle activity in intact subjects (Powers et al. 1992; Uribe et al. 1992; Halseth et al. 1995; Powers et al. 1997) and that locomotor and respiratory rhythms are coupled (Kawahara et al. 1989a; Kawahara et al. 1989b; Romaniuk et al. 1994). Further, voluntary exercise has been shown to increase the production of several neurotrophins (Gomez-Pinilla et al. 2002; Ying et al. 2003) which may play significant roles in motor recovery and synaptic plasticity post-SCI (Ying et al. 2008). Although the effects of locomotor training on cough specifically have not been tested, the combined literature suggests it may have contributed to the substantial cough function seen in the current study. It will be important to test the effects of locomotor training on cough function following SCI in future studies.

Despite complete disruption of the brainstem descending expiratory projections to the left side of the spinal cord below T9, cats in the current study showed substantially normal cough function and abdominal muscle activity bilaterally. Although only one of the four primary expiratory muscles was assessed (rectus abdominis), its normal activity level alone would not be sufficient to generate the normal Pes seen. This suggests that the other three abdominal muscles also were contributing to force generation. These results suggest that the neural substrate(s) underlying the respiratory defense mechanism of cough has substantial capacity for plasticity and/or preservation of function in the abdominal motor system following spinal hemisection.

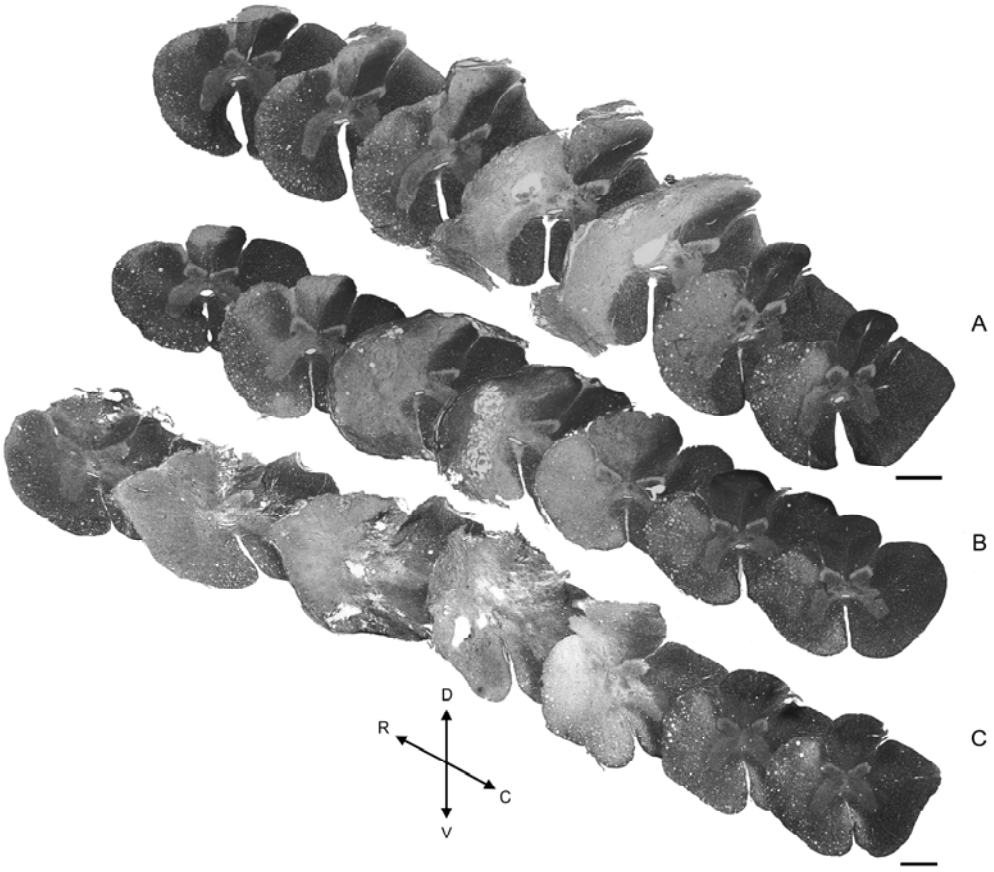


Figure 2-1. Range of lesion magnitudes. The smallest lesion was an under-hemisection with ipsilateral ventro-medial white matter sparing (A). In a complete-hemisection the ipsilateral gray and white matter are damaged and the contralateral gray and white matter spared (B). The largest lesion was an over-hemisection with contralateral gray and white matter damage (C). The size bar is the same for A and B. Dorsal (D), ventral (V), rostral (R), and caudal (C) are indicated for orientation.

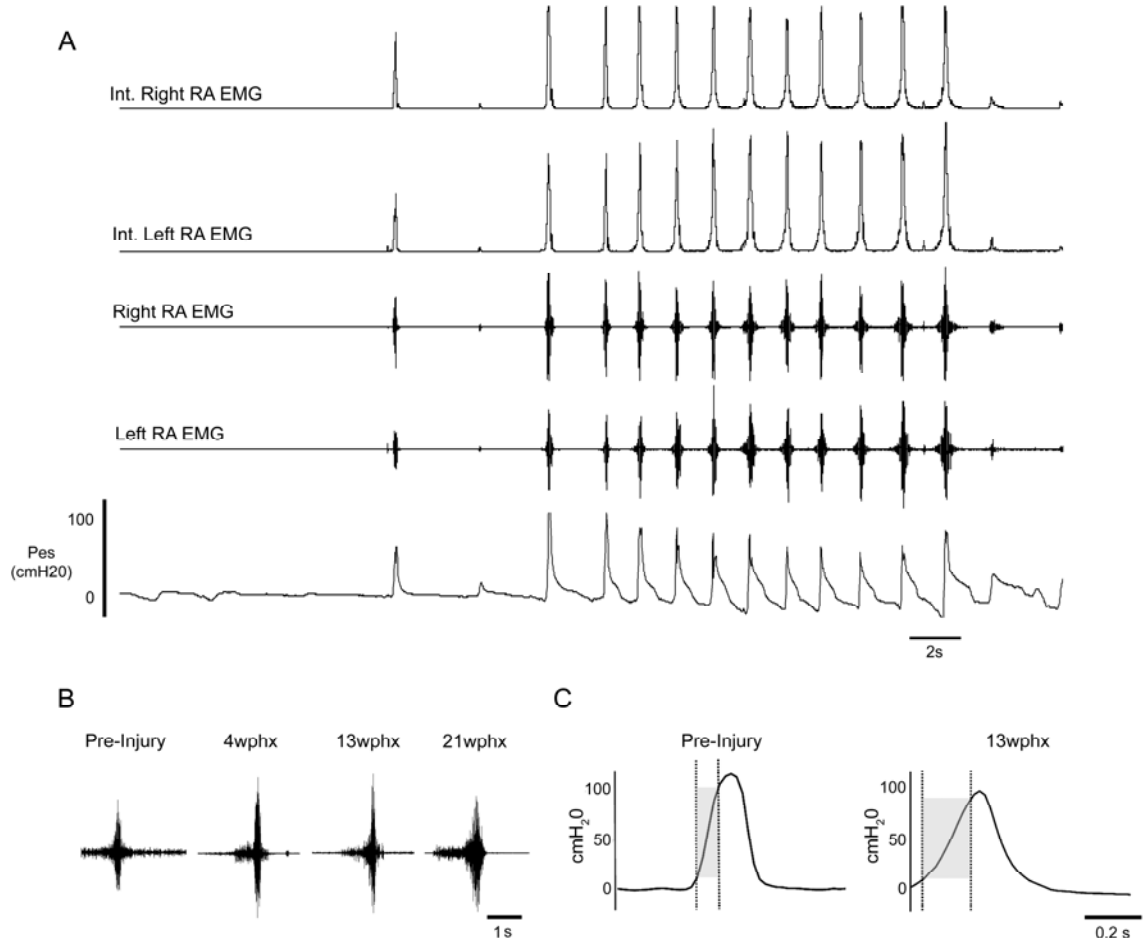


Figure 2-2. Cough characteristics. Representative filtered and moving average integrated electromyograms of the right rectus abdominis and left rectus abdominis with corresponding esophageal pressures (cmH_20) during cough in a cat 13 weeks post-hemisection (A). The single cough, as well as the subsequent bout of coughing, shows robust esophageal pressures and EMG activities bilaterally which are similar to those seen in normal cats. Representative filtered RA EMGs show the pre-expulsive behavior seen during some coughs at all post-SCI timepoints during the inspiratory phase (B). The duration of pre-expulsive EMG activity was similar to previously reported studies. A prolonged Pes rise time was seen in some coughs at 13wphx compared to other time points (C). When this prolonged rise time occurred, it was not accompanied by an increase in Pes.

CHAPTER 3
EFFECTS OF THORACIC SPINAL HEMISECTION AND CHONDROITINASE ABC TREATMENT ON BASIC LOCOMOTION AND THE COUGH REFLEX IN THE CAT

Introduction

Following traumatic spinal injury, axons in the spinal cord have a minimal capacity for axonal regeneration due to the presence of the glial scar, de-myelination, and the up-regulation of potent inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs) (Schwab and Bartholdi 1996). Despite this inhibitory environment, significant spontaneous recovery of locomotor function following spinal lesions has been described in the mouse (Steward et al. 2008), rat (Li et al. 1994; Fouad et al. 2001b; Weidner et al. 2001; Bareyre et al. 2004; Courtine et al. 2008), cat (Murray and Goldberger 1974; Bregman and Goldberger 1983; Kato et al. 1984; Eidelberg et al. 1986; Alstermark et al. 1987; Barbeau and Rossignol 1987; Pettersson et al. 1997; Rossignol et al. 1999), monkey (Lawrence and Kuypers 1968; Courtine et al. 2005), and human (Dietz et al. 1998; Dobkin et al. 2007; Fawcett et al. 2007). Reinnervation of denervated areas may occur via growth of lesioned fibers and/or sprouting from intact fiber systems.

Tapping into these plastic systems via combinatorial therapeutic and rehabilitative interventions may further induce axonal sprouting and regeneration, and therefore increase functional motor recovery. Degradation of the inhibitory chondroitin sulfate glycosaminoglycan (CS-GAG) side chains of CSPGs with the bacterial enzyme Chondroitinase ABC (Ch'ase ABC) has been shown to enhance axonal growth and/or behavioral recovery in numerous rodent models of SCI (Yick et al. 2000; Moon et al. 2001; Bradbury et al. 2002; Yick et al. 2003; Chau et al. 2004; Caggiano et al. 2005; Barritt et al. 2006; Houle et al. 2006; Massey et al. 2006; Cafferty et al. 2007; Carter et al. 2008; Iseda et al. 2008; Massey et al. 2008) as well as in our cat model (Tester and Howland 2008).

Previous research from our laboratory following a low thoracic spinal hemisection in the adult cat, illustrated that CS-GAG degradation with Ch'ase ABC enhanced qualitative recovery of bipedal and overground locomotion (Tester and Howland 2008). In the present study, we quantitatively examined the effects of low thoracic spinal hemisection on numerous aspects of the step cycle and ipsilateral hindlimb stepping ability during bipedal treadmill and overground locomotion in adult cats. Quantitative assessments of ipsilateral hindlimb movements before and after injury at multiple timepoints allowed us to examine, in detail, locomotor performance deficits as well as the degree of spontaneous recovery following thoracic hemisection in the adult cat during two distinct locomotor tasks; bipedal treadmill and overground locomotion. We then examined whether delivery of Ch'ase ABC would enhance locomotor recovery on these tasks in our model. Due to the significant spontaneous recovery in our cat model following T9/10 hemisection during basic locomotion, we hypothesized that Ch'ase ABC would have variable effects on the specific temporal components of the gait cycle as well as the cough reflex.

Our results show that there is significant preservation of many temporal aspects of the locomotor cycle following low thoracic spinal hemisection in the adult cat during bipedal treadmill and overground locomotion. Despite this resiliency, many features sustain persistent, quantifiable deficits. Aspects of the step cycle and of ipsilateral hindlimb function that were affected by low thoracic hemisection, across the two tasks assessed, were generally not improved with intraspinal Ch'ase ABC treatment.

Methods

Animals

Twelve adult SPF female cats (6-8 lbs) were used in this study. All cats were spayed to ensure that behavioral data collection was not influenced by postural changes induced by hormone alteration during estrus (Sribnick et al. 2003). All animal procedures were conducted in

accordance with the NIH guidelines for the care and use of experimental animals and were approved by the University of Florida's Institutional Animal Care and Use Committee.

Surgical Procedures

Spinal T9/10 hemisections were performed as previously published (Tester and Howland 2008). Penicillin G, procaine (40,000U/kg BW IM) was given the day before, the day of, and the day after surgery. Prior to all surgeries, cats received 0.1cc atropine sulfate (0.04-0.06 mg/kg SQ) and 0.1cc acetylpromazine (0.4-0.5 mg/kg SQ). Following orotracheal intubation, anesthesia was maintained with isoflurane (1-3.5%) and an IV was placed for fluid administration (Lactated Ringers, 10 ml/kg/h). Respiratory rate, expired CO_2, SPO_2, blood pressure, body temperature, as well as the general plane of anesthesia were closely monitored.

A left lateral hemisection was made using iridectomy scissors at spinal T9 or T10. Light suction with a pulled glass pipette was used if necessary to lift any fibers adhering to the dura to facilitate the execution of a complete hemisection without compromising the integrity of the dura. Thrombin and gel foam were used to stop any bleeding. Micro-implantable infusion ports (Harvard Apparatus, Holliston, MA) were placed sub-cutaneously and sutured to muscle lateral to the vertebral column. Port tubing was held in place by suturing it to the muscle at several points until it reached midline. VetBond (Webster Veterinary Supply, Inc., Sterling, MA) then was used to secure it to the lamina caudal to the laminectomy. The end of the port tubing was secured in the lesion cavity by suturing the dura (8-0 Prolene). Prior to dural suturing, the port reservoir and tubing (~5µl total volume) was filled with protease free Ch'ase ABC (1 U/200 µl saline, pH 7.8, Seikagaku Corp., Tokyo, Japan). Injury-only animals did not receive port placement or delivery of any fluid into the lesion area. Durafilm (Codman-Shurtleff, Inc., Randolph, MA) and gelfoam (Pharmacia and Upjohn, Inc., Peapack, NJ) were placed over the dural sutures and the muscle and skin sutured. Anesthesia was terminated, cats were extubated,

and then placed in temperature controlled recovery chambers (ThermoCare, Las Vegas). Buprenorphine (0.01 mg/kg SQ) was administered TID for 48 hours.

Procedures used to maintain the general health of the cats were similar to those described in our previous studies (Howland et al. 1995b; Howland et al. 1995a). Once body temperature stabilized to a minimum of 100° F, cats returned to their home cages with 5-7 inch egg crate foam cushions covering the entire cage floor to prevent peripheral nerve compression, pressure sores, and skin breakdown. None of the animals developed any of these complications. For the first few days post-op bladders were expressed manually using Crede's method. Animal health was continuously monitored throughout the study, including maintenance of food intake and body weight.

Chondroitinase ABC Delivery

Cats were placed into two groups following injury: injury-only (n=6) and injury+Ch'ase ABC (n=6). Commercial Ch'ase ABC may not retain its stability at body temperature for extended periods (Tester et al. 2007), therefore 50 microliters of protease-free Ch'ase ABC (1 U/200µl), or vehicle were injected every other day for 1 month. The concentration delivered was identical to our previous study (Tester and Howland 2008). The volume is ~4x that used in rodent studies (Bradbury et al. 2002) due to the larger size of the cat spinal cord. For treatment administration, the cats were anesthetized (1-3% isofluorane and 1.5LO2) for 15 minutes while the Ch'ase ABC was delivered (0.14L/min) using a microinjection syringe pump (Harvard Apparatus, Holliston, MA).

General Locomotor Training

Cats were trained daily (5 times/week) on a variety of basic and skilled locomotor tasks requiring different levels of input from the neural axis including bipedal treadmill, 12" and 2" overground, obstacle, and pegboard locomotion. For the current studies, we focused specifically

on effects on multiple components of the gait cycle during bipedal treadmill and 12" overground locomotion following thoracic hemisection and Ch'ase ABC treatment.

All locomotor tasks were conditioned to a food reward. When all tasks were performed consistently, baseline data was collected pre-injury. All cats then received a left spinal T10 hemisection, and locomotor training was re-initiated within 24 hours and continued daily for the remainder of the study. A left T10 hemisection primarily affects locomotor function of the ipsilateral, left hindlimb (LHL). Therefore, quantitative assessments were done on the LHL.

Bipedal Treadmill and Overground Locomotion

Bipedal locomotion was performed on a motor driven treadmill 5 times/week at 0.5m/s for a food reward. Immediately following spinal hemisection cats could not walk at this speed, but all recovered the ability to consistently walk at 0.5m/s by the first behavioral assessment time point of 2wphx. Cat forelimbs were placed on a stationary platform and their hindlimbs were allowed to freely move over the treadmill belt while receiving a liquid food reward in a raised bowl in front of them. Ten consecutive step cycles at each time point/cat were used and averaged within Peak Motus to 51 frames in length each for quantitative assessments.

Cats were also trained and assessed during overground locomotion (12" wide runway). Cats crossed the overground runway for a food reward given at each end. The comfortable fast walk/slow trot speed was determined pre-injury for each individual cat, and post-injury crossings were carefully chosen for each cat to closely match their pre-injury crossing times. Therefore, any quantitative locomotor changes cannot be attributed to speed changes within animals following spinal hemisection. Ten contiguous step cycles from two step cycles at each time point/cat were used and averaged within Peak Motus to 51 frames in length each for quantitative assessments and then averaged across cats.

Step Cycle, Swing, and Stance Durations

The left hindlimb step cycle duration was defined from paw contact with the treadmill belt or overground runway until the subsequent paw contact. Step cycle duration was based on the raw number of frames the LHL spent in an entire gait cycle. The gait cycle was further delineated into its two main phases: stance, which begins with initial paw contact, and swing, which begins when the paw leaves the contact surface. Swing and stance were calculated as a percentage of the entire duration of the step cycle for both behavioral tasks. Ten step cycles for each cat were assessed to define the percentage of the step cycle that was spent in swing and stance, and then these percentages were averaged across all 10 step cycles per cat, giving an average percentage of the step cycle for swing and stance per each cat at each timepoint. These averages per cat where then averaged across cats to give a final percentage of the step cycle spent in swing and stance.

Paw Drag and Kinematic Locomotor Assessment

Paw drag was assessed during bipedal and overground locomotion for each cat. Paw drag was defined as the duration of frames the paw was in dorsal contact with the treadmill belt or overground surface during the swing phase of the step cycle. Paw drag was assessed during ten step cycles and averaged for each cat during bipedal and overground locomotion and subsequently averaged across cats within each group for statistical comparisons.

The cats performances were recorded at multiple timepoints until 20 weeks post-injury. The cats hindquarters were shaved and reflective spheres were placed on the iliac crest, greater trochanter, lateral malleolus, and the base of the 5th metatarsal. A fifth marker was placed on the fibula approximately one inch above the lateral malleolus marker in order to generate a unit vector, which in combination with the measured length from the lateral malleolus to the head of the lateral condyle, created the knee position. These spheres allowed for the tracking of the hip,

knee, and ankle joints across the step cycle. Locomotor kinematics were assessed using Motus software (Vicon Peak). The maximum hip and ankle angle at the transition from stance to swing was found during ten step cycles for each cat at each timepoint. The angles during the ten step cycles were averaged within a cat and then across cats at each timepoint. The minimum knee angle during swing was also found during ten step cycles for each cat at each timepoint, averaged within a cat, and then across cats at each timepoint.

Cough Stimulation and Assessment

For this portion of the study 14 cats were assessed: Ch'ase ABC treated (n=8), and control (n=6). Cats were initially given atropine sulfate (0.1 mg/kg) subcutaneously to block salavation and reflex tracheal secretions. Pre-injury and at multiple post-operative timepoints, spontaneously breathing cats were gaseously anesthetized (2-3% isofluorane in 1.5 LO_2). End-tidal CO2 was monitored during the procedures and anesthesia was adjusted accordingly. Cats were placed in the supine position and the abdomen was shaved and sterilized. Paired bipolar Teflon-coated stainless steel wire electrodes were placed bilaterally approximately 2-3 mm apart in the left and right rectus abdominis muscles below the level of the spinal hemisection and approximately one cm lateral to the midline in the mid-pelvic region. A ground electrode was also placed in the left hamstring muscle. An esophageal balloon catheter was placed into the esophagus and inflated with a syringe. Cough was elicited by mechanical stimulation of the vocal folds and epiglottis with a small length of flexible plastic tubing.

Cough analysis was dependent on a clear bilateral RA EMG signal and cough amplitude larger than 5cm H_20. All coughs generated at each data collection point were used if they met these criteria. Esophageal pressure (P_{ES}) and left and right rectus abdominis (LRA and RRA) electromyograms (EMGs) were recorded. P_{ES} (cmH_20), LRA/RRA cough amplitudes (normalized percentages), esophageal rise times (seconds), and rectus abdominis rise times

(seconds) were calculated. EMG amplitudes per cat were normalized to the largest EMG burst at a given time point on the same side, expressed as percentages, and then averaged across animals at each time-point. Rise times were calculated by subtracting 10 % of the total rise time from the peak and the baseline in order to standardize the data. Cough rise times and amplitudes were measured pre-injury, 4weeks post hemisection (wphx), 13wphx, and 21wphx. Individual cough rise times for each cat were averaged at each time-point and then averaged across animals.

Histological Confirmation of T10 Hemisection

Before tissue was cut on the cryostat it was placed in a solution containing 30% sucrose and 70% fixative overnight. The dura was removed, while maintaining the spinal cord moist, and then immediately placed into dry ice to freeze. Tissue was placed in mounting media and cut with a tissue thickness of 25µ. Sections were cut into tubes filled with 0.1M PBS. The tissue was then organized by cutting into series of 10. One section of every ten was mounted onto subbed slides and stained with cresyl violet (cresyl violet with acetate, Sigma) and myelin (Eriochrome Cyanine R, Fluka, New York) for assessment of lesion extent and damage.

Statistical Analyses

Statistical Analyses were performed for characterists of locomotion using Statistical Package for the Social Sciences (SPSS) v. 17 (Chicago, IL). To assess changes over time Mixed 2 factor ANOVAs were used and the Huynh-Feldt correction was used if sphericity could not be assumed. Independent t-tests were used to assess individual changes across two timepoints and across groups at one particular timepoint. Paired two-tailed t-tests were also done to compare individual changes across timepoints within the same group. Post hoc analyses were done using Bonferonni corrections. For characteristics of cough, Graphpad Instat softwar was used. To assess changes over time, repeated measures ANOVA using Bonferonnie post hoc analyses were used.

Results

Extent of Spinal Hemisection

Spinal T9/T10 left hemisections were performed on all cats. A representative spinal cord section depicting the greatest cross-sectional extent of lesion damage for each animal is shown in Figure 3-1. The size bar paired with cat lesion A1 also corresponds to every lesion without a matching size demarcation. Cat lesions A4 and B1 were sized accordingly in order to match lesion cross-sectional area across cats for visual clarity. Lesion epicenter representations were produced from light microscope examination of cresyl violet and myelin stained spinal cord cross sections at the level of the greatest extent of spinal damage. Cat lesion A1 was cut longitudinally and the cross-sectional diagram was created by examination of serial sections through the dorso-ventral extent of the lesion and subsequent superimposition over cat lesion A6 as a template. In all animals, the ipsilateral lateral funiculus was entirely damaged, eliminating the corticospinal and rubrospinal tracts. The ipsilateral dorsal funiculus also was completely affected in all cats. The extent of damage incurred to the ipsilateral ventral funiculus ranged from complete to minimal damage or complete sparing. A range of damage to the contralateral gray matter, dorsal funiculus, and ventral funiculus occurred across all cats.

Locomotor Recovery

Behavioral recovery during bipedal treadmill and overground locomotion was assessed for lesion only (no port placement) and lesion + Ch'ase ABC (port placement with Cha'ase ABC diluted in saline). For all animals assessed, regardless of treatment paradigm, the ability to perform bipedal locomotion independently at 0.5 m/s with integration of the left hindlimb occurred within the first week post-injury, and often times occurred within the first few days. Similarly, this rapid recovery was also seen during overground locomotion where all animals could independently perform the task with left hindlimb integration within one week post-injury.

Across all lesion only and lesion + Ch'ase ABC cats the ability to place the affected left hindlimb primarily utilizing plantigrade paw position, versus dorsal paw placement, occurred within two to three weeks post-injury. This behavioral timeline is consistent with previous research utilizing the hemisection model (Helgren and Goldberger 1993; Kuhtz-Buschbeck et al. 1996; Tester and Howland 2007).

Recovery of Characteristics of Basic Locomotion

The left hemisection lesion primarily affects the ipsilateral, left hindlimb and therefore all analyses were assessed on this limb. All twelve cats acquired the ability to walk independently during bipedal locomotion and overground locomotion by 2 weeks post-hemisection (wphx) and therefore this time point was used as the first post-injury assessment time.

Step-cycle duration

The step cycle duration and its variability (average ± SEM) at pre-injury and post-injury time points for lesion-only and lesion + Ch'ase ABC cats during bipedal treadmill and overground locomotion are illustrated in Figure 3-2. The step cycle duration during bipedal treadmill locomotion was very consistent at every post-injury timepoint compared to pre-injury, with no significant effects across time ($p=0.571$), treatment ($p=0.794$), or time x treatment ($p=0.405$) (Mixed 2 factor ANOVA). During overground locomotion, there was a significant effect of time ($p=0.02$) on step cycle duration showing an increase in duration after injury. Post hoc comparisons isolated the differences between pre-injury and 8wphx ($p=0.041$) and between pre-injury and 20wphx ($p=0.036$), suggesting a sustained injury effect on step cycle duration during overground locomotion. There was also a trend towards an effect of treatment ($p=0.058$), with these animals also showing a trend towards increased step cycle duration at post-injury timepoints.

Stance duration

The percentage of the step cycle spent in the stance phase for injury-only and Ch'ase ABC treated cats during bipedal treadmill and overground locomotion can be seen in Figure 3-3. There was a significant effect of time (p=0.000) on stance duration during bipedal treadmill locomotion, showing an overall decrease in the percentage of time spent in stance at all post-injury timepoints. Post hoc comparisons isolated the differences between pre-injury and 2wphx (p=0.040), pre-injury and 8wphx (p=0.046), and between pre-injury and 20wphx (p=0.031), illustrating a persistent injury effect on stance duration. Stance duration as a percentage of the step cycle was not significantly effected across time x treatment (p= 0.0642) or treatment alone (p=0.941).

There was also a significant effect of time (p=0.002) on stance duration during overground locomotion. Post hoc comparisons isolated an increase in stance duration between pre-injury and 8wphx (p=0.002) and between pre-injury and 20wphx (p=0.012), suggestive of a delayed injury effect during overground locomotion on stance duration. Interestingly, there was a significant increase in stance duration post-injury during overground locomotion, whereas during bipedal locomotion there was an overall decrease in stance post-injury.

Swing duration

The percentage of the step cycle spent in the swing phase for injury-only and Ch'ase ABC treated cats during bipedal treadmill and overground locomotion can be seen in Figure 3-3. There was a significant effect of time (p=0.000) on swing duration during bipedal treadmill locomotion, showing an overall increase in the percentage of time spent in swing at all post-injury timepoints. Post hoc comparisons isolated the differences to be between pre-injury and each post-injury timepoint (p=0.04, p=0.046, and p=0.031 respectively), demonstrating a persistent injury effect on swing duration during bipedal treadmill. Swing duration as a

percentage of the step cycle was not significantly effected across time x treatment (p=0.642) or treatment alone (p=0.941).

There was also a significant effect of time (p=0.007, Huynh-Feldt correction was applied for violations of the assumption of sphericity), of the swing duration during overground locomotion, showing an overall decrease in the percentage of time spent in swing post-injury. Post hoc comparisons isolated the differences between pre-injury and 8wphx (p=0.002) and between pre-injury and 20wphx (p=0.012), suggestive of a delayed injury effect during overground locomotion on swing duration.

Paw drag

Following spinal hemisection the occurrence of paw drag during the swing phase significantly increased during both bipedal treadmill and overground locomotion (Figure 3-5). Stick figure diagrams from a representative animal during bipedal treadmill and overground locomotion at pre-injury, 2wphx, and 20wphx are shown in Figure 3-4. During the pre-injury swing phase, the paw is lifted off the treadmill belt or overground runway in a very precise and efficient manner. Immediately following injury during both behavioral tasks, the paw is dragged dorsally during the majority of the swing phase. By 20wphx, during both tasks, cats are now able to lift the paw off the treadmill belt or overground runway, but not as efficiently as pre-injury, with visible knee hyperflexion occurring during both tasks.

During bipedal treadmill there was a significant effect of time (p=0.000) on paw drag, showing an increase at all post-injury timepoints. Post hoc comparisons were found between pre-injury and 2wphx (p=0.000), 4wphx (p=0.001), 8wphx (p=0.011), 16wphx (p=0.006) and between 20wphx (0.038). Following spinal hemisection, paw drag during bipedal treadmill locomotion does not return back to pre-injury values even by 4-5 months post-injury. There

were no effects seen across time x treatment (p=0.116) or treatment alone (p=0.899) on paw drag during bipedal treadmill locomotion (Figure 3-5).

During overground locomotion there also was a significant effect of time (p=0.000, Huynh-Feldt correction was applied for violations of the assumption of sphericity) on paw drag, but post hoc comparisons found differences only between pre-injury and 2wphx (0.027) and pre-injury and 4wphx (0.442). Paw drag had normalized back to values similar to pre-injury sometime between one and two months post-injury. Therefore paw drag significantly improved following injury during overground locomotion much faster than during bipedal locomotion. This suggests that input from supraspinal centers can significantly improve paw drag following thoracic hemisection. There were also no effects seen across time x treatment (p=0.700) or treatment alone (p=0.460) on paw drag during overground locomotion. Ch'ase ABC treatment does not appear to improve paw drag deficits following thoracic hemisection (Figure 3-5).

Left hindlimb angular kinematics

Angular kinematics were assessed at the hip, knee, and ankle across all cats to determine how the specific joint angles were affected by thoracic hemisection in the cat model. Maximum hip angle was quantitatively assessed at the transition from stance to swing during pre-injury and at 20wphx during bipedal treadmill and overground locomotion. Two-tailed independent t-tests revealed that there was no injury effect on maximal hip extension during bipedal treadmill (p=0.773) or overground locomotion (p=0.932). Injury-only pre-injury hip maximal extension angles were not significantly different from Ch'ase ABC treated pre-injury values during bipedal treadmill (p=0.169) or overground locomotion (p=0.522), and the same was true when comparing their 20wphx values (p=0.079, p=0.281 respectively). In the Ch'ase ABC treated cats there was not an injury effect. Despite this, when comparing the absolute degree change of hip maximal extension at 20wphx compared to pre-injury values, there was no significant difference

between injury-only and Ch'ase ABC treated cats during bipedal treadmill (p=0.062) or overground locomotion (p=0.405). Therefore, this specific characteristic of the gait cycle is stable following thoracic hemisection and is not affected by Ch'ase ABC treatment.

Minimal knee angle during swing, reflective of maximal knee flexion during swing, was also quantitatively assessed during pre-injury and at 20wphx during bipedal treadmill and overground locomotion. Two-tailed independependent t-tests revealed there was no injury effect on maximum knee flexion during bipedal treadmill (p=0.101), but there was a significant effect of injury on this parameter during overground locomotion (p=0.048) (Figure 3-6). Injury-only pre-injury maximal knee flexion angles were not significantly different from Ch'ase ABC treated pre-injury values during bipedal treadmill (p=0.350) or overground locomotion (p=0.687), and the same was true when comparing their 20wphx values (p=0.944, p=0.802 respectively). It can therefore be stated that Ch'ase ABC treated cats also sustained an injury effect of maximal knee flexion during swing during overground locomotion but not during bipedal treadmill. When comparing the absolute degree change of maximal knee flexion during swing at 20wphx compared to pre-injury values, there was no significant difference between injury-only and Ch'ase ABC treated cats during bipedal treadmill (p=0.732) or overground locomotion (p=0.207). Therefore, maximal knee flexion during swing is affected by thoracic hemisection during overground locomotion but not during bipedal locomotion, and there are no apparent effects of Ch'ase ABC treatment based on the analyses assessed.

Finally, we quantitatively assessed the maximum ankle angle at the transition from stance to swing during pre-injury and at 20wphx during the same two simple behavioral tasks. Statistical tests revealed that there was no injury effect on maximal ankle extension during bipedal treadmill (p=0.632) or overground locomotion (p=0.623). Injury-only pre-injury ankle

maximal extension angles were not significantly different from Ch'ase ABC treated pre-injury values during bipedal treadmill (p=0.917) or overground locomotion (p=0.549), and the same was true when comparing their 20wphx values (p=0.622, p=0.164 respectively). Also, in the Ch'ase ABC treated group there was also not an injury effect on maximum ankle extension during the step cycle. When comparing the absolute degree change of maximal ankle extension during the step cycle at 20wphx to pre-injury values, there was no significant difference between injury-only and Ch'ase ABC treated cats during bipedal treadmill (p=0.425) or overground locomotion (p=0.801). Therefore, maximal ankle extension at the transition from stance to swing is not affected by thoracic hemisection during overground or bipedal treadmill locomotion, and there are not apparent effects of Ch'ase ABC treatment on this parameter based on the analyses assessed.

Recovery of the Cough Reflex

Our laboratory has previously found that following a low thoracic hemisection, the general characteristics of the cough reflex are preserved, including left and right rectus abdominis electromyogram (EMG) amplitudes and rise times, as well as esophageal pressure amplitudes and rise times (Jefferson S 2008). Following Ch'ase ABC treatment, there were no significant changes from pre-injury of the esophageal pressure rise times (p=0.1750), left rectus abdominis rise times (p=0.1508), right rectus abdominis rise times (p=0.5604), left rectus abdominis amplitudes (p=0.4278), or in right rectus abdominis amplitudes (p=0.4372). However, with Ch'ase ABC treatement, esophageal pressure amplitudes at all post-injury timepoints tested were significantly increased compared to pre-injury values (p=0.0381) (Figure 3-7).

Discussion

This study assessed the effects of thoracic hemisection in the cat on specific components of the gait cycle during a task that requires no descending input, bipedal treadmill, and a task that

integrates descending input with spinal pattern generation, overground locomotion. We also assessed the effects of Ch'ase ABC treatment on the cough reflex. Following complete thoracic spinal transection, cats maintain coordinated hindlimb walking on a treadmill. This demonstrates that intraspinal networks in the caudal spinal cord, in isolation from descending supraspinal inputs, can generate rhythmic hindlimb motor behaviors (Grillner and Zangger 1979; Barbeau and Rossignol 1987; Howland et al. 1995a; Howland et al. 1995b; Rossignol et al. 1996). However, one persistent deficit that remains is paw drag during swing (Belanger et al. 1996). Similarly, overground locomotion requires input from the caudal cord, but also depends on descending supraspinal input for completion.

Overall, the characteristics assessed during bipedal and treadmill locomotion were affected by our injury paradigm but were not affected by Ch'ase ABC administration. There is a tremendous amount of intrinsic plasticity in the cat motor system in response to thoracic spinal hemisection, but there are still persistent, quantifiable deficits.

Our results show that step cycle duration during bipedal treadmill is very stable following thoracic hemisection in the adult cat, whereas during overground locomotion step cycle duration is significantly increased up until four or five months post-hemisection. This could be due to slight decrease in speed during overground locomotion at post-injury timepoints. Overground crossing speeds were closely matched within cats to their pre-injury values, but small changes in time could affect this parameter. It has been well documented that locomotor cycle durations can be affected by descending, as well as sensory, input to the spinal cord (Armstrong 1988; McCrea 2001). During normal overground gait, an increase in speed of locomotion is usually accompanied by a decrease in step cycle duration, mostly due to the shortening of the stance phase (Murray 1967; Goslow et al. 1973), or inversely a decrease in locomotor speed would be

accompanied by an increase in step cycle duration and a lengthening of the stance phase. We see this exact effect following injury during overground locomotion; the step cycle duration was seen to increase with a correlative increase in stance duration. The complete spinal animal maintains consistent relationships between the step cycle duration and the swing and stance duration (Barbeau and Rossignol 1987; Belanger et al. 1996), which was also generally seen in our injury model.

Following thoracic lesion of the lateral and dorsal descending pathways cats exhibit substantial hindpaw drag during swing, an increased HL cycle duration, a disruption of stance/swing transition, and alteration in intralimb coupling, and a severe intralimb uncoupling during quadrupedal treadmill locomotion (Courtine et al. 2005). In all cats assessed in this study, the most obvious locomotor deficit was the substantial swing phase paw drag during bipedal treadmill locomotion as well as overground locomotion following spinal hemisection. Paw drag during overground locomotion returned to normative values within one month of injury, whereas there was significant increases in paw drag during bipedal locomotion at all post-injury timepoints. Integration of descending systems such as the cortico- and rubro- spinal systems during overground locomotion may have contributed to the diminished paw drag following thoracic hemisection. During bipedal treadmill, a task that can be accomplished without descending control the central pattern generator alone was unable to compensate for the thoracic injury and therefore paw drag never returned to normative values post-injury. Studies in the cat have shown that locomotor training can significantly enhance behavioral recovery following spinal cord injury (Barbeau and Rossignol 1987), and likely upregulates a variety of growth factors that are likely to enhance plasticity within the spinal cord (Gomez-Pinilla et al. 2002).

Therefore, we may be getting a training effect during the parameters assessed following injury-only because our animals are extensively trained daily on a variety of locomotor tasks.

In our injury model, the hip, knee, and ankle angles post-injury were generally conserved, except that there wss an injury-effect of time on the maximum knee flexion during swing during overground locomotion. Injury-only animals had a significantly increased knee flexion during swing correlated with a visible hypermetric gait that was not as apparent during bipedal treadmill locomotion. Ch'ase ABC did not affect this result positively or negatively.

Although basic voluntary forms of locomotion require supraspinal input to spinal networks, it is the skilled tasks requiring greater balance and control of limb trajectory that demand the greatest supraspinal contributions. Spinal hemisection of the thoracic cord did not prevent the cats from performing bipedal treadmill or overground locomotion as early as a few days post-lesion, although previously we have shown that this lesion significantly delays the ability to complete skilled locomotor tasks (Tester and Howland 2008). Skilled tasks are the most affected by our lesion paradigm, and therefore it is logical that Ch'ase ABC would be more beneficial during locomotor tasks that are greater affected by our lesion paradigm.

Ch'ase ABC administration did not affect any locomotor features assessed in this study, however Ch'ase ABC treatment significantly increased esophageal pressures at all post-injury timepoints compared to pre-injury values and all injury-only values. This may not be relevant in our current injury model, but in a cervical lesion model where the respiratory deficits are greater, this increase in esophageal pressure may allow for a more efficient cough production.

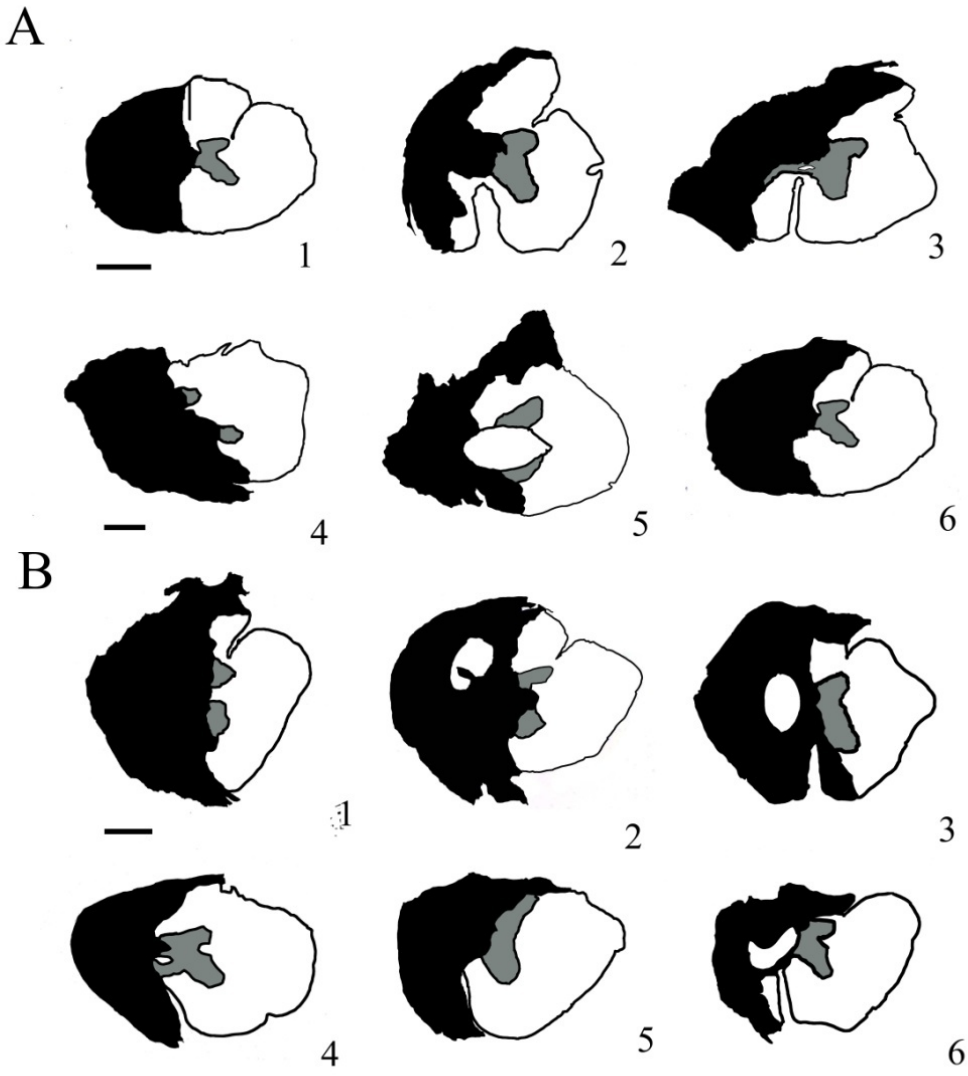


Figure 3-1. Cross section lesion extents. Pictorial representations of the greatest extent of cross-sectional lesion damage for all lesion-only (A1-A6) and Ch'ase ABC treated cats (B1-B6). All images were generated within Adobe Photoshop from light microscope photomicrographs of cresyl violet and myelin stained spinal cord cross-sections at the lesion epicenter. White; intact white matter undamaged by the spinal lesion; Black, severely or moderately damaged grey and white matter as well as gliotic scar tissue; Gray, intact grey matter.

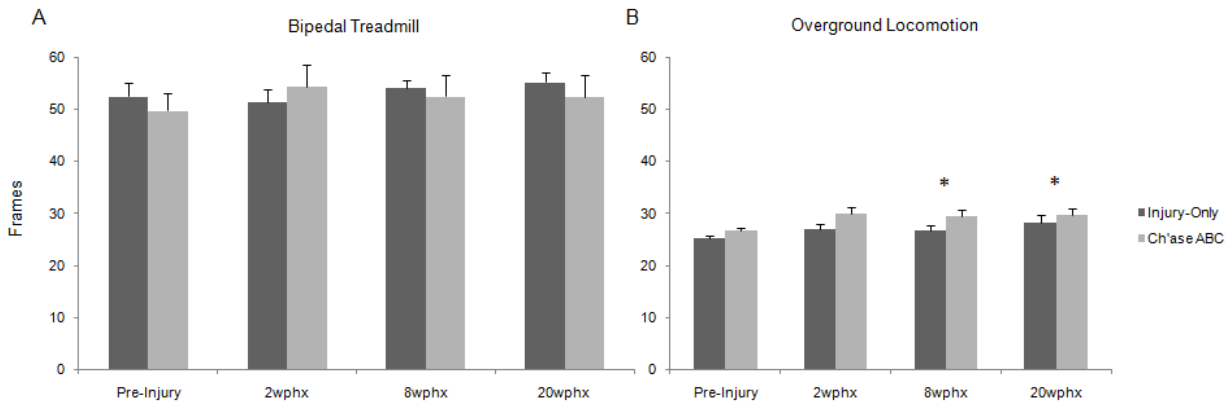


Figure 3-2. Step cycle duration during bipedal treadmill and overground locomotion. Step cycle durations of the left hindlimb represented as raw frames are shown for injury-only and Ch'ase ABC treated cats at pre-injury, 2, 8, and 20 weeks post-injury during bipedal treadmill and overground locomotion. Step cycle durations of the left hindlimbs of injury only and Ch'ase ABC treated cats are not significantly altered following injury at any post-injury timepoints. A delayed increase in the step cycle duration during overground locomotion is seen at 8 and 20 weeks post hemisection that is not significantly altered with Ch'ase ABC treatment. Data represent averages ± SEM. Asterisks indicate significant changes compared to pre-injury values. * Indicates a significant change from pre-injury based on post hoc analysis.

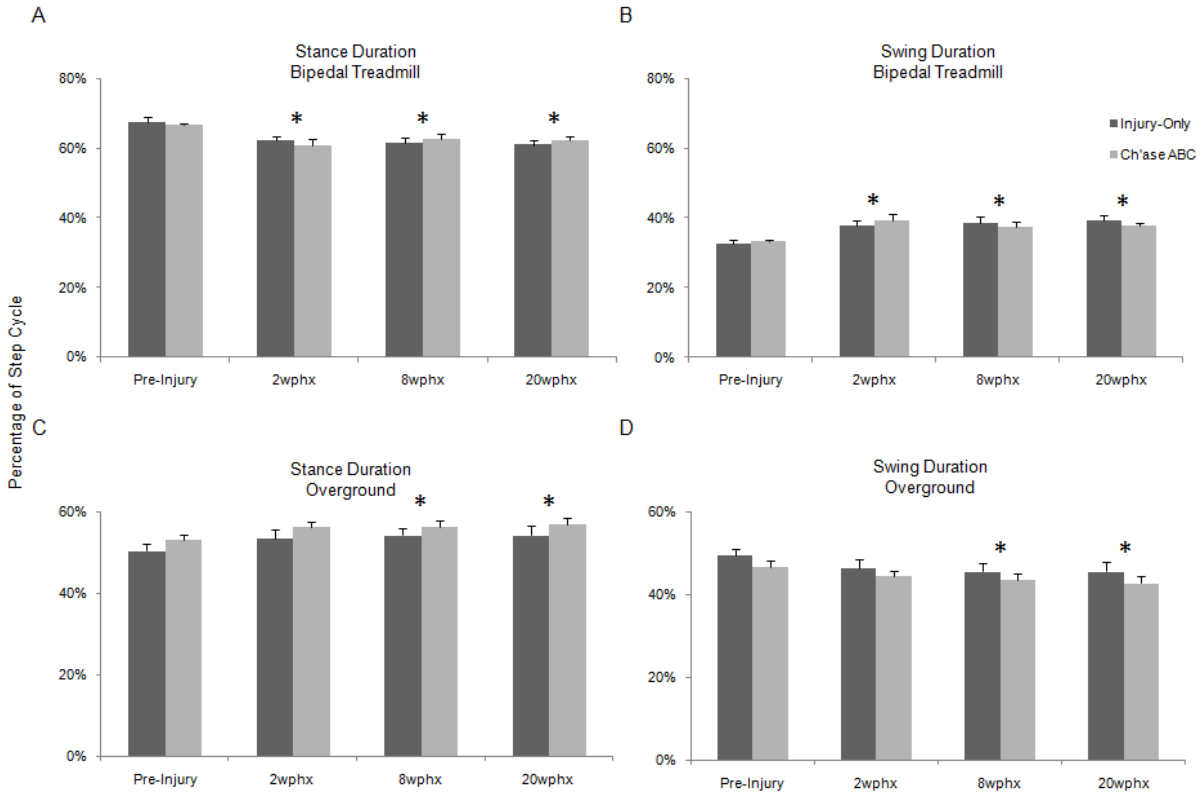


Figure 3-3. Swing and stance durations during bipedal treadmill and overground locomotion. Stance and swing durations of the left hindlimb represented as the percentage of the entire step cycle are shown for injury-only and Ch'ase ABC treated cats at pre-injury, 2, 8, and 20 weeks post-injury during bipedal treadmill and overground locomotion. Stance duration significantly decreases at all post-injury timepoints during bipedal treadmill locomotion and swing has a correlative increase post-injury. No treatment effects were seen. Swing duration significantly increases at 8 and 20 weeks post-injury during bipedal treadmill locomotion and swing has a correlative decrease at these post-injury timepoints. No treatment effects were seen during overground locomotion as well. Data represent averages ±SEM. Asterisks indicate significant changes compared to pre-injury values. * Indicates a significant change from pre-injury based on post hoc analysis.

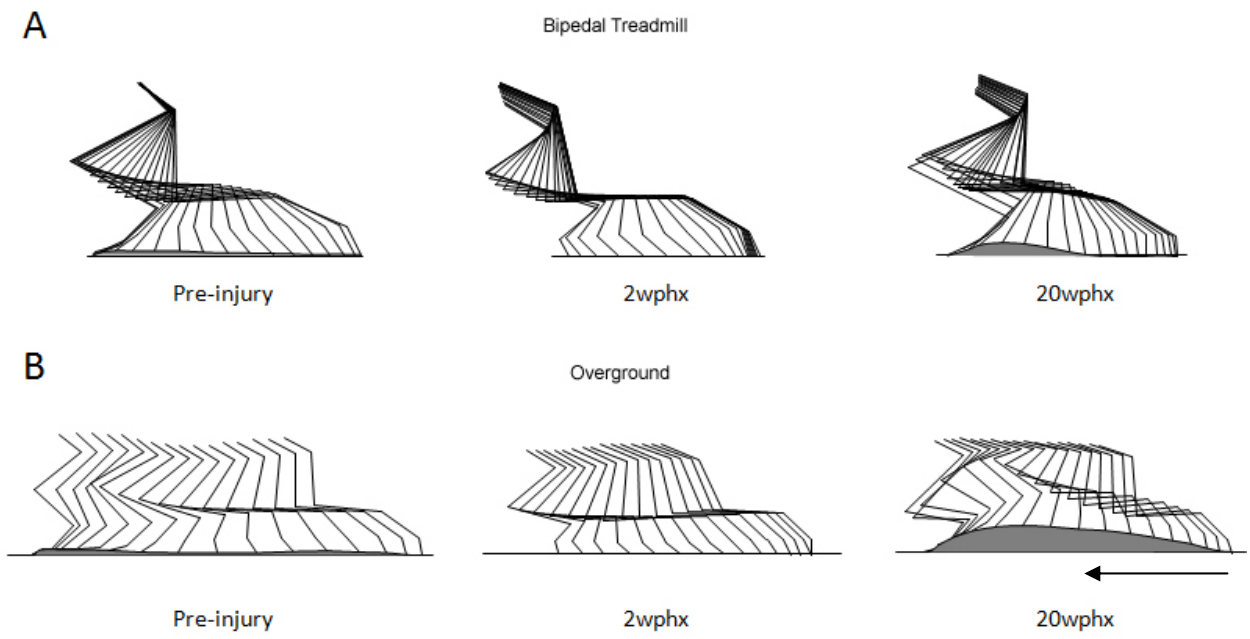


Figure 3-4. Stick figure representations of paw drag during swing. Representative stick figure diagrams of ipsilateral, left hindlimb kinematic movements during the swing phase on bipedal treadmill at 0.5 m/s (A) and overground locomotion (B) at pre-injury, 2 weeks post-injury, and 20 weeks post-injury in cat B1. The gray shaded region illustrates when the ipsilateral hindpaw was not dragging along either the treadmill belt or overground runway during the swing phase. The direction of movement moves from right to left in the direction of the arrow.

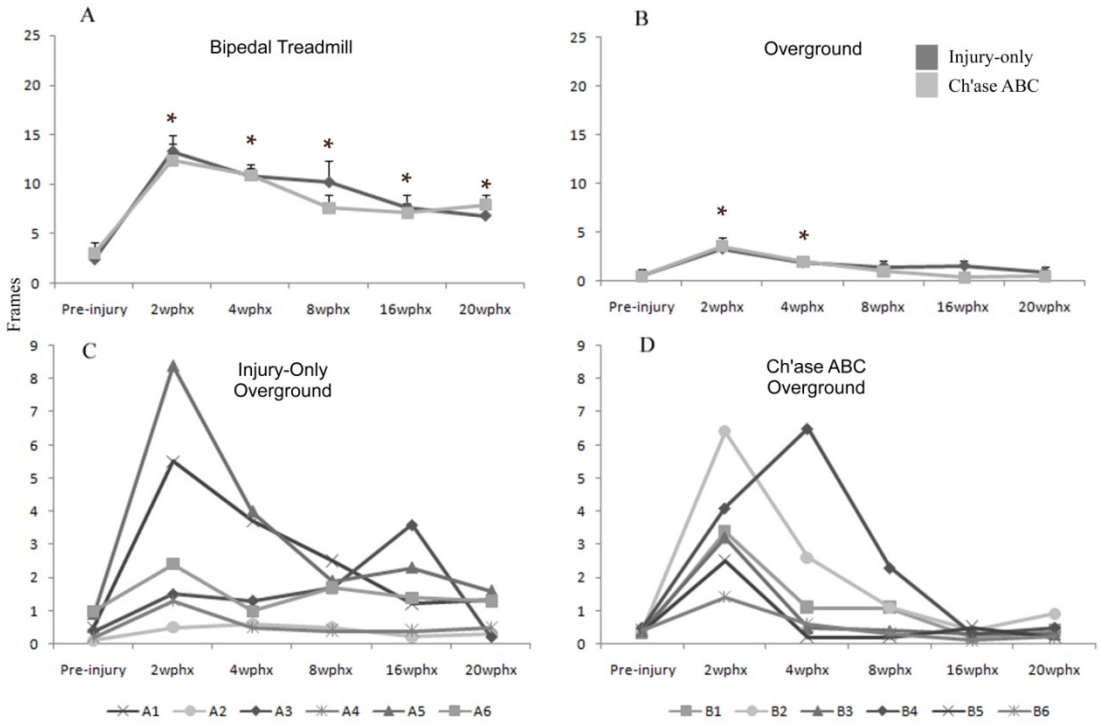


Figure 3-5. Paw drag during bipedal treadmill and overground locomotion. Paw drag during the swing phase (frames ± SEM) is shown for injury-only and Ch'ase ABC treated animals during bipedal treadmill (A) and overground (B) locomotion. Individual data for each injury only cat (C) and each Ch'ase ABC treated cat (D) are shown during overground locomotion. Overall, more Ch'ase ABC treated cats have decreased paw drag at 16 and 20 weeks post-hemisection than the injury-only cats during overground locomotion. There is a significant increase in paw drag at all post-injury timepoints during bipedal treadmill. During overground locomotion there is a transient increase in paw drag within the first month after injury, but paw drag returns to normative values within two months following injury. There is no significant treatment effect across paw drag during either task. Asterisks indicate significant differences from pre-injury.

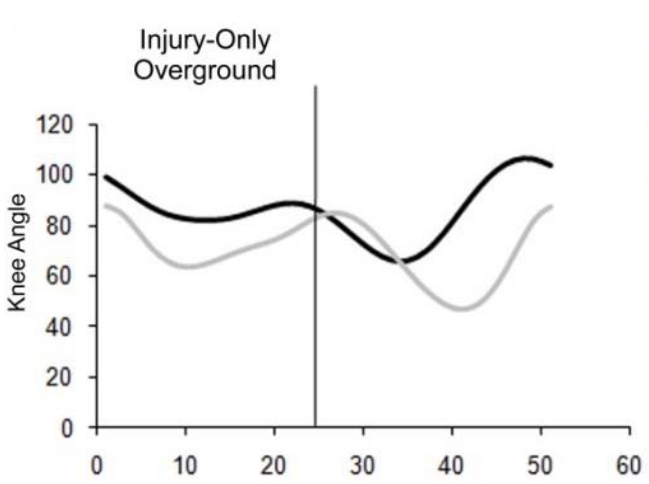


Figure 3-6. Swing knee flexion during overground locomotion. Knee angle plots for an entire step cycle normalized in length across animals to 51 frames are shown during overground locomotion for a representative injury-only cat at pre-injury (black lines) and at 20 weeks post-hemisection (gray lines). The vertical black line represents the transition from stance to swing during pre-injury locomotion. The maximum knee flexion during swing was significantly decreased following injury, and this change was not affected by Ch'ase ABC treatment.

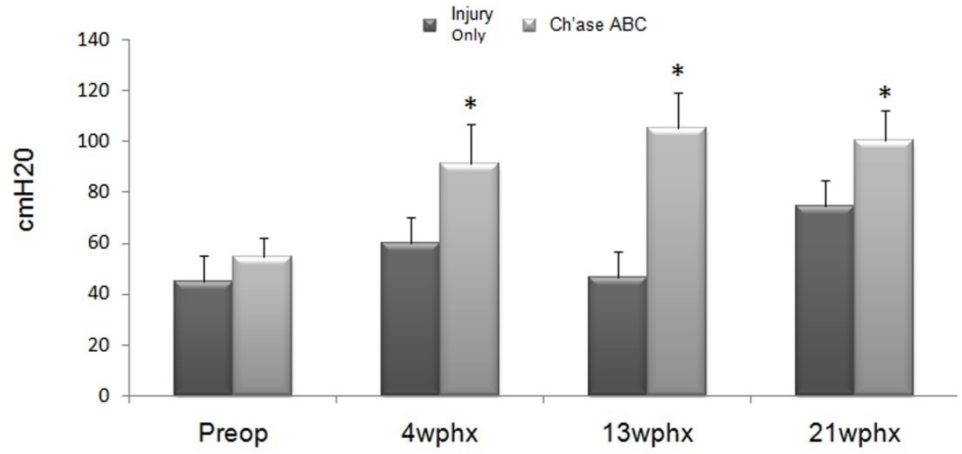


Figure 3-7. Ch'ase ABC increases esophageal pressure amplitudes following hemisection. Esophageal pressure amplitudes (cmH_20) are shown for injury-only and Ch'ase ABC treated cats during pre-injury coughing and during coughing at 4, 13, and 21 weeks post-hemisection. Ch'ase ABC treated cats have a significantly greater esophageal pressure during all post-injury coughing session compared to pre-injury values and compared to injury-only animals at the same timepoints. Asterisks indicate a significant change from pre-injury values.

CHAPTER 4
CHONDROITINASE ABC PROMOTES RECOVERY OF SKILLED LIMB MOVEMENTS AND PLASTICITY OF THE RUBROSPINAL TRACT IN THE CAT

Introduction

Chondroitinase ABC (Ch'ase ABC) has been used experimentally to cleave the growth inhibitory chondroitin sulfate glycosaminoglycans (CS-GAGs) *in vitro* (Snow et al. 1990a; McKeon et al. 1995) and *in vivo* models of spinal cord injury to cleave the growth inhibitory chondroitin sulfate glycosaminoglycans (CS-GAGs) (Kwok et al. 2008). Disruption of CS-GAGs with Ch'ase ABC *in vivo*, either alone or in combination with other treatments in several models of SCI, has been associated with behavioral recovery as well as enhancement of axonal growth (Yick et al. 2000; Moon et al. 2001; Bradbury et al. 2002; Yick et al. 2003; Chau et al. 2004; Caggiano et al. 2005; Barritt et al. 2006; Houle et al. 2006; Massey et al. 2006; Cafferty et al. 2007; Carter et al. 2008; Iseda et al. 2008; Massey et al. 2008).

Previous work by our laboratory illustrated that Ch'ase ABC also appears to enhance motor function following SCI in the cat (Tester and Howland 2008). Some benefits of the cat model include its remarkable locomotor capacity, well characterized circuitry associated with different features of locomotion, the large size of its spinal cord, the similarity between its CS GAG sulfation patterns to that of the human (Tester and Howland 2008), and its use as a platform for prior translation of experimental animal work to the human SCI condition (Young 1991; Hodgson et al. 1994; Behrman and Harkema 2000; de Leon et al. 2001). Of particular interest, our prior work showed that intraspinal delivery of Ch'ase ABC across one month following a low thoracic hemisection promotes recovery of ipsilateral hindlimb use during skilled motor tasks (Tester and Howland 2008). Although not tested anatomically, this type of recovery suggests the involvement of the descending rubrospinal system. In the normal cat, the rubrospinal tract contributes to coordinated, multi-articular movements (Gibson et al. 1985;

Mewes and Cheney 1994), gait adaptations and interlimb coordination (Widajewicz et al. 1994; Lavoie and Drew 2002) which are all important during skilled locomotion. There also is precedent in the rat to suggest that this system may respond favorably to Ch'ase ABC treatment (Yick et al., 2004). We hypothesized that Ch'ase ABC treatment would significantly enhance recovery of skilled locomotion and also enhance axonal growth caudal to the lesion and promote growth of the rubrospinal tract, which is known to be important during skilled locomotion.

The current study focuses on the recovery of the ipsilateral hindlimb following thoracic hemisection during the peg board crossing task. The specific movement patterns of the ipsilateral hindlimb including how it is integrated using angular kinematics, scoring of limb placement, and interlimb coordination are characterized for 16-20 weeks post-injury. Further, pNF-H is used to assess general axonal growth, and retrograde tract tracing with Fluorogold (FG) to assess the presence of rubrospinal axons, below the level of the lesion. Significant behavioral and anatomical differences are found between Ch'ase ABC treated cats and controls. The movement patterns of the Ch'ase ABC treated cats are predictable and similar across animals as well as distinctly different from recovery in control animals. In addition, significant differences are found with regards to axonal growth caudal to the lesion; pNF-H is greater in the ipsilateral white and contralateral gray matter in Ch'ase ABC treated cats and an average of 23% of the axotomized red nucleus neurons have axons caudal to the lesion in Ch'ase ABC treated cats compared to 8% in control animals.

Methods

Animals

Twenty two adult female cats (6-8 lbs), purchased from specific-pathogen-free vendors and housed in the AALAC accredited animal facility in the McKnight Brain Institute, were used. All animal procedures were conducted in accordance with the NIH guidelines for the care and use of

experimental animals and were approved by the University of Florida's and the Malcom Randall VA Medical Center's Institutional Animal Care and Use Committees.

Surgical Procedures

Spinal T9/10 hemisections were performed as previously published (Tester and Howland 2008). Penicillin G, procaine (40,000U/kg BW IM) was given the day before, the day of, and the day after surgery. Prior to all surgeries, cats received 0.1cc atropine sulfate (0.04-0.06 mg/kg SQ) and 0.1cc acetylpromazine (0.4-0.5 mg/kg SQ). Following orotracheal intubation, anesthesia was maintained with isoflurane (1-3.5%) and an IV was placed for fluid administration (Lactated Ringers, 10 ml/kg/h). Respiratory rate, expired CO_2, SPO_2, blood pressure, body temperature, as well as the general plane of anesthesia were closely monitored.

A left lateral hemisection was made using iridectomy scissors at spinal T9 or T10. Light suction with a pulled glass pipette was used if necessary to lift any fibers adhering to the dura to facilitate the execution of a complete hemisection without compromising the integrity of the dura. Thrombin and gel foam were used to stop any bleeding. Micro-implantable infusion ports (Harvard Apparatus, Holliston, MA) were placed sub-cutaneously and sutured to muscle lateral to the vertebral column. Port tubing was held in place by suturing it to the muscle at several points until it reached midline. VetBond (Webster Veterinary Supply, Inc., Sterling, MA) then was used to secure it to the lamina caudal to the laminectomy. The end of the port tubing was secured in the lesion cavity by suturing the dura (8-0 Prolene). Prior to dural suturing, the port reservoir and tubing (~5µl total volume) was filled with either protease free Ch'ase ABC (1 U/200 µl Tris-HCl or saline, pH 7.8, Seikagaku Corp., Tokyo, Japan) or vehicle control (Tris-HCl pH 7.8). Durafilm (Codman-Shurtleff, Inc., Randolph, MA) and gelfoam (Pharmacia and Upjohn, Inc., Peapack, NJ) were placed over the dural sutures and the muscle and skin sutured. Anesthesia was terminated, cats were extubated, and then placed in temperature controlled

recovery chambers (ThermoCare, Las Vegas). Buprenorphine (0.01 mg/kg SQ) was administered TID for 48 hours.

Procedures used to maintain the general health of the cats were similar to those described in our previous studies (Howland et al. 1995b; Howland et al. 1995a). Once body temperature stabilized to a minimum of 100° F, cats returned to their home cages with 5-7 inch egg crate foam cushions covering the entire cage floor to prevent peripheral nerve compression, pressure sores, and skin breakdown. None of the animals developed any of these complications. For the first few days post-op bladders were expressed manually using Crede's method. Animal health was continuously monitored throughout the study, including maintenance of food intake and body weight.

Treatment Administration

Ten cats were treated with Ch'ase ABC and twelve cats were controls. Of the controls, 8 received hemisections-only (no port; 2 were non-behavior cats) and 4 received vehicle. Commercial Ch'ase ABC may not retain its stability at body temperature for extended periods (Tester et al. 2007), therefore 50 microliters of protease-free Ch'ase ABC (1 U/200µl), or vehicle were injected every other day for 1 month. The concentration delivered was identical to our previous study (Tester and Howland 2008). The volume is ~4x that used in rodent studies (Bradbury et al. 2002) due to the larger size of the cat spinal cord. For treatment administration, the cats were anesthetized (1-3% isofluorane and 1.5LO2) for 15 minutes while the Ch'ase ABC or vehicle was delivered (0.14L/min) using a microinjection syringe pump (Harvard Apparatus, Holliston, MA).

Behavioral Training and Quantitative Locomotor Assessments

Cats were trained daily (5x/week) on a variety of locomotor tasks; for the purposes of this paper only the skilled pegboard task will be presented. The horizontal pegboard was 4.5 meters

in length. Alternating pegs on the right and left side of the pegboard, 12 pegs on the right side and 11 on the left side, were spaced evenly along the length of the board at 20 cm intervals. The width between the right and left pegs was 15cm. The surface of each peg was 3.8cm^2 and the height 30.5cm. Crossing of the pegboard was conditioned to a food reward, and when performance was consistent, baseline data was collected pre-injury. All cats then received a left T9/10 hemisection and were placed into the control (n=10) or ch'ase ABC treated groups (n=10). Basic locomotor training was re-initiated within 24 hours and peg board crossing after the first post-operative week. Manual trainer assistance was given as necessary to assist with weight support, postural control and paw placement during pegboard crossings until independence was achieved. Training continued daily. The cats' performances were filmed every two weeks until 16-20 weeks post-injury. For video recording, the cats' hindquarters were shaved and reflective spheres placed on the iliac crest, greater trochanter, lateral malleolus, and the base of the 5th metatarsal. A fifth marker was placed on the fibula approximately one inch above the lateral malleolus marker in order to generate a unit vector, which in combination with the measured length from the lateral malleolus to the head of the lateral condyle (length of the fibula), was used to automatically calculate the knee position using Motus Software (Vicon-Peak, Englewood, CO). Following a hemisection, the ipsilateral hindlimb is primarily affected; therefore assessments predominantly focused on the left hindlimb (LHL).

Post- injury, assistance with weight support was provided until a cat could complete the pegboard task independently. Recovery onset of independent crossing and integration of LHL placement were determined for each cat. These were defined respectively as at least two independent full crossings and a minimum of one LHL placement/crossing. Placement required that the LHL be positioned and maintained with weight support on a peg. Once independent

crossing recovered, the percentage of LHL placements was quantified from the three best crossings at each remaining timepoint. Interlimb coordination patterns between the four limbs were assessed during independent crossings using support (footfall) pattern and stance-swing diagrams during 10 step cycles/cat/timepoint. For these assessments, the step cycle was divided into the swing and stance phases and each phase tracked for all four limbs relative to each other. These analyses indicate if there is a predictable pattern with regards to the timing of these phases across limbs within and across cats. Interlimb coordination and limb placement data were analyzed frame-by-frame using a remote search controller. Angular kinematics were assessed from a minimum of 10 step cycles per time point using Motus software (Vicon-Peak, Englewood, CO).

Retrograde Tracing

The retrograde tracer Fluorogold (FG, 0.5% in sterile water, Fluorochrome, Inc., Denver, CO) was used to label rubrospinal tract neurons with axons extending below the level of the hemisection. One to two weeks following the last behavioral data point collection, cats were anesthetized as for the hemisection surgery and the lesion site re-exposed. Using a 33 gauge Hamilton syringe, 2µl s of FG was injected bilaterally 15 millimeters below the caudal aspect of the lesion. In order to ensure adequate spread of the tracer across the entire cross-section of the spinal cord, the total 2µl volume of FG was delivered into 4 injection sites (0.5µl each site). Within each site, half of the volume (0.25 µl) was placed in the ventral half of the spinal cord and half into the dorsal half. No cats used in this study had FG spread into the lesion site.

Tissue Processing and Histology

Thirteen days following fluorogold injections, cats were anesthetized with an overdose of sodium pentobarbital (>40mg/kg, IP) followed by 1 cc of heparin (IV; 1000U) followed 20 minutes later by an injection of 1% sodium nitrite (1 cc) intravenously. Cats were perfused

transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The brain and spinal cords were removed, blocked, post-fixed with 4% paraformaldehyde overnight, and then placed in 30% sucrose in 4% paraformaldehyde for cryoprotection. Frozen longitudinal sections (25μ) of the FG injection sites were cut serially on a cryostat, mounted onto Superfrost/Plus Fisherbrand microscope slides (Fisher Scientific, Hampton, NH) and coverslipped (Vectashield Hard Set Mounting Media for Fluorescence Vector Laboratories, Inc., Burlingame, CA). FG autofluorescence was assessed to verify that the tracer was distributed across the width and depth of the spinal cord but did not spread into the lesion site. Mid-brain and spinal cord lesion segments were cut serially in cross-section at 25μ on a cryostat and collected in a 0.1M phosphate buffer saline (PBS) solution (pH 7.2, saline 0.9%)). Every tenth section of the lesion and mid-brain, were stained with cresyl violet (cresyl violet with acetate, Sigma-Aldrich, St. Louis, MO) and myelin stains (Eriochrome Cyanine R; Fluka, New York, NY) as described previously (Howland et al. 1995a; Tester and Howland 2008). The remaining sections were processed for immunohistochemistry.

Immunohistochemistry

To identify p-NF-H within and caudal to the lesion, sections were processed using the monoclonal chicken anti-phosphorylated-neurofilament heavy chain antibody (1:10,000, pNF-H Gift from G. Shaw and Encore Biotechnology, Gainesville, FL). Endogenous enzyme activity was quenched using 30% H2O2 in PBS for 30 minutes and then the sections were rinsed with PBS. Sections were rinsed in 1% goat serum in PBS containing 0.4% Triton (1%-S-PBS-T), blocked in 5% goat serum in PBS-T (5%-S-PBS-T) for 1 hour at room temperature, and incubated with the primary antibody diluted in 1%-S-PBS-T overnight at 4ºC. The next day tissue sections were rinsed with 1%-S-PBS-T before and after a one hour incubation with Alexa

Fluor 488 (1:400, Molecular Probes, Eugene, OR). Tissue sections were mounted onto charged slides coverslipped using the ProLong Anti-Fade kit (Molecular Probes).

To identify FG labeled rubrospinal neurons, midbrain sections containing the red nuclei were rinsed with 1%-S-PBS-T, blocked with 5%-S-PBS-T at room temperature for one hour and incubated overnight at room temperature with rabbit anti-FG (1:10,000, Fluorochrome, Inc, Denver, CO). Tissue sections were rinsed with 1%-S-PBS-T the next day followed by incubation with an anti-rabbit mouse secondary antibody (Vector laboratories, Burlingame, CA). Signal amplification was accomplished with the avidin-biotin-complex method (ABC, Vector Laboratories, Burlingame, CA) and visualized with 3-3 Diaminobezidine (DAB) reaction (Sigma, Saint Louis, MO) to produce a brownish stain.

To visualize pre-synaptic terminals on FG labeled rubrospinal tract neurons Double immunoperoxidase immunohistochemistry was done using monoclonal mouse anti-synaptophysin (1:1,000, Sigma, Saint Louis, MO) and anti-fluorogold antibodies. Tissue was processed as above for FG staining and then rinsed with 1%-S-PBS-T and blocked with 5%-S-PBS-T at room temperature for one hour. This was followed by incubated in primary anti-synaptophysin overnight at 4ºC. The next day tissue sections were rinsed with 1%-S-PBS-T followed by incubation with an anti-mouse secondary (Vector Laboratories, Burlingame, CA). Signal amplification was accomplished with the ABC method and visualized with the Vector VIP peroxidase substrate kit (Vector Laboratories, Burlingame, CA) to give a purplish reaction product. Sections were mounted onto chromium potassium sulfate and ply-L-Lysine subbed slides, allowed to dry, exposed to 4% paraformaldeyde fumes for at least 30 minutes to enhance bonding to the slide coating, dehydrated through increasing alcohol concentrations, placed into xylene and coverslipped using DPX (Fluka, Buchs., Switzerland).

Stereological Analyses of pNF-H

Three spinal cord cross-sections 300μ apart from each other, were used from control (n=4) and Ch'ase ABC (n=4) treated cats in order to assess the area fraction of pNF-H in four distinct areas of the spinal cord: (1) ipsilateral gray matter (2) ipsilateral white matter (3) contralateral gray matter and (4) contralateral white matter. The first of these sections in each animal was 1200μ caudal to the lesion epicenter. Stereoinvestigator software (MBF Biosciences, Colchester, VT) was used to assess the fractional area of pNF-H immunoreactivity within these contours, across each section, across cats using the area fraction fractionators probe (Cavalieri spacing: 250x500 for the gray matter areas and 375x750 for the white matter areas, Grid spacing: 15μ, and Frame size: 100μ x 100μ). The data was calculated using two dimensional dissectors on a single focal plane with systematic random sampling. To define the contour areas ipsilateral to the lesion, tissue caudal to frank lesion damage was included. Inversely, contour areas contralateral to the lesion included tissue areas caudal to areas not damaged at the lesion epicenter. This minimized the effect of any differences in sparing at the lesion site. The fractional area of pNF-H immunoreactivity within each section was obtained per cat and statistically assessed across groups.

Quantification of Rubrospinal Neurons

The left (spared) red nucleus (RN) was used as an internal control in each animal. FG labeled rubrospinal tract (RST) neurons in the left and right RN were quantified in each animal from every 8th section (200μ) throughout the rostro-caudal extent of each RN. Only neurons with visible punctate FG staining throughout their soma were counted. In addition to calculating the total numbers of labeled neurons in each nuclei, the number of neurons in the right (experimental) RN also were calculated and expressed as a percentage of the left (spared) RN as an internal control for individual animal differences.

Statistical Analyses

Statistical Analyses were performed using Statistical Package for the Social Sciences (SPSS) v. 17 (Chicago, IL). For the categorical data the Fisher's exact Test was used due to the occurrence of cells with frequencies <5. Discrete, ordinal data for independent samples (Ch'ase versus Tris) was assessed using the Mann-Whitney U test. All Fisher's Exact and Mann-Whitney U tests were two tailed and a value of $P<.05$ was considered significant. Discrete, ordinal data for dependent samples (Pre- compared to post-op performance within a group) was assessed using the Wilcoxon Sign-Rank Test. All tests for this assessment were one tailed as performance could change in only one direction and significance was set at $P<0.05$.

Results

Narrow Range of Spinal Hemisections

Cresyl violet and myelin stained serial sections were used to determine the extent of spinal cord damage. The lesions of all 22 cats were similar, and typically showed complete ipsilateral gray and white matter damage. Variability across lesions was limited and three spinal cord sections representing the entire range of spinal hemisections are shown in Figure 1. The smallest lesion had some ipsilateral ventromedial white matter sparing and the largest had some contralateral damage (Figure 4-1). Critical to the RN counts, the ipsilateral lateral funiculus where the rubrospinal tract is located was completely severed and the contralateral completely spared in all cats.

Ch'ase ABC Enhances Multiple Features of Pegboard Performance

All cats showed an initial period (<24 hrs) of flaccid paralysis followed by reflex activity and then voluntary movement of the left hindlimb within 48-72 hours of injury. Consistent with previous reports from our lab and others, basic LHL stepping during bipedal treadmill and voluntary overground locomotion began to recover by the end of the first post-operative

(Eidelberg et al. 1986; Helgren and Goldberger 1993; Tester and Howland 2008) indicating the similarity across lesions.

Although all cats quickly, efficiently and independently crossed the pegboard prior to injury, the ability to accomplish this task was disrupted by hemisection (Figure 4-2). At two weeks post-injury, few cats (1/10 controls, 3/10 Ch'ase ABC) could independently cross.the pegboard and there was no significant difference in number of cats within each group accomplishing this task (p= 0.582, Fisher's Exact). The number of Ch'ase ABC treated cats crossing at 4 weeks post-SCI (8/10) however, was significantly greater than the number seen in the control group (2/10; p=.023 Fisher's Exact). This significant difference between the two groups' performances also was seen at 6 weeks (p=.005, Fisher's Exact). By 8 weeks the number of Ch'ase ABC treated (9/10) compared to control (4/10) cats was not significantly different (p=.057, Fisher's Exact). The differences between groups at 16 weeks (9/10 v 5/10; p=.141 Fisher's Exact) and 20 weeks (10/10 v.7/10; p=0.211, Fisher's Exact) also were not significant. These results suggest that Ch'ase ABC treatment significantly accelerates recovery of crossing but that performance on this ability levels out between the two groups around 8 weeks post-injury.

Prior to injury, all cats crossed the pegboard using four limbs. Following hemisection, however, cats might or might not place their LHL on a peg while crossing (Figure 4-2). When the LHL was not integrated in this manner, cats would cross by placing only their other three limbs onto the pegs. Recovery onset of the ability to integrate the LHL by placing it onto a peg was assessed in all cats (Figure 4-2). At two weeks, although several cats could independently cross, none of the cats placed their LHLs onto pegs. By 4 weeks, using the Fisher's Exact Test, a significantly greater number of Ch'ase ABC treated cats were integrating their LHL than in the

control group (p=.011). This significant difference between the two groups continued to be seen at 6 weeks (p=.001), 8 weeks (p=.020), 16 weeks (p=.020) and 20 weeks (p=.033) post-SCI.

To determine how effectively cats integrated their LHLs during crossings and whether or not Ch'ase ABC enhanced recovery of this feature, the number of LHL placements onto a peg were quantified (Figure 4-2). No significant differences in performance were seen pre-injury between the cats that would be placed into each group (p=1.0, Mann Whitney U) as all cats placed their LHL onto a peg 100% of the time. There also was not a significant difference between the two groups at two weeks post-injury (p=1.0) as none of the cats placed their LHLs onto a peg. By 4 weeks, however, the average percentage of LHL placements was significantly greater in the Ch'ase ABC treated group (22%) compared to the control group (0%; p=.005, Mann Whitney U). The performance of the Ch'ase ABC treated animals continued to show significantly greater LHL placements at 8 weeks (28% v. 5%; p=.012) and 16 weeks (44% v. 9%, p=.009). Only 10 cats (4 Ch'ase treated and 6 controls) remained in the study out to 20 weeks. Using the Mann Whitney U, significant differences also were seen in this smaller number at 20 weeks post-injury (100% v. 9%, p=.005).

To understand how this recovery occurs between time points within each group, additional assessments of the data using the Wilcoxon Matched Pair Sign Rank Test were performed. The control group of cats showed a significant decrease in LHL peg placements at all time points compared to pre-injury performance: 2 weeks (.002), 4 weeks (p=.002), 8 weeks (p=008), 16 weeks (p=.004) and 20 weeks (p=.020). As seen in the control group, the Ch'ase ABC group showed significant decreases in the number of LHL placements at 2 weeks (p=.002), 4 weeks (p=.005), 8 weeks (p=.008) and 16 weeks (p=.018) post-injury compared to their pre-injury performances. In contrast to the control group, the LHL performance of Ch'ase ABC treated

group at 20 weeks was not significantly different from pre-injury (p=1.0) indicating that performance was similar to that seen pre- injury. Assessments also indicated that significant increases in performance were seen from 2-to-4 weeks (p=.028) and from 8-to-16 weeks (p=.012) in the Ch'ase ABC group. No significant improvements in performance were seen between any post-injury time points in the control group. Collectively these data on the use of the LHL suggest that Ch'ase ABC has significantly enhances the general integration of the hindlimb as well as the accuracy with which the limb is used.

Ch'ase ABC Treated Cats Develop a Novel Interlimb Coordination Pattern

Prior to injury, cats typically place their left limbs (fore and hind) on the pegs on the left side of the board and their right limbs (fore and hind) on the right side pegs (Figure 4-3). This basic placement strategy is not seen post-injury. Cats that do not re-integrate their LHLs simply do not place them (Figure 4-3). However, the cats that do reintegrate the limb, cross the body midline with the LHL to place it onto a peg on the right side of the board (Figure 4-3). Further, the LHL is now typically paired with the right forelimb (RFL), for at least initial peg contact. Thus, the LHL and RFL share a peg for some amount of time on the right side of the pegboard (Figure 4-3).

Pre-injury, when the majority of cats (16/20) consistently kept their left and right limbs on the left and right sides of the pegboard respectively, their performances were characterized by a single, consistent footfall pattern that was repeated with the beginning of each step cycle (Figure 4-3). During the majority of the step cycle, only one limb at a time was in swing. The very brief periods in which the swing of two limbs coincided were typically 1-2 video frames in length which is equivalent to 33-66 milliseconds. The limbs paired during these brief swing phase overlaps showed a consistent, repeating pattern. Overlap of the ipsilateral fore- and hindlimb swing phases occurred first followed by overlap of the contralateral fore- and hindlimb swing

phases. Additionally initiation of the stance phases of the four limbs also occurred in a predictable order beginning with the LHL, followed by the LFL, then right hindlimb (RHL) ending with the right forelimb (RFL). Approximately 60% of the stepcycle was characterized by triple limb support time and ~40% by double limb support. Analyses using support pattern diagrams also indicate the use of a consistent pattern interlimb pattern (Figure 4-3). The support pattern diagrams showed a consistent 3-2-3-2-3-2-3-2 support formula (Figure 4-3). Thus, as has been shown in many studies for quadrupedal treadmill and voluntary overground locomotion, steps on a pegboard show a consistent interlimb coordination pattern.

Three Ch'ase ABC treated cats capable of integrating their LHLs consistently at 16 weeks post-injury were evaluated to determine if any showed a predicable interlimb coordination pattern. Interestingly, not only was a consistent interlimb coordination pattern seen but it was consistent across the three cats and distinctly different in several ways from that seen pre-injury. The total support time of all limbs was increased and contributed to the introduction of a quadrupedal support period. In particular, the stance phase of the left hindlimb was lengthened such that it overlapped with at least two stance phases of each of the other limbs. The LHL swing phase also was lengthened. The increased time spent in each of these phases resulted in a 1:2 ratio of LHL stepcycles to the stepcycles of each of the other limbs. The general order of the stance and swing phases for each limb relative to the others showed a similarity to the pre-injury pattern with the exception of the LHL. The support pattern diagrams (Figure 4-3) also suggested a consistent interlimb coordination patterm. A repeating support formula of 3-2-3-4-3-2-3 for the first set of step cycle and 4-3-2-3-4-3-2 for the subsequent step cyclewas seen.Thus, footfall patterns and support diagrams both indicate that a unique pattern of interlimb coordination is seen in Ch'ase ABC treated cats which recover LHL placement on the pegboard.

The LHL proximal angular kinematic patterns showed distinctly different ranges of movement in cats that placed their LHLs on pegs versus those that did not. Although control animals typically crossed the pegboard on three limbs, the LHL was not passive. It alternated between flexion and extension, but its active range of movement was much smaller than seen pre-injury (Figure 4-4). In dramatic contrast, the angular kinematics of the proximal LHL of Ch'ase ABC treated cats that placed post-injury showed nearly twice the range of angular excursion at the hip and the knee in comparison to pre-injury values (Figure 4-4). The increased angular excursion is consistent with the placement of the LHL on the right side pegs and the skipping of a peg due to the 1LHL:2 other limb stepcycle ratio seen post-injury. The peg skipping of the LHL post-injury was in contrast to LHL placement onto every peg pre-injury. Collectively these results suggest that Ch'ase ABC cats develop unique but consistent and effective new LHL movement strategies post-hemisection for crossing the pegboard.

Enhancement of Axonal Densities at the Spinal Level in Cats Receiving Ch'ase ABC

Axonal growth was assessed qualitatively within the lesion epicenter using an antibody against the 200kD phosphorylated axonal form of the neurofilament heavy chain (pNF-H) positive axonal profiles were visible throughout the lesion scar in all animals assessed (Figure 4-5). In all Ch'ase ABC treated animals the pNF-H positive axons appeared highly fasciculated and densely packed in the lesion environment (Figure 4-5). This staining profile was observed in only one control animal (Figure 4-5). Axons positive for pNF-H in the lesion epicenter of the remaining three control animals were not highly fasciculated or densely packed and had a blunted (Figure 4-5) or thin filamentous appearance (Figure 4-5). pNF-H staining density was not assessed as the size of the scar area varied notably across cats and would have confounded any findings.

The density of pNF-H profiles were quantitatively assessed in eight cats, four controls and four Ch'ase ABC treated cats, throughout the ipsilateral and contralateral gray and white matter caudal to the hemisections. Three spinal cord cross-sections starting 1200μ caudal to the lesion epicenter as defined by cresyl-violet myelin staining, and 300 μ apart from each other, were used from control (n=4) and Ch'ase ABC (n=4) treated cats. The area fraction of pNF-H in four distinct contoured areas of these sections was assessed: (1) ipsilateral gray matter (2) ipsilateral white matter (3) contralateral gray matter and (4) contralateral white matter. Ipsilateral contours are caudal and on the same side as the spinal hemisection, and contralateral contours are caudal and on the spared side of the spinal cord.

The area fractions of pNF-H immunoreactivity within the caudal ipsilateral gray matter (Figure 4-6) and contralateral white matter (Figure 4-6) were not significantly different between Ch'ase ABC and control treated cats as assessed by the Mann Whitney U (p=0.166 for each) . In contrast, Ch'ase ABC treated cats had a significantly greater area fraction of pNF-H immunoreactivity in the caudal ipsilateral white matter as compared to controls (p=.033 Mann Whitney U; Figure 4-6. Ch'ase ABC treated cats also had a significantlygreater area fraction of pNF-H in the caudal contralateral gray matter as compared to controls (p=.003; Figure 4-6).

More Rubrospinal Neurons have Axons Caudal to the Hemisection in Ch'ase ABC Treated Cats

To determine if rubrospinal axons contributed to the increased pNF-H immunoreactivity seen caudal to the lesion, retrograde tract tracing studies were conducted using Fluorogold (FG) in 10 cats (5 controls and 5 Ch'ase ABC treated cats). Bilateral injections of FG were made approximately 1 1/2-2 segments below the original left, T9/10 lateral hemisection. The injections sites of all 10 cats showed a good distribution of the tracers across the entire cross-sectional area, but did not spread into the lesion site .The hemisection of every cat in this study

completely interrupted the rubrospinal tract on the side of the lesion at the lesion epicenter FG-labeled neurons in the left (control) RN were found throughout the hindlimb region of th RNm (Figure 4-7). The number of FG-labeled RN neurons in the left (control) RN was not significantly different between Ch'ase ABC and control animals (740±40 and 894±109 respectively). The number of retrogradely neurons in the right (axotomized) RN however was greatly decreased compared to the left (Figure 4-7). Ch'ase ABC treated cats had an average of 308±59 compared to 137±43 in the control group. . These averages were then expressed as a percentage of the number of labeled neurons in the left RN. The percentage of neurons in the Ch'ase treated cats (23%) was significantly greater than the than in the control cats (9%) as determined with the Mann Whitney U (p=032 Figure 4-7).

Brainstem sections stained for FG throughout the RN also were double labeled with synaptophysin, a pre-synaptic terminal marker. Synaptophysin labeled puncta were visualized on the perimeter of FG labeled neurons in the left (control) RN (Figure 4-7). Synaptophysin co-localized around FG labeled neurons in the right (axotomized) RN in Ch'ase ABC as well as control treated cats (Figure 4-7), suggesting all of these neurons were receiving input.

Discussion

In the present study, we demonstrated that degradation of CS-GAGs via intraspinal delivery of Ch'ase ABC promoted axonal growth caudal to the lesion as well as regeneration and/or collateral sprouting of approximately 22% of the axotomized rubrospinal tract neurons. The RST is a major descending pathway associated with the control of skilled locomotion, and correlatively, Ch'ase ABC treated cats also had significantly enhanced recovery during skilled pegboard locomotion. Ch'ase ABC treated animals were able to integrate the ipsilateral LHL earlier and significantly more at all post-injury timepoints compared to controls, and this recovery was paralleled by the use of a novel, complex hindlimb movement pattern that

integrated the LHL kinematically different than pre-injury. We further demonstrated that Ch'ase ABC treated animals had an increased pNF-H area fraction within the caudal ipsilateral white matter and contralateral gray matter, indicating that Ch'ase ABC promoted axonal growth through the lesion environment as well as around the lesion that maintained projections 1-2 segments below the original lesion. This axonal growth may have included axons of RST origin following axotomy and Ch'ase ABC treatment.

Skilled Pegboard following Thoracic Hemisection and Ch'ase ABC Treatment

The RST is principally involved with modulating motor control and has a specific role in skilled motor functions (Whishaw et al. 1998). Lesions of the RST have been shown to affect forelimb function during skilled locomotion (Schrimsher and Reier 1993; Whishaw and Gorny 1996; Whishaw et al. 1998; Muir et al. 2007), and there also is evidence that the RST contributes to hindlimb function as well (Orlovsky 1972a; Lavoie and Drew 2002). Rubrospinal neurons have been proven to aid in precise limb modification during skilled locomotion, regulation of locomotion during gait adaptation to environmental demands, and for the regulation of intra- and interlimb coordination in the normal cat (Widajewicz et al. 1994; Lavoie and Drew 2002). Pegboard locomotion requires precise intra- and interlimb coordination as well as accurate limb targeting. Our hemisection model axotomized the RST unilaterally and consequently caused substantial deficits in pegboard locomotion.

Similar to the ladder rung walking test used in rats (Metz and Whishaw 2002), the pegboard task used in our cat model is exceptionally challenging. Immediately following spinal hemisection, cats must precisely adapt their weight support and compensate for the ipsilateral limb deficits by adjusting postural control and shifting of the body weight to the less affected limbs. There is considerable evidence that animals with lesions of motor pathways maintain the ability to compensate for lesion-induced deficits in skilled locomotion (Miklyaeva et al. 1994;

Whishaw et al. 1997a; Whishaw et al. 1997b; Kleim et al. 1998; Whishaw et al. 1998; Metz and Whishaw 2002). Despite a notable compensatory change in behavior, there are still substantial impairments present. Similarly, in the present study, control animals sustained residual impairment of LHL placement onto pegboard pegs, but some were able to compensate by crossing the pegboard on three limbs. Ch'ase ABC treated animals had substantially decreased impairment of LHL placement onto the pegs at all post-injury timepoints compared to controls. The appearance of a common compensatory response occurred in Ch'ase treated cats post-injury, where the LHL was no longer placed onto the left side of the pegboard like pre-injury placement, but it was instead paired with the RFL on the right side of the pegboard. This unique limb placement strategy altered the support pattern of all four limbs when compared to pre-injury placement. Notably, this post-injury compensatory strategy was equally as efficient as the pre-injury strategy. Muir et al. hypothesized that following unilateral lesions affecting multiple descending pathways, a common plastic response may arise to mask deficits that are specific to the loss of each different pathway (Muir et al. 2007). Since our lesion paradigm affects multiple descending motor systems, it is therefore possible that this could be occurring with the emergence of the unique compensatory strategy in Ch'ase ABC treated animals. The ability to re-integrate the impaired limb during skilled pegboard locomotion correlated with increased axonal growth caudal to the lesion and an increase in growth of axotomized RST axons, illustrates that Ch'ase ABC treatment affected the plasticity of a critical motor system and subsequently caused enhanced skilled behavioral function.

Training in all animals in the present study involved treadmill walking ,as well as crossing of simple and challenging runways 5x/week (for examples see (Tester and Howland 2008). Substantial evidence suggests that locomotor training can improve hindlimb/lower

extremity motor functions post-spinal cord injury in animals and humans respectively (Edgerton et al. 2004; Thomas and Gorassini 2005; Behrman et al. 2006; Frigon and Rossignol 2006). Voluntary exercise also has been shown to increase the production of several neurotrophins (Gomez-Pinilla et al. 2002; Ying et al. 2003) which may play significant roles in motor recovery and synaptic plasticity post-spinal cord injury (Ying et al. 2008). This possible increase in neurotrophins such as BDNF from our rigorous training regimen may have fascilitated skilled locomotor recovery in our animals, as well as enhancement of RST axonal growth. Control animals in this study had approximately an 8% increase in axotomized RST axons caudal the spinal lesion. This increase could have partially been due to an upregulation of neurotrophic support from locomotor training, and interestingly the percentage increase in RST growth was similar to studies where cells genetically modified to express BDNF were transplanted into cervical lesions in the rat (Liu et al. 1999; Liu et al. 2002b). Furthermore, tracing studies have demonstrated that an ipslateral rubrospinal pathway exists in the feline (Hayes and Rustioni 1981; Holstege and Kuypers 1982; Holstege 1987). Therefore, it is plausible that a small percentage of retrogradely labeled RST neurons in the experimental, right red nucleus of our control and Ch'ase ABC treated animals came from ipsilateral projections.

Strategies to Promote Rubrospinal Tract Growth after Cervical Axotomy

Spontaneous plasticity of multiple axonal tracts has been established after partial lesions of the spinal cord across a variety of species (Murray and Goldberger 1974; Li et al. 1994; Fouad et al. 2001b; Weidner et al. 2001; Bareyre et al. 2004; Steward et al. 2008). It has been shown previously that following a cervical hemisection in the adult rat, RST axons approach the rostral lesion edge but do not have the capacity to spontaneously re-grow through or caudal to the lesion (Houle and Jin 2001), therefore numerous experimental strategies to specifically promote RST axonal growth have been conducted in animal models of spinal cord injury.

Acute transplantation of olfactory ensheathing glia or fibroblasts genetically modified to express the brain-derived neurotrophic factor (BDNF) following cervical lesions of the lateral funiculus in the adult rat promoted minimal sprouting of RST axons distal to the lesion site (Ruitenberg et al. 2003), and modest growth of 7-10% of axotomized RST axons (Liu et al. 1999; Liu et al. 2002b). Intrathecal delivery of NEP1-40, a Nogo receptor (NgR) antagonist proved even less effective, inducing RST axonal growth rostral and at the level of the lesion but not caudal (Cao et al. 2008). Transplants of fibroblasts modified to express BDNF and NT-3 elicited modest regeneration of RST axons when the transplants were delayed for 6 weeks following cervical hemisection (Tobias et al. 2003), and less growth was observed than when grafted acutely (Liu et al. 1999) or after 4 weeks of delay following lesion (Jin et al. 2002). Infusion of BDNF directly to the RST neuronal cell bodies one week post-axotomy at the cervical level induced less than 5% growth of axotomized rubrospinal axons into a peripheral nerve graft (Kobayashi et al. 1997) and approximately 3% when applied one year after cervical injury (Kwon et al. 2002).

Fetal spinal cord transplanted into a cervical hemisection promoted occasional RST axons to grow into the transplant but none caudal (Mori et al. 1997), and peripheral nerve grafted into the lesion epicenter alone promoted the growth of approximately 2% of RST axons (Kobayashi et al. 1997; Harvey et al. 2005). Transplantation of human adult olfactory neuroepithelial neurosphere forming cells (NSFCs) induced growth of RST axons 4-8 segments caudal to the graft and re-established synaptic connections with distal targets (Xiao et al. 2007). All together, these experimental strategies elicited minimal RST axonal growth following partial lesions of the cervical spinal cord.

Ch'ase ABC Treatment to Promote Spinal Plasticity and Rubrospinal Tract Growth

Disruption of CS-GAGs with Ch'ase ABC *in vivo*, either alone or in combination with other treatments, has been shown to enhance axonal growth and/or functional behavioral recovery after SCI (Yick et al. 2000; Moon et al. 2001; Bradbury et al. 2002; Yick et al. 2003; Chau et al. 2004; Caggiano et al. 2005; Barritt et al. 2006; Houle et al. 2006; Massey et al. 2006; Cafferty et al. 2007; Carter et al. 2008; Iseda et al. 2008; Massey et al. 2008; Tester and Howland 2008). Degradation of CS-GAGs using Ch'ase ABC has been shown to effectively enhance the regeneration of injured nigrostriatal axons (Moon et al. 2001) as well as of crushed CST axons (Bradbury et al. 2002). Previous studies utilizing the enzymatic degradation of CS-GAGs with the administration of Ch'ase ABC into the lesion cavity following cervical hemisection in the adult rat, resulted in a 22% re-growth of RST axons caudal to the original lesion, and in combination with lithium chloride (LiCl), growth of RST axons was enhanced to 42% (Yick et al. 2004). Until the present study, experiments have not been conducted that assess the growth of RST axons following axotomy of the thoracic spinal cord, and correlatively in an even more translational model of spinal cord injury.

Previous studies have shown that implantation of peripheral nerves into the adult rat spinal cord following cervical transection of the RST resulted in only a small percentage (1-2%) of rubrospinal axon growth into the peripheral nerve graft (Richardson et al. 1984; Houle 1991; Tetzlaff et al. 1994), whereas descending axons rarely regenerated following thoracic or lumbar injury, implicating that the distance from cell body to injury was a strong determinant of potential axonal growth (Richardson et al. 1984). Fernandes et al. confirmed and extended their results illustrating that RST neurons have the growth capacity to extend their axons into a peripheral nerve transplant after cervical but not after thoracic axotomy and that after cervical and not thoracic axotomy, regeneration-associated gene expression was enhanced (Fernandes et

al. 1999). Based on the poor growth potential following thoracic spinal injuries, the growth of 22% of axotomized RST neurons following Ch'ase ABC treatment in our study is remarkable, especially considering that in our feline model of thoracic injury the distance from cell body to axotomy is much larger than it would be in the rat.

Axonal growth was also assessed in our studies witin the lesion qualitatively and caudal to the spinal hemisection by quantitatively. Degradation of neurofilament proteins occurs following spinal cord injury (Banik et al. 1982; Martin et al. 1990; Banik et al. 1997; Schumacher et al. 1999; von Euler et al. 2002; Liu et al. 2009) and traumatic brain injury (Nakamura et al. 1992; Kaku et al. 1993; Saatman et al. 1998; Huh et al. 2002). It has previously been reported that 15 weeks following ischemic spinal cord injury that axons positive for pNF-H are present within the lesion cavity (von Euler et al. 2002). In the present study, Ch'ase ABC treated cats had dense, fasciculated pNF-H immunolabeling profiles within the lesion environment as opposed to control animals that had some swollen axonal profiles and an overall less dense pNF-H appearance. Caudal to the lesion Ch'ase ABC treated cats had an increase in the area fraction of pNF-H within the ipsilateral white matter and contralateral gray matter as compared to controls. These results indicate that Ch'ase ABC enhances axonal plasticity through and around a thoracic hemisection. The increased axonal growth in the ipsilateral white matter following Ch'ase ABC treatment may have lead to functional re-connectivity of the RST and in turn behavioral recovery in our Ch'ase ABC treated animals.

In conclusion, Ch'ase ABC promotes axonal growth caudal to a thoracic spinal hemisection in the adult cat, and correlatively increases the regeneration and/or collateral sprouting of rubrospinal tract axons caudal to the lesion. Furthermore, these growth

enhancements of the RST with Ch'ase ABC treatment may have affected the skilled locomotor recovery also seen.

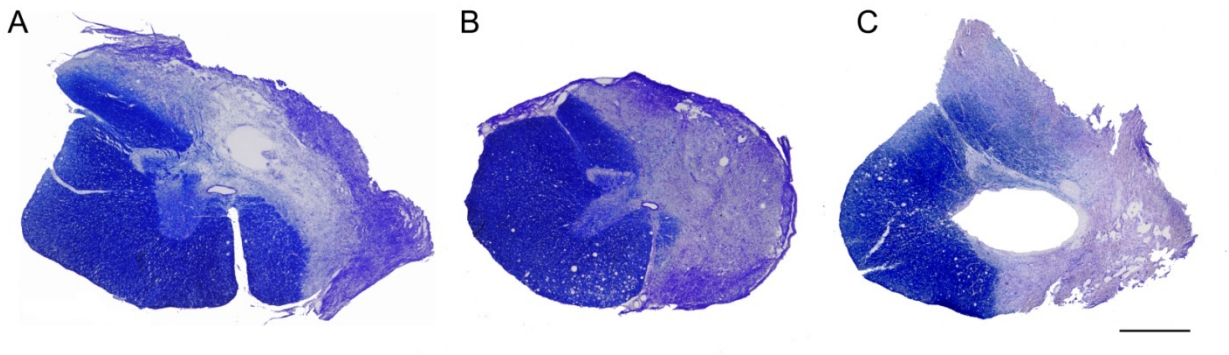


Figure 4-1. Representative range of spinal hemisections. Horizontal sections through the lesion epicenter stained with cresyl violet and myelin show that lesions ranged from an under-hemisection with ipsilateral ventromedial sparing (A), to a complete lesion with interruption of all ipsilateral gray and white matter and no damage to the contralateral tissue (B), to an over-hemisection with interruption of contralateral gray and white matter and possible cyst formation or enlargement of the central canal (*C*). Animals used in this study had slight variations of tissue sparing and damage as compared to these examples. Scale bar: 1mm.

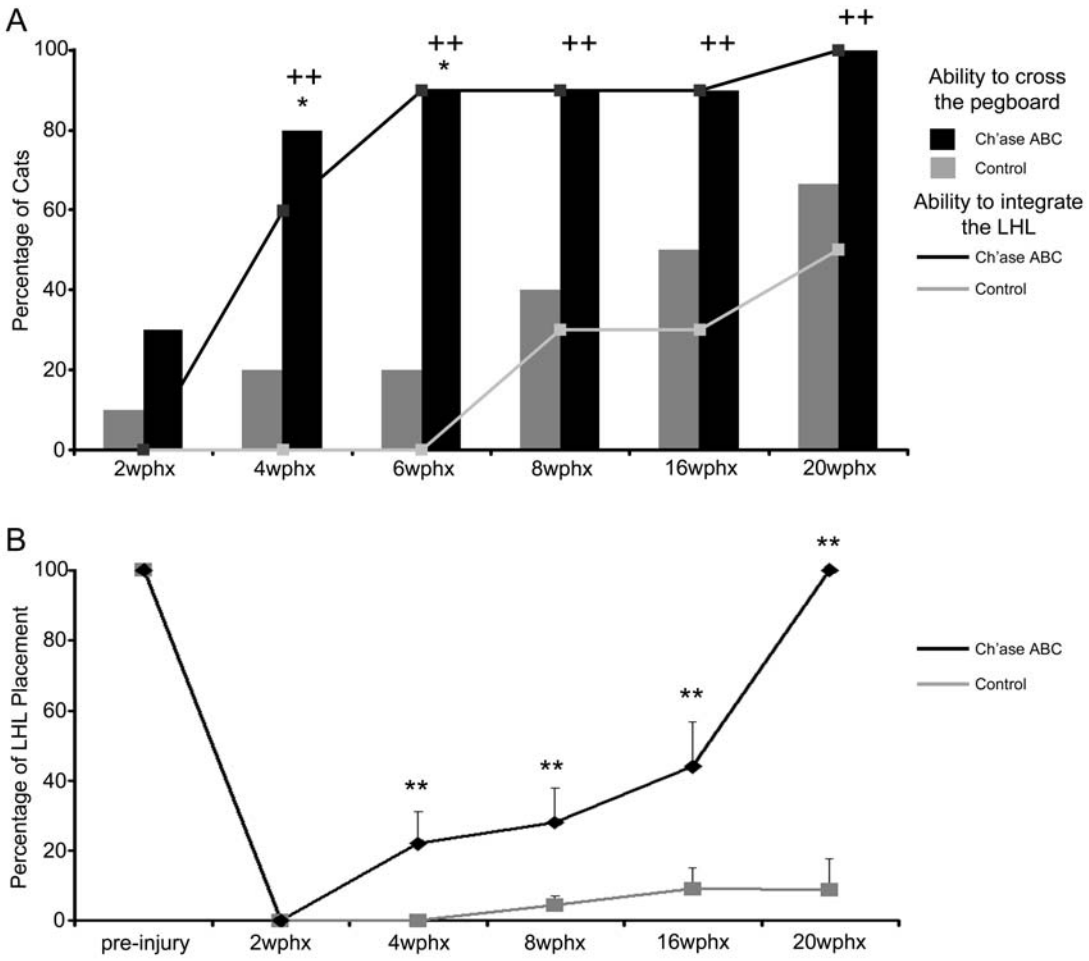


Figure 4-2. Pegboard locomotor recovery is improved with Ch'ase ABC administration. The ability to cross the pegboard (bar graphs) and place the left hindlimb (LHL) on the pegboard (line graphs) was assessed at multiple post-injury timepoints (A). During pre-injury pegboard locomotion, all cats were able to cross the pegboard and place the LHL onto the pegs with 100% accuracy. Our lesion paradigm causes significant locomotor impairment in the ipsilateral LHL. Following injury, significantly more Ch'ase ABC treated cats could cross the pegboard independently at 4 and 6 weeks post-injury compared to controls (*). Ch'ase ABC treated cats at every post-injury timepoint compared to controls could integrate the LHL into placement significantly more (++). The percentage of step cycles that the LHL was accurately placed on the pegboard was assessed at multiple post-injury timepoints (B). Ch'ase ABC treated cats integrate the LHL a greater percentage of time compared to controls at 4, 6, 8, 16, and 20 weeks post-injury (**). Error bars denote SEM. * Indicate a significant change across groups when assessing the ability to cross the pegboard. ++ Indicate a significant change across groups at a particular timepoint when assessing the ability to integrate the LHL. ** Indicate a significant change across groups at each timepoint when assessing the percentage of LHL placement onto the pegboard

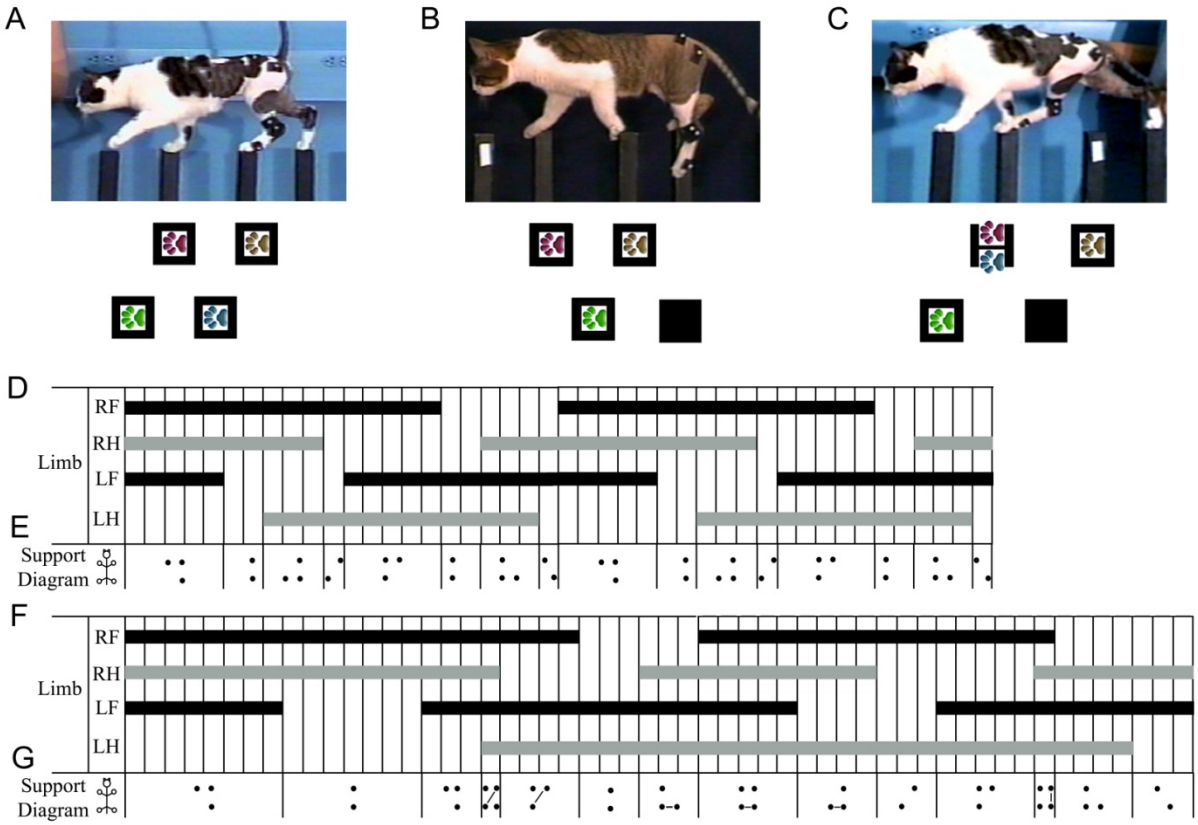


Figure 4-3. Limb placement strategies during pegboard locomotion. During pre-injury crossing of the pegboard, the majority of cats place their four limbs in a specific spatial pattern. The left limbs stay on the left side of the pegboard and the right limbs stay on the right side of the pegboard during crossing, and no two limbs are ever placed onto the same peg (A). Following injury, control animals primarily cross the pegboard using three limbs and are not able to incorporate their LHL into pegboard placement (B). Post-injury, Ch'ase ABC cats are able to incorporate the LHL into placement onto the pegboard (C) but differently than pre-injury. The LHL now pairs with the right forelimb at initial contact on the right side of the pegboard (A, C). Footfall pattern diagram during pre-injury pegboard crossing (D) and post-injury LHL placement at 16wphx by a representative Ch'ase ABC treated cat (F). The solid bars indicate when the limb is in the stance phase and the open areas indicate when the limb is in the swing phase. Two complete step cycles are shown in D and F. Time between vertical lines is one frame (33.3ms). Pre-injury, the support pattern diagram shows a 3-2-3-2-3-2-3-2 support formula (E). The support pattern diagram for 16wphx crossing of the pegboard shows a repeating two-step cycle support formula of 3-2-3-4-3-2-3 and 4-3-2-3-4-3-2 (G). Lines drawn between limbs in the support pattern diagram indicate that the two limbs are being paired on the same peg. LF, left forelimb; RF, right forelimb; LH, left hindlimb; RH, right hindlimb.

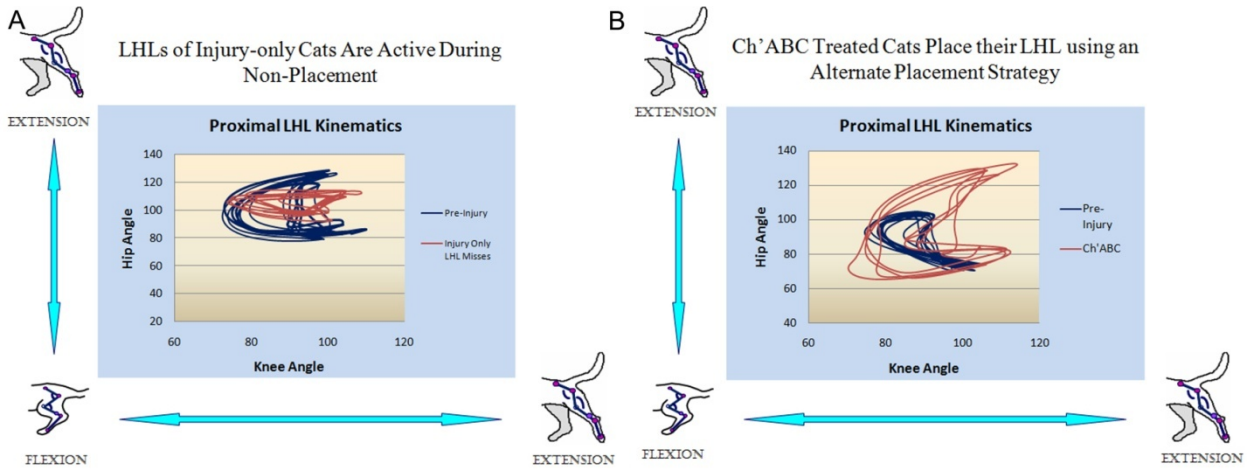


Figure 4-4. Proximal left hindlimb angular kinematics during different pegboard crossing strategies. LHLs of control cats are active during post-injury non-placement (A). Angle-angle kinematic pattern of the proximal LHL of a control treated cat is shown during pre-injury placement of the LHL on the pegboard (blue) and during non-placement at 16wphx (red). At 16wphx, the majority of injury-only animals are unable to integrate their LHL into placement on the pegboard. Despite this, during non-placement, when the control animals are crossing on three limbs, the LHL is still flexing and extending with a similar kinematic pattern to pre-injury. The range of excursion of the proximal limb is smaller, but the overall kinematic pattern is preserved. The angular kinematics of the proximal LHL of Ch'ase ABC treated cats has a larger excursion at 16wphx during placement (red) on the pegboard than during pre-injury placement (blue), due to the use of an alternate placement strategy post-injury (B). Smaller joint angles on either axis represent increased flexion of the joint being assessed, and larger angles represent increased joint extension. At each timepoint 10 representative step cycles are shown.

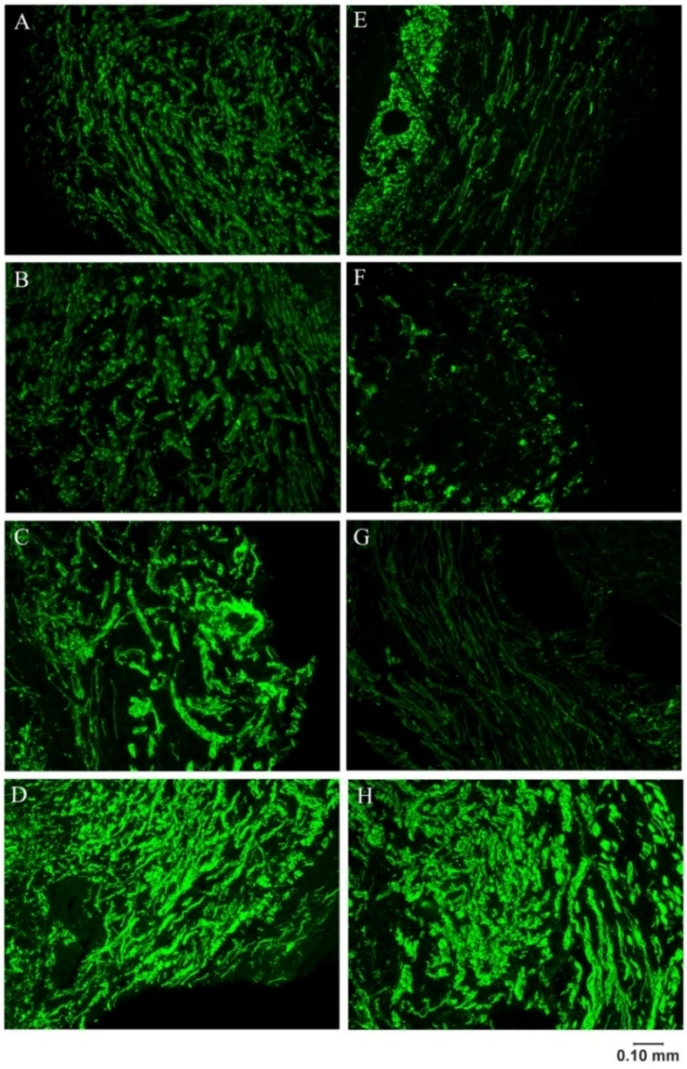
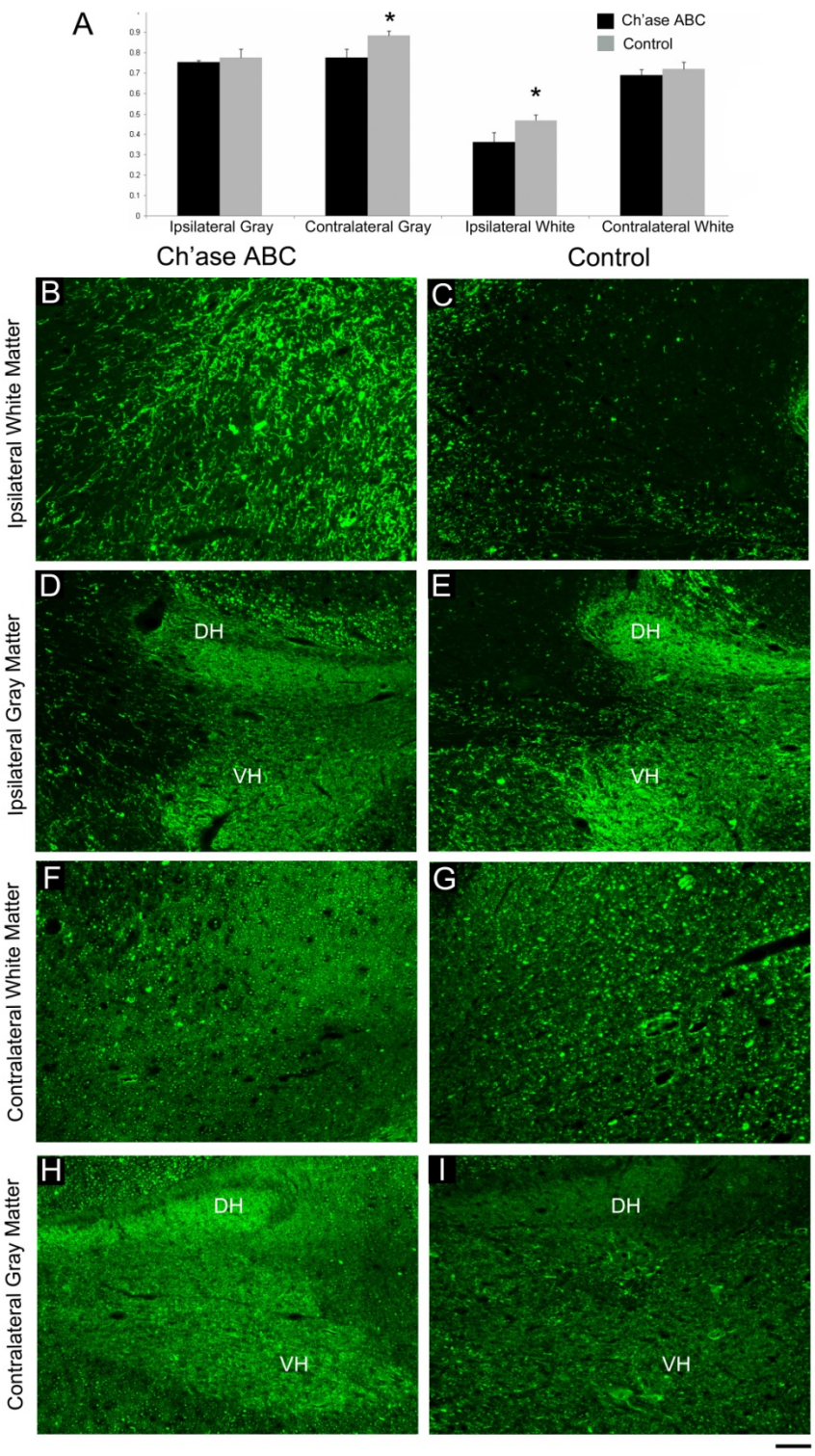


Figure 4-5. Phosphorylated neurofilament heavy chain (pNF-H) immunoreactivity within the lesion epicenter. pNF-H within the lesion epicenter of Ch'ase ABC treated cats (A, B, C, D) and control cats (E, F, G, H). pNF-H axonal growth appears to be stunted and more finely distributed in the majority of control animals (E, F, G) as compared to all Ch'ase ABC treated animals where the pNF-H immunoreactivity in the lesion is dense and highly fasciculated. Scale bar: 0.1mm.

Figure 4-6. Increases in phosphorylated neurofilament heavy chain (pNF-H) caudal to the spinal hemisection with Ch'ase ABC treatment. Quantification of pNF-H caudal to the spinal hemisection (A). Non-biased stereological quantification of the area fraction of pNF-H was assessed in four distinct contours of the caudal spinal cord; ipsilateral white matter (B,C), ipsilateral gray matter (D, E), contralateral white matter (F, G), and contralateral gray matter (H, I). Quantification revealed that Ch'ase ABC treated animals (B, D, F, H) had a significant increase in the area fraction of pNF-H as compared to control animals (C, E, G, I) in the ipsilateral white matter (B, C) and in the contralateral gray matter (H, I). There was no significant difference seen in the area fraction of pNF-H across groups in the ipsilateral gray matter (D, E) or the contralateral white matter (F, G). Error bars denote SEM, * Indicates a significant change across groups. Photomicrographs of the ipsilateral and contralateral white matter (B, C, F, and G) are within the lateral funiculus, where the RST axons traverse. Scale bar: 0.1mm. Y axis values in A are the area fraction of pNF-H staining as a percentage of the contoured area.


A
Ch'ase ABC
Control
*
*
0.9
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0.3
0.2
0.1
0
Ipsilateral Gray
Contralateral Gray
Ipsilateral White
Contralateral White
Ch'ase ABC
Control
B
C
Ipsilateral White Matter
D
E
DH
VH
DH
VH
Ipsilateral Gray Matter
F
G
Contralateral White Matter
H
I
DH
VH
DH
VH
Contralateral Gray Matter

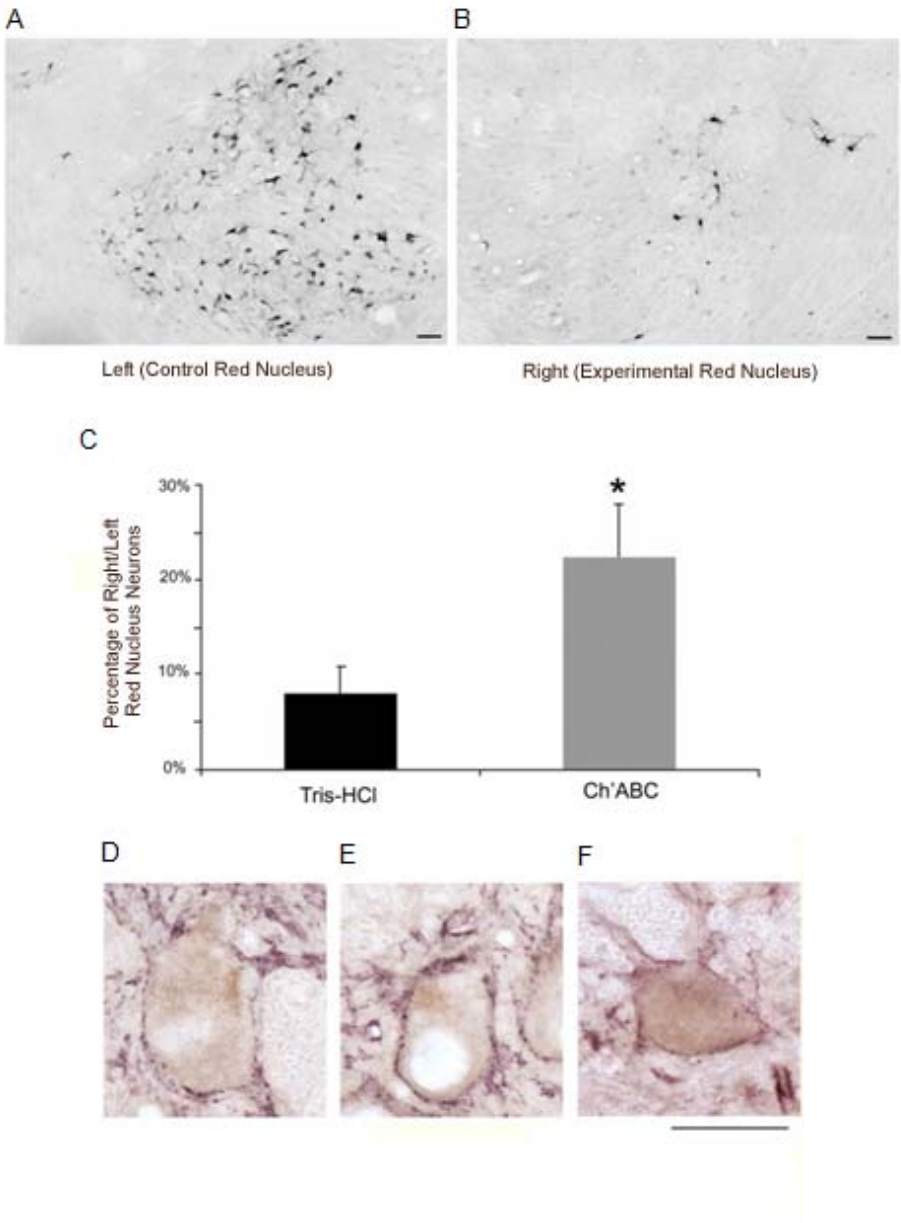

Figure 4-7. Retrograde labeling in axotomized red nucleus neurons is increased with Ch'ase ABC administration. Representative retrogradely labeled neurons in the control red nucleus (A), and in the experimental red nucleus (B) after fluorogold (FG) injections bilaterally and caudal to the original spinal hemisection. Ch'ase ABC treated cats had a greater number of retrogradely labeled neurons in the experimental red nucleus expressed as a percentage of the neurons labeled on the control side as compared to controls (C), * Indicate a significant change between groups. Error bars denote SEM. Scale bar: 1mm. Retrogradely labeled control red nucleus neurons co-stained for the pre-synaptic terminal marker synaptophysin (D), and FG labeled red nucleus neurons in the experimental red nucleus of control (E) and Ch'ase ABC treated animals (F) also co-localized with synaptophysin.

CHAPTER 5
SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Following traumatic spinal injury, axons have a minimal capacity for regeneration due to the presence of the glial scar, de-myelination, and the up-regulation of inhibitory molecules including chondroitin sulfate proteoglycans (CSPGs) (Schwab and Bartholdi 1996). Synthesis of several members of the family of CSPGs is increased and these proteins are concentrated in the area of the glial scar following injury (McKeon et al. 1995; Fitch and Silver 1997; Lemons et al. 1999; Asher et al. 2000). These proteins have been shown to define barriers to migrating neurons and restrict the extension of axons *in vitro* (Hynds and Snow 1999; Snow et al. 2001; Johnson et al. 2002). Specifically, the chondroitin sulfate glycosaminoglycan (CS-GAG) side chains have been shown to inhibit axonal regeneration and plasticity following SCI (Bandtlow and Zimmermann 2000). Degradation of CS-GAGs with Chondroitinase ABC (Ch'ABC), a bacterial enzyme isolated from *proteus vulgaris*, disrupts their inhibitory properties *in vitro* (Snow et al. 1990a; McKeon et al. 1995) and within the last eight years has been shown to enhance axonal growth and behavioral recovery in rodent models of SCI (Yick et al. 2000; Bradbury et al. 2002; Yick et al. 2003; Caggiano et al. 2005; Houle et al. 2006) and in our cat model (Tester and Howland 2007).

The cat presents an excellent translational model in which to study the effects of therapeutic interventions such as Ch'ABC treatment on systems underlying locomotor recovery. The benefits of this model include its remarkable locomotor capacity, well characterized circuitry associated with different features of locomotion, the larger size of its spinal cord, and its use as a platform for prior translational work in spinal cord injury (Hodgson et al. 1994; Behrman and Harkema 2000; de Leon et al. 2001). Previous research from our laboratory has shown that following a low thoracic spinal hemisection in the cat model, CS-GAG degradation

with Ch'ABC enhanced recovery of basic and skilled locomotion (Tester and Howland 2007; Tester and Howland 2008). My studies have further assessed the intrinsic behavioral plasticity following a low thoracic hemisection during many features of basic and skilled locomotion as well as assessed the effects of this lesion model on another motor system, the cough reflex. These studies are the first to demonstrate that Ch'ase ABC promotes axonal growth caudal to a thoracic spinal hemisection in the adult cat, and correlatively increases the regeneration and/or collateral sprouting of rubrospinal tract axons caudal to the lesion. Furthermore, these growth enhancements of the RST with Ch'ase ABC treatment may have affected the skilled locomotor recovery also seen.

Future studies could further assess the plasticity within the cough reflex after low thoracic spinal hemisection. Assessing the cough reflex at early timepoints directly after injury could help us to understand the timeline of plasticity that occurs in this particular motor system. We could assess whether the cough motor system is resilient following low thoracic hemisection or whether it is extremely plastic to the induced spinal lesion. The rectus abdominiis electromyogram recordings also could be further assessed by analyzing such things as burst duration, inspiratory activity burst duration, as well as the relationship between the esophageal pressure records and rectus abdominis electromyogram records in a temporal fashion.

This work has also shown that thoracic hemisection affects many temporal components of the gait cycle such as swing and stance duration during overground and bipedal treadmill. It would be interesting to further breakdown the step cycle into the 4 epochs of time defined by Phillipson (E2, E3, F, and E1) in order to assess at what particular part of the step cycle the ipsilateral hindlimb was making the most alterations in response to the injury. Assessments of possible Ch'ase ABC affects on the temporal components of the gait cycle such as step cycle

duration, swing duration, stance duration, and the duration of the four sub-phases during a more skilled task such as ladder or pegboard locomotion may also be future projects to pursue. It would also be helpful to assess if the velocity or acceleration of the proximal or distal limb was affected by Ch'ase ABC application during basic or skilled locomotor tasks, which may help attribute to the emergence of the unique intralimb pairing pattern during pegboard locomotion following Ch'ase ABC treatment.

Primarily, studies assessing locomotor function following partial lesions of the spinal cord have focused on ipsilateral limb recovery (Webb and Muir 2002; Courtine et al. 2005). To better understand the effects of thoracic hemisection in our lesion model on locomotor recovery, assessment of the contralateral limb could elucidate any compensatory strategiesor mechanisms of recovery that could affect the ipsilateral limb. The contralateral limb likely plays an important role in the recovery of locomotor function following thoracic hemisection. Preliminary data by our laboratory show that the contralateral hindlimb, following thoracic hemisection in the adult cat may stabilize its locomotor pattern during bipedal treadmill and overground locomotion post-injury in order to allow for functional improvement in the ipsilateral hindlimb that acquired the most deficits following injury. It has been shown that when you behaviorally train the unimpaired limb following nervous system injury, the more impaired limb does not recovery to its full potential (Allred and Jones 2008). Therefore, kinematic stabilization of the RHL following spinal cord injury may be a compensatory mechanism used to help regain function in the more impaired limb. It would also be lucrative to assess the contralateral limb during more skilled tasks such as pegboard and ladder locomotion. Whether Ch'ase ABC treatment affects any parameters of the RHLduring basic or skilled locomotion would also be important to assess.

Lastly, many novel assessments could continue the retrograde tract tracing study conducted utilizing bilateral, caudal injections of the regrograde tracer Fluorogold for assessment of rubrospinal tract axons that had grown through or around the original hemisection in response to the injury alone or to Ch'ase ABC treatment. The current results do not identify whether the increase in rubrospinal neurons in Ch'ase ABC treated cats in the experimental red nucleus was due to collateral sprouting of intact axons proximal or distal to the injury site or regeneration of axotomized axons. A double retrograde tract tracing study could help elucidate this quandary. A retrograde tracer such as Fast Blue could be placed at the site of injury at the original time of spinal hemisection to label any cut axons. A second axonal tracer could be added at the end of the study, such as Fluorogold, to label any axons that are at the caudal site of injection. Double labeled neurons in the red nucleus would indicate that an axon was originally axotomized during the injury and then regrew through or around the injury site. The corticospinal tract is also a very important motor tract involved with the control of skilled locomotion. Neuron counts of Fluorogold labeled neurons within the motor cortex of cats following thoracic hemisection and Ch'ase ABC treatment could further elucidate whether the corticospinal tract may also be involved in the significant skilled locomotor recovery we see following Ch'ase ABC treatment.

Many different strategies could be implemented that assess different delivery systems for Ch'ase ABC in our model system, including cells genetically engineered to secrete Ch'ase ABC into the scar environment, nanosphere technology to deliver the enzyme after injury, as well as viral vector delivery systems. Combinatorial treatment approaches could also be undertaken utilizing stem cell delivery, delivery of neurotrophic factors such as BDNF and/or NT-3, application of olfactory ensheathing cells to provide scaffolding for cellular remodeling, as well as combining Ch'ase ABC treatment with peripheral nerve grafts in our cat model.

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BIOGRAPHICAL SKETCH

Stephanie Christine Jefferson was born in Bellevue, Washington, to Rawle and Janet Jefferson, in the summer of 1981. She had the privilege of growing up on the beautiful, and quaint Bainbridge Island, which is located only a 35 minute ferry-ride away from the Emerald City, Seattle. She graduated in 1999 in the top of her class from Bainbridge High School in Bainbridge Island, Washington. She then attended Sweet Briar College in Sweet Briar, Virginia, where she received her B.S. in biology, magna cum laude. During her undergraduate education she was very active in the Sweet Briar academic community. She was inducted into Alpha Lambda Delta, the national honors society that honors freshman achieving academic excellence, and then served as the organizations' president the following year. She was also proudly inducted into Iota Sigma Pi, the national honors society for women in chemistry, as well as Phi Beta Kappa, the oldest undergraduate honors organization in the United States. She also served as a biology teaching assistant during most of her undergraduate education, which ignited her passion for scientific research. Dr. Linda Fink, a biology professor at Sweet Briar College and her academic advisor, provided Stephanie with much needed guidance, support, and friendship that aided in her decision to pursue a career in the biological sciences. Stephanie participated in multiple summer undergraduate research programs during her time at Sweet Briar College that ranged in breadth from molecular cloning of a gene responsible for retinal degeneration to induction of insulin production in type 1 diabetic patients. She found her niche in scientific research, and in August 2003 entered into the Interdisciplinary Program (IDP) in Biomedical Sciences at the University of Florida. This interdisciplinary program allowed her to experience a vast array of scientific research across a broad spectrum of disciplines, and eventually led her to the fascinating field of neuroscience. In the Spring of 2004, she joined the laboratory of Dr. Dena R. Howland where she conducted laboratory research to understand the basic pathobiology

and inhibitory substrates that halt neural regeneration and functional recovery following spinal cord injury. She worked directly to associate evidence of regeneration with behavioral motor changes following spinal cord injury in a complex model that is translatable to clinical applications. Specifically, her dissertation research focused on the effects of intraspinal delivery of the bacterial enzyme Chondroitinase ABC in the cat model following low thoracic spinal hemisection. She assessed the effects of this therapeutic treatment on two motor systems, locomotion and the cough reflex, and correlated their enhancements with anatomical plasticity at the spinal and supraspinal level. Her dissertation research will be published in three first author manuscripts that will hopefully enhance the field of neuroscience and in particular neuronal plasticity. She is grateful for all of the knowledge and wisdom she has acquired along this journey and wants to thank each individual who helped her achieve her goals.