PLASTICITY OF THE CENTRAL NERVOUS SYSTEM AND FUNCTIONAL RECOVERY FOLLOWING SPINAL CORD INJURY

By

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To my fiancé, my parents, and my sister, thank you for everything

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The focus of these studies was on plasticity of the central nervous system and functional recovery following a low thoracic hemisection in the cat. Functional recovery was assessed by characterizing the obstacle negotiation task which can be achieved using supraspinal and/or intraspinal networks for voluntary clearance or the stumbling corrective response respectively. Following thoracic hemisection, significant changes were seen in the cat's ability to clear an obstacle and the partial recovery seen suggested underlying plasticity was likely. To identify potential substrates that may contribute to the recovery, populations of neurons that bridged the lesion segment were identified using a retrograde tracer. Populations of long and short propriospinal neurons (PSNs) and rubrospinal tract (RST) neurons were assessed. All systems showed an immediate, significant decrease in the number of neurons with axons below the level of the lesion. The decrease was permanent in the long PSNs. However, the number of short PSNs increased significantly post-injury. The number of neurons contralateral to the lesion was significantly greater than that seen in normal controls suggesting that in addition to regeneration and collateral sprouting of axons in passage, neurons with axons that normally terminate rostral to the lesion were extending branches bridging the lesion site. Similar significant changes were seen in the non-axotomized red nucleus. These neurons represent a novel substrate that may

have contributed to new circuitry which supported the functional recovery seen. Traditionally, studies focus on the lesion environment when identifying substrates that inhibit axonal growth following injury. However, areas distant to the lesion undergo Wallerian degeneration. To understand how this may affect the distant spinal substrates, the glial responses and extracellular matrix changes in the cervical and lumbar spinal cord following a thoracic injury were assessed. Using immunohistochemical techniques, increases in activated glial cells as well as chondroitin sulfate proteoglycans were identified in areas undergoing Wallerian degeneration. Furthermore, these changes were delayed with respect to the timing of these same changes at the lesion site. This identifies the importance of considering injury-induced changes in substrates distant to the lesion when assessing plasticity and designing treatments to promote long distance regeneration. Understanding the potential for plasticity and the putative impact of cellular and extracellular matrix changes along the length of the spinal cord following injury are important to understand recovery that is seen and critical to the development of therapeutic interventions to enhance functional recovery.

CHAPTER 1
BACKGROUND

Spinal cord injury (SCI) is a life altering event with approximately 13,000 new cases each year (NSCISC, 2009). Individuals who survive the trauma to their spinal cord often suffer a significant loss of sensory and motor function below their injury level. The extent of their disability corresponds with the level of spinal injury and the degree of damage. Injury to the cervical spinal cord causes deficits in the upper and lower extremities, as well as breathing, bowel, and bladder control. Injury to more caudal areas of the cord corresponds with loss of function in the lower extremities as well as bowel and bladder control and sexual function (NSCISC, 2009). Thus, the ultimate goal of any therapeutic strategy for spinal cord injury is to facilitate functional recovery whether through, cellular, molecular, or rehabilitative means. Understanding the intrinsic plasticity of spinal and supraspinal systems that may control these lost functions is likely to facilitate the development of effective therapeutic and rehabilitative interventions. Basic scientific research can provide an initial understanding of the efficacy of these treatments and ultimately support their transition into the clinic (Jackson et al., 2004; Thuret et al., 2006).

Inhibitory Environment of the Central Nervous System after Spinal Cord Injury

For many decades, spinal cord injury was viewed as irreversible and the inability of central nervous system (CNS) neurons to regenerate was widely accepted. It was Santiago Ramon y Cajal, considered one of the fathers of neuroscience, who said, “In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this hard decree (Ramon y Cajal, 1928).” Through observations of the injured nervous tissue, he showed a lack of fibers and swollen endbulbs that appeared to withdrawal from the injury site over time. He believed that it was a

combination of the intrinsic properties of the neurons and extrinsic environmental factors that caused damaged axons to degenerate rather than elongate (Cajal, 1928). This viewpoint was the predominant theory for many decades.

Cajal hypothesized that the lack of regeneration in the CNS was due to an intrinsic inability of adult neurons to grow. This hypothesis was disproven by a study in which a piece of spinal cord was removed and replaced with a sciatic nerve graft connecting the severed ends. Axons originating from the spinal cord grew at least 10 millimeters (mm) into the peripheral nervous system (PNS) graft (Richardson et al., 1980). Next a 35mm long piece of sciatic nerve was used as a bridge between the medulla and lower cervical spinal cord. Axons from the brainstem grew the entire length of the bridge, but again failed to grow into the CNS (David and Aguayo, 1981). Together these studies show that at least some CNS neurons are capable of regenerating long distances when provided with a more permissive environment. Assessment of the molecules present following CNS injury revealed two major impediments to neuron outgrowth, myelin-associated molecules and the fibrotic glial scar matrix.

Myelin-Associated Molecules

Cajal also postulated that the limited regenerative capacity of the CNS was due its inhibitory environment. CNS myelin was first postulated to inhibit neurite growth when isolated neurons failed to grow on immobilized CNS myelin. When primary culture neurons were plated on myelin isolated from adult rat spinal cord neurite growth failed, but when placed on a substrate isolated from the sciatic nerve, the environment was growth permissive (Schwab and Caroni, 1988). Thus CNS myelin contains potent inhibitors of neurite growth. *In vivo* myelin formation ceases axon growth during a critical period in development. A study done in neonatal rats showed that X irradiation of their lumbar spinal cord prevented myelination during development. After the developmental period was complete these rats underwent unilateral

lesions of the corticospinal tract (CST) and sprouting was assessed. Rats that were irradiated showed pronounced sprouting of uninjured CST fibers into the denervated area of the spinal cord compared to animals that were not irradiated. This sprouting lasted well past the "critical period" of axon growth and suggested that CNS myelin contained growth inhibitory components (Vanek et al., 1998).

Two specific membrane molecules, NI-250 and NI-35, which have highly non-permissive properties, were identified by antibodies IN-1 and IN-2 that neutralize myelin inhibition (Caroni and Schwab, 1988). Among these, Nogo-A (NI-250) has been well characterized because of its abundance in CNS oligodendrocytes. Since Nogo-A is an antigen for IN-1 it has become the target of many studies that try to reduce the inhibition of myelin after spinal cord injury and induce regeneration of disrupted tracts (Chen et al., 2000). In order to neutralize the inhibitory nature of the myelin-associated molecules, hybridoma cells, which secrete IN-1, were injected into the dorsal frontal cortex of rats 7-10 days before complete transection of the CST was made in the thoracic cord. Rats that received the IN-1 producing cells had labeled fibers 7-11mm caudal to the lesion site whereas control rats had fibers only 0.5-1mm away (Schnell and Schwab, 1990). Along with the increase in collateral sprouting and regenerative growth associated with IN-1 secreting hybridoma cells, behavioral recovery was characterized for a variety of different motor tasks associated with the injured CST (Thallmair et al., 1998; Z'Graggen et al., 1998).

Although, this treatment has shown promising results in the rodent injury model there are many issues associated with the implantation of tumor forming hybridoma cells. Another issue with this particular treatment is the instability of the IN-1 antibody. Brosamle et al., (2000) developed a recombinant, humanized IN-1 fragment to enhance translational potential. This

fragment was injected through an osmotic mini-pump directly into the intrathecal space of the spinal cord near the lesion site. When compared to the hybridoma cell studies, similar results were seen with regards to enhancement of axonal growth. (Brosamle et al., 2000).

Following the identification of Nogo-A, a number of myelin-associated molecules have been characterized. Studies done *in vitro* showed that myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) are inhibitors of axonal growth (McKerracher et al., 1994; Wang et al., 2002b). MAG, OMgp, and Nogo-A share a common receptor, Nogo receptor (NgR). Thus interfering with the downstream signaling pathway may allow lesioned axons to overcome much of the inhibition caused by CNS myelin (Wang et al., 2002a).

In addition to these myelin-associated molecules, developmental guidance molecules that repulse developing axons also are present in mature CNS myelin. One of these is Sema4D/CD100 which is expressed on developing and mature oligodendrocytes. Studies using stripe assays showed that Sema4D/CD100 forms an inhibitory substrate by causing the collapse of adult growth cones. *In vivo*, there is some evidence that after a thoracic hemisection, Sema4D/CD100 increases around the lesion site and may play a role in the inhibition of axonal regeneration (Moreau-Fauvarque et al., 2003).

Fibrotic Glial Scar Formation in Response to Central Nervous System Injury

Following SCI, a fibrotic, glial scar consisting of reactive astrocytes and other extracellular matrix molecules is formed at the lesion epicenter. The primary cell types present in the scar environment are astrocytes, microglia, and oligodendrocyte precursor cells (OPCs) (reviewed by Fawcett and Asher, 1999). Some of the most compelling evidence that the scar environment is a major inhibitor of axonal regeneration was demonstrated by Silver and colleagues. Axotomized axons regenerated up to the scar matrix where they formed dystrophic endbulbs and ceased growing (Davies et al., 1999). In an *in vitro* model of the glial scar, time lapse videos show that

dystrophic endbulbs constantly try, but cannot advance (Tom et al., 2004). When the scar was suppressed after SCI by application of an iron chelator, regeneration of corticospinal tract neurons past the scar and into the caudal cord occurred resulting in functional recovery (Klapka et al., 2005).

The first cells to respond to the injury are microglia and macrophages. There is conflicting evidence as to whether these cells play a neurotoxic or neuroprotective role. Microglia can produce free radicals, nitric oxide, arachidonic acid derivatives, as well as other neurotoxic molecules (reviewed by Fawcett and Asher, 1999). However, Rabchevsky and Streit showed that implanted microglia, with and without astrocytes, enhanced axonal regeneration following spinal cord injury (Rabchevsky and Streit, 1997). Microglia also have been shown to degrade myelin inhibitory molecules (David et al., 1990). Thus, the role of microglia following injury still is being debated.

Oligodendrocyte precursor cells also are recruited to the site of injury. These cells have been shown to express a variety of proteoglycans including neurocan, phosphacan, versican, and NG2. These proteoglycans contribute to the inhibitory nature of the scar environment following injury (discussed in later section).

A major cellular constituent of the scar is the reactive astrocyte. Reactive astrocytes are characterized by hypertrophic processes and increased expression of glial fibrillary acidic protein (GFAP). Numerous studies, *in vitro* and *in vivo*, have shown that the astroglial environment is extremely inhibitory to axonal regeneration following injury (Reier and Houle, 1988; McKeon et al., 1991). When a small number of axons were axotomized with minimal damage to the astroglial framework in the adult rat brain, regeneration occurred far from the lesion, but not in the vicinity of the injury (Davies et al., 1996).

Extracellular Matrix Molecule: Chondroitin Sulfate Proteoglycans

The inhibitory extracellular matrix molecules known as chondroitin sulfate proteoglycans (CSPGs) are a major component of the scar. At least two cell types, astrocytes and OPCs, contribute to their increase following CNS injury. The CSPG family includes aggrecan, brevican, neurocan, phosphacan, versican, and NG2. These molecules consist of a core protein and large unbranched sugar side chains, known as glycosaminoglycans (GAGs). The composition of the GAG chains defines which family of proteoglycans a molecule belongs to. The members of the CSPG family have GAG chains that are defined by chondroitin sulfate (CS) glycosylation. The presence of different disaccharide units along the GAG chains results in the formation of different structural motifs known as CS-A (mono-sulfated at the 6 position), CS-C (mono-sulfated at the 4 position), CS-D and CS-E. These sulfation patterns define the binding capabilities of the GAGs and thus the overall function of CSPGs (reviewed by Bandtlow and Zimmermann, 2000; Properzi et al., 2003).

Multiple studies using *in vitro* stripe assays demonstrated the inhibitory nature of CSPGs. Neurites were grown on alternating strips of laminin and laminin/CSPG substrate. Robust outgrowth of embryonic chick and rodent neurons was seen on the growth permissive laminin substrate, but neurites came to an abrupt stop and some turned away from the substrate containing CSPGs (Snow et al., 1990b). In contrast to the single stripe assay described above, growth cones grew onto and up a step-wise increase of CSPGs bound to the growth permissive laminin. The neurites that reached the CSPG substrate reduced their rate and amount of outgrowth along the increasing gradient. These data suggest that the barrier function of CSPGs during development is dependent upon the balance of growth permissive and inhibitory molecules which regulate direction and timing of axonal outgrowth (Snow and Letourneau, 1992).

Developmental role

The developing visual system clearly illustrates CSPGs' role as a barrier molecule in axonal pathfinding and navigation during development (reviewed by Laabs et al., 2005). The gradual regression of CSPGs from the center to the periphery of the embryonic rat retina is correlated with maturation and appears to help guide axons to the optic nerve. Digestion of these CSPGs with the enzyme Chondroitinase ABC (Ch'ABC) resulted in disorganized growth of retinal ganglion cells (Brittis et al., 1992). CSPGs also were present in the optic chiasm of embryonic mouse retinas. Similar experiments with Ch'ABC in the developing mouse optic chiasm suggest that CSPGs play an axonal guidance role (Chung et al., 2000a; Chung et al., 2000b).

CSPG expression guides axons during development of the brain and spinal cord. During the development of the chick forebrain, CSPGs were expressed around large extracellular spaces in the subplate of the forebrain during a period when growing axons were absent. Axon growth into this area later in development correlated with a reduction of CSPGs (Snow et al., 1990a). Similarly, development of the avian hippocampus is influenced by the CSPG, neurocan. The pattern of neurocan expression relative to the cell adhesion molecule L1 demonstrated a potential interaction that could help guide developing hippocampal neurons through the alveus (Wilson and Snow, 2000). During embryonic rat neurulation, migration of the neural crest cells was characterized by a spatial and temporal gradient of CSPGs that remained until the completion of the neural tube (Morriss-Kay and Tuckett, 1989). Thus, CSPGs appear to perform an important barrier function during development, but their distribution and levels are decreased in the adult.

Role in neural regeneration

Davies et al (1997) used a microtransplantation technique, which minimized scarring, to inject adult dorsal root ganglia (DRG) neurons into the white matter of the corpus collusum or

fimbria of adult rats. These studies suggested that myelin is not inhibitory to axonal growth and that CSPGs are more inhibitory than myelin in some instances. Successful transplants showed minimal upregulation of CSPGs at the host/transplant interface allowing DRG neurons to extend their axons for long distances in white matter. Transplants with increased levels of CSPGs had axons that failed to leave the transplant either stopping in the CSPG rich area or turning back into the interior of the transplant (Davies et al., 1997). Robust regeneration also was seen in areas of active Wallerian degeneration when DRG neurons were injected distal to a lesion of the dorsal columns in the cervical spinal cord. Once the growing axons entered the CSPG rich lesion site growth cones stopped and became dystrophic (Davies et al., 1999). Therefore, areas outside of the CSPG rich lesion site are capable of supporting axonal regeneration.

Following injury, CSPG expression is rapidly increased at the lesion epicenter. Early studies showed that implanting a piece of nitrocellulose into the cerebral cortex of the adult rat induced the infiltration of GFAP positive astrocytes to which CSPGs were co-localized. CSPGs were not found around GFAP positive astrocytes away from the injury site (McKeon et al., 1991). In the spinal cord, immunohistochemistry showed that CSPGs increased after contusion injury as early as 4 days post-injury and persisted at least 40 days. Further, GFAP immunoreactivity was closely associated with this increase in CSPGs (Lemons et al., 1999). Although these studies revealed an increase in CSPGs after injury in the adult CNS, the specific CSPG family members that were being expressed were unknown.

Western blot and immunohistochemical analyses suggest that a variety of CSPG family members are differentially expressed after SCI. NG2 was expressed as early as 24 hours post-SCI and increased levels maintained out to 6 months in the lesion area. Macrophages and OPCs are the major sources of NG2 (Jones et al., 2002). Versican also is produced by OPCs. Versican

was upregulated after a knife wound injury to the cerebral cortex and in some instances SCI (Asher et al., 2002; Jones et al., 2003). Tang et al. (2003) reported a significant decrease in versican after SCI; this difference is potentially due to a differing cellular response in the two cases. Other types of CSPGs, including astrocyte derived neurocan and brevican, showed moderate increases in expression levels days after SCI. Interestingly, there was an initial decrease in phosphacan levels that was eventually recovered and surpassed normal levels for at least 2 months post-SCI (Jones et al., 2003; Tang et al., 2003). These studies defined phases of different CSPGs' expression levels in the developing scar after SCI. Thus, there is a combination of CSPGs responsible for the inhibitory nature of the scar after CNS injury.

Overcoming inhibition after central nervous system injury

There is ample evidence, both *in vitro* and *in vivo*, to suggest that CS-GAG chains are the main inhibitory component of CSPGs (McKeon et al., 1991; Brittis et al., 1992; Davies et al., 1999; Chung et al., 2000b). Therefore, many therapeutic interventions have focused on removing the CS-GAGs in order to create a more permissive environment after injury. Chondroitinase ABC (Ch'ABC) is a bacterial enzyme that cleaves the CS-GAGs leaving a sugar stub on the core protein. Lemons et al. (1999) were the first to show that gelfoam soaked in Ch'ABC could successfully cleave CS-GAGs *in vivo*, followed by Moon and colleagues who also showed successful regeneration. Treatment with Ch'ABC after unilateral axotomy of the nigrostriatal tract in adult rats led to an increase in dopaminergic nigral axons re-growing through the lesion site back to their original target. In animals treated with Ch'ABC there was a decrease in the staining by an antibody for the CSPG core protein with sulfated GAG chains illustrating the effectiveness of Ch'ABC treatment in cleaving the CS-GAGs (Moon et al., 2001). Intrathecal Ch'ABC application after bilateral dorsal column lesions resulted in the growth of both ascending sensory and descending motor tracts and the upregulation of GAP-43, a

regeneration-associated protein. Furthermore, this treatment led to the restoration of locomotor and proprioceptive function (Bradbury et al., 2002). Restoration of function also was characterized in the cat after low thoracic hemisection. Ch'ABC treatment increased functional recovery in skilled motor tasks that incorporated both descending and intraspinal input, such as ladder and pegboard walking (Tester and Howland, 2008). Ch'ABC has proven to be a successful therapy in making the scar environment more permissive, but the mechanism(s) promoting functional recovery is/are still being determined.

Inhibitory Molecules Distant From a Spinal Cord Lesion

Following injury to the CNS, activation of astrocytes and microglia is not solely limited to the lesion epicenter, but also can be present in areas of ongoing Wallerian degeneration (WD) or denervated target nuclei (Koshinaga and Whittemore, 1995; Crowe et al., 1997; Liu et al., 1997; Shuman et al., 1997; Massey et al., 2008). Wallerian degeneration involves the breakdown and phagocytosis of damaged axons and their myelin sheaths distal to the injury site, but in the CNS removal of broken down axons and myelin is extremely slow (reviewed by Vargas and Barres, 2007). Experiments have shown that individual components of myelin were degraded at differing rates during WD of white matter spinal tracts. The persistence of growth inhibitory proteins, such as Nogo-A, in areas of degenerating fiber tracts distal to the lesion could contribute to the maintenance of a non-growth permissive environment (Buss and Schwab, 2003; Buss et al., 2005). Along with the presence of myelin inhibitory proteins, there was a delayed and prolonged increase in GFAP-positive astrocytes forming a dense astrocytic scar along the length of the human spinal cord (Buss et al., 2004). Recently, one study provided a temporal and spatial timeline of major glial and axonal responses in areas of WD in two distinct pathways in the rat, CST and dorsal ascending tract (DAT). A dorsal funiculotomy was made at T8 and these tracts were studied along the length of the spinal cord. Increased number of macrophages and

astrocytes and decreased number of oligodendrocytes and axons were seen in areas of WD at least 10mm rostral and caudal of the lesion site. These changes persisted throughout the different time points studied (Wang et al., 2009). A follow-up study in the monkey demonstrated a much slower process than in the rat (Shi et al., 2009). The presence of activated astrocytes, microglia, and other inhibitory molecules in areas distal to the lesion site could be important for treatment of SCI.

Most studies assessing the anatomical effects of spinal cord injury have focused on delivering therapeutic interventions to the site of injury alone, but the aforementioned studies suggest other areas must be considered. Recently, Massey et al. (2006, 2008) showed a significant increase in CSPGs in the denervated dorsal column nuclei after cervical SCI. Injection of Ch'ABC into the dorsal column nuclei resulted in enhanced reinnervation by regenerated axons and increased collateral sprouting by spared axons. These results led to behavioral recovery illustrating that functional synaptic connections were made to the denervated target (Massey et al., 2006; Massey et al., 2008). Thus, treatment strategies promoting axonal regeneration might need to be applied not only to the lesion site, but to outlying areas.

Plasticity of the Central Nervous System

The view of the CNS as hardwired has changed. The proof that undamaged axons in the CNS are capable of generating collateral sprouts provided evidence to contradict Cajal's theory. Following partial denervation of the feline spinal cord, dorsal root afferent axons formed new processes termed collateral sprouts. The quantity of this sprouting was proportional to the amount of damage; the more damage the more sprouting that occurred (Liu and Chambers, 1958). This seminal research proved that undamaged axons can fill in areas that were devoid of synapses, but the next step was to determine whether functional synapses were formed. Similar to Liu and Chambers, Raisman (1969) showed in the rat, de-afferentation mediated sprouting of

intact fibers. Further, he provided evidence that they also made functional synaptic connections (Raisman, 1969). The formation of functional synapses post-injury also was supported by synaptic potential recordings and electron microscopy studies in both the monkey (McCouch et al., 1958; Bernstein and Bernstein, 1973) and cat (McCouch et al., 1958).

It is now accepted that multiple neuronal systems are capable of collateral sprouting in both the adult mammalian and lamprey CNS. These studies include, tract tracing studies in the adult monkey after unilateral pyramidotomy or spinal cord hemisection which showed collateral sprouting of corticospinal tract axons distal to the lesion (Kucera and Wiesendanger, 1985; Aoki et al., 1986). Following a transection of the dorsal corticospinal tract at C3 in the adult rat ventral CST fibers sprouted collaterals resulting in improved forelimb function (Weidner et al., 2001). The reticulospinal tract also has been shown to sprout collaterals caudal to the lesion resulting in functional recovery (Ballermann and Fouad, 2006).

Recently, attention has focused on the plasticity of propriospinal neurons (PSNs). Early evidence from spinal cord transected lampreys suggested that brainstem projections were not the only system contributing to functional recovery. Within 4 weeks after spinal transection, the lamprey CNS repairs itself and swimming behavior recovers to pre-injury levels along the length of the body. Although brainstem projections are restored to the rostral spinal cord, they do not extend into the area of caudal spinal pattern generator needed for swimming. Thus, following spinal transection, the lamprey does not require bulbospinal projections to recover swimming behavior (Davies and McClellan, 1994; McClellan, 1994).

In order to determine what underlying system was modulating this recovery, a retrograde horseradish peroxidase (HRP) approach was used to trace descending PSNs. Lampreys have short descending PSNs along the entire length of the spinal cord with larger concentrations in the

rostral and caudal regions than in the middle region of the body. Following a transection in the rostral spinal cord there was a substantial increase in PSNs labeled at 40% of the body length or directly below the lesion. At the same time period the restoration of descending projections below the lesion was still incomplete, but motor recovery was complete. The increase in PSNs below the lesion worked in concert with the descending brain projections in order to restore swimming function (Rouse and McClellan, 1997).

After a thoracic compression injury in rats, long and short PSNs were differentially affected. There was a considerable amount of axonal damage to short, thoracic PSNs, but many long PSNs survived. Even though the axons of short PSNs were damaged their cell bodies of origin remained intact out to 2 weeks post-axotomy. These data illustrate the potential regenerative capacity of both the short and long PSNs in a mammalian model that could enhance locomotor recovery (Conta and Stelzner, 2004).

Two studies, in particular, have shown the plastic abilities of the short and long PSNs in rodents. The first, performed by Bareyre et al. (2004), focused on sprouting of the corticospinal tract onto long descending PSNs in the rat. In this study a dorsal hemisection in the thoracic spinal cord transected the dorsal CST. Collateral sprouting of the CST increased following injury. These collaterals made contact with both types of PSNs, but the contacts made onto long PSNs remained throughout the course of the study suggesting that long PSNs had a role in mediating the resulting behavioral recovery. Along with the increase in sprouting from the motor cortex there also was an increase in collateral sprouting of the long PSNs themselves onto motoneurons of the lumbar spinal cord. This study provided strong evidence that a new functional circuit was formed utilizing long PSNs (Bareyre et al., 2004; reviewed by Blight, 2004).

Following this work, Courtine et al. (2008) showed locomotor recovery following a series of hemisection injuries mediated by short PSNs in the mouse model. The first lesion was made at T12 followed by a second lesion on the contralateral side at T7 weeks later. The staggered time frame first allowed for behavioral recovery to reach a plateau then disrupted the rest of the descending input to the lumbar spinal cord. Even with a complete disruption of descending input, these mice recovered the ability to step on a treadmill. This strongly suggests that sprouting of short PSNs around the lesion is capable of re-innervating the area below the level of the lesion sufficiently to support basic stepping (Courtine et al., 2008; reviewed by Stelzner, 2008). Following medial myelotomy in the cat, transected commissural interneurons regenerated across the midline re-forming functional synapses (Fenrich and Rose, 2009). Because the plastic changes of the propriospinal neurons were able to facilitate some level of functional recovery, they are a good substrate for therapeutic interventions in order to enhance this recovery.

A Motor System Effected by Spinal Cord Injury: Locomotion

Feline Model of Locomotion

The general organization of the locomotor system has been well conserved among vertebrates. Since the beginning of the 20th century the cat has been one of the most advantageous translational models for analysis of locomotor control due to their size and sophisticated motor control system. Cats have a well defined step cycle and kinematic analyses are easily performed. They are capable of performing a variety of behaviors that require different levels of the neural axis making them an ideal model for studying therapeutic interventions following central nervous system injury. The functional organization of the locomotor system includes circuits contained completely in the spinal cord, descending supraspinal input, efferent input and peripheral afferent input (reviewed by Orlovsky et al., 1999).

Intraspinal Circuits

The existence of rhythmic patterns generated by the lumbosacral cord was first described by TG Brown in 1911. His experiments using the cat showed that alternating ankle extensor and flexor movements can be produced without afferent or supraspinal input. From these experiments he developed a model in which intrinsic "half-centres" control the rhythmic pattern of locomotion (Brown, 1911). This intrinsic circuit is now known as the central pattern generator for locomotion (CPG).

Numerous studies have been done to support the existence of the CPG for locomotion in many vertebrate and mammalian species. A series of studies using spinalized kittens showed that the caudal spinal cord could mediate basic stepping and transitions to speed changes (Forssberg et al., 1980a; Forssberg et al., 1980b). Even more striking was the ability of the adult T13 spinalized cat to step with their hindlimbs on the treadmill. The EMG activity and the stepping kinematics were similar to that of normal cats indicating that the isolated lumbar spinal cord can produce complex motor movements without descending input. Further, lesions in the midlumbar region of the spinal cord abolished all stepping activity that was present after spinalization (Belanger et al., 1996; Rossignol et al., 2002).

The lumbar spinal cord in humans also has the potential to generate rhythmic stepping. During development, the descending input from the brain to the caudal cord is not complete until after the infant stage. Subjects under one year of age have the ability to step on a split-belt treadmill and can modulate their stepping to speed changes as well as directional changes. The lumbar cord is highly organized even before the descending inputs are fully mature (Yang et al., 2004). Following a severe, incomplete SCI in a child, stepping was recovered without the recovery of isolated voluntary movements of the lower extremities (Behrman et al., 2008). After SCI, subjects characterized as an American Spinal Cord Injury Association (ASIA) classification

A have no sensory or motor function below their lesion. These subjects were capable of modulating their walking by using cues from the amount of loading on their lower limbs also illustrating the role of afferent input (Harkema et al., 1997).

A key element in all quadrupedal locomotor CPGs is left-right and fore-hindlimb coordination. The connections between the CPGs that make this coordination possible are propriospinal neurons whose axons lie solely in the spinal cord. There are two different populations of PSNs, long and short. Long PSNs run the length of the spinal cord connecting the spinal enlargements at the cervical and lumbar levels of the spinal cord by coursing through the ventral and lateral funiculi (Barilari and Kuypers, 1969; Molenaar and Kuypers, 1978). Since long PSNs' pathways are intermingled with descending supraspinal systems and short PSNs, determination of their function has proven to be complicated. Lesions of the ventral lateral funiculi and ventral columns caused severe deficits in interlimb coordination in the cat (Miller and Burg, 1973), rat (Loy et al., 2002), and turtle (Samara and Currie, 2008) suggesting their role in limb coordination.

Short PSNs are located along the entire length of the spinal cord and connect segments through ascending, descending, and commissural projections. Their cell bodies are distributed throughout most of the lateral gray matter, including laminas V, VI, and VII (Molenaar and Kuypers, 1978; Kostyuk and Vasilenko, 1979). This widespread distribution allows many long descending motor tracts responsible for locomotion to terminate on propriospinal fibers (Scheibel and Scheibel, 1966). Along with long PSNs, short PSNs, especially commissural PSNs, contribute to left-right coordination (reviewed by Butt et al., 2002a; Butt et al., 2002b; Butt and Kiehn, 2003). They also play a role in postural control in the quadrupedal animal (reviewed by Deliagina et al., 2008).

Supraspinal Control

While the central pattern generator can produce the basic locomotor rhythm, spinal input from brainstem structures is necessary to initiate and regulate the rhythm. Input from many areas of the brainstem converge onto reticulospinal neurons in the medullary reticular formation (MRF). These reticular axons travel in the ventrolateral funiculus to all levels of the spinal cord and contribute to gross locomotor movements and posture. For example, the mesencephalic locomotor region (MLR) activates a great number of reticulospinal neurons in the MRF. Increasing stimulation in the area causes an increase in the firing frequency of the neurons and the intensity of the locomotor movements. Lesions or inactivation of the reticulospinal pathway can block MLR induced locomotion (Shik, 1983; reviewed by Whelan, 1996; Orlovsky et al., 1999). Other areas of the brainstem that project to the MRF can evoke locomotor activity. These include the subthalamic locomotor region (SLR) and the pontomedullary locomotor strip (PLS) (reviewed by Whelan, 1996). Complex locomotor behaviors, such as visually guided limb placement and locomotor adaptations, are controlled by pathways originating in the motor cortex and the red nuclei.

Anatomy of the corticospinal and rubrospinal tracts

The corticospinal tract (CST) is the longest, largest locomotor tract. In humans, the CST originates primarily in the motor cortex. It courses through the internal capsule, the cerebral peduncles, the pons, and into the pyramids of the caudal medulla. In the medulla 80% of the fibers decussate to form the lateral corticospinal tract, which then terminates in the intermediate and dorsolateral regions of the ventral horn. The remaining 20% of fibers do not cross, but remain ipsilateral as the medial corticospinal tract which terminates in the ventral medial region of the spinal gray matter.

Multiple studies done in the cat model, recorded the electrical activity of pyramidal tract neurons at either the cell body (antidromic) or at the level of the medulla or spinal cord (orthodromic) to determine their relay activity. The recorded conduction velocities from the cortex to the brainstem, corresponded with the presence of two fiber groups that comprise the corticospinal tract, fast conducting fibers with velocities of 48-57m/s and slow conducting fibers at 22-25 m/s. Further, these two groups were recorded down the contralateral side of the spinal cord when the brainstem was stimulated. The fast conducting group maintained a consistent velocity down the spinal cord suggesting a direct relay of information, whereas the velocity of the slower group decreased with distance (Lance, 1953; Lance and Manning, 1954).

These recording studies along with the tracing of degenerating axons identified the origin of the corticospinal tract in the cat cortex. Following different cortical ablations, the degeneration of pyramidal tract fibers were studied at multiple time points. Importantly, these studies took into account that thick and thin fibers degenerate at different rates. Following ablation of the frontal cortex, nearly all CST fibers had degenerated where as the ablation of other cortical lobes caused no degeneration of these fibers (Van Crevel and Verhaart, 1963b, a). These studies concluded that the CST is composed of two sets of fibers arising from the precentral sulcus of the cat frontal cortex.

In order to determine where CST fibers terminate in the spinal cord, Scheibel and Scheibel used the rapid Golgi stain. Collateral and terminal fibers enter the gray matter at right angles from the white matter and terminate in a fan-like fashion in laminas IV through VII. Many of the fibers that enter the gray matter terminate upon propriospinal neurons, with few fibers terminating on the motoneurons of lamina IX. Propriospinal neurons are able to propagate the

influence of a single corticospinal fiber onto many levels of the spinal cord and across the midline (Scheibel and Scheibel, 1966).

Running adjacent to the CST in the spinal cord is the rubrospinal tract (RST), which originates in the magnocellular region of the red nucleus. As RST fibers leave the red nucleus they immediately decussate and terminate in the spinal cord similar to the lateral CST.

In lower mammalians, including cats and some non-human primates, the RST is a very well developed tract. Nyberg-Hanson and Brodal in 1964 created several lesions of the red nucleus in adult cats and used silver impregnation to determine the exact course of the rubrospinal tract. Degenerating fibers were observed at all levels of the spinal cord. These fibers were located in the dorsal half of the lateral funiculus and shifted dorsolaterally during the descent down the cord. Fibers and collaterals were observed entering the gray matter laterally in laminas V-VII. Similar to corticospinal fibers, rubrospinal fibers radiate in a fanlike fashion reaching the lateral half of lamina V and VI and the dorsal and central parts of lamina VII. These fibers then terminate onto interneurons located in these lamina (Nyberg-Hanson and Brodal, 1964).

Postsynaptic potential experiments in the cat revealed that very few rubrospinal fibers, similar to CST fibers, make direct contact with motoneurons. Instead many contact interneurons via a number of different spinal reflex pathways. These interneurons include both first and last order neurons integrating commands from many other descending systems (Jankowska, 1988). The few direct contacts made by the rubrospinal tract were visualized after an anterograde tracer, wheat germ agglutinin (WGA)-HRP, was injected into the red nucleus of the cat. Rubrospinal fibers were seen terminating on motoneurons of lamina IX at levels C8 and T1, which control

distal forelimb musculature although no other levels of the spinal cord showed staining of rubrospinal fibers in lamina IX (McCurdy et al., 1987).

Role of the corticospinal and rubrospinal tracts in locomotion

In order to determine the function of the CST and RST in the cat, electrophysiological techniques were used to record activity of either pyramidal tract neurons in the motor cortex or rubral neurons in the red nucleus. These recordings took place as the cat walked on a treadmill or performed a task that required voluntary modifications in limb trajectory. One such task is obstacle negotiation which is accomplished by a voluntary increase in flexion during the swing phase of the step cycle (Lavoie et al., 1995).

As cats walked on a treadmill with intermittent obstacles, activity of pyramidal tract neurons (PTNs) increased in groups during four phases of the step cycle when the obstacle was encountered. The largest burst of activity was seen during swing phase when the flexors of the hip, knee, and ankle joints were at their maximum. As the cat exited swing phase and extended its limb another group of PTNs became active as the paw was repositioned and stabilized on the surface of the treadmill. Activity of two smaller groups of PTNs increased when extensor muscles were activated during stance before and after the modified step to ensure stability (Widajewicz et al., 1994; Drew et al., 2002). During normal treadmill locomotion, rhythmic increases in PTN activity also were evoked, but there were no bursts of increased activity as seen in the obstacle negotiation task (Armstrong and Drew, 1984).

These studies showed that PTN activity was increased during obstacle negotiation, but the actual cause of the increase in activity was still unknown. To further study PTN activity, another locomotor task requiring alteration in limb trajectories, ladder walking, was used. This task requires very accurate paw placement without the substantial increase in flexion required in stepping over an obstacle. Transitions from runway walking to ladder walking caused an

increase in the firing rate, as well as peak discharge, of recorded PTNs when the limbs were targeting the ladder rungs. Rungs that were differentially spaced caused a greater increase in cortical neuron firing about 400 milliseconds before the paw landed correctly onto the ladder rung (Amos et al., 1990). Inactivation of the motor cortex did not affect normal runway locomotion, but it did affect accurate paw placement as the cats attempted to cross a ladder or step over barriers (Beloozerova and Sirota, 1993). Thus the motor cortex has little direct control in normal locomotion, but plays a major role in accurate limb placement and voluntary limb modifications.

The RST has a similar, but not identical, role to the CST in the control of locomotion in the cat. Microstimulation of both tracts causes a large overlap in EMG recordings of the extensor and flexor muscles during uninterrupted locomotion. For example, normal treadmill walking caused neurons in the red nucleus to phasically fire during both swing and stance phase and, like cortical neurons, discharge increased during swing phase when the limb was fully flexed (Orlovsky, 1972; Arshavsky et al., 1988). On the other hand, microstimulation of the red nucleus did not cause substantial changes in the timing of the step cycle, whereas microstimulation of the motor cortex reduced stance duration causing the initiation of a new swing phase (Rho et al., 1999). There are subtle differences between the function of the corticospinal tract, which dictates the movements and the rubrospinal tract, which modulates the level of activity in the corresponding muscles.

To determine the exact role of the red nucleus during locomotion, studies again were conducted using visually guided obstacle negotiation. As a cat stepped over an obstacle there was an increase in intensity as well as phasic modifications of the RST neurons during both swing and paw down phases of the step cycle. In addition to phasic firing, there was a slight

increase in neuronal activity in the ipsilateral limb suggesting that the RST could also be involved in the control of intralimb coordination during locomotion (Lavoie and Drew, 2002).

At the spinal level, lesions of the cortico- and rubrospinal tract in the dorsolateral cord at T13 showed slight deficits in normal treadmill walking, but abolished the cats' ability to step over an obstacle (Drew et al., 1996; Drew et al., 2002). Thus, visually triggered, voluntary gait modifications in the cat use the complementary roles of the cortico- and rubrospinal tracts in order to complete these tasks.

Beneficial Effects of Locomotor Training

Studies have shown that the spinal cord has the ability to relearn tasks and strengthen newly formed pathways through repetitive training (reviewed by Edgerton et al., 2008). Many of the early training studies focused on improving locomotor performance in spinalized cats. These cats recovered the ability to perform full weight bearing steps during bipedal treadmill locomotion. When compared to spontaneous recovery, trained cats reached faster treadmill speeds and sustained longer bouts of full weight bearing steps (Lovely et al., 1986, 1990). Further studies showed the importance of the type of training spinalized cats received. When cats were trained to stand on a treadmill they were unable to step or had poor stepping quality, whereas step trained cats were unable to stand for long periods of time (Hodgson et al., 1994). Accompanying the change of neural networks, exercise increases the release of certain neurotrophins. One of the most susceptible trophic factors to regulation by activity is brain derived neurotrophic factor (BDNF). BDNF not only regulates the cells in the developing CNS, but plays a role in axonal regeneration and neuronal plasticity following CNS injury (reviewed by Vaynman and Gomez-Pinilla, 2005).

These seminal studies done in the cat model led to the development and study of body weight supported treadmill training in the human population of SCI. Body weight supported

treadmill training takes advantage of spared descending fibers in the spinal cord as well as sensory input the subjects receive from walking on the treadmill to induce stepping and increase recovery. Recently, results from locomotor training studies have shown improvement in walking ability in both the clinic and home life of SCI subjects, but to this day complete recovery has yet to be attained (Behrman and Harkema, 2000; Thomas and Gorassini, 2005; Behrman et al., 2008; reviewed by Dietz, 2009). Thus, locomotor training to enhance neuronal plasticity in combination with regenerative treatment strategies may help individuals gain a greater recovery in functional loss and increase their quality of life.

CHAPTER 2
ALTERED OBSTACLE NEGOTIATION FOLLOWING LOW THORACIC HEMISECTION IN THE CAT

Introduction

Locomotor adaptations are essential for safe walking. Successful negotiation of environmental change requires modification in limb trajectories controlled by different levels of the neural axis. Specifically, visualization of an obstacle allows voluntary step cycle modifications, which are mediated by the integration of signals from descending supraspinal and intraspinal pathways (Mohagheghi et al., 2004; Wilkinson and Sherk, 2005; Patla and Greig, 2006). The corticospinal and rubrospinal tracts have been identified as critical descending motor pathways in the control of voluntary obstacle negotiation (Beloozerova and Sirota, 1993; Widajewicz et al., 1994; Drew et al., 1996; Lavoie and Drew, 2002). When an unanticipated obstacle, with no preceding visual cues, is encountered changes in limb trajectory can occur as result of foot contact with the obstacle. Limb trajectory changes initiated by stimuli directly to the surface of the foot or leg are referred to as the stumbling corrective response (Forssberg, 1979; Schillings et al., 1996; McVea and Pearson, 2007). In contrast to the voluntary response, work in the low spinally transected cat indicates that this response can be mediated by spinal circuitry (Forssberg et al., 1975).

Our work (Tester and Howland, 2008), as well as that of others (Eidelberg et al., 1986; Helgren and Goldberger, 1993; Basso et al., 1994; Kuhtz-Buschbeck et al., 1996), shows substantial motor recovery occurs following thoracic hemisection in the cat. Despite this recovery, quantifiable deficits still exist. The hindlimb ipsilateral to the lesion is reintegrated during basic overgound locomotion, and kinematic patterns of hindlimb stepping are similar to those seen pre-injury. However, performance deficits are obvious while crossing a ladder or pegboard, which require a high level of limb accuracy (Helgren and Goldberger, 1993; Tester

and Howland, 2008). Further, there is a difference in the time course of recovery between basic overground locomotion and locomotion requiring accurate paw placement. Thus, more adaptive features of locomotion have a longer recovery time and recovery is less complete. This is similar to what is seen in humans with Brown-Sequard syndrome (McKinley et al., 2007). Surprisingly, the voluntary and spinal responses to obstacle negotiation have yet to be evaluated in this model.

The current study was designed to determine if a thoracic hemisection injury alters voluntary obstacle negotiation of the ipsilateral hindlimb. This injury interrupts ipsilateral descending input while preserving all contralateral input. Cats were trained pre- and post-injury, to cross a basic overground runway with and without an obstacle. Ipsilateral hindlimb performance was evaluated out to 16 weeks post-injury. Following injury, significant changes were seen in the cat's ability to clear an obstacle. Initially, there was a significant decrease in effective voluntary responses. Interestingly, the stumbling corrective response was not evoked at this early time point, but emerged between 2 and 4 weeks post-injury. By 16 weeks post-injury, both the voluntary and spinally mediated responses were equally represented. Thus, voluntary and spinally mediated responses to the presence of an obstacle are altered by the disruption of ipsilateral, descending input although a substantial recovery was seen.

Methods

All animal procedures were conducted in accordance with the National Institutes of Health guidelines for the care and use of experimental animals and were approved by the University of Florida and the Malcom Randall Veteran's Affair's Institutional Animal Care and Use Committees.

Five, purpose bred, specific pathogen free (SPF), female, adult cats were used. Cats were spayed to remove potential hormone effects associated with the estrus cycle on behavior and lesion size (Sribnick et al., 2005; Stein, 2008; Sribnick et al., 2010).

Spinal Hemisection Surgery

All cats received a left spinal thoracic (T) 10 hemisection as described in our previous studies (Tester and Howland, 2008; Jefferson et al., 2010). In summary, animals received 0.1 cubic centimeter (cc) of both atropine sulfate (0.04-0.06 milligrams (mg)/ kilograms (kg)) and acetlypromazine (0.4-0.5mg/kg) subcutaneously (SQ), were anesthetized in a gaseous chamber with an isoflurane and oxygen mixture (2-5% isoflurane, 1-2 liters of oxygen (LO_2)), intubated, and maintained at a surgical plane of anesthesia with isoflurane (2-3% isoflurane). Throughout the duration of the surgical procedure, temperature, electrocardiogram (EKG), respiratory rate, and expired carbon dioxide (CO_2) were monitored and maintained within normal physiological limits.

The spinal cord was exposed by bilateral laminectomies at the vertebral T9-T10 level. A left lateral hemisection was made at the spinal T10 level using iridectomy scissors. Any fibers adhering to the dura were gently lifted with suction using a pulled glass pipette and cut. Once the hemisection was complete, the dura was sutured and durafilm (Codman-Shurtleff, Inc., Randolf, MA) and then gelfoam (Pharmacian and Upjohn, Inc., Peakpack, NJ) placed over the sutures. Muscle and skin were sutured in layers. Buprenorphine (0.01 mg/kg, SQ) was given every 6-12 hours for the first 48 hours after the surgery.

Procedures used to maintain the general health of the animals followed those used previously (Tester and Howland, 2008; Jefferson et al., 2010). Cats were housed singly or in pairs on thick beds of shredded newspaper or egg crate foam. The Crede maneuver was used to assist with bladder emptying for the first few days following injury until voluntary voiding recovered. Cats' food intake, weight and behavior were monitored closely throughout the study.

Training and Assessments

Pre- and post-injury, cats were conditioned to perform a variety of basic and skilled locomotor tasks for food rewards. These included crossing of a basic runway (0.305 x 4.5 meters (m)) with and without an obstacle (0.077 m high x 0.025 m depth) placed at the midpoint. Cats were trained to walk at their most comfortable fast walk/slow trot speed pre-injury. Post-injury, speeds were closely matched to pre-injury speeds for each cat. Preferred speeds did vary across cats and reflect normal biological variations including leg lengths. Post-injury, training resumed within 2-3 days. Initially, trainer assistance was given, if needed, and ceased as soon as weight support and postural control were sufficient to allow independent crossing. On training days, cats were fed exclusively in the behavior room. On non-training days, cats were fed ad lib in their cages. Water was provided ad lib throughout the study. Performances were filmed pre-injury and at 2, 4, 8, and 16 weeks post-injury for quantitative assessments. The ipsilateral hindlimb shows the most obvious motor deficits following hemisection. Thus, focus was on recovery of ipsilateral hindlimb movement.

Response type

Sixteen to 24 obstacle encounters were assessed per cat, at pre- and all post-injury time points. The range of crossings used reflects a fatigue factor during the early post-injury time points. Each obstacle encounter was identified as 1) voluntary response, 2) stumbling corrective response, or 3) no response. The following criteria were used. During a voluntary response the ipsilateral hindlimb clears the obstacle without contact. The stumbling corrective response is characterized by hindlimb contact followed by an immediate obvious flexion response is evoked. No response occurs when hindlimb contact does not evoke an obvious flexion response. These responses were averaged across animals at each time point and compared across time points. To

determine if the lead limb over the obstacle affected their ipsilateral hindlimb's negotiation, these crossings were classified by which hindlimb, left versus right, initially crossed the obstacle.

3-dimensional (3-D) angular kinematics

To illustrate normal hindlimb adjustments during obstacle negotiation, 3-D angular kinematics of ten basic steps and ten obstacle encounters for each cat were compared pre-injury. To understand how injury impacts performance, angular kinematics of 10 steps per time point during obstacle negotiation were compared across all time points. Each cat's post-injury performance was always compared to its pre-injury baseline, because cats, like humans, show individual variation. These variations in baseline may mask important changes post-injury if data is averaged across cats. To generate angles, reflective spheres were placed on 4 bony landmarks (iliac crest, greater trochanter, lateral malleolus and base of the fifth metatarsal). A fifth sphere was placed on the fibula above the lateral malleolus as a vector mark, which in combination with the length of the fibula, allowed for the automatic calculation of the knee joint using Motus software (Vicon Peak, Englewood, CO). Joint angles for the hip, knee, and ankle were calculated throughout the step cycle using the Peak Performance Analysis System (Vicon Peak, Englewood, CO). For representative purposes steps were normalized to 51 frames in order to facilitate qualitative joint angle comparisons during different subphases.

Approach and maximum heights

To illustrate the efficiency of limb lift during normal stepping versus obstacle negotiation the maximal vertical displacement was determined for both tasks pre-injury. The maximal vertical displacement was calculated by finding the overall range of movement (10 basic steps and 10 obstacle negotiations per cat) using the Y coordinates of the base of the 5^{th} metatarsal marker. To determine negotiation efficiency, two height measurements were assessed. The height of the paw when it first reaches the obstacle was measured in order to reflect limb

trajectory adjustments in anticipation of clearing the obstacle. This was termed the *approach height.* The paw height also was measured at the maximum height it reached during the obstacle encounter. This was termed the *maximum height.* To achieve these measurements, the following was done. The Y coordinate of the runway surface was set to equal 0. The height of the obstacle was defined by the Y coordinate of its top edge and the height of the foot defined as the Y coordinate of the marker on the base of the 5th metatarsal. The *maximum height* was calculated by subtracting the obstacle height from the maximum value of the base of the 5th metatarsal (equal to the maximal vertical displacement of the paw). The *approach height* was defined as the Y coordinate of the base of the 5th metatarsal as it reaches the vertical plane of the face of the obstacle minus the height of the obstacle. The maximal vertical displacement of the limb from the surface of the runway also was determined in order to compare limb displacements between basic locomotion and obstacle negotiation.

Vertical limb displacement

The vertical displacement of three points on the hindlimb representing the hip, knee, and ankle was used to determine if there was a change in their mobility during obstacle negotiation suggesting a change in the stability of the ipsilateral hindlimb. The Y coordinates of the hip (greater trochanter), knee (calculated point), and ankle (lateral malleolus) joints were calculated using Motus software. The maximum and minimum Y coordinates were found for each step and the difference was taken to define the vertical displacement.

Histology

Procedures used for basic lesion morphology were the same as those used in our previous studies (Tester and Howland, 2008; Jefferson et al., 2010). In brief, 5 months post-injury, all cats were anesthetized with an overdose of sodium pentobarbital (>40 mg/kg, intraperitoneal (IP)) and given 1.0 cc of both heparin (1000 units (U)) and sodium nitrite (1%) intravenously 20

minutes apart. Immediately following sodium nitrite administration, cats were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 molar (M) phosphate buffer (pH 7.4). The spinal cords were removed, the lesion segments blocked, and cryoprotected in 30% sucrose, 4% paraformaldehyde (pH 7.4). Tissue was sectioned at 25 microns (μm) using a cryostat. The first section of every 10 was mounted onto subbed slides and processed with cresyl violet (cresyl violet with acetate, Sigma) and myelin (Eriochrome Cyanine R, Fluka, New York) stains to view basic lesion morphology on the Nikon Eclipse E600 microscope.

Statistical Analyses

Using SPSS software (Chicago, IL), non-parametric Kruskal-Wallis tests were conducted to identify significance. Once significance was determined, Mann-Whitney U tests were performed to isolate the significance using a p value of ≤ 0.05.

Results

Basic Locomotion and Obstacle Negotiation

Movement patterns during basic locomotion and obstacle negotiation were compared (Figure 2-1). The movement of the hindlimb during basic locomotion and obstacle negotiation was very efficient; however there were distinct differences in hindlimb movement patterns during each task (Figure 2-1). Qualitatively the most apparent change was the pronounced knee flexion that occurred as the hindlimb approached and cleared the obstacle (Figure 2-1). Less pronounced qualitative changes were observed in the hip (Figure 2-1). A larger range of hip movement was seen during obstacle negotiation due to greater extension during the transition from stance to swing and more pronounced flexion in mid-late swing. In contrast to the hip and knee, the ankle joint was held in a more extended posture throughout obstacle clearance, despite this, the range of angular excursion was very similar to that seen during basic locomotion (Figure 2-1). These data are in agreement with Lavoie et al. (1995) who showed larger changes in the

knee and ankle joint angles and to a lesser extent at the hip in cats that were trained to step over an obstacle on a moving treadmill belt. To quantify differences between basic locomotion and obstacle negotiation, the maximum vertical displacement of the hindlimb was assessed (Figure 2-1). Specifically, the distance between the marker on the base of the 5^{th} metatarsal and the walking surface was averaged across 50 step cycles (10 steps from 5 cats). During basic locomotion the maximum vertical displacement averaged 3.98 centimeters (cm) while during obstacle negotiation the average was 12.84 cm. Because the length of the longest metatarsal was ~3cm, this indicated that the paw cleared the walking surface with ~1cm, and the 7.7 cm high obstacle with ~2cm of space. The difference in the maximum vertical displacements between these two tasks showed that a significant increase occurred during obstacle negotiation ($p=0.008$). The vertical displacement of the paw also illustrated the efficiency of limb lift during both types of locomotion, albeit efficiency was greatest during the less adaptive task.

Qualitative Characterization of Post-Injury Obstacle Negotiation

Following a lateral hemisection, changes in the ipsilateral hindlimbs' response to the obstacle were seen. Three general responses were characterized: no response, the stumbling corrective response, and the voluntary response (Figure 2-2). The no response was characterized by no apparent voluntary alterations ipsilateral hindlimb trajectory. Typically, during a no response the ipsilateral hindlimb appears to be dragged over the obstacle and in many instances was trapped between the body of the cat and the obstacle (Figure 2-2). All three joints were extended while passing over the obstacle as the limb was trapped between the body of the cat and the obstacle (Figure 2-2). After the limb was no longer in contact with the obstacle one of two things occurred; either the limb dropped to the runway landing on the dorsum paw or the knee flexed allowing for plantigrade placement. In the later instance the knee appears to play a role in repositioning the limb underneath the body.

The stumbling corrective response was characterized by a large increase in flexion evoked by contract of the dorsal surface of the paw with the obstacle (Figure 2-2). This response caused hypermetric knee flexion allowing successful obstacle clearance in that it did not interrupt forward progression (Figure 2-2). The observed hypermetric flexion of the knee is in agreement with EMG recordings that show activation of knee flexors after stimulation of the dorsal paw (Forssberg et al., 1977; Buford and Smith, 1993; McVea and Pearson, 2007).

The voluntary response was defined by any encounter with the obstacle during which the hindlimb does not make contact. Thus, all responses seen pre-injury were categorized as voluntary (Figure 2-1). The voluntary responses seen post-injury, however, showed distinct differences (Figure 2-2). Comparison of pre-injury voluntary responses with those seen 16 weeks post-injury (the most chronic time point) identified long term changes in voluntary obstacle negotiation(Figure 2-2). The hip and ankle joints remained in a slightly more flexed position as the limb transitioned from swing to stance post-injury (Figure 2-2). The knee, which showed the greatest chronic changes post-injury, was characterized by increased flexion throughout the step over the obstacle (Figure 2-2). Thus, a voluntary response was seen post-injury, but the joint angles were altered.

Quantification of Post-Injury Obstacle Negotiation

To evaluate recovery over time, the number of voluntary, no, and stumbling corrective responses were quantified. Pre-injury, because the obstacle was visualized and anticipated, hindlimb trajectories were modified and the obstacle consistently cleared (equals 100% voluntary response; Figure 2-3). Following injury, initially, there was a significant decrease in voluntary responses as well as total responses (defined as the sum of voluntary and stumbling corrective responses; $p=0.008$ for both). Thus, this initial 2 week time point was predominantly characterized by “no response” of the hindlimb to the obstacle (Figure 2-3). The small number

of voluntary responses seen (20%) at 2 weeks post-injury comprised the entirety of total responses. The stumbling corrective response did not emerge until 4 weeks post-injury, when it became the predominant response (65%) and was significantly greater than the number of voluntary responses (Figure 2-3; p=0.016). The emergence of the stumbling corrective response combined with the voluntary responses significantly increased the total responses from 2 weeks to 4 (p=0.008) and 8 weeks (p=0.008). Although, the voluntary response remained significantly decreased throughout the time course of this study (p=0.008 at 4 and 8, 0.032 at 16 weeks), there was a strong recovery trend with an increase in the number of voluntary responses from 2 to 8 weeks (p=0.056). Since the stumbling corrective response was not present pre-injury and 2 weeks post-injury a significant increase was seen from these time points out to the most chronic time point (p=0.008 at both 4 and 8, p=0.032 at 16 weeks), but no changes were seen between 4 and 16 weeks. A plateau in recovery appears between 8 and 16 weeks post-injury when total responses reach ~90% and approximately half were represented by the stumbling corrective response.

Efficiency of Obstacle Negotiation

In order to understand the efficiency in which animals were able to negotiate the obstacle, the height of the paw was assessed at two points as the limb passed over the obstacle (Figure 2-4). In contrast from the maximal vertical displacement (Figure 2-1), these displacements were calculated from the top surface of the obstacle. The approach height was defined as the height of the paw when it broke the vertical plane of the obstacle illustrating the anticipation of the obstacle. The maximum height was the highest point that the paw reached during obstacle negotiation. The more similar the two values are the more efficient the animal was at anticipating and clearing the obstacle. Pre-injury, the approach height (4.52 cm) and the maximum height (5.14 cm) were very similar and not significantly different (Figure 2-4,

p=0.095). Following injury, at 2 weeks there was a significant decrease in the approach height (2.36 cm), due to the increase in crossings during which the paw simply dragged over the obstacle (Figure 2-3, p=0.008). This also caused the maximum height to remain similar to pre-injury values (4.86 cm) since the width of the paw (~3 cm) filled the space between the obstacle and the marker on the base of the 5th metatarsal. As the voluntary response was recovered so was the approach height defined by a significant increase from 2 weeks to 16 weeks post-injury (4.08 cm; p=0.008), with no difference from pre-injury values (p=0.151). On the other hand, the maximum height showed long lasting changes. At 4 weeks, characterized by the emergence of the stumbling corrective response (Figure 2-3), the maximum height began to increase, becoming significant at 8 weeks (7.89 cm) from both normal and 2 week post-injury (p=0.008 from pre-injury, 0.016 from 2 weeks). During many obstacle encounters the maximum height occurred after the hindlimb had cleared the obstacle as a response to the initial paw contact, which can be defined by significantly smaller approach heights at all post-injury time points (p=0.008 for all time points).

Categorization of responses to the obstacle made it apparent that paw heights were correlated with response type (Figure 2-4). The largest approach heights were seen during the pre-injury voluntary response because animals were able to lift their limb to the maximum height needed to clear the obstacle without any alterations. By 16 weeks, the approach height (4.08 cm) continued to be less then pre-injury values. Post-injury, encounters during which the paw contacted the obstacle, the no responses and the stumbling corrective responses, initially decreased the approach height. By 8 (3.76 cm) and 16 weeks (3.55 cm), though, the no response encounters were characterized by approach heights that were very similar to voluntary response approach heights. When further examined, these responses had full active limb participation, but

where just short of clearing the obstacle. Based upon this analysis, the 10% of "no response" encounters present at 8 and 16 weeks post-injury (Figure 2-3) would be more appropriately termed a voluntary response with toe drag. During these encounters the tip of the paw dragged over the obstacle not severely interrupting forward progression therefore not evoking the stumbling corrective response. The increased flexion of the stumbling corrective response contributed to the significant increase in maximum height at the 8 week time point.

Joint Contribution

The vertical range of movement of the hip, knee and ankle joints was assessed using the marker on the base of the 5^{th} metatarsal to determine joint contribution and ipsilateral hindlimb stability during obstacle negotiation. Pre-injury, the vertical movement of the knee (7.13 cm) and ankle (10.35 cm) joints had the greatest contribution to limb lift over the obstacle (Figure 2-5). At 2 weeks post-injury, significant increases in the movement of the knee (9.33 cm) and hip (7.40 cm) were accompanied by no change in the ankle joint (10.56 cm) movement (knee $p=0.032$, hip $p=0.016$). Thus, it is evident that the ankle joint was not being actively modified over the obstacle causing the observed passive drag (Figure 2-2). By 4 weeks post-injury, all three joints show significantly increased vertical range of movement, which remained out to 16 weeks post-injury (hip $p=0.032$, 0.008, 0.008, knee $p=0.032$, 0.008, 0.008, and ankle $p=0.032$, 0.016, 0.008). The sustained increase in joint movement suggests that the stability and efficiency of the hindlimb did not fully recover following injury.

Leading Limb Preference

Pre-injury, cats crossed the obstacle without any bias in limb and were equally efficient independent of which limb lead (Figure 2-6). Following injury, preference to lead with the left hindlimb showed a strong trend ($p=0.056$) at 8 weeks, but overall no change was seen over time.

Again categorization of the responses to the obstacle highlighted a distinct bias dependent on response type. As mentioned above during the pre-injury voluntary response the cats had no bias toward the leading hindlimb (Figure 2-6). During the post-injury voluntary response there was a strong bias toward the left hindlimb crossing over the obstacle first with the amount of these crossing being significantly or strongly increased from right crossings at all time points ($p=0.016, 0.056, 0.008, 0.008$). In contrast, the stumbling corrective response, which emerged at 4 weeks post-injury, appeared to have a right hindlimb bias although there were no significant differences over time or between the left and right hindlimb at any given time point. The crossings that were classified as no responses of the ipsilateral hindlimb (Figure 2-6) had an initial right hindlimb trend ($p=0.056$), but switched to a left hindlimb bias at the 8 week time point ($p=0.008$). This switch at the later time points is similar to the bias that was seen during voluntary response encounters. As the voluntary response was being recovered the few no responses were similar to a voluntary response, but with the presence of toe drag. Thus, the most successful post-injury responses led with the left hindlimb first while leaving the right hindlimb on the runway.

Discussion

The present results characterize the recovery of voluntary obstacle negotiation following low thoracic hemisection in the cat. Although recovery occurred, persistent deficits were seen even 4 months after injury. Initially, the ipsilateral hindlimb typically was dragged behind the body. Little to no response was elicited by anticipation or contact with the obstacle. Between 2 and 4 weeks, the spinally mediated response, the stumbling corrective response, emerged and the ability to voluntarily modify limb trajectory in response to the obstacle began to recover. Despite voluntary recovery, decreased initial limb lift evoked the stumbling corrective response ~50% of the time. Thus, efficiency of paw lift and clearance never fully recovered. By 16

weeks post-injury, animals still were unable to successfully clear the obstacle 100% of the time. In addition, there was no preference for leading with the hindlimb that typically increased negotiation success. Thus, thoracic hemisection injury does allow for substantial recovery of voluntary modifications in response to an obstacle, but persistent deficits in certain aspects of this negotiation remain.

Translational Impact

Motor incomplete spinal cord injuries in humans are very common. Because a large amount of the descending input is spared in these injuries there is the potential for voluntary functional recovery. One example of an incomplete spinal cord injury in humans is Brown-Sequard syndrome (BSS), characterized only by ipsilateral motor and proprioceptive impairments and contralateral loss in pain and temperature sensitivity. The pure form of BSS is very rare. More commonly individuals exhibit Brown-Sequard-plus syndrome (BSPS). BSPS also is characterized by asymmetrical impairments, but the loss may be less on the involved side or involve both sides. Traditionally, BSS or BSPS were associated with stab wounds to half of the spinal cord, but there are a variety of etiologies including tumors, herniated disks and other traumatic events that may results in the same functional asymmetries (Koehler and Endtz, 1986; Roth et al., 1991; reviewed by McKinley et al., 2007). This asymmetrical impairment and the rapid, early recovery are similar to that seen following a lateral hemisection injury in the cat (Eidelberg et al., 1986; Helgren and Goldberger, 1993; Basso et al., 1994; Tester and Howland, 2008; Jefferson et al., 2010). Recovery from Brown-Sequard syndrome begins within 24 hours following injury and typically progresses until 1-6 months post-injury when the recovery plateaus. Despite substantial recovery, deficits in skilled movements still exist (Little and Halar, 1985). This is consistent with the recovery progression seen in the current study. Two weeks post-injury all animals had regained the ability to independently cross the runway, but recovery

of skilled obstacle negotiation lagged behind. This recovery which required specific balance and limb adaptations showed a different time course from basic locomotor recovery (Helgren and Goldberger, 1993; Tester and Howland, 2008) in that it did not plateau until 2-to-4 months post-injury. Further, although substantial recovery occurred, persistent deficits were apparent in the ability to voluntarily clear an obstacle post-injury.

Pathways Involved in Functional Recovery

Voluntary obstacle negotiation requires the cortico- and rubro-spinal tracts (Beloozerova and Sirota, 1993; Widajewicz et al., 1994; Drew et al., 1996; Drew et al., 2002; Lavoie and Drew, 2002; Beloozerova et al., 2010). During the first two weeks post-injury obstacle negotiation typically was characterized by an unresponsive ipsilateral hindlimb dragging over the obstacle. In a few instances (~10%) the limb cleared the obstacle without contact. These early successful clearances were likely mediated by spared tissue. In particular, the preserved corticospinal tract collaterals that cross in the commisures along the length of the spinal cord in the cat (Satomi et al., 1991) and monkey (Lacroix et al., 2004) are obvious candidates. The recovery in voluntary control that occurs after two weeks post-injury, however, suggests that mechanisms, other than spared substrates, are responsible. A number of studies suggest that plasticity of spared descending systems may occur following spinal cord injury. Following incomplete spinal cord injuries in rodents, substantial increases in collateral sprouting of the corticospinal tract have been reported during the first two months post-injury (Li et al., 1994; Bareyre et al., 2004). Although some aspects of recovery are reported to occur fairly rapidly, activation of dennervated muscles with cortical stimulation is delayed until after 4 weeks post-injury correlating this aspect of recovery with corticospinal plasticity (Bareyre et al., 2004). In the current study, voluntary control of the ipsilateral hindlimb during obstacle negotiation began to show substantial recovery by 4 weeks post-injury which is consistent with a dependence upon

new connections. Following injury, corticospinal input may not be direct, but relayed through plasticity of other systems. Likely relay candidates include the propriospinal systems. Corticospinal axons axotomized in the rat thoracic spinal cord have been shown to sprout collaterals at the cervical level which synapse onto long propriospinal neurons. These long propriospinals, already connected with the caudal cord, serve as an indirect pathway capable of mediating function (Bareyre et al., 2004). Short propriospinal neurons in the thoracic spinal cord of the mouse also have been reported to serve as a bridge and support basic stepping activity (Courtine et al., 2008; Courtine et al., 2009). To understand the plasticity and potential contributions of these intraspinal systems to recovery in the cat, tract tracing studies were pursued (see chapter 3).

In the normal cat, cutaneous afferents are responsible for triggering the stumbling corrective response (Prochazka et al., 1978; Forssberg, 1979; Wand et al., 1980). Thus, it is classified as a contact/tactile placing response and mediated by cortical and cerebellar connections to the rubrospinal tract and spinal circuitry (Batson and Amassian, 1986; Amassian and Batson, 1988; Fleshman et al., 1988). Consistent with our findings, others have reported that this response is abolished following hemisection (Murray and Goldberger, 1974; Helgren and Goldberger, 1993). However, in the current study recovery of a modified stumbling corrective response was seen beginning around 4 weeks post-injury. Although, the exact amount of force was not measured, the observed limb displacement that occurred prior to eliciting the stumbling corrective response far exceeded contact (Forssberg, 1979; McVea and Pearson, 2007). Prochazka et al. (1978) suggests that the stumbling corrective response has an underlying proprioceptive stretch reflex in the ankle flexors, which surfaces when cutaneous sensory input to the dorsum of the paw is blocked (Prochazka et al., 1978). Our results are consistent with a

proprioceptive response and its delayed onset is likely due to a change in the main afferent control. Further, the lack of a cutaneous response is consistent with the disruption of the dorsal columns.

Studies in the rodent show that immediately following spinal transection, persistent inward currents (PICs) associated with the dendrities of motoneurons are greatly decreased. This results in reduced motoneuron excitability and leads to complete or partial disruption of reflex activity for >1month (Bennett et al., 2001; Li and Bennett, 2003). In the chronically injured spinal cord, however, the motoneurons recover the ability to generate large PICs which results in motoneuron hyperexcitability and muscle spasms (reviewed by Bennett et al., 2001; Li and Bennett, 2003; Heckman et al., 2005). Although the hemisection model used in the current study only partially disrupts the descending monoaminergic input believed to be critical for normal levels of PICs, this may be sufficient to alter PIC levels and contribute to the initial suppression of the stumbling corrective responses for several weeks post-injury. Further, the change in threshold (tactile to proprioceptive) for eliciting the response post-injury also is consistent with altered mononeuron excitation thresholds associated with a change in PICs.

Adaptive Strategies

Pre-injury, cats exhibited no leading limb bias. Post-injury however, negotiation of the obstacle was most effective when the ipsilateral hindlimb led over the obstacle. This approach increased the cats' stability by leaving the contralateral hindlimb in a weight supporting position as the ipsilateral hindlimb negotiated the obstacle. Postural adjustments, such as this, are extremely important during locomotion where the number of supporting limbs and their positions constantly change. Postural control requires the integration of spinal mechanisms controlled by somatosensory input and descending systems including the corticospinal and rubrospinal tracts (reviewed by Deliagina et al., 2008). In both the rabbit (Lyalka et al., 2005) and cat (Helgren

and Goldberger, 1993; Tester and Howland, 2008), postural control of the hindquarters during walking recovers within the first two weeks following a thoracic lateral hemisection (Lyalka et al., 2005). In contrast, the more challenging tasks of beam and ladder crossing which require greater balance and precision, take longer to show some recovery (Helgren and Goldberger, 1993; Tester and Howland, 2008). Obstacle negotiation also requires adaptations in limb trajectory, posture and balance. Thus, it is not surprising that its recovery also is similarly delayed and incomplete compared to walking.

Locomotor Training Effects

One of the most promising approaches to the treatment of chronic spinal cord injury in the human population is the use of body weight supported treadmill training and overground locomotor training (Harkema et al., 1997; Behrman et al., 2005; Thomas and Gorassini, 2005; Norton and Gorassini, 2006). Clinical studies using this approach are based upon work done in the low spinal cat which showed that task specific locomotor training increased basic stepping recovery (Lovely et al., 1986, 1990; De Leon et al., 1998). Locomotor training also increases neurotrophin levels which in turn can effect synaptic plasticity (Gomez-Pinilla et al., 2002; Vaynman and Gomez-Pinilla, 2005). The most efficacious time to start training or exercise post-spinal cord injury however is not clear. Positive outcomes associated with early initiation of training following spinal cord injury (De Leon et al., 1998; Rossignol et al., 2002; Dupont-Versteegden et al., 2004; Boyce et al., 2007; Engesser-Cesar et al., 2007) are somewhat tempered by reports of negative effects following both spinal cord (Smith et al., 2009) and brain injury (Griesbach et al., 2004a; Griesbach et al., 2004b). Although the effects of training in the current study are unknown, it is an important consideration and, we believe, likely contributed positively to performance levels.

Conclusions

The data from this study show substantial, yet incomplete recovery of obstacle negotiation following low thoracic hemisection. Interestingly, initially post-injury there was a depression of the spinally mediated stumbling corrective response, which when recovered resembled more of a proprioceptive versus contact placing response. This recovery was incomplete despite sparing of half of the spinal cord and rigorous task specific training. It will be important in future studies to begin to understand the role of training and if the timing of training initiation is critical to recovery. The improvements in functional recovery do suggest robust and prolonged intrinsic plasticity of the systems that control obstacle negotiation. Understanding the underlying intrinsic plasticity of spinal and supraspinal systems is critical to the development of therapeutic and rehabilitative interventions for spinal cord injury.

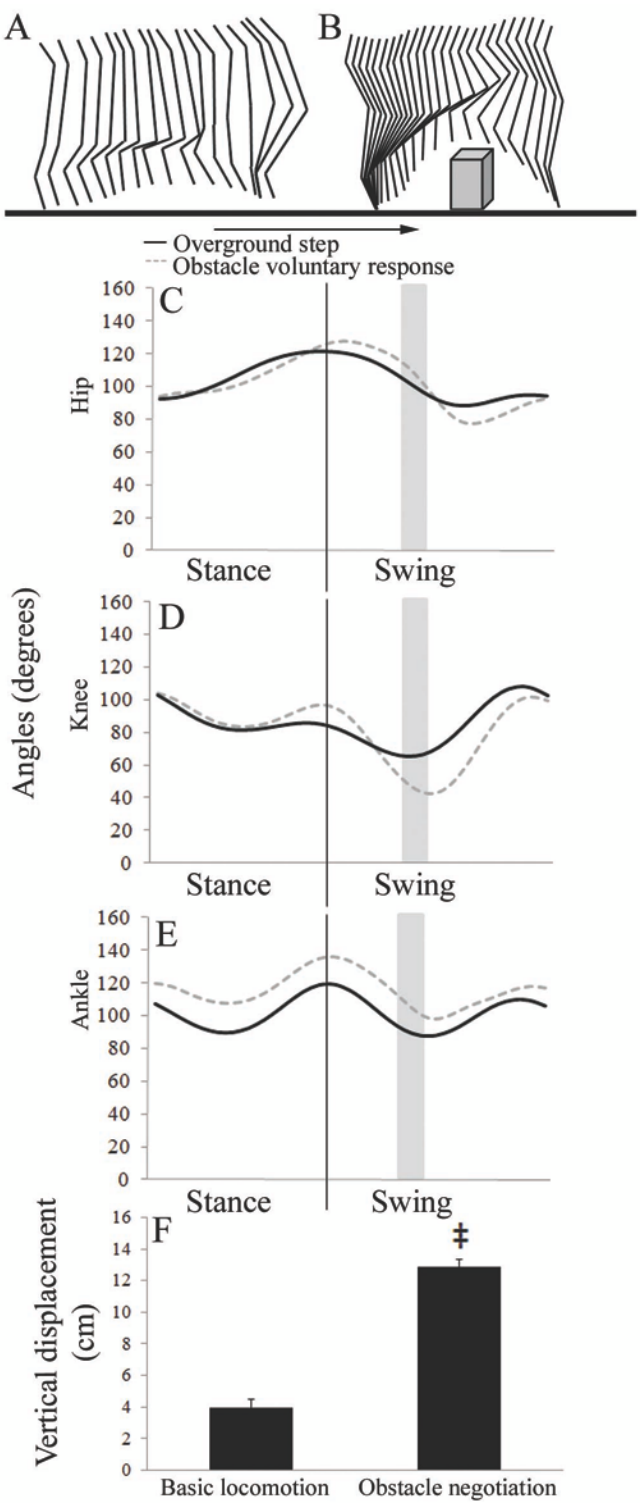


Figure 2-1. Kinematic data recorded from hindlimbs during runway crossing and obstacle negotiation pre-injury. Stick figures of one representative swing phase during basic locomotion (A) and obstacle negotiation (B) illustrate qualitative differences in hindlimb movement patterns between the two tasks. Arrow indicates direction of

Figure 2-1. Continued. movement (A, B). Waveform graphs are of a single step or obstacle negotiation from a representative animal. Each is shown from paw down to paw down. The hip (C), knee (D), and ankle (E) joints are compared between tasks. The position of the obstacle is represented by the vertical thick gray line. Angular changes were seen at all joints, with the greatest occurring at the knee during clearance of the obstacle. Maximal vertical displacement of the limb was significantly greater (‡) during obstacle negotiation compared to basic locomotion (F). Error bars denote standard deviation (SD).

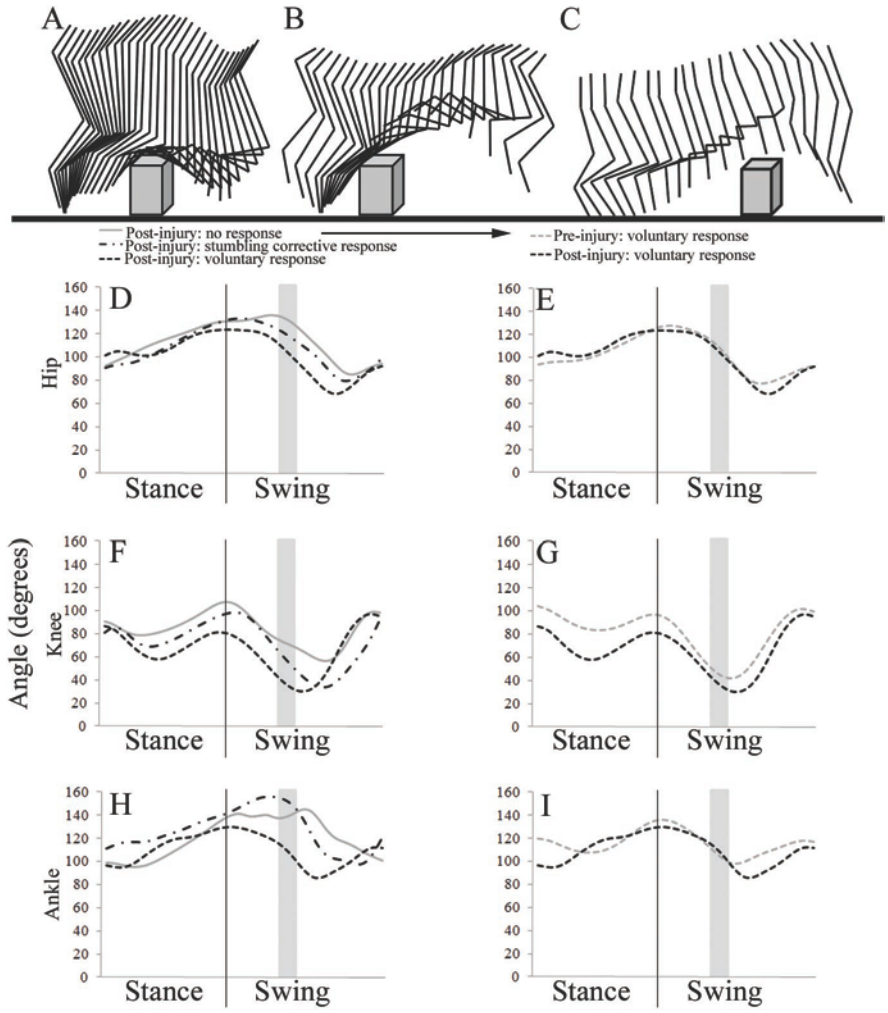


Figure 2-2. Kinematic data from the ipsilateral (left) hindlimb following injury during obstacle negotiation. Stick figures of one representative swing phase when the limb was not being actively modified (A, a "no response"), during the stumbling corrective response (B), and during a voluntary response (C). Arrow indicates direction of movement (A, B, C). Waveform graphs depict post-injury changes in hip (D, E), knee (F, G), and ankle joints (H, I). Long lasting changes in joint angles were seen even out to the most chronic time point. The gray shaded area represents the placement of the obstacle. The thin line indicates the division between stance and swing.

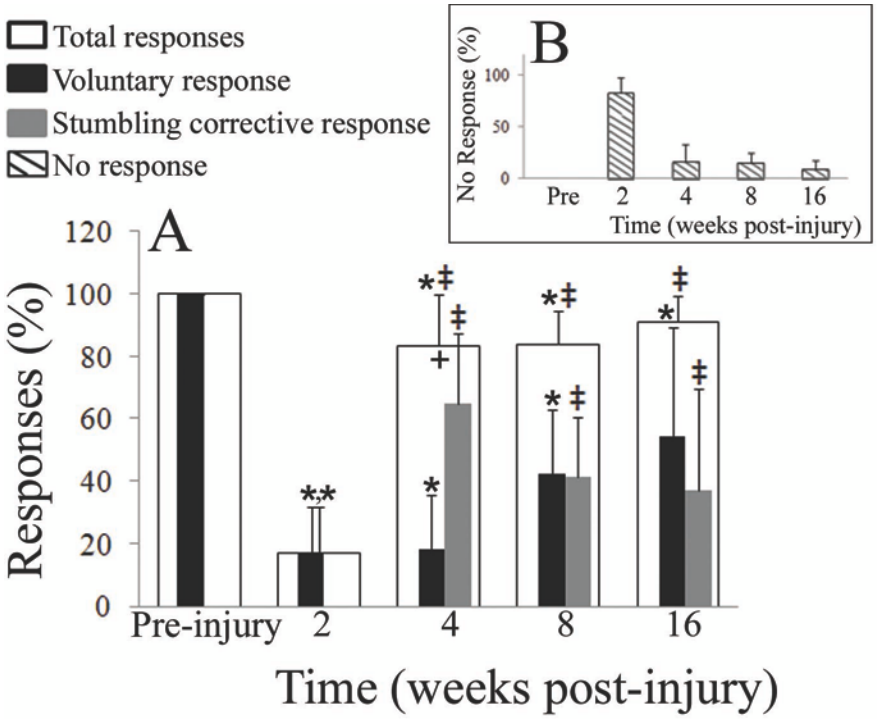
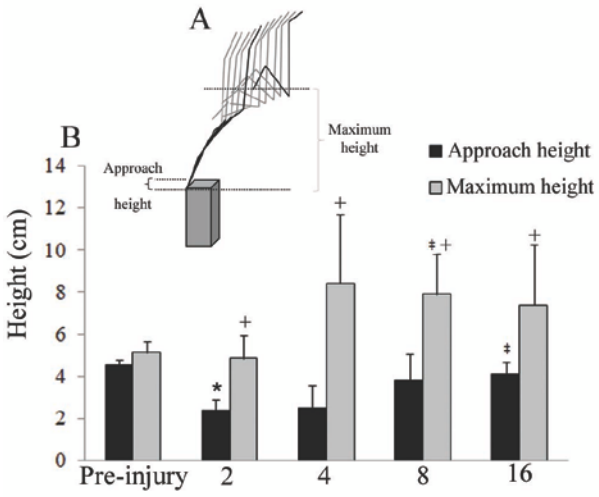


Figure 2-3. Recovery time course of ipsilateral hindlimb responses during obstacle negotiation. Total responses (A), as defined by the sum of voluntary and stumbling corrective responses, were significantly decreased at 2, 4, and 8 weeks post-injury compared to pre-injury (*). Despite this, significant recovery in total responses was seen from 2 weeks to 4, 8, and 16 weeks post-injury (‡). Voluntary responses were significantly decreased at all post-injury time points (*) from pre-injury levels. The stumbling corrective response initially emerged as the predominant response at 4 weeks post-injury (+) and was significantly increased from pre-injury and 2 weeks out to 16 weeks (‡). The percent of no response encounters is shown in B. Error bars denote SD.



C

Approach Height (cm)	Pre-injury	2 weeks	4 weeks	8 weeks	16 weeks
VR	4.52 ± 0.27	4.48 ± 2.96	3.60 ±1.22	4.04 ± 2.01	4.08 ± 1.39
SC	N/A	N/A	2.14 ± 1.47	3.29 ± 5.24	3.01 ± 1.79
NR	N/A	2.04 ± 1.49	1.92 ± 2.48	3.76 ± 0.78	3.55 ± 0.72
Maximum Height (cm)					
VR	5.14 ± 0.52	6.00 ± 3.43	4.69 ± 0.76	6.87 ± 2.56	5.05 ± 1.89
SC	N/A	N/A	8.92 ± 3.79	11.91 ± 3.72	11.54 ± 2.13
NR	N/A	4.67 ± 1.92	3.91 ± 0.72	4.91 ± 1.98	4.74 ± 0.77

Figure 2-4. Ipsilateral hindlimb efficiency during obstacle negotiation. The stick figure of the left hindlimb over the obstacle depicts the distances used for the maximum and approach heights (A). Pre-injury, cats were very efficient in their limb lift and clearance during obstacle negotiation (B). At 2 weeks post-injury, there was a significant decrease in the approach height from pre-injury values (*), which by 16 weeks almost equaled pre-injury values (‡). The maximum height was significantly increased at 8 weeks from both pre-injury and 2 weeks post-injury (‡). Pre-injury maximum and approach heights were equal, but at all post-injury time points the maximum height was significantly greater (+). When the responses were characterized it was evident which response was contributing to the totals at each time point (C, see text). Error bars denote SD.

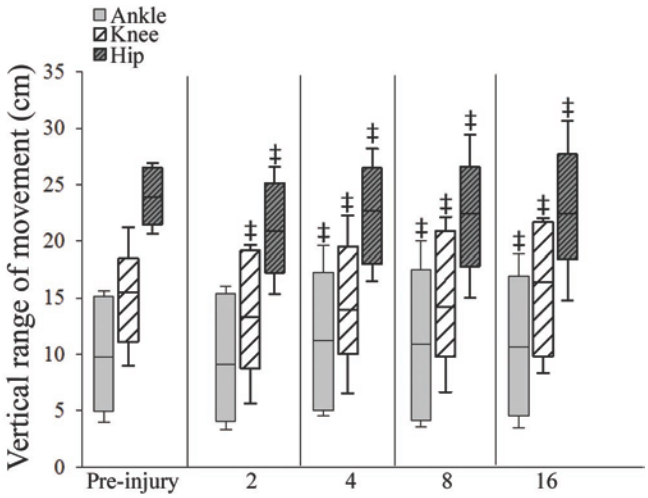


Figure 2-5. Vertical range of movement of individual joints during obstacle negotiation. Pre-injury, the knee and ankle joints show the greatest movement ranges. Post-injury, significant increases in the vertical movement of the hip and knee were seen at all time points (‡). The ankle does not show an initial change, as it is constrained between the obstacle and the body of the animal. By 4 weeks, however, a significant increase is seen and remains at subsequent times (‡). Error bars denote SD.

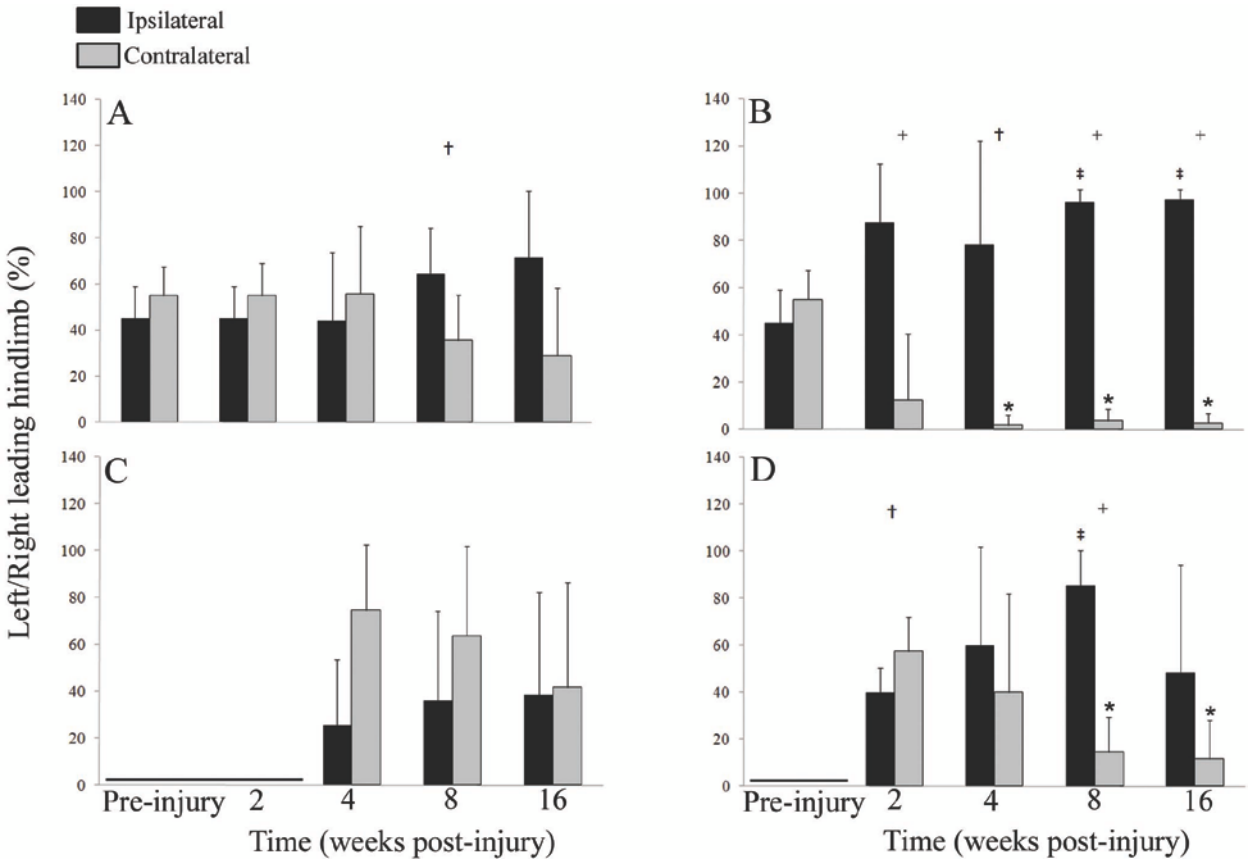


Figure 2-6. Leading limb preference during obstacle negotiation. Overall, animals had no bias, although there was a strong trend to a left hindlimb preference at 8 weeks post-injury (†; A). During voluntary responses (B), the ipsilateral hindlimb was the dominant leading limb at all post-injury time points (+,†) as crossings that were led with the contralateral hindlimb were significantly decreased (*). No hindlimb bias was present during stumbling corrective responses (C). During no responses the contralateral hindlimb showed a trend toward being the dominant leading limb at 2 weeks post-injury (D,†). Following 2 weeks, though, the preference for leading with the ipsilateral hindlimb emerged. By 8 weeks there were significantly more crossings that were led with the ipsilateral hindlimb than the contralateral (‡, +) as these crossings were significantly decreased from 2 weeks (*). Error bars denote SD.

CHAPTER 3
PLASTICITY OF MULTIPLE PATHWAYS CONTRIBUTE TO AXONAL BRIDGING OF THE LESION SITE FOLLOWING INCOMPLETE SPINAL CORD INJURY IN THE ADULT CAT

Introduction

To promote functional recovery of voluntary locomotor features following spinal cord injury, information must flow across the lesion site. Three potential mechanisms for achieving this connectivity are regeneration, spared fiber tracts, and collateral sprouting. Early studies first suggested that the CNS was capable of sustaining growth of intact dorsal root afferents following target denervation and that these collateral sprouts formed functional synapses (Liu and Chambers, 1958; McCouch et al., 1958; Bernstein and Bernstein, 1973). Next, long distance regenerative growth of injured CNS axons was demonstrated when they were placed in a more growth permissive environment (Richardson et al., 1980; David and Aguayo, 1981). Recent attention on propriospinal neurons (PSNs) suggests that they are capable of bridging a spinal lesion. Work in the rodent suggests that following incomplete spinal cord lesions, PSNs form novel circuitry that effectively re-innervated the caudal spinal cord with information from descending systems resulting in functional recovery (Bareyre et al., 2004; Courtine et al., 2008). Another population of neurons, commissural interneurons, following a medial myelotomy in the adult feline regenerated through a highly inhibitory environment re-forming functional connections across the midline of the spinal cord (Fenrich and Rose, 2009). Thus, these resulting collaterals and/or regenerated neurons have the ability to travel rostral, caudal and across the midline in an attempt to restore information below the level of a lesion. However, the specific populations of PSNs, i.e. contralateral or ipsilateral, spared or axotomized, contributing to this novel circuitry, and other neuronal populations that may show similar plasticity have not been identified.

Our lab (Tester and Howland, 2008; Jefferson et al., 2010), and others (Eidelberg et al., 1986; Helgren and Goldberger, 1993; Basso et al., 1994), have shown that substantial functional recovery of multiple motor systems occurs following a low thoracic hemisection in the cat. Locomotor recovery includes recovery of trunk balance and interlimb coordination as well as skilled features such as accuracy of limb placement (Helgren and Goldberger, 1993; Tester and Howland, 2008). Another motor system that effectively recovers following hemisection is the cough motor system (Jefferson et al., 2010). Recovery of both of these systems requires the integration of supraspinal and intraspinal pathways. The goal of the current study was to determine if descending systems, which complement functional recovery in these cats, can bridge a low thoracic hemisection and if the number of contributing neurons changes over time post-injury.

Using a retrograde tracing approach, the current study focused on long and short propriospinal neurons as well as rubrospinal tract neurons that project below the level of a low thoracic hemisection in the cat. These three very different systems potentially play a role in recovery of locomotor and cough motor system function. Long PSNs were assessed at cervical spinal level 5, short PSNs at thoracic 6 and 8 and rubrospinal tract (RST) neurons at the level of the red nuclei. Immediately following injury, there was a significant decrease in all populations of PSNs and contralateral (axotomized) RST neurons with axons below the level of the lesion. The decrease in long PSNs and axotomized RST neurons remained significant out to 16 weeks post-injury. However, both populations of short PSNs showed substantial plasticity with numbers of contributing neurons increasing significantly by 16 weeks. In fact, the number of contralateral short PSNs at T8 was significantly greater than that seen in normal controls. Similar significant changes also were seen in the ipsilateral (non-axotomized) RST neurons.

These results suggest that a novel substrate of axons normally terminating rostral to a lesion, are recruited in addition to regeneration and collateral sprouting.

Methods

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 and the Malcom Randall Veteran's Affair's Institutional Animal Care and Use Committees. Three groups of female, adult, SPF cats were used in this study: normal controls (n=7), acute thoracic hemisections (n=7), and chronic thoracic hemisections (n=6). All cats were part of a locomotor study in which they were trained daily to walk on a treadmill and a variety of runways.

Surgical Procedures

Spinal hemisection injury and general care

All spinal hemisection procedures were described previously (Tester and Howland, 2008; Jefferson et al., 2010). In summary, animals received 0.1cc of both atropine sulfate (0.04-0.06mg/kg) and acetylpromazine (0.4-0.5mg/kg) subcutaneously (SQ). Next, they were anesthetized with a combination of isoflurane (2-5%) and oxygen (1-2 LO_2) and were intubated and maintained at this level of anesthesia throughout the surgical procedure. Bilateral laminectomies were performed to expose the spinal cord and a left, lateral hemisection was made with iridectomy scissors at spinal T9. Any fibers adhering to the ventral or lateral dura were gently lifted with suction using pulled glass pipettes and cut. The dura was sutured, durafilm (Codman-Shurtleff, Inc., Randolf, MA), and gelfoam (Pharmacian and Upjohn, Inc., Peakpack, NJ) placed over the sutures, and muscle and skin closed in layers. Throughout the duration of the surgical procedure temperature, EKG, respiratory rate, and expired CO_2 were monitored and maintained within normal physiological limits. Cats were recovered in temperature controlled incubators. Buprenorphine (0.01 mg/kg, SQ) was given after termination of anesthesia and every

6-12 hours for the first 48 hours after the surgery. Cats were housed singly or in pairs on thick beds of shredded newspaper or egg crate foam. Bladders were emptied using the Crede maneuver for the first few days after surgery and their health was monitored closely.

Tract tracing

To examine the amount of propriospinal and rubrospinal tract neurons that received input caudal to the hemisection injury, the retrograde tract tracer Fluoro-Gold (FG, 0.5% in sterile water, Fluorochrome, LLC, Denver, CO.) was used. As above, animals received 0.1cc of both atropine sulfate (0.04-0.06mg/kg) and acetlypromazine (0.4-0.5mg/kg) subcutaneously (SQ). Next, they were anesthetized with a combination of isoflurane (2-5%) and oxygen (1-2 LO_2) and were intubated and maintained at this level of anesthesia throughout the surgical procedure. All cats received bilateral laminectomies at the injection site. Injections were performed using a 33 gauge Hamilton syringe (Hamilton Company, Reno, NV) at spinal T11 (normals) or 14mm (~T11) below the lesion at the time of injury (acute) or 16 weeks post-injury (chronic)(Figure 3-1). Four needle tracks were made. Within each track ¼ µl was injected ventrally followed ~3 minutes later by injection of ¼ µl dorsally. A total of 2 µls (8 injections) were distributed across the spinal cord. In order to determine normal ipsilateral versus contralateral projections of these neuronal populations, 4 of the 7 normal cats, received injections of FG on the left side of the spinal cord (4 injections, totaling 1 µl). Following these procedure bladders were emptied using the Crede maneuver for the first few days after surgery and their health was monitored closely.

Immunohistochemistry and Neuron Counts

All cats were transcardially perfused with saline (0.9%), followed by 4% buffered paraformaldehyde (pH 7.4) 13 days post-injections. The spinal cords and brains were removed and blocked into segments. Three spinal segments, C5, T6, and T8 along with the red nuclei

were cryoprotected in 30% sucrose in 4% paraformaldehyde (pH 7.4) and sectioned on a cryostat (25 μms). Every 40th section (1000 μms) through each spinal segment and every 8th (200 μms) section through the red nuclei was processed for immunohistochemistry using an antibody against Fluoro-Gold (Fluorochrome, LLC, Denver, CO) and the ABC Vectastain kit (Vector Labs, Burlingame, CA). Stained neuronal somas with Fluoro-Gold labeling were counted using a Nikon Eclipse E600 microscope keeping left and right sides counts separate. All injection sites and lesions were sectioned (25 μms). Fluoro-Gold spread was assessed by auto-fluoresence in these sections to verify that the tracer had covered the entire cross sectional extent of T11, but had not spread into the lesion site. No cats were included in this study if Fluoro-Gold spread was identified in the lesion site. All cats included in the study showed similar tracer spread rostral and caudal to the injection sites. With the exception of 3 animals, all had tracers spread across the entire areal extent of T11. In these 3 cats, (2 chronic animals and 1 normal), the tracer did not cover the ventral funiculi which may have affected labeling of the long propriospinal neurons. Thus, these three animals were not used in the C5 counts. The first section out of every 10 throughout the lesion segment were mounted onto subbed slides and processed with cresyl violet (cresyl violet with acetate, Sigma) and myelin (Eriochrome Cyanine R, Fluka, New York) stains to view basic lesion morphology.

Statistics

Statistical analyses were run using SPSS software (Chicago, IL). The nonparametric Mann-Whitney U test was performed to compare the number of neurons between time points. Significance level was set at $p \leq 0.05$.

Results

Propriospinal and Rubrospinal Tract Projections in the Normal Spinal Cord

Unilateral injections (n=4) were performed to determine the populations of ipsilaterally and contralaterally projecting neurons. Bilateral injections (n=3) were done to suggest if a neuron could have both ipsilateral and contralateral projections and to establish baseline neuronal counts for comparison with injured animals. Similar to early reports (Matsushita et al., 1979; Skinner et al., 1979), long propriospinal neurons appear to have a greater number of contralaterally projecting neurons (Figure 3-2). Short propriospinal neurons appear to have a more equal number of ipsilaterally and contralaterally projecting neurons at both T6 (Figure 3-2) and T8 (Figure 3-2). However, both must have neurons with bilateral projections. The neuronal counts of each population of neurons were compared to the normal animals receiving bilateral injections in order to assess whether an n of 7 could be used in our analysis. For the propriospinal neurons, the number of neurons ipsilateral and contralateral to the injection were added together and compared to each side of the bilaterally injected animals. When statistical analyses were run comparing the two groups of numbers (added unilateral versus bilateral), there were no significant differences. Therefore, the two normal groups were combined when compared to the injured groups. Because the RST is primarily a crossing tract (Pompeiano and Brodal, 1957; reviewed by Massion, 1967; Bruce and Tatton, 1981), unilateral injections yielded neurons in the contralateral red nucleus (Figure 3-2). Following bilateral injections, there was an equal amount of neurons in both red nuclei. This number also was equal to the number in the unilaterally injected contralateral red nucleus. Thus, the ipsilateral red nucleus was set equal to the contralateral red nucleus when these two groups of normal animals were combined.

Quantification of Propriospinal Neurons

The contribution of descending propriospinal neuronal populations below the T9 hemisection was assessed. The ipsilateral (left) and contralateral (right) side neuronal counts remained separate in order to determine the specific population contributing to the observed plasticity. In comparison to uninjured animals, the number of long propriospinal neurons at C5 (Figure 3-3) with axons below the level of the lesion was significantly decreased on both the ipsilateral ($p=0.01$) and contralateral ($p=0.01$) side acutely following injury. This significant decrease persisted bilaterally at the chronic time point when compared to normal numbers ($p=0.03$ on ipsilateral, $p=0.04$ on contralateral). No significant changes were seen between the acute and chronic time points. Therefore, the population of long propriospinal neurons with axons below the lesion remained unaltered following injury.

Two populations of short propriospinal neurons were assessed; three segments above the lesion at T6 (Figure 3-4) and one segment above the lesion at T8 (Figure 3-4). In contrast to the long propriospinal neurons, both populations of short propriospinal neurons, exhibited marked post-injury changes in neuronal contribution below the lesion. Both populations of short propriospinal neurons showed significant bilateral decreases in labeled neurons at the acute time point (T6: $p=0.001$ on ipsilateral, $p=0.048$ on contralateral, T8: $p=0.001$ on ipsilateral, $p=0.034$ on contralateral) in comparison to uninjured animals. This was followed by a significant increase in the number of short propriospinal neurons with axons below the lesion site at the chronic time point. These increases were characterized bilaterally at both T6 ($p=0.014$ on ipsilateral, $p=0.035$ on contralateral) and T8 ($p=0.005$ on ipsilateral, $p=0.001$ on contralateral). At T6 there was no longer a significant difference on the contralateral side at the chronic time point compared to normal animals ($p=0.534$), but the left side remained significantly decreased from normal ($p=0.014$). Notably, contralateral T8 neuron numbers were significantly greater

than the same uninjured population (p=0.010) and ipsilateral numbers were no longer significantly different from normals (p=0.295).

Quantification of Rubrospinal Tract Neurons

The rubrospinal tract has been shown to be a primarily crossed tract (data not shown). Following injury, as expected, there was a significant decrease of neurons in the contralateral (axotomized) red nucleus in comparison to normal animals (Figure 3-5, p=0.006 at acute, p=0.004 at chronic). As mentioned, the rubrospinal tract is primarily a crossing tract (Figure 3-2). Thus, even though these numbers were significantly decreased from normal controls, the presence of neurons in the axotomized red nucleus suggests an increase in their post-injury contributions into the injection site. These post-injury changes remained unaltered between the two time points. Similar to the contralateral T8 neuron population, neuron numbers in the ipsilateral (non-axotomized) red nucleus were significantly greater than normal numbers at both the acute (p=0.004) and chronic time points (p=0.004). Thus, both axotomized and non-axotomized rubrospinal tract neurons undergo plastic changes in neuronal contribution below the lesion following injury.

Discussion

Following a low thoracic lateral hemisection in the cat there is substantial functional recovery of voluntary features of locomotion and the cough motor response (Tester and Howland, 2008; Jefferson et al., 2010). In this study we provide an understanding of neuronal populations that are contributing axons caudal to the lesion, potentially underlying recovery. The number of long propriospinal neurons whose axons run below the level of the lesion was permanently and significantly decreased post-injury. In contrast, populations of short propriospinal neurons, within 1-3 segments of the lesion, show substantial anatomical plasticity post-injury. There were significant bilateral increases in the number of short propriospinal

neurons between the acute and chronic time points. Interestingly, at the chronic time point, the contralateral population of short propriospinal neurons at T6 returned to normal. At T8 this population of neurons was significantly greater than the amount seen in uninjured cats. The rubrospinal tract showed a similar phenomenon. The ipsilateral or non-axotomized rubrospinal tract showed significant increases in neuron number over uninjured cats at both the acute and chronic time points. The number of neurons in the contralateral or axotomized red nucleus were permanently and significantly decreased. These data suggest that both injured and healthy substrates undergo substantial plasticity although not enough to support full functional recovery (Chapter 2).

Limitations of the Normal Propriospinal Neuron Populations

In this study, we assessed two groups of control animals, unilaterally (n=4) and bilaterally (n=3) injected at T11 (Figure 3-2). The unilateral injections were used to establish the population of neurons that normally project ipsilaterally or contralaterally to compare to early studies that were done using different tracing techniques. The bilateral injections were performed in order to establish baseline numbers to compare to the injured, bilaterally injected animals used in this study. In all instances the amount of ipsilateral and contralateral projections determined by the unilateral injections was similar to early reports in the literature. Long propriospinal neurons demonstrated more contralaterally projecting neurons versus ipsilateral (Matsushita et al., 1979; Skinner et al., 1979). Following bilateral injections the left and right side of the spinal cord had similar numbers, but they were less than double the number observed following unilateral injections. If they were double then each side of the spinal cord had an equal amount of projections. Since this was not the case, there must be a population of long propriospinal fibers that project bilaterally. Short propriospinal neurons had a similar amount of neurons that projected ipsilaterally and contralaterally (Rustioni et al., 1971; Molenaar and

Kuypers, 1978), but again the bilateral injections yielded numbers that were less than double the numbers that the ipsilateral injection yielded suggesting bilateral projections. In order to compare the normal group to the injured groups (n=6 or 7) we combined the unilateral and bilateral normal animals to equal an n of 7 (explained in results section). The method by which we did this did not take into account the bilaterally projecting neurons and most likely over-estimated the normal number of neurons in these populations. This over-estimation in number makes our chronic comparisons in the short PSN population with normal numbers even more impressive (Figure 3-4). These numbers on the contralateral side either equal or surpass that of normal controls. Using only animals with bilateral injections would likely decrease the normal population enhancing these differences even more. Incorporating two additional bilaterally injected normal control animals should be considered. This would address concerns regarding accuracy of normal numbers by removing the need to combine the data from unilaterally and bilaterally injected controls as currently done.

Intraspinal and Supraspinal Reorganization Through Sprouting and/or Regeneration of Injured and Healthy Neurons

Plasticity of multiple neuronal populations, both spinal and supraspinal, has been demonstrated in a variety of species (Murray and Goldberger, 1974; Aoki et al., 1986; Li et al., 1994; Weidner et al., 2001; Bareyre et al., 2004; Courtine et al., 2008; Fenrich and Rose, 2009). Previously, following a cervical spinal cord injury in the adult rat, axotomized RST neurons approached the rostral edge of the lesion, but none extended further (Houle and Jin, 2001). Because the RST primarily is a crossed pathway with very few if any ipsilateral fibers (Figure 3-2), the presence of neurons in the contralateral (axotomized) red nucleus at the acute and chronic time points suggests that some, albeit a small amount, of regeneration or sprouting of the axotomized tract is occurring.

This is the first study, to our knowledge, that has looked at the population of axotomized long propriospinal neurons that are contributing axons below the lesion. Previously, axotomized corticospinal tract fibers were shown to increase their number of contacts onto spared long propriospinal neurons following thoracic spinal cord injury and these long propriospinal neurons increased their terminal contacts onto motoneurons in the lumbar spinal cord (Bareyre et al., 2004). This study extended these observations by quantifying the number of long propriospinal neurons contributing axons below the level of a thoracic spinal cord injury over time in the cat model. There was a significant and permanent decrease in the number of contributing neurons suggesting that any anatomical changes happening in this population of neurons is occurring at the terminal ends, which could not be assessed with this tracing paradigm. These data also do not discount the possibility that these neurons may be forming a novel bypass circuit for descending input to the caudal spinal cord as has been reported in the rat (Bareyre et al., 2004).

Following a thoracic hemisection in the mouse spinal cord, the number of short propriospinal neurons in the thoracic spinal cord with axons below the level of the lesion acutely decreased, but increased over time reaching about 40% of the normal population (Courtine et al., 2008). Both populations of short propriospinal neurons observed in this report show an even greater potential for circuit amplification by surpassing in some cases the normal numbers by the chronic time point.

Incomplete Spinal Cord Injury Induces Recruitment of a New Subset of Neurons

The motor system is highly organized with a definitive hierarchy, containing parallel pathways controlling both sensory and motor function. Because of the hierarchical organization, it has been proposed that recovery through collateral sprouting also occurs in a hierarchical fashion. Competition to reinnervate the spinal cord below a lesion occurs due to proximity of the neurons and overlap of injured and healthy substrate (Goldberger and Murray, 1978; Goldberger,

1988). Early studies on collateral sprouting, demonstrated the ability of intact dorsal root afferents to sprout collaterals in the central nervous system into denervated areas of the spinal cord (Liu and Chambers, 1958). Thus far, reorganization of the non-axotomized rubrospinal tract following corticospinal tract transection has only been shown in rats treated with the Nogo-A neutralizing antibody, IN-1 (Raineteau et al., 2002). The present data illustrate the ability of the central nervous system to increase the number of non-axotomized RST neurons contributing axons below the level of an incomplete spinal cord injury when compared to the normal population by 2 weeks post-injury in the cat. This significant increase remains out to the chronic time point. Because the number of neurons surpasses that of the normal population, a novel subset of RST neurons is probably being recruited to sprout collaterals bridging the lesion. The most likely source of these collaterals is neurons that originally terminate above the T11 injection site (Figure 3-6). Following injury, these rostrally terminating neurons sprout collaterals through the injection site. A similar phenomenon was seen in the T8 short propriospinal neuron population on the contralateral side of the spinal cord. Again, there was a significant increase in neuron number when compared to normal control numbers. Even though the number of ipsilateral short propriospinal neurons did not surpass that of normal controls, the increase between the acute and chronic time points could be the result of sprouting of axons that normally terminated rostrally and/or regeneration of axotomized connections (Figure 3-6). The tracing paradigm used in this study does not distinguish between these possibilities. These data, however, strongly suggest that a novel substrate is being recruited to re-innervate the caudal spinal cord following incomplete spinal cord injury.

Implications of Remodeling on Functional Recovery

Following circuitry reorganization functional recovery of either treadmill stepping (Courtine et al., 2008) or hindlimb placing (Bareyre et al., 2004) in the rodent model occurred.

Thus, this study extended these observations by analyzing the contributions of different neuronal populations below an incomplete spinal cord injury in the cat that could subserve the partial recovery of voluntary features of locomotion, such as modifications of limb trajectory and accuracy of limb placement (Tester and Howland, 2008) as well as cough (Jefferson et al., 2010) seen in our prior studies. Since recovery of skilled locomotor tasks, such as obstacle, ladder and pegboard, progresses with increasing voluntary control over time it is likely that recovery is not solely controlled by commissural neurons that were spared by the hemisection, but by reorganization of supraspinal and intraspinal systems.

The recruitment of a healthy substrate from a different region of the spinal cord suggests that a shift in areas controlling different motor functions could be occurring similar to the shifting of the cortical motor map following stroke (Allred and Jones, 2008b) and spinal cord injury (Bareyre et al., 2004; Ghosh et al., 2009; Martinez et al., 2010). Following unilateral injuries, the ipsilateral sensory and motor cortices undergo a reorganization that coincides with the increasing compensatory activity of the unaffected side and the partial recovery on the affected side (Allred and Jones, 2008b; reviewed by Allred and Jones, 2008a; Ghosh et al., 2009). A shift in motor control also has been well illustrated in the somatotopically organized red nucleus following lesions to the corticospinal tract (Belhaj-Saif and Cheney, 2000). Dermatomes organize the sensory input of the spinal cord localizing each nerve to a certain area of the body. Although motor functions of the spinal cord have yet to be mapped out by segment it could be assumed that a motor map does exist. The data here suggests that the central nervous system can compensate for axotomized spinal neurons by sending axons from other regions into denervated areas, shifting motor control areas, and increasing functional recovery.

Implications of Locomotor Training on Remodeling

Substantial evidence suggests that locomotor training in both humans and animals following spinal cord injury improves lower extremity/hindlimb function (reviewed by Behrman et al., 2005; Thomas and Gorassini, 2005; Edgerton et al., 2008). Neural activity due to exercise restores normal levels of neurotrophic factors caudal to the lesion (Ying et al., 2005). Following a thoracic dorsal hemisection in the rat the CST increased its connections with both long and short PSNs in the cervical spinal cord. The connections made onto the long PSNs that actively re-innervated the caudal spinal cord persisted throughout the study where as the connections made onto short PSNs that did not bridge the lesion were lost suggesting an activity dependent selection and maintenance occurring (Bareyre et al., 2004). All animals in the present study were trained 5 times a week starting 24-48 hours following injury on a variety of locomotor tasks. The potential increase in neurotrophins from the locomotor training given to these animals most likely enhanced their functional recovery and mediated the plastic changes in the studied neuronal populations to some extent.

Conclusions

The results of this study in combination with our previous behavioral recovery studies (Tester and Howland, 2008; Jefferson et al., 2010) (Chapter 2) suggest that functional recovery is likely mediated by the plasticity of multiple pathways. Here we show that the short propriospinal neurons and rubrospinal tract neurons increase their neuronal contributions below the level of the injury. Not only do they increase their contribution of axotomized axons, but significant increases in the number of healthy axons suggest recruitment of a novel substrate that was not present at the injection site pre-injury. The most likely source of these contributing axons are neurons that normally terminate more rostrally to the injection site, but now sprout collaterals past the lesion and below/into the injection site. In future studies it will be important

to definitively determine the source of these axons and whether locomotor training plays a substantial role in the observed plasticity. Understanding the intrinsic plasticity of spinal and supraspinal systems is critical to the development of therapeutic interventions that capitalize on plasticity and identifying potential target neuronal populations for these therapies.

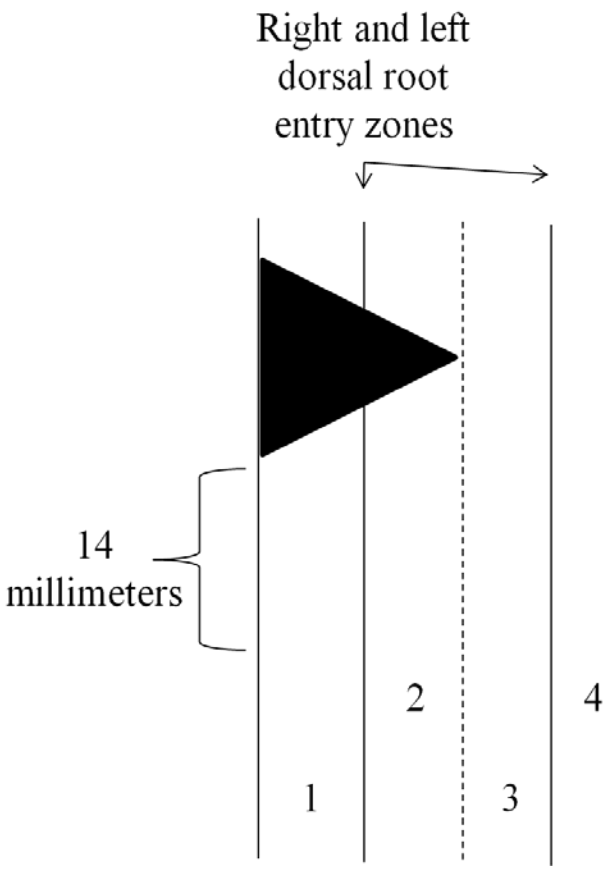


Figure 3-1. Diagram of the injury and Fluoro-Gold injection site paradigm. The hemisection injury was made at thoracic level (T)9/T10 and injections were made ~14 millimeters below the lesion. The numbers represent the sites of the needle tracks.

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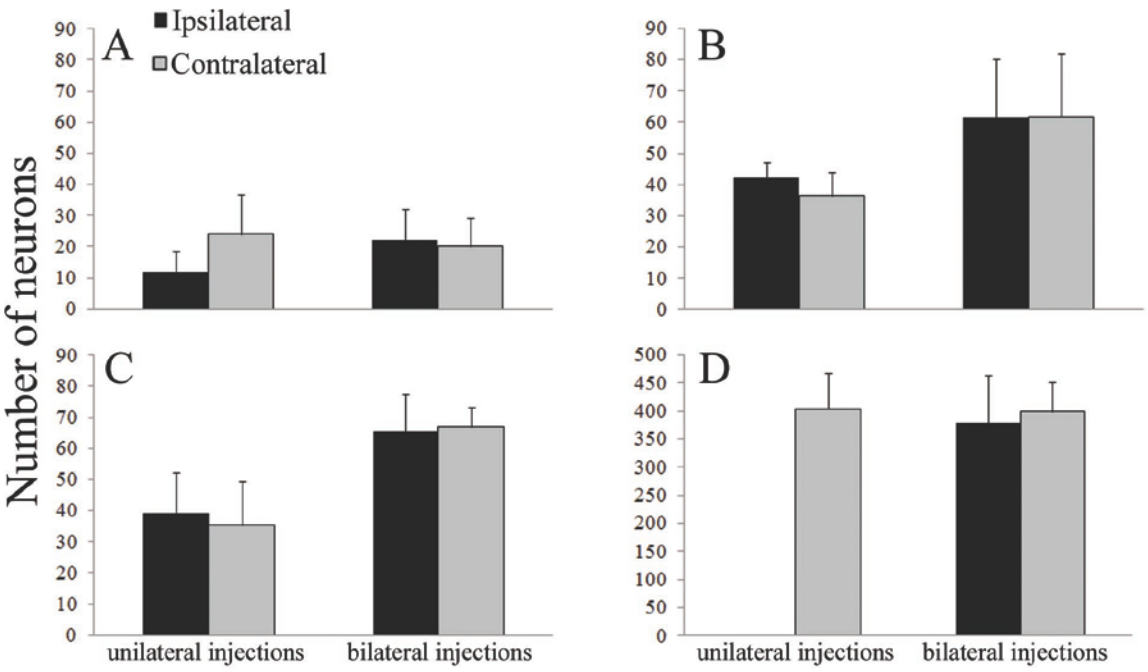

Figure 3-2. Ipsilateral and contralateral projections in the normal spinal cord. Unilateral injections yielded more long propriospinal neurons on the contralateral side than ipsilateral, whereas bilateral injections yielded an equal number on both sides (A). Short propriospinal neurons at both T6 (B) and T8 (C) have an equal number of neurons that project contralaterally and ipsilaterally. In both cases bilateral injections yielded more neurons. Following the unilateral injection labeled neurons were only seen in the contralateral red nucleus (D). Bilateral injections labeled neurons in both red nuclei equal in number to the unilaterally injected red nucleus. Error bars denote SD.

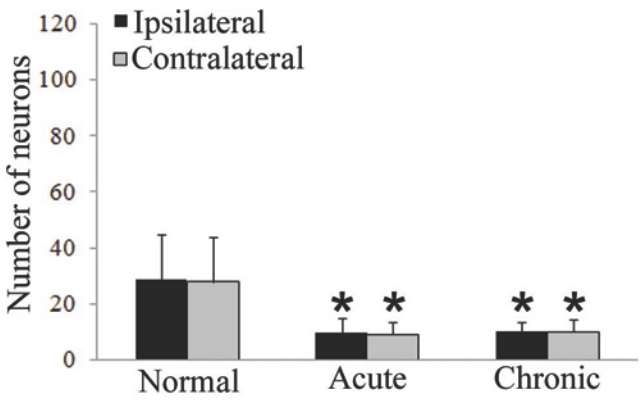

Figure 3-3. Average number of retrogradely labeled ipsilateral and contralateral long propriospinal neurons at cervical (C) level 5. Following injury, the number of the long propriospinal neurons significantly decreases (*) bilaterally at both acute and chronic time points from normal numbers. Error bars denote SD.

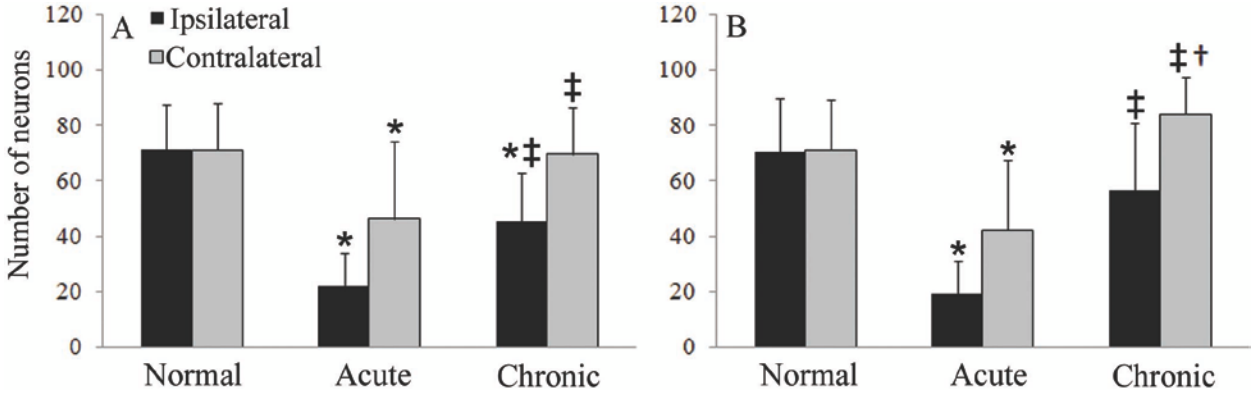

Figure 3-4. Average number of retrogradely labeled ipsilateral and contralateral short propriospinal neurons. Short propriospinal neurons were assessed at T6 (A) and T8 (B). T6 short propriospinal neurons decrease significantly on both sides of the spinal cord (*) acutely, but chronically there were bilateral significant increases (‡) from the acute time point. T8 short propriospinal neurons also had significant bilateral decreases (*) acutely with significant bilateral increases (‡) from the acute time point. The right side chronic numbers were significantly greater than normal numbers (†). Error bars denote SD.

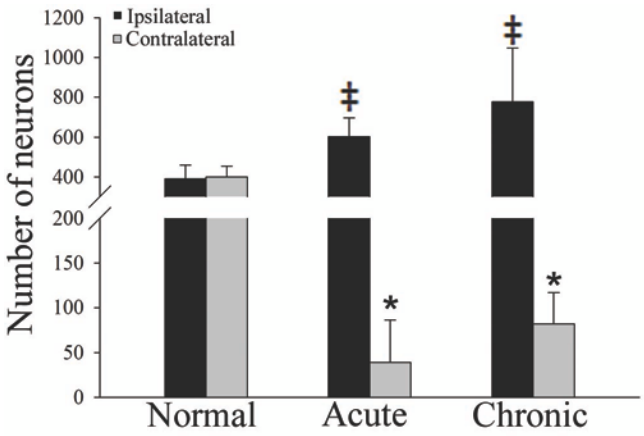

Figure 3-5. Average number of retrogradely labeled ipsilateral and contralateral rubrospinal tract neurons. The left (non-axotomized) red nucleus had significantly more (‡) neurons at both time points than normal neuronal numbers. The right (axotomized) red nucleus had significantly less (*) neurons at both time points when compared to normal numbers. Error bars denote SD.

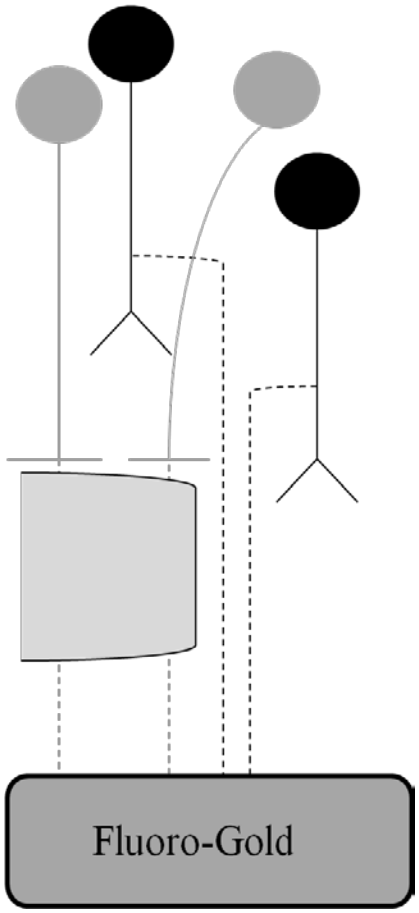

Figure 3-6. Proposed anatomical changes following injury. Increases in the number of contributing neurons at the chronic time points can occur by at least 2 potential mechanisms. The first is regeneration of neurons that were originally labeled (gray), axotomized and regenerated bridging the lesion to the injection site. The second mechanism is recruitment of a new, healthy subset of neurons (black). These neurons have cell bodies at the characterized levels that were not originally labeled because their termination sites were more rostral to the injection site. Following injury, they extended axons which bridged the lesion into the injection site.

CHAPTER 4
SCAR ASSOCIATED MOLECULES IN AREAS DISTANT FROM A SPINAL CORD INJURY IN THE CAT

Introduction

Spinal cord injury (SCI) results in motor and sensory deficits below the level of the injury. In order for functional recovery to occur, axons must bridge the lesion site. Following traumatic injury to the central nervous system (CNS), extracellular matrix molecules are rapidly upregulated in areas surrounding the lesion producing a very dense, fibrotic, glial scar (reviewed by Silver and Miller, 2004; Yiu and He, 2006). The breakdown of the blood-brain-barrier (BBB) causes infiltration of inflammation associated molecules, such as cytokines and macrophages (Fitch and Silver, 1997; Rhodes et al., 2006). The inflammatory response triggers a cascade including the hypertrophy of astrocytes and upregulation of oligodendrocyte progenitor cells (OPCs). These glial cells are implicated in the production and increase of chondroitin sulfate proteoglycans (CSPGs) following injury to the CNS (Lemons et al., 1999; Jones et al., 2002; Jones et al., 2003; Tang et al., 2003). CSPGs are the main impediment to spontaneous axonal regeneration in the scar matrix (McKeon et al., 1991; Dou and Levine, 1994; Davies et al., 1999). In the absence of CPSGs, there is some suggestion that both axotomized and intact axons are capable of long distance regeneration through areas once rich in CSPGs (Moon et al., 2001; Bradbury et al., 2002; Corvetti and Rossi, 2005).

Although much of the aforementioned work described the inflammatory response surrounding the lesion site, injury associated markers are not isolated to this area. Microglial activation and oligodendrocyte apoptosis were observed several millimeters away from the lesion as well as in the area of denervated targets (Koshinaga and Whittemore, 1995; Crowe et al., 1997; Liu et al., 1997; Shuman et al., 1997). Recently, Massey and colleagues (2008) demonstrated the presence of specific CSPGs in denervated dorsal column nuclei following

cervical spinal cord injury. These increases in CSPGs were correlated with activated glial cells and astrocytes in an area distant from direct trauma (Massey et al., 2008). Digestion of the CSPGs in the nuclei increased collateral sprouting of spared axons as well as promoted re-innervation by microtransplanted dorsal root ganglion cells (Massey et al., 2006; Massey et al., 2008). Thus it seems likely that the presence of CPSGs away from the lesion also plays a role in inhibiting axonal growth and thus decreasing the potential for functional recovery.

Even though the presence of CSPGs and glial activation has been characterized at the lesion and in denervated target nuclei little has been characterized in between these two sites. In this preliminary study, which uses 9 cats (n=3/time point) shared with studies in Chapter 3, we demonstrate using immunohistochemical techniques, that areas undergoing Wallerian degeneration far rostral and caudal from a spinal cord injury in the cat are characterized by the presence of activated glial cells and increases in levels of CSPGs. This raises the importance of considering the environment of the entire spinal cord when attempting to promote reconnectivity following spinal cord injury.

Methods

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. Nine purpose bred, SPF, female, adult cats were used. These animals also were part of a tract tracing study in which Fluoro-Gold injections were made at T11 (see Chapter 3). One representation section was chosen for the figures for each stain.

Surgical Procedures

All cats received a left spinal T9/T10 hemisection as described in our previous study (Tester and Howland, 2008; Jefferson et al., 2010). In summary, animals were anesthetized in a gaseous chamber with an isoflurane and oxygen mixture (2-5% isoflurane, 2 LO_2), then

intubated and maintained at a surgical plane of anesthesia with isoflurane (2-3% isoflurane) throughout the surgery.

The spinal cord was exposed by a bilateral laminectomy and a left lateral hemisection was made at approximately the spinal T10 level using iridectomy scissors. Light suction with pulled glass pipettes was used to gently lift remaining fibers so that they could be cut. Dura was sutured closed. Durafilm (Codman-Shurtleff, Inc., Randolf, MA) and gelfoam (Pharmacian and Upjohn, Inc., Peakpack, NJ) were placed on top of the dura and the muscle and skin were sutured in layers. Buprenorphine (0.01 mg/kg, SQ) was given every 6-12 hours for the first 48 hours after the surgery.

Procedures used to maintain the general health of the animals in this study were similar to those used in our other study (Tester and Howland, 2008; Jefferson et al., 2010). Cats were housed singly or in pairs on thick beds of shredded newspaper or egg crate foam. Bladders were emptied using the Crede maneuver for the first few days following the hemisection injury. Cats' food intake, weight and behavior were monitored closely throughout the remainder of the study.

Tract Tracing

A tract tracer was placed in the caudal spinal cord of these cats at the time of injury or 16 weeks post-injury. These methods and results are reported in Chapter 3.

Histology

Two or 16 weeks post-hemisection, cats were anesthetized with an overdose of sodium pentobarbital (>40 mg/kg, intraperitoneal (IP)) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Cats without hemisections, which also were part of a tract tracing study, were perfused for use as controls. The spinal cords were dissected and blocked into segments. The blocks were cryoprotected in a mixture of 30% sucrose in 4% paraformaldehyde (pH 7.4). The cryoprotected C3, T8, L3, and

lesion segments were frozen and cut at 25μm thickness on a cryostat. Sections were processed using monoclonal glial fibrillary acidic protein (GFAP, 1:3200; Sigma), phosphorylated-neurofilament heavy chain (pNF-H, 1:10,000; courtesy of Dr. Gerry Shaw), monoclonal anti-oligodendrocyte (1:1000; Chemicon, Temecula, CA), and polyclonal coronin-1a (1:100; courtesy of Dr. Gerry Shaw). Briefly, tissue was rinsed in 1% goat serum in phosphate buffered saline containing 0.4% Triton (PBS-T), blocked with 10% goat serum in PBS -T for 1 hour at room temperature, and incubated with the primary antibody overnight at 4^{o}C in serum PBS-T. The next day, the tissue sections were rinsed with 1% serum PBS-T before and after a 1 hour incubation with Alexa Fluoro 488 (1:400; Molecular Probes, Eugene, OR) or 594 (1:1000; Molecular Probes) secondary antibodies. Sections were mounted onto charged slides and coverslipped using ProLong Anti-Fade Kit (Molecular Probes).

In some cases biotinylated secondary antibodies (goat anti-mouse or anti-rabbit; Vector Laboratories, Inc., Burlingame CA) were applied for 1 hour at room temperature followed by a 2 hour incubation with ABC reagent (Vectorstain ABC kit; Vector Laboratories). The antibody complex was visualized with 3,3,8-diaminobenzidine (DAB; Sigma). In all cases, controls were run without incubation with primary antibody to confirm the specificity of the antibody labeling. Following the microglia, astrocyte, and oligodendrocyte staining, some sections were counterstained with cresyl violet. Sections were mounted on chrom-alum (chromium potassium sulfate; Sigma) and poly-L-lysine (Sigma) coated slides and then dehydrated through increasing alcohols, xylene, and coverslipped with DPX (Sigma). The protocol for the monoclonal anti-chondroitin-6-sulfate (C6SPG, 1:1000; MP Biomedicals, Irvine, CA) antibody was used as previously reported in detail by Tester and Howland, 2008. In order to visualize degenerating fibers the FD NeuroSilver Kit was used (FD NeuroTechnologies, Baltimore, MD). Some

sections were mounted directly onto subbed slides and processed with cresyl violet (cresyl violet with acetate, Sigma) and myelin (Eriochrome Cyanine R, Sigma) stains to view basic morphology.

Stained sections were visualized using the Zeiss AXIO Imager.D1/Z1 (Carl Zeiss Microimaging Inc., Thornwood, NY). Representative photographs were taken with either the high resolution microscopy camera AxioCam HRc or MRm (Zeiss) using the AxioVision Rel. 4.7 software (Zeiss).

Results

Lesion Epicenter

Cats that received lateral hemisections were sacrificed at two different time points following injury, 2 weeks (acute) and 16 weeks (chronic) post-injury. These hemisections ranged from over or under hemisections to complete hemisections. In all cases Wallerian degeneration (WD) was seen in areas distant from the lesion. The ensuing scar was characterized in order to determine a baseline in changes that are typically seen following this type of injury before comparing these changes to distant areas rostral and caudal segments.

The extent of the lesion first was assessed using a combination of cresyl violet and myelin stains (Figure 4-1). Demyelination of the injured side of the spinal cord was seen within the first 2 weeks of injury and remained demyelinated out to 16 weeks post-injury.

To assess the time course of WD, the axonal marker phosphorylated neurofilament heavy chain (pNF-h) was used (Figure 4-1). pNF-h staining on the spared side of the lesion was distributed evenly across the white matter at both time points. In contrast, the 2 week scar was characterized by a decrease in pNF-h staining and a loss in the normal pattern of staining. By 16 weeks, fewer axonal profiles were present.

Glial fibrillary acidic protein (GFAP) immunoreactivity was used to determine the fate of astrocytes (Figure 4-1). Within the first 2 weeks following injury, an increase in GFAP staining was seen in the scar area bordering spared tissue. Increased GFAP staining was distributed throughout the entire scar by 16 weeks. On the spared side, low levels of GFAP were seen at both time points with no obvious changes in staining intensity between the two time points. Along with the presence of reactive astrocytes, the presence of chondroitin sulfate proteoglycans (CSPGs) also was characterized. Increased CSPG presence was seen in the scar at both 2 and 16 weeks with little to no increase in the spared area of the spinal cord. Although increases were not seen in these particular sections the tract tracer could have caused increases in the spared tissue in other sections of the lesion.

The changes in glial cell morphology also were assessed using an actin binding protein, coronin-1a, as a marker for microglia (Ahmed et al., 2007) and an anti-oligodendrocyte antibody, which stains both mature and early oligodendrocytes and their myelin sheaths (Figure 4-1). Normal microglia are characterized by the presence of ramified processes whereas activated microglia have shortened processes and large amoeboid like soma. In the 2 week scar many microglia presented with round or amoeboid somas suggesting that they are in their activated state. This was not seen in the 16 week scar or on the spared side of the spinal cord. These microglia showed a normal morphology with ramified processes. Oligodendrocytes have been shown to undergo apoptosis following spinal cord injury (Crowe et al., 1997). These sections also were counterstained with cresyl violet (CV) in order to quickly assess the number of stained oligodendrocytes. Few oligodendrocytes were seen in the scar at the acute time point, but by the chronic time point normal oligodendrocyte morphology.

Rostral Dorsal Columns

A large amount of fibers in the dorsal column (DC) ipsilateral and rostral to the thoracic T10 spinal hemisection undergo WD. The DCs at C3 were characterized to determine if there was a difference in the presence of scar associated markers that was dependent on direct trauma versus distant degeneration.

At 2 and 16 weeks post-injury, most of the pNF-h staining relative to controls was diminished in the ipsilateral dorsal column, with a slight decrease in staining also observed contralateral to the injury (Figure 4-2). At the lesion epicenter there was an almost complete loss of pNF-h staining in the DCs. A complementary silver stain was employed to determine the timing of the degenerative processes underlying the loss of phosphorylated neurofilaments (Figure 4-2). At 2 weeks many of the fibers in the left DC were positively stained with silver and therefore degenerating. By 16 weeks post-injury, silver staining was absent leaving an area void of any fibers as illustrated with pNF-h staining.

The presence of myelin was examined (Figure 4-2). Similar to the pNF-h stain, myelin was evenly distributed throughout the white matter of the C3 spinal cord in the controls. Interestingly, 2 weeks post-injury showed no change in the density of myelin staining in the DC areas that lacked pNF-h staining. Even with the presence of myelin, the absence of pNF-h staining showed that viable axons were no longer present. By 16 weeks post-injury, myelin staining was absent in the ipsilateral DC indicating that clearance of myelin debris had occurred. This indicates that myelin removal distant to the lesion is delayed compared to the lesion epicenter where no myelin was seen at 2 weeks post-injury.

The microglial population and morphological changes were assessed (Figure 4-2). In the control and 2 week tissue, a small amount of coronin-1a staining was observed throughout the DCs. In contrast, the 16 week tissue had an increased amount of coronin-1a staining. When

counterstained with the nuclear marker, cresyl violet, it was evident that the increase in staining corresponded with a large increase in the amount of microglia present due to the overlap of the 2 stains. When looked at more closely, the control tissue showed many quiescent microglia throughout the dorsal columns, but no activated profiles. By 2 weeks there was a slight change in the soma shape, but larger overall changes were not seen until 16 weeks. At 16 weeks many soma were elongated again showing that these changes occurred after those seen at the lesion epicenter.

Next, the fate of astrocytes and oligodendrocytes was determined (Figure 4-3). The DCs did not show a large increase in GFAP expression until 16 weeks post-injury. In normal white matter, oligodendrocytes were sparse and evenly spaced through the observed region. Little to no staining was seen in the 2 week tissue suggesting that apoptosis of these cells occurred. In contrast, the 16 week tissue showed an increase in the amount of anti-oligodendrocyte staining. Also, present was an increase in the overlap of cresyl violet and oligodendrocyte staining indicating an increase in the number of oligodendrocytes present. Since astrocytes and oligodendrocyte precursor cells are the 2 main producers of CSPGs following injury, CSPG expression was examined next. The pattern of CSPG expression was a little different from the pattern of increased GFAP and oligodendrocyte immunoreactivity. Increases in staining were seen at 2 weeks and remained out to 16 weeks.

Changes in morphology also were seen in these glial cells (Figure 4-3). Astrocytes are seen in the control DCs evenly spaced out with long processes illustrating the expected stellate morphology. Although, there was no general increase in GFAP staining at 2 weeks, slight morphological changes can be seen. Astrocyte distribution is no longer evenly spaced out and shorter processes are seen. By 16 weeks, there is a large increase in GFAP staining in the

processes suggestive of a reactive state. Mature oligodendroctyes were present in the control spinal cord. They were evenly spaced throughout the white matter and were highly branched. Very few oligodendrocytes, if any, were present in the 2 week post-injury spinal cord. Instead, oligodendrocyte precursor cells were present in abundance 16 weeks. Because the antibody used stains both mature and developing oligodendrocytes, morphology was used to define the stage of the oligodendrocytes present the different time points. First, there was an increase in cells, as seen with the cresyl violet stain overlap. Instead of highly branched mature oligodendrocytes that were seen in the control tissue, these developing oligodendrocytes show large cell bodies and very few branching processes.

Dorsolateral Region Rostral and Caudal to the Lesion

Another region of the spinal cord was chosen due to the heterogeneity of fibers present. The dorsolateral region of the spinal is a mix of both ascending and descending fibers thus this area has both intact fibers and fibers undergoing Wallerian degeneration. The dorsolateral region was assessed both rostral (C3) and caudal (L3) to the spinal (Figure 4-4). The time course of decreases and increases of staining as well as the morphological changes that were seen in the dorsal columns was similar to what was seen in this region. Changes in this region were not as drastic or widespread as the dorsal columns.

Discussion

The intent of this preliminary study was to qualitatively compare major glial and axonal responses in areas of Wallerian degeneration following a low thoracic hemisection in the cat model. Comparisons were made at two post-injury time points, acute and chronic, as well as to tissue from cats without hemisections. Although, the cellular responses leading to the establishment of the fibrotic glial scar have been well characterized at sites of direct trauma, this study also assessed changes seen in areas distant to the lesion. In these distant areas an increase

in scar associated molecules was seen at both post-injury time points. In general, there was a delay in the upregulation and morphological changes that occurred in areas distant from the lesion epicenter when compared to changes at the lesion site. However, the cats without hemisections showed staining above anticipated baseline levels for some of these markers (e.g. CSPG, Figure 4-3) suggesting that the tracers used in these cats for another study confounded the findings. Thus, this must be considered when interpreting the results from this preliminary work.

The Distant Environment Following Spinal Cord Injury

Following injury to the central nervous system axons distal to the injury are broken down in a proximal to distal fashion beginning at the lesion epicenter and spreading along the degenerating pathway (George and Griffin, 1994). We examined axonal profiles in the dorsal columns as well as the dorsolateral white matter and found a large decrease in pNF-h staining in both areas by 2 weeks post-injury, which remained out to the chronic time point. Although the fiber staining was still decreased from control tissue there was more staining in the dorsolateral regions of both C3 and L3 when compared to the dorsal columns. Full demyelination of the areas undergoing Wallerian degeneration did not occur until after the 2 week time point suggesting that myelin clearance is much slower than axonal degeneration illustrated by the large amount of silver stained fibers acutely following injury. The timing of this process is very similar to the timing that has been previously described in the monkey (Shi et al., 2009), where as in the rat these processes happen much faster (Wang et al., 2009).

This delayed myelin breakdown in the degenerating areas may be inhibitory to axonal growth because of the long lasting presence of inhibitory myelin associated proteins and may contribute to the maintenance of an inhibitory environment (Buss and Schwab, 2003; Buss et al., 2005). Previous studies have shown that areas undergoing active Wallerian degeneration may not be as inhibitory as originally postulated. Microtransplanted adult sensory neurons were

capable of long distance regeneration along degenerating white matter tracts (Davies et al., 1999), although it is not known whether native neurons to this area show the same results. The findings from the current preliminary work add to this by showing that GFAP immunoreactivity and, in some areas, CSPG levels are likely to be relatively low in areas of WD for multiple weeks post-injury. Further, a study using the Wallerian degeneration slow (WLD^s) mice suggests that the delay in the start and slower rate of Wallerian degeneration causes a slower rate of functional recovery when compared to wildtype mice (Zhang et al., 1998). These studies imply that the cellular processes of Wallerian degeneration have some sort of interaction or correlation with processes that support functional recovery. Thus, studies using microtransplantation and WLD^s suggest that there is some potential for growth in areas undergoing Wallerian degeneration and that it may be the scar-related molecules and not myelin inhibitory molecules that are the most inhibitory.

Delayed Increases in Inhibitory Scar Associated Molecules

Inhibitory molecules such as chondroitin sulfate proteoglycans are upregulated in areas of gliosis following traumatic injury to the central nervous system. This response is typically enhanced in areas of blood brain barrier breakdown, but evidence also suggests that degeneration of severed axons leads to gliosis in areas distant from the lesion (Barrett et al., 1981; Fitch and Silver, 1997; Massey et al., 2006). Specifically, increases in CSPGs were observed in the perineuronal nets and white matter of target nuclei that were denervated by cervical spinal cord injury (Massey et al., 2006; Massey et al., 2008). These dramatic increases in CSPGs in the dorsal column nuclei occurred within 2 days post-spinal cord injury and remained increased up to 3 weeks (Massey et al., 2008). Chondroitinase ABC (Ch'ABC) was injected into the denervated dorsal column nuclei resulting in functional collateral sprouting. Thus, in areas remote from the lesion that are undergoing gliosis CSPGs play an inhibitory role in axonal

regeneration, possibly allowing for surviving synaptic connections to increase their recovery potential and/or minimizing the formation of aberrant pathways by creating a border around the denervated area. In contrast to the rodent model, the data presented here shows a differential time line of expression of GFAP positive astrocytes. Increases in GFAP staining occurred between the 2 and 16 week time points in areas denervated by the thoracic hemisection independent of distance from the lesion. Furthermore, morphological changes in astrocytes began within the first two weeks, but were not widespread until the chronic time point. Similarly, oligodendrocyte progenitor cells were not observed in the tissue until the16 week time point. Interestingly, the increase in CSPGs occurred much sooner in these distant areas. This could be due to other cells, that were not characterized, which also can produce CSPGs such as fibroblasts (Suwan et al., 2009). Based upon the higher than anticipated levels of CSPG in the dorsal columns of the controls animals, it also may be due to the tracer used in these cats for another study. This must be clarified in future work. However, increases in GFAP-immunoreactivity associated with reactive astrocytes in areas undergoing Wallerian degeneration in cat tissue is delayed when compared to results reported with rodent tissue. This may indicate an extended window of opportunity for axonal growth in the cat.

Implications for the Timing and Location of Therapeutic Interventions

An important issue raised by this work is the timing and location of therapeutic interventions that reduce the inhibition of scar associated molecules. One such treatment is Ch'ABC that cleaves the GAG chains on CSPGs rendering the environment more permissive to axonal regeneration. Increases in CSPGs and GFAP positive astrocytes occur within the first two weeks at the lesion epicenter (Figure 4-1) (Lemons et al., 1999; Jones et al., 2003). Traditionally, studies that look at the efficacy of therapeutic interventions assess their effectiveness at the lesion epicenter only. Our lab in particular showed that intraspinal Ch'ABC,

into the lesion immediately following a hemisection injury in the cat increases functional recovery compared to control-treated animals (Tester and Howland, 2008). Another study showed that treatment starting 7 days post-injury was equally effective on basic stepping, but not as effective as immediate treatment on a skilled reaching task (Garcia-Alias et al., 2008). Even though functional recovery does occur, in all cases it was not complete (Caggiano et al., 2005; Barritt et al., 2006; Tester and Howland, 2008). These data together suggest that treating other areas along the length of the spinal cord in combination with the lesion epicenter could help promote long distance regeneration enhancing functional recovery.

Conclusions

In summary, this preliminary study described changes of major cellular and axonal components in areas of Wallerian degeneration following spinal cord injury in the cat. These distant areas also are characterized by the presence of increased scar associated molecules and the increase in some of these markers is delayed when compared to the lesion epicenter. The delay in myelin breakdown and clearance, the lack of early increases in GFAP positive astrocytes is more similar to that described in the monkey (Shi et al., 2009) and human (Buss et al., 2004; Buss et al., 2005) than the rat (Buss and Schwab, 2003; Wang et al., 2009). Understanding the changes in these scar associated molecules distal to a spinal cord injury is likely to provide important insights into the target areas and timing of novel therapeutic strategies aimed at enhancing long distance growth and functional recovery.

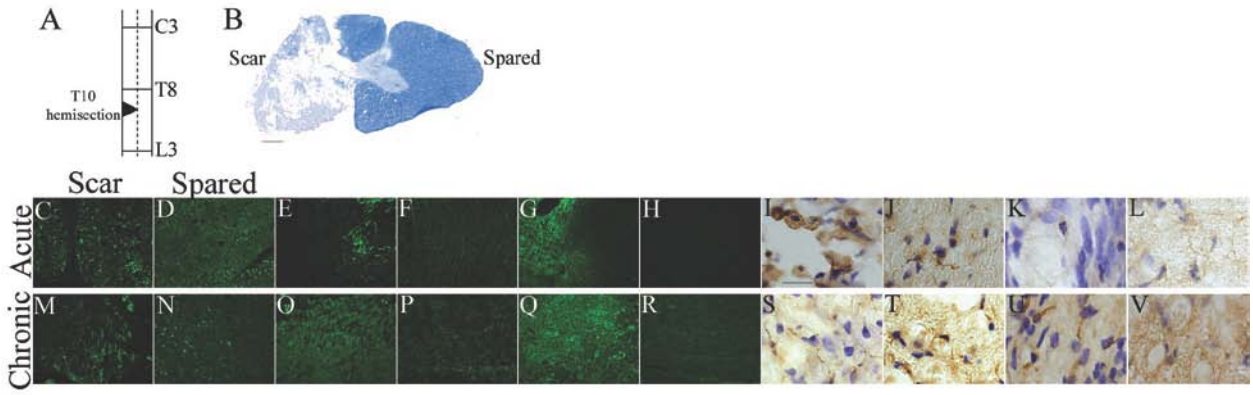

Figure 4-1. Lesion characterization. Following a left thoracic (T) 10 lesion, the cervical (C) 3, T8 and lumbar (L) 3 segments were examined (A). This cresyl violet and myelin (CVM) stain characterized the extent of the lesion (B, scale bar=500 microns (μ)). The injured (scar) and spared side of the spinal cord were compared at acute and chronic time points. The following antibodies were used phosphorylated neurofilament heavy chain (pNF-h, C, D, M, N), glial fibrillary acidic protein (GFAP, E, F, O, P), chondroitin-6-sulfate proteoglycan (C6SPG, G, H, Q, R), coronin-1a (I, J, S, T), and anti-oligodendrocyte (K, L, U, V). Scale bar for fluorescent pictures equals 100μ. Scale bar for brightfield pictures equals 20μ.

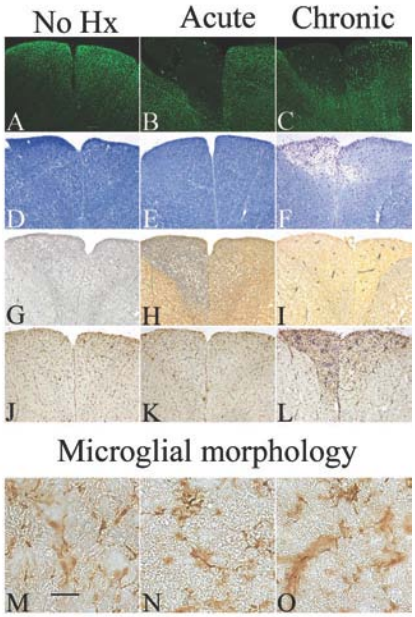

Figure 4-2. Rostral dorsal columns. The dorsal columns (DCs) far rostral from the lesion were characterized in animals without hemisections and in acutely and chronically hemisected animals. The following antibodies were used pNF-h (A-C), CVM (D-F), silver degeneration stain (G-I), and coronin-1a (J-L) (scale bar=100μ). The ipsilateral DC showed the most changes in morphology following injury. Acutely, there were decreases in pNF-h (B) and the silver degeneration stain (H). Myelin staining (F) decreased at the chronic time point when there was an increase in microglial straining (L). The microglial morphology was assessed at 63x magnification (scale bar=20μ) using coronin-1a (M-O). Morphological changes began at the acute time point (N), but increased greatly at the chronic time point (O). No hx=no hemisection.

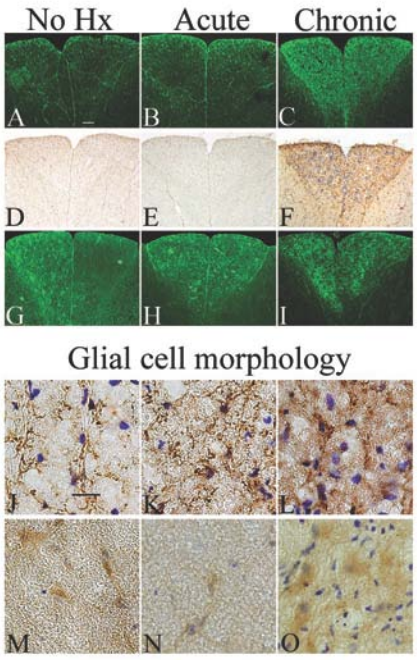

Figure 4-3. Characterization of scar associated molecules in the rostral dorsal columns. The following antibodies were used GFAP (A-C), anti-oligodendrocyte (D-F), and C6SPG (G-I) Scale bar=100μ. 63x magnification (scale bar=20μ) assessed the changes in cell morphology of astrocytes (J-L) and oligodendrocytes (M-O). Wide spread changes in scar associated molecules were not seen until the chronic time point.

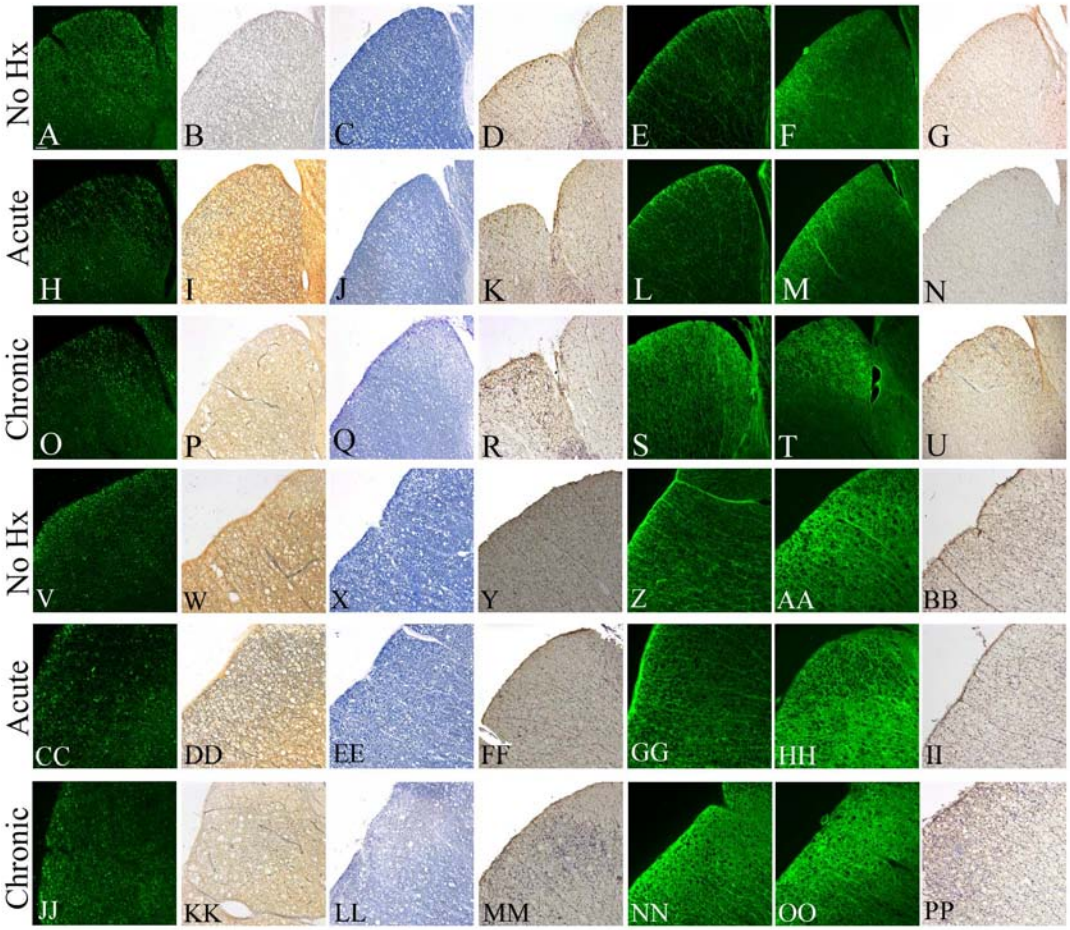

Figure 4-4. The characterization of the dorsolateral funiculus at the third cervical and third lumbar levels. The presence of scar associated molecules also were assessed in the dorsolateral regions of both C3 (A-U) and L3 (V-PP). The following antibodies or stains were used pNF-h (A, H, O, V, CC, JJ), silver degeneration (B, I, P, W, DD, KK), CVM (C, J, Q, X, EE, LL), coronin-1a (D, K, R,Y, FF, MM), GFAP (E, L, S, Z, GG, NN), C6SPG (F, M, T, AA, HH, OO), and anti-oligodendrocyte (G, N, U, BB, II, PP). The changes in immunoreactivity observed in the dorsolateral areas were similar to those seen in the dorsal columns, but to a lesser degree and they encompass a smaller area. Scale bar=100μ.

CHAPTER 5
SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

The majority of human spinal cord injuries are incomplete and may be midline or lateralized. One example of a lateralized injury is Brown-Sequard syndrome, which is can be modeled in experimental animal models as the lateral hemisection. Following a lateralized lesion in both humans (Little and Halar, 1985; Koehler and Endtz, 1986; Roth et al., 1991) and cats (Helgren and Goldberger, 1993; Tester and Howland, 2008), there is substantial, but incomplete locomotor recovery of basic and voluntary features. Recovery of voluntary locomotor features following spinal cord injury requires information flow across the lesion site. Following injury to the central nervous system (CNS), axons have a minimal capacity for regenerative growth due to the presence of the glial scar, demyelination, and the upregulation of inhibitory extracellular matrix molecules such as CSPGs. The formation of the glial scar has been well characterized in the area of and adjacent to the lesion (reviewed by Silver and Miller, 2004), but recently the upregulation of CSPGs at the denervated target nuclei show that scar associated molecules are located away from direct trauma sites (Massey et al., 2006; Massey et al., 2008).

The cat is an advantageous translational model in which to study the effects of spinal cord injury on motor systems such as locomotion. The benefits of this model include well characterized locomotor circuitry associated with both simple stepping and more complicated skilled tasks, the larger size of the spinal cord and its prior use as a translational model following SCI (reviewed by Hodgson et al., 1994; Behrman and Harkema, 2000). Previous work in our lab (Tester and Howland, 2008; Jefferson et al., 2010) and others (Eidelberg et al., 1986; Helgren and Goldberger, 1993; Basso et al., 1994) have described functional recovery following a low thoracic hemisection in the cat model. Much of the locomotor recovery occurred during basic

stepping and flat overground locomotion, but when cats were placed on tasks that required accurate limb placement and trajectory modifications deficits persisted out to chronic time points (Helgren and Goldberger, 1993; Tester and Howland, 2008). My studies further assessed the behavioral recovery following a low thoracic hemisection during a task that requires both intraspinal and supraspinal control, obstacle negotiation. This is the first study to characterize the recovery of obstacle negotiation using the hemisection lesion paradigm. Although all cats recovered the ability to inconsistently clear the obstacle without paw contact, limb efficiency and stability never fully recovered. Due to the incomplete recovery and in depth characterization that were performed, the results presented indicate that obstacle negotiation is an ideal task to test the efficacy of therapeutic interventions targeted at improving adaptive motor control following incomplete spinal cord injury .

All of the cats in these studies were extensively trained on a variety of tasks beginning 24-48 hours following injury. One of the most promising approaches to the treatment of spinal cord injury in the human population is the use of body weight supported treadmill training and overground locomotor training (Harkema et al., 1997; Behrman et al., 2005; Thomas and Gorassini, 2005; Norton and Gorassini, 2006). Locomotor training following spinal cord injury increases neurotrophic factors known to enhance synaptic plasticity (Gomez-Pinilla et al., 2002; Vaynman and Gomez-Pinilla, 2005). Although, exercise was beneficial in these studies, the best timing for initiation of training following CNS injury is still being debated. Early initiation of training, compared to initiation at a chronic stage, has been shown to be more effective in enhancing locomotor output following spinal cord injury (De Leon et al., 1998; Rossignol et al., 2002; Dupont-Versteegden et al., 2004; Boyce et al., 2007; Engesser-Cesar et al., 2007). However others report that initiating training acutely following traumatic brain injury (Griesbach

et al., 2004a; Griesbach et al., 2004b) and spinal cord injury (Smith et al., 2009) interferes with locomotor recovery and increased growth factor expression, as well as exacerbates the extent of the lesion. Although it is doubtful, it is unknown whether the same functional recovery would have been achieved in the current study without training or delayed training. It will be important in future studies to begin to understand the role of training and if the timing of training initiation is critical to the recovery reported in obstacle negotiation.

The partial functional recovery characterized during the obstacle negotiation task led to assessment of potential underlying mechanisms. Anatomical assessment showed that the number of short propriospinal neurons as well as rubrospinal tract neurons that contribute axons below the level of the lesion increases significantly over time following hemisection injury. In contrast, the number of long propriospinal neurons permanently decreased, although this does not imply that these neurons are not plastic. Studies in the rat suggest that long propriospinal neurons are capable of afferent plasticity in that they increase their synaptic contacts with lumbar motoneurons following a discrete funicular spinal cord injury (Bareyre et al., 2004). The current studies did not assess the terminal arborizations of these neurons. This could be examined by the use of an anterograde tracer in future studies. The current studies also do not allow us to determine whether the increase in projections below the lesion were made by intact axons sprouting collaterals around the lesion or regenerating axotomized axons. A double retrograde tract tracing study would help to determine this. One retrograde tracer would be placed at the lesion site at the time of injury to label cut axons. At the end of the study a second retrograde tracer would be placed caudal to the lesion to label any neurons whose axonal projections were below the lesion as done in the study presented here. A double labeled neuron would indicate that this neuron was originally axotomized and regenerated through or around the lesion site.

Finally, other descending motor tracts, such as the corticospinal tract in the motor cortex, involved in the control of voluntary locomotion should be studied to understand their underlying plasticity in order to develop targeted therapeutic interventions.

Traditionally, studies have focused on the environment adjacent to the lesion site when assessing inhibitory components of the central nervous system. Early studies, showed that dorsal root ganglia microtransplated into cortical white matter exhibit robust regeneration in areas of active Wallerian degeneration distal to a lesion of the cervical dorsal columns. Once the growing axons entered the CSPG rich lesion site growth cones stopped and became dystrophic (Davies et al., 1999). Although these studies showed the ability of DRG axons to grow throughout the degenerating white matter they are not the axons that typically are found in this area. Other studies should be done to determine whether neurons that originally course through these white matter tracts have the ability to regenerate in the presence of Wallerian degeneration or scar associated molecules. Our studies presented here (Chapter 4) and others (Massey et al., 2006; Massey et al., 2008) suggest that CSPGs are increased in areas undergoing Wallerian degeneration along the length of the spinal cord and denervated target nuclei. Other scar associated molecules such as hypertrophied astrocytes and oligodendrocyte precursor cells also are increased along the length of the spinal cord. Some of these increases appear to occur later than increases seen at the lesion site. Thus, it is important to consider the environmental conditions throughout the entire spinal cord when devising therapeutic strategies, such as Chondroitinase ABC, which target long distance regeneration. These studies, illustrate the intrinsic abilities of neurons to regenerate and/or grow new collaterals that extend below the level of a spinal cord injury. Because the functional recovery following a spinal hemisection is incomplete the intrinsic plasticity is not sufficient for normal performance. Understanding the

plasticity underlying these endogenous substrates is critical to the development of effective therapeutic interventions and identification of appropriate therapeutic targets to enhance recovery.

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BIOGRAPHICAL SKETCH

Adele Elizabeth Blum was born in Easton, Pennsylvania to Cheryl and Fred Blum in March of 1982. When she was two years old they moved to sunny Jacksonville, Florida. Adele graduated from the International Baccalaureate program at Stanton College Preparatory School in Jacksonville in 2000. She then attended Emory University in Atlanta, Georgia, where she received her Bachelor's of Science in biology. During her undergraduate studies, Adele was a member of the varsity cross-country and track and field teams. It also was during these years that her passion for scientific research began to flourish. Under the mentorship of Dr. Heather Patisaul, Adele worked on a study that identified the role of soy phytoestrogens in female rodents (Patisaul et al., 2005). She was privileged enough to be a second author on the paper that came out of this study. In 2004, Adele entered the Interdisciplinary Program in Biomedical Sciences at the University of Florida. Following her rotations, she began work toward her Doctor of Philosophy in 2005 in the laboratory of Dr. Dena Howland where she studied neural plasticity following spinal cord injury. In addition to the work presented within this dissertation, Adele is a co-author on a published study assessing recovery of cough following hemisection (Jefferson et al., 2010) and is co-author on other manuscripts in preparation in the Howland laboratory. Adele successfully defended her doctoral dissertation orally on April 13th and received her Ph.D. May 2, 2010.