ASSESSMENT AND PROMOTION OF PLASTICITY AND LOCOMOTOR RECOVERY FOLLOWING SPINAL CORD INJURY

By

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

UNIVERSITY OF FLORIDA

2012

To my Parents, who gave me the space and opportunity to determine my genuine goals, as well as the encouragement and resources to achieve them

ACKNOWLEDGMENTS

Obtaining my doctoral degree was a long and challenging journey that could not have been completed without the support and guidance of several specific individuals. First and foremost, I would like to thank my mentor Dr. Dena Howland, as she donated massive amounts of her time to offer critical advice, guidance, and support through each portion of this project. Her understanding and patience throughout the earlier learning stages of my doctoral degree were invaluable, and I can only hope she will continue to be a source of support in the years to come.

I can not say thank you enough to my lab peers: Wilbur O'Steen, Nicole Tester, Stephanie Jefferson, Adele Blum, Eric Brunk, Megan Black, Kenny Maskell, Rhea Chattopadhayay, and Valerie Tainsh. As lab manager, Wilbur helped with absolutely every part of my project, from ordering supplies necessary for experiments, helping with surgeries, and being an overall handy-man, to also helping maintain my sanity through humor and kind words. I cannot imagine getting through this process without his help. Nicole Tester, Stephanie Jefferson and Adele Blum took the time to teach me the majority of technical skills necessary for this project and as the more senior graduate students, never stopped offering valuable advise despite having moved on to different laboratories. Eric Brunk, Megan Black, Kenny Maskell, Rhea Chattopadhayay, Valerie Tainsh, and Martina Spiess were past laboratory team members that helped with various experiments and analyses. Additionally, their humor acted as essential comedic relief during stressful times.

My committee members in addition to Dr. Dena Howland: Dr's. Paul Reier, Floyd Thompson, Andrea Behrman, and Mark Lewis, offered extremely helpful guidance and advice throughout this entire process. They have helped me understand the process of

research and science overall, and have pushed me to become a strong scientist. They each deserve many thanks for their priceless time and efforts.

My family has been extremely supportive and helpful as well. My parents spent hours of their lives listening to me vent and offering guidance whenever they could. My older sister Donna and brother in-law Chuck always opened up their home in NY to me, providing critical breaks and opportunities to recharge. Both my younger sister Amanda and best friend Annie have lent strong, yet sensitive ears over the past few years, primarily in regards to non-academic issues. Their support helped me to stay on task.

Lastly, and so importantly I would like to thank all of the priceless friends I have made along the way outside the laboratory. My happiness, sanity, and maintained perseverance throughout the majority of my time spent on this project was a result of their constant understanding, caring, and thoughtful words offered throughout every success and failure along the way.

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By

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August 2012

Chair: Dena Ruth Howland
Major: Medical Sciences – Neuroscience

Damage to the spinal cord causes sensorimotor loss that is permanent. The resulting functional losses are debilitating, may be life threatening, and affect an individual's ability to be independent in the home and community. Unfortunately, there are no effective therapeutics to reduce the functional deficits caused by spinal cord injury (SCI). Identification of effective treatments has been complicated by the limited regenerative capacity of the central nervous system in addition to SCI being a multifarious problem likely to require a complex, combinatorial treatment approach.

Some individuals with incomplete SCIs may recover basic walking abilities but continue to have difficulty with more challenging forms of locomotion including those that require greater balance and alterations in leg trajectories. In the current studies, a cat low thoracic hemisection model was used. This incomplete, asymmetrical injury is similar to Brown Sequard Syndrome (BSS) described in a subpopulation of humans with SCIs. In the first set of studies, a battery of gait features were assessed to compare performances during a basic locomotor task (flat overground walking) and an adaptive locomotor task (horizontal ladder crossing). Gait features critical for successful performance of adaptive locomotion pre- and post-SCI were identified. In the second

study, results from previous chondroitinase abc (ch'abc) studies in the lab were extended to determine the effects of different treatment durations on anatomical plasticity and functional recovery. The results from this study contribute important information relative to treatment duration for the ultimate translation of ch'abc to a clinical setting. In the final study, retrograde tract tracing with Fluorogold (FG) was optimized for use in a large animal model and will contribute to future assessments of circuitry disruption and plasticity in the injured spinal cord.

Collectively, the body of work presented in this dissertation contributes to our understanding of the anatomical and behavioral changes that occur following SCI, how functional performance might be enhanced with a promising therapeutic treatment, and how methods to assess anatomical plasticity can be improved to enhance future studies.

CHAPTER 1
BACKGROUND

The Spinal Cord Is Inhibitory to Axonal Growth After Injury

The devastating consequences of spinal cord injury (SCI) and the permanence of the resulting effects in humans are well documented. The earliest documentation of SCI dates back to 1550 B.C. in the Egyptian papyrus known as the "Edwin Smith Papyrus". In that document it is stated that "If you examine a man with a neck injury…and find he is without sensation in both arms and both legs, and unable to move them…it is due to the breaking of the spinal cord caused by dislocation of the cervical vertebra. This is a condition which cannot be treated." Almost 400 years later, one of the most notable neuroscientists, Ramon y Cajal wrote: "In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated (Cajal, 1928)". These concepts of the impossibility of functional recovery and the absence of axonal growth after SCI dominated the scientific community until the 1940's, when another type of post-SCI growth was discovered. Collateral sprouting, the growth of nerve fibers from intact axons after injury was first identified in experiments using nerve fibers supplying the skin in rabbits (Weddell et al., 1941). Similar growth was subsequently identified in the spinal cord by Liu and Chambers in 1958. Using a spared root prep, they denervated several dorsal roots from one side of the cord, waited several months, then transected the remaining roots from the chronically injured side in addition to the matching roots from the non-injured side. Two weeks later tissue was assessed with a neurodegeneration stain, which showed a greater number of degenerating profiles in the chronic compared to the acute side. This was attributed to connections between the chronic and acute sides by way of collateral sprouting (Liu and

Chambers, 1958; For Review See Guth, 1975). Subsequently, the concept that regeneration of axotomized fibers does not occur was disputed by several studies which reported that severed axons returned to active states if stimulated properly (Li and Raisman, 1995; Kobayashi et al., 1997). For example, a study completed by Yi and colleagues determined that damaged rubrospinal tract fibers one year after injury could grow into a peripheral nerve graft if first treated with brain derived neurotrophic factor (BDNF) (Kobayashi et al., 1997). These combined findings, in addition to many others, suggested that the spinal cord has a much greater potential for axonal plasticity and functional recovery after injury than previously believed.

The greatest functional restoration most likely will be obtained through a combination of regeneration and collateral sprouting. Research to better understand and enhance these types of plasticity is being actively pursued using experimental animal models. A multitude of different approaches such as locomotor training (For review see Battistuzzo et al., 2012), cellular replacement, introduction of matrix substrates, as well as numerous different pharmacological interventions (For review see Jeffery and Blakemore, 1999; Lu and Tuszynski, 2008; Boulenguez and Vinay, 2009) all have shown some enhancement of axonal growth after injury. For this new growth to be functionally effective after an injury, it must make functional connections. The motor system is an extremely complex combination of many tracts each playing their own independent, as well as complementary roles in locomotion. Understanding the function of these pathways during locomotion in an uninjured system, as well as their contributions to locomotor recovery after injury is important for designing approaches to enhance locomotor recovery post-SCI.

The Multiple Motor Pathways that Control Locomotion

In 2004, the report of a survey taken by 681 SCI individuals ranked seven specific functions in order of their importance for quality of life. The ability to walk ranked fourth highest, indicating the need to determine methods for recovering locomotor function after injury (Anderson, 2004). The underlying neural control of walking and other types of locomotion has been well studied over the course of several centuries and provides a strong foundation for guiding present and future investigators towards promoting recovery of locomotor function after SCI (For review see Clarac, 2008).

Central Pattern Generator

The underlying circuitry responsible for locomotion is complex and involves all levels of the neural axis spanning from intraspinal networks to supraspinal tracts (D.A, 1975; Eidelberg and Yu, 1981; Yu and Eidelberg, 1981). The most notable locomotor related intraspinal systems are the central pattern generators (CPGs); a well organized circuit of interneurons and motoneurons located in the cervical and lumbar areas of the spinal cord. These circuits fire in specific patterns leading to alternating bilateral flexion and extension of the arms and legs to produce stepping. This CPG activity can occur spontaneously without descending supraspinal or sensory inputs and is considered to be the neural basis for the basic stepping pattern. Circuitry can be modulated in response to external stimuli through spinal interneurons allowing for some degree of adaption to changes in the environment (For review see Grillner and Zangger, 1979; Rossignol and Frigon, 2011).

Propriospinal System

Propriospinals (PSNs) make up a large portion of spinal interneurons and play a significant role in locomotion. This system contributes to trunk control, inter- and intra-

limb integration, and modulates input from both the descending supraspinal systems and peripheral afferents. Additionally, they synchronize motor circuitry throughout the entire length of the spinal cord (For review see Flynn et al., 2011). The PSN system is primarily contained within the spinal cord, linking different spinal segments together allowing for complex movements. However, some project to supraspinal centers like the lateral reticular nucleus (Alstermark et al., 1981b; Skinner et al., 1989), and cerebellum (Skinner et al., 1989). Depending on their length PSN pathways are classified as either “short”, 1-6 segments or “long”, >6 segments (Conta Steencken and Stelzner, 2009). Thus, short PSNs modulate activity occurring relatively local to the cell of origin and long PSNs make connections to distant regions of the spinal cord. In fact, evidence indicates that the long PSNs may be responsible for connecting the cervical and lumbar CPGs for quadrupedal stepping/synchrony across the forelimbs and hindlimbs in the cat (Miller et al., 1973), and possibly the arms and legs in humans (For review see Dietz, 2002; Huang and Ferris, 2009; Tester et al., 2012). PSN projections can occur either in the rostral or caudal plane from their cell of origin, located in the spinal cord gray matter (Skinner et al., 1989). This flexibility allows for the complex circuitry that underlies the production and control of elaborate multi-segmental movements.

Supraspinal Motor Pathways

Voluntary locomotor control is mediated by supraspinal tracts that originate in the brain or brainstem. Primary supraspinal motor tracts include the corticospinal (CST), rubrospinal (RuST), reticulospinal (ReST), and vestibulospinal (VST) tracts. These tracts are separated into a medial system: ReST, VST, and a lateral system: RuST, CST. Approximately 87.6% of the CST is located dorsolaterally while 12.4% of the CST

(anterior CST) travels in the ventromedial funiculi of the spinal cord (Kwon et al., 2011). These two systems, the CST and RuST, are essential for producing fine-tuned, precise movements like stepping over an obstacle (Mohagheghi et al., 2004), grasping food (Alstermark et al., 1981a; Alstermark et al., 1987; Whishaw et al., 1998), and paw placement (Batson and Amassian, 1986; For review see Drew et al., 2002). The loss of these systems causes difficulties maneuvering through environments and completing day-to-day activities.

The medial system is responsible for creating the necessary tone in postural musculature allowing for upright locomotion. Additionally, the ReST has been shown to initiate stepping (Jell et al., 1985). The VST, originating in the vestibular nuclei within the medulla, is primarily controlled by the utricles and saccules of the vestibular system and allows for the body's musculature to quickly respond and recover from sudden displacements (Markham, 1987). In this way, the VST is critical for maintaining balance. Disruption to either of the medial or lateral systems has detrimental effects that vary depending on the magnitude of the lesion itself.

Locomotor Recovery following Spinal Cord Injury

Complete transection of the spinal cord, which removes all supraspinal input below the level of the lesion, results in immediate and complete loss of motor function and reflexes below the injury. This period, known as "spinal shock", is present in experimental animals as well as humans. It lasts for approximately two weeks in humans after which some reflexes begin to return (For review see Riddoch, 1917; Eidelberg, 1981). Some investigators argue that the period of spinal shock is much shorter on the magnitude of minutes to hours (For review see Ditunno et al., 2004). Regardless, there is an overall period of motor depression after an injury that lasts for

several weeks. Several months following complete spinal transection spasticity may occur, characterized by hypertonus, clonus, involuntary somatic reflexes, and muscle spasms that are often extremely painful (For review see Rabchevsky and Kitzman; Adams and Hicks, 2005). This secondary impairment as a result of SCI, is believed to be due to aberrant persistent inward currents (PIC). In the uninjured nervous system, PICs are depolarizing inward currents intrinsic to motoneurons that can self-sustain firing as long as the cell remains depolarized. Cessation requires inhibitory synaptic input, often led by supraspinal centers. PICs are controlled by the monoamines 5HT and norepinephrine, in which their primary sources are the raphe nucleus and locus coeruleus. Acutely after an injury, motoneuron excitability is reduced due to a loss of supraspinal connectivity and thus, monoamines. However, over time motoneuronal excitability returns to somewhat normal levels, and is particularly monoamine-sensitive. Residual monoamines present in the spinal cord and vasculature are able to activate PICs, however the remaining lack of supraspinal connectivity prevents these PICs from being inhibited, causing spasticity (For review see ElBasiouny et al., 2009). There are currently several treatment options available for spasticity including baclofen, tizanidine, and botulinum neurotoxin, however they have varying degrees of efficacy (Rabchevsky and Kitzman). Multiple animal models of spasticity are used in order to improve our understanding of this impairment and improve treatment options (Thompson et al., 2001).

Complete versus Incomplete Spinal Cord Injury

Although voluntary stepping does not recover after an anatomically complete SCI, CPG based spinal stepping can be elicited as seen in humans (Dietz and Colombo, 2004), cats (Grillner and Rossignol, 1978; Eidelberg et al., 1980), rats (references),

dogs (Hart, 1971), and possums (Hinsey and Cutting, 1936). Incomplete SCI, like anterior cord syndrome, central cord syndrome, posterior cord syndrome, and brown sequard syndrome (BSS), result in substantially greater locomotor recovery due to the sparing of some tissue (Eidelberg, 1981). Individuals with incomplete SCI typically have notable recovery of basic locomotion like walking overground or on a treadmill, however the presence of some common deficits remain. Often, these individuals have decreased gait speed (Knutsson and Richards, 1979; Dietz et al., 1981; Conrad et al., 1983; Wainberg et al., 1986; Wagenaar and Beek, 1992; Lajoie et al., 1999) and cycle duration (Lajoie et al., 1999; Barbeau et al., 2002). Additionally, a variety of different joint alterations have been reported such as increased hip angular excursion, and increased knee flexion during either foot touchdown alone, or the entire step cycle. The ankle also has been reported as either being dorsiflexed, or plantar flexed causing foot drag (Conrad et al., 1983). Changes in muscle activation during walking also have been shown to occur after injury. Typically, an abnormal co-activation of antagonist muscles occurs (Fung and Barbeau, 1989, 1994) in addition to altered shape and timing of activation patterns (Fung and Barbeau, 1989; Domingo et al., 2007).

Individuals with incomplete SCI often have greater struggles with challenging types of locomotion. This can be illustrated by studies looking at functional recovery in cats with a hemisection injury similar to BSS. Studies completed by the Howland laboratory have shown that following a T10 hemisection, cats were able to recover bipedal treadmill and crossing of a 12” wide basic overground runway well, but had greater difficulties when performing challenging tasks like crossing of a horizontal ladder, peg walkway, or 2” wide narrow beam (Tester and Howland, 2008; Jefferson et

al., 2011). The disparity between functional recovery in individuals with complete versus incomplete SCI suggests that these two populations require different rehabilitation strategies.

The Effect of Spared Tissue on Locomotor Recovery

The degree of recovery in individuals with incomplete SCI varies greatly across individuals and is partially due to the different types of pathways spared by the injuries. Animal studies in which specific cuts were made in order to axotomize select tracts both reveal individual tract functions through the process of elimination, as well as determines the contributions of different tracts on locomotor recovery after SCI.

Extensive damage to the medial systems often is accompanied by severe and permanent postural control and locomotor impairments (Lawrence and Kuypers, 1968; Brustein and Rossignol, 1998). This suggests a critical role in basic locomotion for these systems (Lawrence and Kuypers, 1968; Brustein and Rossignol, 1998). In contrast, studies in which injuries were isolated to the CST and RuST showed only transient locomotor deficits and a quick recovery of overground locomotion (Laursen and Wiesendanger, 1967; Muir and Whishaw, 2000; Kanagal and Muir, 2009) food grasping (Blagovechtchenski et al., 2000) and other skilled finger movements (Hepp-Reymond et al., 1970). However, components of these movements remained impaired, indicating that these lateral systems are most important for more skillful, precision-based movements. Damage to these lateral pathways paired with sparing of the ventromedial cord has been associated with substantial locomotor recovery after SCI (Windle et al., 1958; Nathan and Smith, 1973; Afelt, 1974; Eidelberg et al., 1981; Schucht et al., 2002; Krajacic et al., 2010) indicating that alternate pathways can contribute to functional recovery after injury.

Pathway-Specific Potential for Plasticity

The contributions of certain pathways to recovery after injury are largely due to their innate responses to injury and plastic properties. These properties differ across systems. Axonal die-back is a major response to injury that occurs in all transected fibers (Busch et al., 2009) which affects their participation in new circuit formation after injury; the further an axon retracts, the more distance regenerating and/or sprouting fibers must cover to bridge the lesion site and restore function. Even if a fiber with significant retraction sprouts onto nearby spared circuitry, that circuit itself will have further distance to cover, increasing its chances for failure. Comparing axonal die-back properties across several supraspinal tract systems provides insight into their individual potential for meaningful plasticity. The application of trophic factors like BDNF and neurotrophin 3 (NT-3) have been associated with increased axonal growth of multiple supraspinal pathways after injury (Kobayashi et al., 1997; Hiebert et al., 2002; Kwon et al., 2002; Plunet et al., 2002; Dolbeare and Houle, 2003). The responsiveness of individual tracts to trophic factor application also can provide a general indication of specific tracts' ability to grow after injury.

Axonal Die-Back

Significant evidence indicates that the CST has limited regenerative potential. For example, several studies looking at plasticity in multiple pathways have reported minimal CST growth, but significant growth in the RuST (Richardson et al., 1984a; Houle and Ye, 1999; Decherchi and Gauthier, 2000; Plunet et al., 2002), ReST, and VST (Houle and Ye, 1999; Oudega et al., 1999). One reason CST growth is limited after injury is likely related to its significant and prolonged axonal die-back as depicted in several studies. Specifically, these studies found that following injury the proximal

ends of CST axons formed dystrophic endbulbs and retracted for several millimeters and for up to eight weeks (Pallini et al., 1988; Oudega et al., 1999). Meanwhile, axonal dieback only lasted for ~ four weeks post-injury in the VST, ReST and RuST tracts with axons measuring approximately 0.5 to 1.5 mm from the lesion site (Houle and Jin, 2001). In contrast to the above-mentioned results, one study also found continued die-back for eight weeks following axotomy of the VST, suggesting this tract may share regenerative and sprouting difficulties similar to the CST (Oudega et al., 1999).

Responses to Trophic Factor Application

Both the RuST (Liu, 1999; Liu et al., 2002; Murray et al., 2002; Jones et al., 2003b), and ReST (Xu et al., 1995; Menei et al., 1998) have shown enhanced post-axotomy growth in response to trophic factor application. However, the CST and VST have been reported to respond less notably in several studies. Specifically, two separate groups found that while the addition of BDNF to red nucleus cell bodies resulted in enhanced regeneration of the RuST into peripheral nerve grafts, the same treatment to motor cortex cell bodies did not cause CST regeneration. Interestingly, they did indicate enhanced collateral sprouting both rostral (Hiebert et al., 2002; Plunet et al., 2002) and caudal (Plunet et al., 2002) to the injury. A similar study comparing regeneration of the RuST, ReST, CST, and VST after application of one of three different growth factors (ciliary neurotrophic factor (CNTF), BDNF, or neurotrophin 3 (NT-3)) found enhanced regeneration of the ReST and RuST in response to all growth factors. However, the VST responded to only one of the growth factors, CNTF, while the CST did not respond to any (Ye and Houle, 1997). Similar results have been found in additional studies indicating that both the CST (Schnell et al., 1994; Tuszynski et al., 1997; Blesch, 1999; Lu et al., 2001) and VST (Xu et al., 1995; Ye and Houle, 1997;

Menei et al., 1998) may be less apt for plasticity after axotomy. However, despite these tracts underwhelming plastic responses to trophic factors, several studies utilizing different growth promoting methods have reported increased growth. For example, administration of leukemia inhibitory factor (Blesch, 1999), and olfactory ensheathing cell transplants (Li et al., 1997) have both led to enhanced CST growth. Even more interesting is the study by Bareyre and colleagues who reported spontaneous sprouting of transected CST axons onto long PSN tracts in the cervical spinal cord following a thoracic SCI in rats. This new circuitry was capable of bridging the lesion site and enhancing functional recovery (Bareyre et al., 2004). Such findings indicate that CST growth may, in fact, be occurring in regions distant from the lesion where plasticity is not typically assessed. A similar phenomena was reported in a study by Rozensweig and colleagues who showed substantial spontaneous CST growth in a primate model of SCI, citing species differences as a potential reason for the lack of CST plasticity commonly reported in rodents (Rosenzweig et al., 2010). Plasticity of the VST, though less well studied, has shown enhancements when growing into an embryonic tissue transplant (Ito et al., 1999). Overall, these findings indicate that the CST and VST have the potential for plasticity, but respond less readily to certain treatments compared to the RuSt and ReST. Alternatively, plasticity in the CST and VST may be occurring as readily as in the ReST and RuST, but in regions distant to the lesion that are not typically assessed.

Propriospinal Plasticity

Although plasticity of the PSN system is currently not as well studied as the supraspinal systems, researchers are beginning to identify it as a key player in creating novel pathways that allow for neuronal signals to bypass the lesion and restore function.

In a study by Courtine and colleagues, they determined that following several carefully timed and placed incomplete lesions, spared PSN axons were independently capable of mediating recovery of stepping without direct input from the brain (Courtine et al., 2008). Spontaneous PSN tract plasticity also has been shown to form novel circuitry after a high cervical injury resulting in recovery of chronic diaphragm activity (Darlot et al., 2012). These findings indicate incredible plastic potential in this PSN system that is capable of functional restoration. However, based on detailed investigations by the Stelzner group the potential for plasticity appears to differ across the short and long PSNs. In a collection of studies, they showed that long and short PSNs respond differentially to axotomy in that there are fewer surviving short PSNs after injury compared to long PSNs (Conta and Stelzner, 2004; Siebert et al., 2010b; Conta Steencken et al., 2011). Furthermore, while short PSNs have an initial upregulation of growth factor receptor gene expression, as well as immune, inflammatory, and pro-apoptotic gene expression transiently after injury, these same genes are downregulated in the long PSNs. This translates into more short PSN cell death compared to long. However, surviving short PSNs have genetic profiles that return to normal by one month (Siebert et al., 2010a; Siebert et al., 2010b) and their neuronal size does not significantly change between two weeks and the course of the study (eight weeks). The authors suggest that the surviving short PSNs could be a specialized population with “sustaining collaterals” making them more resilient to axotomy (Siebert et al., 2010a). This theory was validated in a separate study which reported significant short PSN axonal growth across a midline injury to form functional synaptic connections with motoneurons after SCI (Fenrich and Rose, 2009).

Although the above mentioned set of studies shows an initial and permanent decrease in the number of short PSNs over the course of a 16 week study (Conta Steencken and Stelzner, 2009), studies from my own lab have shown an initial yet transient decrease in short PSNs followed by a significant increase by 16 weeks post-injury (Blum, 2010). This difference in short PSN plasticity across studies may be a result of species differences, as our study was completed in feline and theirs in rat. However, it is most likely due to the extensive training our animals underwent and the lack of training in their study as training increases BDNF production (Beaumont et al., 2008) which in turn increases plasticity (Girgis et al., 2007). Additionally, the Stelzner group used a contusion injury model, while a a hemisection model was used in ours. Hemisections typically have been associated with greater axonal growth (Iseda et al., 2008). These contradictory findings indicate that the PSN system has plastic potential, however requires certain stimuli to activate this potential after injury.

Enhancing Propriospinal Plasticity

Although there are few studies that have specifically focused on the effects of trophic factors on PSN plasticity results following Schwann cell transplants into the lesion site showed an enhancement in long and short PSN plasticity however, the greatest amount of plasticity was in the short PSNs (Xu et al., 1997; Takami et al., 2002; Doperalski et al., in preparation). Additionally, one study found that the combination of GDNF and Schwann cell seeded channels applied to the lesion site led to enhanced plasticity of short PSNs. Collectively, the above studies suggest that although there is substantial PSN cell death after injury, those that survive are capable of substantial plasticity that can mediate and support recovery after SCI (Xu et al., 1995; Menei et al., 1998).

The Effects of Training on Functional Recovery after Injury

Training itself has been shown to enhance plasticity and functional recovery after injury (For review see Marsh et al., 2010). Multiple studies using training as the sole therapeutic after injury have reported enhancements of the function being trained. Examples of these functions include basic stepping (Harkema et al., 2011), treadmill stepping in both humans (Thomas and Gorassini, 2005), and cats (Lovely et al., 1986; Barbeau and Rossignol, 1987), wheel-based stepping (Beaumont et al., 2008), staircase climbing (Singh et al., 2011), single pellet reaching, and horizontal ladder walking in rats (Girgis et al., 2007; Starkey et al., 2011). Further assessments regarding how training enhances functional recovery have identified training-induced enhancements of growth factor upregulation, specifically BDNF and Anti-Growth Associated Protein-43 (GAP-43) (Girgis et al., 2007; Beaumont et al., 2008; Ying et al., 2008). These growth factors enhance axonal plasticity (Liu, 1999; Murray et al., 2002), and help shape synaptic plasticity (Ying et al., 2008). Training also has been shown to enhance motoneuronal electrophysiological properties like resting membrane potential, spike trigger levels (Beaumont et al., 2008), and increase excitability of the CST underlying leg muscle activity (Thomas and Gorassini, 2005).

The way training is conducted after injury has drastic affects on functional outcomes. Task-specific training of one type of task has been shown to enhance recovery of that task, but in some cases does not transfer to other tasks. For example, swim training was shown to enhance swimming kinematics, but had no effect on overground walking (Magnuson et al., 2009). Additionally, stair climbing ascent training led to enhanced recovery of this specific task, but caused only partial improvements on overground and grid stepping (Singh et al., 2011).

Task-specific training of one task also has been shown to have detrimental and/or positive effects on the recovery of other tasks. For example, rats that were trained specifically on single pellet reaching had significant functional improvements on this task after injury, but were significantly worse at crossing a horizontal ladder compared to their untrained rat counterparts (Girgis et al., 2007). Another example is one study completed by Garcia-Alias and colleagues, who found that rats trained to walk on a grid had significantly enhanced recovery of skilled pellet reaching, but were significantly worse at horizontal ladder stepping compared to their untrained counterparts (Garcia-Alias et al., 2009). The potential for task-specific training on one task to transfer to other tasks most likely relates to the kinematic similarities across tasks as specific neuromuscular activity patterns lead to differential muscle responses. This concept was assessed and confirmed in a study that compared the muscle activity of spinalized cats trained to either step on a treadmill or stand after injury. Those cats trained to stand had increased maximum rate of shortening in their medial gastrocnemius muscles, as well as greater muscle mass when compared to cats that were only trained to step on a treadmill. These same cats also had an overall greater shift of muscle fiber type towards fast fibers (Roy et al., 1999). Overall, these findings demonstrate that the neuromuscular system is extremely sensitive to different training regimens, and that there is a strong need to enhance our understanding of how different training paradigms affect functional recovery after injury in order to maximize recovery.

Cats as a Translational Model of Spinal Cord Injury

Cats are considered to be a highly translational SCI model as they share numerous similarities with humans in regards to neuro-anatomical control of movement, gait mechanics, and interlimb coordination (For review see Vilensky, 1987; Dietz, 2002;

Majczynski and Slawinska, 2007). The overall organization of the large ascending and descending tracts within the spinal cord are similar across species (Majczynski and Slawinska, 2007; Watson, 2009). Additionally, the presence of a central pattern generator, which underlies stepping patterning, has been confirmed in the cat (Miller et al., 1973; Smith et al., 1983), and indirectly identified in humans (Harkema et al.; Dimitrijevic et al., 1998; Jilge et al., 2004; Calancie, 2006). Evidence from both species also has indicated the presence of flexible networks in the spinal cord that are responsible for independent movement of each limb, suggesting that both species have a “half centre” model of locomotion (For review see Brown, 1914; Prokop et al., 1995). In regards to reflex characteristics, both cats and humans share certain reflexive responses to afferent stimuli (Lisin et al., 1973) and also have several shared speed-related gait changes (Vilensky, 1987). Postural control in response to perturbations also was identified as being similar across cats and humans when humans were positioned in a quadrupedal stance (Macpherson et al., 1989).

Despite the more extreme differences in overall body size, the average cat spinal cord length is only 9 cm shorter than the average human spinal cord. Thus, the distance necessary for axonal plasticity to render functional changes is similar across cats and humans. These species similarities make the cat a valuable animal model for SCI research, therefore the studies to be described in the experimental chapters (2-4) of this dissertation were completed in a cat model of SCI.

The Pathology of Spinal Cord Injury

The peripheral nervous system (PNS) is substantially more conducive to axonal growth after injury than the central nervous system (CNS), and It has been determined that the CNS environment is responsible for this disparity (Richardson et al., 1980;

Benfey and Aguayo, 1982; Richardson et al., 1984a). Injury to the CNS, specifically the spinal cord, creates an upregulation of multiple different physiological responses that further inhibit this system's potential for plasticity.

SCI occurs in a biphasic manner in which there is an initial mechanical injury followed immediately by a period of ongoing damage. This period is commonly known as secondary injury and can continue for months after the mechanical injury (Rowland et al., 2008; Flynn et al., 2011; Kuzhandaivel et al., 2011). Secondary injury begins immediately after insult, in which there is substantial hemorrhagic necrosis, microglial activation (Donnelly and Popovich, 2008), and an upregulation of pro-inflammatory cytokines like interleukin 1β, and tumor necrosis factor α (Pineau and Lacroix, 2007). Within the next 48 hours the Blood Brain Barrier reaches its peak level of permeability (Noble and Wrathall, 1989); neutrophils invade the lesion site, and vasogenic and cytotoxic edema start to set in (For review see Rowland et al., 2008). Additionally, hemorrhaging continues which leads to free radical production and a dysregulation of Ca++ ion concentrations followed by calpain activation and mitochondrial dysfunction (Schanne et al., 1979). Extracellular glutamate eventually reaches toxic levels at the lesion site (Wrathall et al., 1996) and collectively these components will result in cell death (Schanne et al., 1979). Studies indicate that neuronal death is primarily by way of necrosis, although there have been some reports of neuronal apoptosis (Liu et al., 1997; Beattie et al., 2000; Lu et al., 2000). Oligodendrocytes are more prone to apoptosis than neurons (Crowe et al., 1997).

Within the next several days, multiple different inflammatory-related cell types infiltrate the lesion site, including reactive astrocytes, monocytes, and macrophages

(Popovich et al., 1997; Fleming et al., 2006; Donnelly and Popovich, 2008). The axonal death that has been occurring throughout this entire injury period leads to a breakdown of the myelin sheaths that once surrounded them. This myelin debris acts as a strong inhibitor to axonal growth and is more slowly removed by the immune system in the CNS than in the PNS (Filbin, 2003). In addition to the above mentioned responses to injury, a glial scar begins to form. This scar is one of the primary inhibitory components of the lesion environment.

The Glial Scar

The glial scar is primarily formed by reactive astrocytes that are characterized by many intermeshing cytoplasmic processes. Additionally, it consists of reactive microglia, macrophages, fibroblasts, oligodendrocytes, oligodendrocyte precursor cells, Schwann cells, and meningeal cells (Fawcett and Asher, 1999). The fibrous qualities of this scar present as a physical barrier that is extremely difficult for axons to penetrate. An even greater obstacle against axonal growth within the scar is the upregulation of chondroitin sulfate proteoglycans (CSPG). Following SCI, these CSPGs are increased at the lesion site primarily by reactive astrocytes, however reactive microglia, macrophages, fibroblasts and oligodendrocyte precursor cells also play a role (McKeon et al., 1991; Dou and Levine, 1994; Smith-Thomas et al., 1994; Fitch and Silver, 1997; Fawcett and Asher, 1999; Dawson et al., 2000). These CSPGs create a strong chemical inhibition against axonal growth (Rudge and Silver, 1990; McKeon et al., 1991).

The inhibitory nature of chondroitin sulfate proteoglycans

CSPGs are one of the largest and most abundant proteoglycan families in the normal, uninjured nervous system. They make up a large portion of the extracellular matrix present in the intercellular spaces between neurons and glial cells, forming a

tight meshwork with hyaluronate, tenascin, and link proteins (Hook et al., 1984; For review see Kwok et al., 2008; Zimmermann and Dours-Zimmermann, 2008; Hyatt et al., 2010). They also are an important component of the dense perineuronal nets (PNN) that surround neurons and regulate plasticity, neuroprotection, and homeostasis (Deepa et al., 2006). CSPGs also are important during neurodevelopment by interacting with tenascin to help guide axons to the appropriate locations through inhibition and the formation of inhibitory PNNs (Snow et al., 1991; Brückner et al., 2000; Pizzorusso et al., 2002). External to the nervous system, CSPGs are present in cartilage by binding strongly to, and stabilizing its components: Laminin, fibronectin, and collagen (Oldberg and Ruoslahti, 1982; Snowden, 1982).

CSPGs are a large family consisting of seven different members: Brevican, decorin, neurocan, aggrecan, versican, phosphacan and neuron-glial antigen 2 (NG2). Each member has a different core protein with unique characteristics and multiple attachment sites for chondroitin sulfated glycosaminoglycan sugar side chains (CS-GAGs) (Herndon and Lander, 1990). These CS-GAG chains are sulfated repeats of hexonate dissacharides (glucoronate or iduronate) and hexosamines (glucosamine and galactosamine) (Silbert and Sugumaran, 2002). Sulfation occurs primarily on C-2, C-4 and C-6 and the amount of this sulfation varies greatly across core proteins, thus there is substantial heterogeneity within this family (For review see Iozzo, 1998; Kwok et al., 2008). In addition to the CSPGs there are several other families of proteoglycans including Keratan Sulfate-(KSPG), and Heparan Sulfate-(HSPG). These families consist of the same core proteins but in lieu of or in addition to CS-GAGs have keratan or heparan sulfate chain attachments. KSPGs have recently been reported to have

inhibitory properties similar to CSPGs (Imagama et al., 2011; Hilton et al., 2012), while HSPGs have been shown to promote axonal growth (Mammadov et al.; Riopelle and Dow, 1990).

Over the past decade CSPGs have become an area of intense research in regards to their inhibition of axonal growth following nervous system injury. Upregulation of some CSPGs begins within hours following injury and develops over several weeks to months (Fitch and Silver, 1997) leading to the formation of a mature glial scar (McKeon et al., 1991; Levine, 1994; Fawcett and Asher, 1999; Haas et al., 1999; Lemons et al., 1999; McKeon et al., 1999). Not only does this scar act as a physical barrier to axonal growth, but also as a chemical barrier mediated by the CSPGs.

The inhibitory properties of CSPGs have been extensively studied and confirmed across many laboratories (Rudge and Silver, 1990; Snow et al., 1990; McKeon et al., 1991; Dou and Levine, 1994; Milev et al., 1994; Davies et al., 1997; Fidler et al., 1999; Hynds and Snow, 1999; Schmalfeldt et al., 2000; Becker and Becker, 2002). One of the initial in vitro studies to investigate this idea performed a stripe assay which determined that neurite outgrowth of chick dorsal root ganglia would grow abundantly onto a laminin stripe, but would stop or grow along the border of a keratan sulfate (KS)/CSPG stripe. These findings indicated that neurite outgrowth is inhibited by KS/CSPGs (Snow et al., 1990). Extension of these results to a CNS trauma environment was completed in a study by McKeon and colleagues. In this study, they found that only adult, P-30 rats had CSPGs and cytotactin/tenascin (CT) present in their gliotic scar following brain injury, while the neonates with a similar injury did not.

Additionally, it was only these adult rats that could not properly support axonal growth, indicating that the presence of CSPGs and CT correlates with inhibition of axonal growth (McKeon et al., 1991). A more detailed examination of CSPGs in vitro, determined that CSPG inhibition is gradient-dependent with neurite growth inhibition being greatest in the presence of higher CSPG concentrations (Snow and Letourneau, 1992). These findings indicate that the removal of these CSPGs after an SCI may create an environment more conducive to axonal growth and act as a promising potential therapeutic.

Chondroitin sulfate proteoglycan temporal expression patterns after spinal cord injury

The realization that CSPGs inhibit axonal growth after injury led to a much closer examination of their upregulation pattern post-SCI. While CSPGs are considered to be a proteoglycan "family", the post-injury expression of each individual CSPG core protein is unique from one another. There are six different types of CSPGs: neurocan, brevican, versican, phosphacan, NG2, and aggrecan (For review see Kwok et al., 2008). Currently, all studies regarding the expressions of each individual CSPG have been completed in rats.

In an uninjured spinal cord, all CSPGs are expressed at low levels. As quickly as 24 hours post injury there is moderate upregulation of neurocan, brevican, versican (Jones et al., 2003a) and NG2 (Jones et al., 2002; Jones et al., 2003a). Notably, phosphacan expression significantly decreases during this period, possibly because of an increase in proteolytic enzymes that degrade phosphacan, like plasmin (Wu et al., 2005). Expression of aggrecan also was reported to decrease (Lemons et al., 2001; Andrews et al., 2011). Peak expression of neurocan, brevican, versican occurs at 2

weeks post injury. NG2 expression peaks earlier at 1 week post-injury and remains elevated for at least 7 weeks (Jones et al., 2002). Phosphacan expression does not begin to increase until 4 weeks post injury after which it remains elevated for at least 8 weeks and most likely plays a large role in axonal inhibition during chronic injuries. Expression of brevican, versican, and NG2 also were still elevated at 8 weeks post-injury, but only in regions closely surrounding the lesion site. Neurocan expression was reduced to basal levels by 8 weeks (Jones et al., 2002; Jones et al., 2003a) and recovery of aggrecan expression was shown to begin at 2 weeks after hemisection injury (Lemons et al., 2001), but remained decreased after a contusion, as determined by a combination of western blot analysis and immunohistochemistry (Andrews et al., 2011). Overall, these findings demonstrate considerable diversity across the CSPGs in regards to the timing of their upregulation after injury. This is an important consideration in regards to the timing of administration of potential therapeutics that may act on these inhibitory components.

Chondroitin sulfate glycosaminoglycans are the primary inhibitory component of chondroitin sulfate proteoglycans

As previously described, CSPGs consist of two main components: Core protein, and CS-GAG chains. By removing the CS-GAGs from the core proteins using the bacterial enzyme chondroitinase abc (ch'abc), it was determined that CS-GAGs contain the majority of this complex's inhibitory properties (Snow et al., 1990). Ch'abc is a bacterial enzyme purified from *Proteus Vulgaris*, a gram negative bacteria normally present in the intestinal tracts of humans and other animals. It consists of two enzyme components capable of degrading chondroitin sulfate proteoglycans: an endoeliminase, which depolymerises CSPGs, and an exoeliminase which degrades tetra- and hexa-

saccharides resulting in disaccharides (For review see Crespo et al., 2007). Ch'abc has been used extensively as an in vitro tool for understanding the role of CS-GAGs in axonal growth inhibition. Snow and colleagues was one of the first investigators to utilize this new tool and found that the removal of CS-GAGs with ch'abc led to enhanced growth of chick dorsal root ganglia neurites onto KS/CS-PG substrate suggesting that the CS-GAG chains are the primary inhibitory portion of CSPGs (Snow et al., 1990). These studies were later confirmed by numerous others prior to advancing the assessments of ch'abc-mediated CS-GAG removal to an in vivo setting (Snow et al., 1990; Smith-Thomas et al., 1994; McKeon et al., 1995; Zuo et al., 1998; Chung et al., 2000; Yu and Bellamkonda, 2001).

Chondroitinase ABC as a Potential Therapeutic

The confirmation that CS-GAG removal with ch'abc results in enhanced neurite outgrowth in vitro sparked an interest among many investigators to determine this enzyme's effectiveness in vivo. Previous studies completed in the Howland laboratory were the first to show that ch'abc applied in vivo to the injured spinal cord would lead to CSPG cleavage (Lemons et al., 1999). Following this study, Yick and colleagues were the first to show that ch'abc administration to the spinal cord after injury led to enhanced axonal growth. In this study, a peripheral nerve graft implantation was paired with either vehicle, BDNF, or ch'abc application after a T11 hemisection in rats. The vehicle, or BDNF applications did not enhance axonal growth of Clarke's nucleus neurons into the graft, while ch'abc application did lead to enhanced growth (Yick et al., 2000). Further investigations by numerous other groups also found enhanced plasticity within multiple pathways, in addition to functional recovery. Bradbury and colleagues were the first group to show that ch'abc administration led not only to enhanced axonal growth and a

restoration of post-synaptic activity caudal to the lesion, but also enhanced recovery of multiple different functions (Bradbury et al., 2002). Following this study, a multitude of others were completed which also reported enhanced plasticity and functional recovery after injury (Tropea et al., 2003; Barritt et al., 2006; Houle et al., 2006; Massey et al., 2006; Galtrey et al., 2007; Vavrek et al., 2007; Cafferty et al., 2008; Iseda et al., 2008; Massey et al., 2008; Tester and Howland, 2008; Tom and Houlé, 2008; Garcia-Alias et al., 2009; Lee et al., 2009; Tom et al., 2009b; Bai et al., 2010; Karimi-Abdolrezaee et al., 2010; Jefferson et al., 2011). Each of these studies utilized different injury models and treatment administration methods suggesting the robustness of this treatment and its potential as a future therapeutic.

Chondroitinase ABC-Mediated Tract Plasticity

Presumably, ch'abc enhances functional recovery by enhancing axonal growth after injury, however the exact systems underlying this recovery are currently unknown. Multiple groups have assessed the effects of ch'abc application on the plasticity of several different pathways. The most widely studied system has been the CST, whose plasticity seems to be enhanced with this enzyme. Some studies, which used anterograde tracing techniques showed increased CST growth rostral to the lesion site (Karimi-Abdolrezaee et al., 2010) paralleling the results of the trophic factor studies previously described. However, many studies also have found enhanced CST growth into the lesion site (Bradbury et al., 2002), or into tissue bridges (Iseda et al., 2008) suggesting that ch'abc has a stronger affect on enhancing CST growth compared to trophic factors. This conclusion is strengthened by studies which found ch'abc-mediated CST growth traveling closely around the lesion site and connecting caudal to the injury (Barritt et al., 2006; Garcia-Alias et al., 2009). In fact, it has been shown that

inhibition of glycogen synthase kinase 3, a component activated by CSPGs, led to increased CST growth. This may be related to one of the mechanisms by which ch'abc enhances CST growth (Dill et al., 2008). Interestingly, the one study to assess CST plasticity that utilized a retrograde tracing technique did not find any FG-labeled neurons in the motor cortex, despite having found ch'abc mediated enhancement of plasticity in the Rest, VST, and RuST (Bai et al., 2010). In this study, rats received a T10 transection and 12 weeks later, FG was placed into the transection site. The lack of motor cortex labeling in conjunction with enhanced labeling in the other aforementioned systems may be related to the CSTs tendency for extreme axonal dieback after axotomy. Additionally, it is possible that spared CST axons are more likely to sprout than axotomized axons are to regenerate proximal to the lesion site. As depicted by Bareyre and colleagues, it is also possible that sprouting of axotomized fibers might be occurring in regions distal from the lesion site (Bareyre et al., 2004).

Several studies have assessed the effects of ch'abc on plasticity of other supraspinal systems, all of which utilized a retrograde tracing technique. These studies reported significant ch'abc-mediated enhancement of plasticity in the ReST (Houle et al., 2006; Vavrek et al., 2007; Bai et al., 2010) and RuST (Houle et al., 2006; Vavrek et al., 2007; Bai et al., 2010; Jefferson et al., 2011). Additionally, the VST system also has shown an enhancement of plasticity following ch'abc treatment in the majority of studies (Vavrek et al., 2007; Bai et al., 2010). However, one study did not find an enhancement within this system despite seeing enhanced plasticity in the other previously described systems (Houle et al., 2006). These results, in conjunction with those previously described regarding the VST, suggest that this system may not be as plastic as the

other brainstem derived descending pathways or, that plasticity primarily occurs at the terminal ends in this system.

The effect of ch'abc on the plasticity of the PSN system following injury has been minimally assessed. One study identified ch'abc-mediated plasticity within this system, as well as other systems (ReST, RN, VST), indicating that the addition of ch'abc led to further axonal growth into the peripheral nerve graft (Houle et al., 2006). Further assessment of this system's response to enzymatic digestion remains to be completed.

Potential Mechanisms Underlying Chondroitinase ABC-Mediated Effects

Within the past several years, the first CSPG receptors have been identified. The transmembrane receptor protein tyrosine phosphatase sigma (RPTP) is one that has been shown to inhibit axonal growth through the CS-GAG portion of the proteoglycans (Shen et al., 2009). Several studies also have found that the disruption of the genes encoding this receptor results in enhanced axonal growth through CSPG regions after an SCI (Fry et al., 2009; Shen et al., 2009; Duan and Giger, 2010). In addition, both NgR1 and NgR3, two receptors known to mediate myelin associated inhibitor (MAI) inhibition, have been identified as binding with high affinity to the GAG moiety of CSPGs and thus may play a role in CSPG neurite growth inhibition (Dickendesher et al., 2012).

Calcium and its interaction with epidermal growth factor receptors (EGFR) and Protein Kinase C (PKC) also may be involved in ch'abc-mediated plasticity, though it has yet to be directly tested. Both kinase activity of EGFR and PKC activity lead to CSPG inhibition of neurite outgrowth with the blocking of either one of these components causing increased neurite outgrowth. Subsequently, both EGFR phosphorylation and PKC activity are activated by calcium which has been shown to increase transiently in the presence of CSPGs (Snow et al., 1994; Sivasankaran et al.,

2004; Koprivica et al., 2005). Thus, the digestion of CS-GAGs with ch'abc may decrease the amount of calcium within the lesion site, decreasing the activity of these inhibitory factors. Lastly, the Rho/ROCK pathway has been affiliated with neuronal growth cone collapse that is associated with CSPGs, though the exact connection between the two is not well understood (Borisoff et al., 2003; Monnier et al., 2003; Duffy et al., 2009).

Determining Optimal Chondroitinase ABC Application Paradigms

Across the full range of studies, ch'abc administration varies in a number of different ways: Duration of treatment, the period after injury at which treatment begins, volume, concentration, location, and delivery device. Interestingly, the majority of these study paradigms have reported some degree of ch'abc-mediated enhanced functional recovery and/or plasticity. One of the few examples in which ch'abc was not effective was in a study completed by Jakeman and colleagues. In this study, a single dose of ch'abc was administered to the lumbar cord one week after a mid-thoracic contusion injury in mice. This treatment, paired with voluntary wheel running as training, did not lead to enhanced functional recovery (Jakeman et al., 2010). These findings suggest that it is important to determine the optimal administration paradigm(s) in order to progress ch'abc to the clinic.

Biological stability

One critical factor to consider is that ch'abc is not biologically stable at body temperature (Tester et al., 2007). Therefore, ch'abc must be administered multiple times following injury in order to ensure continued cleavage. Since direct application to the spinal cord is critical, many groups implant a catheter system with tubing placed within the lesion site, as well as an injectable port implanted externally for ease of delivery

(Bradbury et al., 2002; Barritt et al., 2006; Houle et al., 2006; Iaci et al., 2007; Vavrek et al., 2007; Carter et al., 2008; García-Alías et al., 2008; Garcia-Alias et al., 2009; Karimi-Abdolrezaee et al., 2010; Carter et al., 2011). Groups utilizing this method tend to use an osmotic mini-pump for injections in order to slowly inject solution to the cord and limit further exacerbation of the injury site. Alternatively, multiple groups have created other ch'abc delivery methods in order to bypass the more long-term invasive catheter system. Examples of this include a slow-release concentrated fibrin gel for slow ch'abc delivery (Hyatt et al., 2010), and a thermostabilized form of ch'abc using sugar trehalose and a hydrogel microtube scaffold system (Lee et al., 2009). All effectively prolong the activity of ch'abc in the spinal cord resulting in greater CS-GAG cleavage. Additionally, an adeno viral tet-on ch'abc vector (Curinga et al., 2007), and mammalian cells modified to secrete ch'abc (Muir et al., 2010; Kluppel, 2011) appear to be promising alternatives to a multiple-injection delivery paradigm though they have yet to be tested in vivo. CS-GAG cleavage by a lentiviral vector encoding ch'abc has been tested both in vitro and in vivo (Jin et al., 2011). Results found both enhanced CS-GAG cleavage and neurite outgrowth in vitro and substantial CS-GAG cleavage in vivo. Although this study did not address in vivo axonal growth, these results suggest that vector-based ch'abc administration have potential therapeutic implications.

Chondroitin sulfate glycosaminoglycan turnover rate

Understanding the turnover rate for CS-GAGs after ch'abc cleavage is a critical consideration regarding treatment duration; the quicker the turnover rate, the shorter ch'abc effects will last and the longer ch'abc needs to be delivered. Unfortunately, this issue is only partially understood as there is no study that has focused specifically on this issue. However, multiple papers have partially addressed it through assessment of

immunoreactivity of either intact CSPGs (CS56 antibody) or 2B6 sugar stubs (ch'abc cleavage byproduct) at the lesion site after ch'abc digestion. Multiple investigators have looked 10-14 days after a single ch'abc treatment, with the results indicating a continued decrease in the amount of intact CSPGs present (Yick et al., 2000), as well as the continued presence of 2B6 sugar stubs (Cafferty et al., 2008; Tom et al., 2009a; Siebert et al., 2011). These combined findings suggest that CS-GAGs remain cleaved and do not completely turnover within a two week period. However, by three weeks post-injury intact CSPG reactivity appears to return back to levels comparable to a vehicle treated animal (Hyatt et al., 2010). While these studies provide valuable clues, it is difficult to make conclusions from these results alone. The continued presence of 2B6 sugar stubs at the lesion site two weeks after injury does not strongly confirm that CSPG turnover has yet to occur, but instead indicates that the stubs from previous ch'abc cleavage have yet to be cleared. Meanwhile, new CSPGs also may be present, but not detectable with the 2B6 antibody. Determining the immunoreactivity for intact CSPGs, as was done by Yick and colleagues, would allow for more accurate assessment.

Delivery period

Determining the optimal delivery period is critical for progressing ch'abc to the clinic. While the majority of ch'abc studies begin treatment the same day as injury, there have been several investigators who have assessed delivery at more clinically relevant, chronic time points. A study completed by Garcia Alias and colleagues was one of the few to assess this using ch'abc alone and no additional interventions. CST regeneration, recovery of contact placing, and stride length were all similar across the acute rats treated at the time of injury, and the delayed rats treated at two, four, or

seven days post-injury. However, skilled reaching performance was significantly better in the acutely treated group. These findings suggest that ch'abc has the potential to be effective in chronic injuries, but that recovery of certain behaviors respond differentially to different treatment periods (García-Alías et al., 2008). Ch'abc treatment delayed by four weeks after an incomplete injury has shown enhanced CST axonal growth (Iseda et al., 2008) and rescue of injured red nucleus neurons (Carter et al., 2011). However, enhanced axonal growth was not present following a larger contusion suggesting that ch'abe effectiveness depends on both period of delivery and lesion size (Iseda et al., 2008). Studies combining ch'abc with either a peripheral nerve graft and glial cell-line derived neurotrophic factor (GDNF) at eight weeks post-injury (Tom et al., 2009b), or stem cell/progenitor cell transplants and GDNF at six weeks post-injury (Karimi-Abdolrezaee et al., 2010) however show enhanced plasticity and recovery of both basic (Tom et al., 2009b) and skilled locomotion (Karimi-Abdolrezaee et al., 2010) despite having larger injuries. These results suggest that for ch'abc application to be most effective at chronic time points and in larger lesions, a combinatorial therapy approach is ideal.

The Important Role of Tract Tracing

Much of what we know about nervous system anatomy and plasticity after injury is a result of tract tracing; it is a powerful technique that allows researchers to understand both the anatomy of their tracts of interest, as well as the responses of those tracts to insult. In regards to enhancing recovery after SCI, this technique is necessary for understanding the circuitry changes that underlie certain functional changes in order to identify what therapeutic and training methods are effective. The Nauta Silver degeneration stain developed in the 1950's, was the first widely used method of

anterograde tract tracing (Nauta and Gygax, 1954). This was followed by the retrograde tracer horseradish peroxidase in the 1970's (Nauta et al., 1975). A wave of new tracers were then developed including but not limited to biotin dextran amines, phaseolus vulgaris-leucoagglutinin, fast blue, and fluorogold. Each tracer comes with its own advantages and disadvantages, and the choice regarding which one to use for a specific study is dependent on the study paradigms and goals (For review see Lanciego and Wouterlood, 2011). Tract tracing in larger animal models, as performed in this work, is difficult. The tracing distance is often longer, and in some cases certain tracers do not work properly. For example, pseudorabies virus traces beautifully in rodents (Lane et al., 2011) and ferrets (Jian et al., 2005), but is unsuccessful in cats. Due to the valuable nature of tract tracing, improving tracing techniques for both small and large animal models would be beneficial for future studies in SCI research, as well as other neuroscience field.

CHAPTER 2
DIFFERENTIAL RECOVERY OF GAIT FEATURES DURING BASIC AND ADAPTIVE LOCOMOTION FOLLOWING SPINAL HEMISECTION IN THE CAT

Introduction

Although it is generally understood that human incomplete spinal cord injuries (SCI) like Brown-Sequard Syndrome (BSS) result in substantial functional recovery, the assessment of this recovery is typically limited to basic locomotion such as walking on a flat, unimpeded surface at a comfortable speed (Schwab and Bartholdi, 1996; Dietz et al., 1998; Rossignol et al., 1999). Reports in both feline incomplete SCI models (Helgren and Goldberger, 1993; Tester and Howland, 2008; Jefferson et al., 2011) and some patients with incomplete SCI (Barbeau et al., 2002; Capaday, 2002; Ladouceur et al., 2003) indicate that more difficult locomotor tasks requiring adaptations of limb trajectories and balance responses lead to poorer recovery. Gait features necessary for the performance of these more complex locomotor tasks have not been thoroughly examined. There is no standard battery to assess the adaptive features necessary during locomotion to adapt to one's environment or one's behavioral goals after an SCI. In the current study, spatiotemporal features, ipsilateral limb targeting and maintenance, hind-to-hindlimb coordination, and trunk and distal limb control were determined both before and after a low thoracic spinal hemisection in cats as they crossed a flat 30.5 cm wide overground walkway requiring minimal alterations to the basic intraspinally-controlled stepping pattern. These features also were determined for the same animals as they were challenged to cross a 30.5 cm wide horizontal ladder requiring greater supraspinal input, limb accuracy, enhanced balance, and postural stability (Bolton et al., 2006; Beloozerova et al., 2010a). With different neural control mechanisms contributing to successful locomotion across 1) a wide level pathway and 2) a horizontal ladder, the

goal of this work was to determine the necessary components of recovery based upon specific task requirements and demands. Developing a test battery that can effectively identify differences in recovery patterns across locomotor tasks will be valuable in assessing new interventions and therapies promoting recovery after motor incomplete SCI.

Methods

All procedures involving animals were performed in agreement with the NIH guidelines for the care and use of experimental animals, which were approved by both the Malcom Randall VA Medical Center and the University of Florida Institutional Animal Care and Use Committees. A total of six cats, with similar lesions, were used in this study to characterize performance and recovery of basic walking on an overground walkway and adaptive locomotor features required for crossing of a horizontal ladder.

Subjects

Cats were purpose bred, SPF, spayed, adult, females. Spays were performed to remove potential hormonal effects on lesion magnitude and behavioral performance (For review see Sribnick et al., 2003; Sribnick et al., 2005). Prior to injury, all animals were trained to perform a number of tasks as in our previous studies (Tester and Howland, 2008). Performance of two of these tasks, 30.5 cm wide, flat runway (overground walkway), and a 30.5 cm wide horizontal ladder were assessed for the current study. Once cats were able to consistently perform all tasks, baseline data was collected. Following injuries, training continued and performances were recorded periodically across five months.

Surgical Procedures

T10 spinal hemisection

Detailed surgical procedures and post-op care are detailed in prior reports (Howland et al., 1995b, a; Tester and Howland, 2008). In brief, fascia and musculature were cleared from T10, after which a laminectomy was performed. The left half of the spinal cord was cut using iridectomy scissors. Durafilm and gelfoam were placed over the lesion site and muscle and skin were closed with absorbable sutures.

Behavioral Tasks, Training Paradigm, and Gait Features

Cats were conditioned to perform locomotor tasks for food rewards. Tasks ranged from simple to challenging: Bipedal (hindlimb) stepping on a treadmill and crossing of a 30.5 cm wide overground walkway, horizontal ladder, peg walkway, and 5 cm wide narrow beam (refer to Tester and Howland, 2008 for full description of tasks). Training continued post-SCI, beginning the second day after injury. Specifically, cats were trained to step bipedally (hindlimbs) on a treadmill, in addition to one of the other tasks, which alternated equally every day for 22-35 weeks after injury. The current study focused on performances on the basic overground runway (30.5 cm wide x 4.5m long), and the horizontal ladder (30.5 cm wide x 4.5 m long, with rungs 2.5 cm wide and spaced 15 cm apart) (Figure 2-1).

Overall assessment of locomotor recovery

A daily log was kept to qualitatively monitor recovery and changes in locomotor characteristics. For quantitative assessment, animals were filmed on each of the tasks using a 3D pan and tilt system (Peak Vicon®) at 60Hz. Baseline data was collected prior to injury. Post injury, performances were filmed at two weeks, 4 weeks and then monthly for at least 20 weeks. Behavioral assessments included onset of task

recovery, limb accuracy, paw placement on ladder rungs (phalanges versus metatarsal), step cycle duration, stride length, footfall patterns, % of stepcycle spent in stance (%stance), double support period, iliac crest mediolateral movement, interval between toe down and positive support, and maximum ankle flexion at yield. These gait features were based on the animal's performances on the overground walkway and horizontal ladder at pre-injury, four, and twenty weeks post-injury. Spatiotemporal analyses were based on 10 steps taken from crossings of similar speeds and were assessed using Motus Software (Vicon Peak®). Overground runway analysis for one of the cats was collected at 22 weeks post injury due to technical issues with filming at 20 weeks.

Onset of recovery

Onset of recovery was defined as the number of days for cats to recover the ability to perform three crossings independently.

Limb accuracy

The number of times each cat was able to accurately place and maintain its ipsilateral hindlimb on a horizontal ladder rung, termed "limb accuracy", was determined from the three best crossings pre-injury as well as four and twenty weeks post injury. These crossing were chosen based upon speed and best performance.

Crossing speed

Crossing speeds were calculated using markers set ~1/2 meter from the end of each walkway and the time required to traverse this known distance (meters/second; m/s). Exclusion of runway ends from these calculations removed acceleration and deceleration affects which occur at the beginning and end of each runway crossing,

respectively. Crossings used for this assessment were those in which the cat was crossing at its typical speed.

Percentage of abnormal foot positioning on rungs

Cats normally walk on their toes (phalanges) during crossing of the basic overground walkway and the horizontal ladder. However, after injury cats also positioned their ipsilateral paw abnormally on the rung, on the metatarsals. For each animal it was determined whether they used an abnormal paw placement and if so, the percentage of times they abnormally placed was calculated.

Step cycle duration

The number of fields for a single ipsilateral hindlimb step cycle to occur was determined, and then converted to seconds, with each field being equivalent to 0.0166 seconds. The cycles/second was then calculated and averaged.

Stride length

Stride length was defined as the length between toe touch downs of the ipsilateral hindlimb. These distances (cm) were determined using the transformed “R” coordinates calculated in Peak Motus, which measures movement in the horizontal axis with respect to the camera, located perpendicular to the walkway.

Footfall patterns

The number of frames in which the contralateral and ipsilateral hindlimbs were in stance (limb in contact with the walking surface) and swing while walking across the overground walkway and horizontal ladder were determined and depicted in diagram form to determine changes in interlimb coordination.

For the horizontal ladder, each rung was assigned a number based on the first rung stepped on. Each time a cat was in stance on a rung, that rung’s assigned number

was recorded onto the footfall pattern diagram. This allowed for an understanding of both the rung skipping pattern of cats prior to and after injury, as well as how often or infrequently cats paired hindlimbs onto the same rung.

Stance percentage

The number of fields the ipsilateral and contralateral hindlimbs were in stance were separately determined for ten step cycles, then the total number of fields for each of the steps for each separate hindlimb was determined. The percentage of fields that were in stance as compared to the entire step was calculated for each hindlimb.

Double support period

The number of fields in which the right and left hindlimbs were in stance together was determined and converted to seconds, with each field equaling 0.0166 seconds.

Iliac crest mediolateral movement

Using the transformed “X” coordinates, which measures movement occurring in the field-of-view plane, the greatest value of the ipsilateral iliac crest in the left direction with respect to the animal’s trunk, was subtracted from the greatest value of the same iliac crest in the right direction for the swing and stance phases, separately.

Duration to support after toe down

The maximum ankle flexion occurring after initiation of stance (toe down) was determined. Then, the number of fields taken to reach the point at which the limb began to extend after this maximum flexion was determined. This was then converted to seconds, with frames being equivalent to 0.0166 seconds.

Ankle flexion during yield after toe down

The maximum ankle flexion occurring after initiation of stance (toe down) was determined.

Tissue Processing

Details of these procedures are described in our previous work (Tester and Howland, 2008).

Perfusions and tissue preparation

At 22-35 weeks after injury cats were deeply anesthetized with an overdose of sodium pentobarbital (>40mg/kg, i.p.). Any supplemental doses were given I.V. Heparin (1cc;1000U/i.v.) was then administered and 20 minutes later 1cc of 1% sodium nitrite IV given. Immediately following injection of sodium nitrite, cats were transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The spinal cords were dissected, blocked, and post-fixed in 30% sucrose and 4% paraformaldehyde (pH 7.4). Lesion blocks were cut on a cryostat at 25 µms and stored in 0.1 M Phosphate Buffered Saline (PBS; pH 7.2, saline 0.9%) at 4º Celsius.

Histology: Cresyl violet and myelin staining

Every 10th section through the lesion block was mounted onto a Superfrost/Plus slide (Fisher Scientific©) subbed with chrom-alum and poly-L-lysine (chromium potassium sulfate and poly-L-lysine, Sigma-Aldrich®; gelatin, Fisher Scientific©). Sections were fume fixed on the slides with 4% paraformaldehyde. Tissue was rinsed in water, then placed in increasing alcohol concentrations (70-100%, 6-10 mins each) and finally, in xylene (10+ mins). Tissue then was rehydrated in decreasing alcohol concentrations, placed in myelin dye (Eriochrome Cyanine R; Fluka™; 10 mins) followed by dye differentiation in 1% ammonium hydroxide. After extensive rinsing, slides were placed in 0.5% cresyl violet (cresyl violet with acetate, Sigma-Aldrich®) for three minutes followed by differentiation with 1% glacial acetic acid in 75% alcohol. Tissue was then

completely dehydrated in increasing alcohol concentrations, followed by xylene (10+ mins), and coverslipped with DPX (Fluka™).

Statistical analysis

Using Analyse-It Software (Microsoft Excel®), the Kruskal-Wallis test was bonferroni corrected in order to compare crossing speeds within tasks and across time points. The Friedman test was used to compare the task onsets for both tasks, as well as the pre-injury values across both tasks for crossing speed. The Mann-Whitney U test was used to compare metatarsal placement percentage and success across the different groups of cats. A p-value of <0.05 was set to determine significance.

Results

Lesion Magnitudes

Cresyl violet and myelin stained sections through the lesion epicenters of each animal were used to determine the extent of tissue sparing and damage. The representative cross-sectional drawing created for each animal from review of multiple sections, showed that the lesions of the six cats were very similar (Figure 2-1). Typically, complete disruption of the ipsilateral gray and white matter was seen with an exception in one cat. Contralateral gray and white matter was completely spared with the exception of some superficial white matter dorsally in four of the six cats. Thus, lesion variability was minimal. The similarity in lesion magnitudes minimized the possibility of differences in functional recovery being related to differences in spared tracts.

Recovery Onset and Effects of Injury on Crossing Speed

Performance both prior to, and after a thoracic hemisection was compared on the overground walkway (Figure 2-2A) and horizontal ladder (Figure 2-2B). Acutely

following injury, all cats showed the expected ipsilateral hindlimb flaccidity followed by some voluntary movement within 48 hours. During this period, the contralateral hindlimb showed good range of motion and substantial or full weight support. On average, cats began independently crossing the overground walkway by three days after injury (Figure 2-2C). During these crossings, the ipsilateral hindlimb began showing partial stepping movements. Collectively, these results are consistent with our prior works and the reports of others (Eidelberg et al., 1986; Helgren and Goldberger, 1993; Tester and Howland, 2008; Jefferson et al., 2011). The ability to independently cross the ladder occurred significantly later than on the overground walkway (Figure 2-2C; p=0.014, Friedman test). On average, cats began crossing the horizontal ladder without assistance at 18 days post-SCI. This was two weeks later than on the overground walkway. The range in initiation of horizontal ladder crossing (15-25 days) also was much greater than seen for overground walkway (two-eight days).

Prior to injury, the typical comfortable crossing speed for all cats were significantly faster on the overground walkway than the horizontal ladder (Figure 2-2D; p=0.014, Friedman test) which is consistent with studies of normal cats (Beloozerova et al., 2010a). After injury, all crossing speeds were significantly slower from pre-injury on the overground walkway at both four (Figure 2-2D; p=0.030, Kruskal-Wallis test) and 20 weeks after injury (Figure 2-2D; p=0.003, Kruskal-Wallis test), and on the horizontal ladder at both four (Figure 2-2D; p=006, Kruskal-Wallis test) and 20 weeks after injury (Figure 2-2D; p=0.003, Kruskal-Wallis test). Evaluation of the absolute changes in crossing speeds from pre-injury values was not significantly different between the locomotor tasks at either post-injury time points (Figure 2-2E).

Phalangeal versus Abnormal Metatarsal Placement onto Ladder Rungs Post-Spinal Cord Injury

Pre-injury, all cats effectively placed their hindlimbs on the ladder rungs. This ability was disrupted for the ipsilateral hindlimb following injury. Over time, the ability to place the ipsilateral hindlimb began to recover. By 20 weeks cats showed an average contactt rate (contact with rung) of 69.3% and an average effective placement rate (contact and maintenance of limb position on rung) of 56.7% (Figure 2-3A). Similar to crossing of the peg walkway described in our prior work (Jefferson et al., 2011), cats crossed the horizontal ladder using either a three or four limb strategy. Typically, a three limb strategy was employed early after injury when cats lacked the ability to accurately target with their ipsilateral hindlimbs. However, as recovery continued, animals began to incorporate their ipsilateral limbs more frequently and a four limb strategy emerged in some animals. Half of the cats evaluated (3/6) were capable of effectively placing and maintaining their ipsilateral hindlimb >75% of the time on the ladder rungs by 20 weeks (75%, 100%, 100%). The other three cats were only able to contact and maintain their ipsilateral hindlimb onto a ladder rung <40% of the time at this chronic time point (12%, 13%, 40%). Thus, the cats fell out into two groups; Group 1 with ineffective limb placements and Group 2 with effective limb placements.

To determine potential differences that might contribute to accurate paw placement and maintenance, frame by frame analyses of toe touch down through weight bearing was done for both groups of cats. Prior to injury, animals stepped onto and weight supported on ladder rungs with their phalanges (on their toes; Figure 2-3B). After injury, two alternate patterns emerged. The first pattern, which occurred less often, consisted of the paw not being brought forward enough and placing on the tip of a

single phalanx. The second, more predominant pattern was placement onto the metatarsals (MT) (Figure 2-3C). The percent of paw placements characterized by positioning on the metatarsals (% MT) of all placements showed a similar range in the lower (Group 1) and higher (Group 2) paw placement performing groups at four (group 1=36-100%, group 2=23-69%) and 20 weeks (group 1=38-85%, group 2=17-95%) (Figure 2-3D,E). However, there was a distinct difference in the two group's abilities to maintain this abnormal placement on the rung as the limb began to weight bear. At four weeks, Group 2 cats were able to maintain their ipsilateral hind paws on the rungs during a significantly greater percent of the placements with abnormal metatarsal positioning (% MT Success) compared to Group 1 cats (Figure 2-3; p=0.05, Mann-Whitney U test). This effect continued to the 20 week post-injury period. These results suggest greater deficits, possibly proprioceptive, in the Group 1 cats.

To further understand crossing recovery of more highly functioning animals characterized by effective paw placement on the horizontal ladder, the remaining analyses focused on the Group 2 cats as they provided a sufficient number of contiguous ipsilateral steps for evaluation. The lesion epicenters of the Group 2 cats are shown in the second row of Figure 2-1.

Injury Effects on Cycle Duration and Stride Length

To understand what step cycle features might contribute to slower crossing speeds, cycle duration and stride length of both the contralateral and ipsilateral hindlimbs were assessed. Prior to injury, and consistent with normal control results from Beloozerova and colleagues, there were no differences in cycle duration between crossings of the overground walkway and horizontal ladder (Figure 2-4A) (Beloozerova et al., 2010a). This may differ across species as the duration of the rat's step cycle is

reported to be longer on the ladder compared to overground (Bolton et al., 2006). At four weeks after injury, all three cats showed changes in their cycle durations (Figure 2-4B). During overground the direction of this change (faster or slower) was mixed across cats (Figure 2-4B). In contrast, all had slower cycle durations on the horizontal ladder. At 20 weeks on overground walkway, cycle duration had almost returned to normal with the absolute change in cycle duration approaching zero. The direction of even this minimal change continued to be different across cats at this time point. In contrast on the horizontal ladder, all cats continued to show slower cycle durations compared to pre-injury and the average absolute change in cycle duration from pre-injury was greater on horizontal ladder compared to on the overground walkway (Figure 2-4B).

Pre-injury, stride length was shorter on horizontal ladder compared to overground walkway (Figure 2-4C). This was most likely a result of rung spacing on horizontal ladder. At four weeks after SCI, some cats had shorter stride lengths, while others had longer ones on both tasks. At 20 weeks, all cats had shorter stride lengths on overground walkway, while on horizontal ladder there continued to be variability in the direction of change. There was no difference in absolute change from pre-injury across tasks (Figure 2-4D).

Effects of Injury on Interlimb Coordination, Stance Duration, and Double Support

Pre-injury, stepping patterns of the hindlimbs on the overground walkway and horizontal ladder were similar (Figure 2-5A,B) which is consistent with reports in a normal, uninjured system (Beloozerova et al., 2010b). Both right and left hindlimbs alternate in a 1:1 fashion. However, after an SCI distinct differences were seen between overground and ladder crossing patterns. On overground walkway, the 1:1 pattern primarily was preserved at both four (Figure 2-5C) and 20 weeks after injury

(Figure 2-5D); however this pattern was disrupted on the ladder (Figure 2-5E-J). At four weeks the contralateral hindlimb of all three cats had a higher stepping frequency relative to the ipsilateral hindlimb. The stance phases of both limbs were generally increased and there was a greater overlap of the stance phases (Figure 2-5E-G). By 20 weeks, a 1:1 stepping ratio was recovered in one of the three cats (Figure 2-5H), while the other two adopted a 2 contralateral:1 ipsilateral ratio (Figure 2-5I,J). The stance phase of the contralateral hindlimb remained increased in all cats (Figure 2-5H-J). Assessment of the rungs used during horizontal ladder crossing identified further alterations in hind-to-hindlimb coordination after injury. Prior to injury, each cat placed their hindlimbs onto every third rung, skipping over two with each swing phase. Additionally, the ipsilateral and contralateral hindlimbs were placed on different rungs (Figure 2-5B). After injury, this pattern was lost. At four weeks, the contralateral hindlimb switched to a relatively consistent pattern of stepping on every second rung, skipping over one. This pattern was seen in each of the three cats (Figure 2-5E-G). The ipsilateral hindlimb in all three animals inconsistently made contact on every three to five rungs, skipping over two to four rungs, but primarily missed the rungs during these attempts (Figure 2-5E-G). Cats were most successful at accurately placing when their ipsilateral hindlimb targeted every other rung, skipping only a single rung. Cats frequently stepped onto the same rung with both hindlimbs which was not seen pre-injury (Figure 2-5B).

By 20 weeks post injury, unique patterns in regards to hind-to-hindlimb coordination and rung placements were seen in each cat. In the first example shown (Figure 2-5H), the cat uses a 1:1 stepping ratio similar to what was seen pre-injury.

However, the hind-to-hindlimb coordination differed greatly from pre-injury; the contralateral and ipsilateral hindlimbs placed onto every other rung, used the same rungs, and typically shared a rung during part of the support phase. The contralateral hindlimb strategy seen at four weeks continued to be used at 20 weeks post injury, placing on every other rung, skipping one. The two other cats used a different approach for crossing the horizontal ladder (Figure 2-5I,J). Their approach showed similarities to the pattern seen at four weeks post injury. Between two and four rungs were skipped by the ipsilateral hindimbs (placing onto every third to sixth rung). However, in contrast to similar attempts made at four weeks post injury, they were successful in accurately placing their ipsilateral hindlimb onto the rungs most of the time. The footfall pattern of the contralateral hindlimb remained identical to the pattern exhibited at four weeks post injury, placing onto every second rung and skipping only one rung. This footfall pattern showed frequent sharing of a rung with the contralateral hindlimb. Collectively, these results show that novel, yet consistent foot placement strategies and hind-to-hindlimb coordination emerged during recovery of horizontal ladder crossing in these three cats. Different cats used different strategies demonstrating that there are multiple ways this task can be successfully completed after injury.

Qualitatively, the footfall pattern analysis suggested there were changes in the support (stance) phase on both locomotor tasks. To assess this, stance as a percentage of the step cycle and hindlimb double support periods were determined. Prior to injury, the percent of time each hindlimb spent in stance over the course of an entire step was similar within a task, but different across the tasks. During horizontal ladder crossing, both hindlimbs showed increased periods of stance relative to

overground walkway in normal, uninjured cats (Figure 2-6A). This increase was consistent with a greater double support period during horizontal ladder compared to crossings of the overground walkway (Figure 2-6C).

Four weeks post-injury, the contralateral hindlimb stance period increased in all three animals during both overground and ladder compared to pre-injury (Figure 2-6B). This was in contrast to the ipsilateral hindlimb which showed relatively little absolute change on either task (Figure 2-6B). The direction of that change from pre-injury was increased in all cats in the contralateral hindlimb, but was increased in some cats and decreased in others in the ipsilateral hindlimb. At 20 weeks post injury, this trend continues with the absolute change in stance period from pre-injury remaining higher in the contralateral hindlimb compared to the ipsilateral hindlimb.

Pre-injury, the period of double support was greater during ladder crossing than on the overground runway (Figure 2-6C). Following injury, the absolute change in the double support period of the hindlimbs showed a very small increase during overground walking. The double support period and absolute change was much greater during ladder crossings compared to overground walkway locomotion at both four and 20 weeks post-injury (Figure 2-6D).

Mediolateral Movement

To determine the effects of SCI on proximal stability, and how this differs across basic and skilled tasks, the change in the range of mediolateral movement of the top of iliac crest was assessed during both overground walkway and horizontal ladder crossings. Prior to injury, mediolateral movement was similar across the two tasks during both the stance and swing periods (Figure 2-7A). Movement was tightly controlled within a ~1.5-2 cm range. Following injury, a ~25% and 50% change in the

range was seen during stance and swing respectively on overground walkway (Figure 2-7A). While all cats had increased mediolateral movement at 4 weeks during stance, the change in direction from pre-injury varied across cats during swing at 4 weeks post-injury, and both swing and stance at 20 weeks after injury. In contrast, mediolateral movement was greater during horizontal ladder crossing compared to overground walkway. This suggests that the cats have much greater difficulty controlling the midline position of their hindquarters during horizontal ladder crossing post-injury in comparison to traversing the overground walkway. Additionally, in contrast to behaviors on the overground walkway, all cats had the same direction of change from pre-injury on horizontal ladder at both timepoints and phases of the step cycle (Figure 2-7B). Interestingly, while the greatest change from pre-injury occurred during the swing phase on overground walkway, it occurred during the stance phase on horizontal ladder (Figure 2-7B). Closer, frame-by-frame examination of this effect on horizontal ladder showed that the majority of iliac crest movement occurred as the contralateral hindlimb was in swing; the entire trunk would shift to the contralateral side. These findings further suggest the importance of the contralateral hindlimb during the performance of the horizontal ladder after injury.

Distal Joint Stability

Distal control of the limb was assessed in two ways: 1) assessment of the period duration between toe touchdown (stance initiation) and initiation of positive support (indicated by ankle extension) and; 2) maximum ankle flexion at yield. Prior to injury, there was no significant difference in the period duration from toe touchdown to positive support between the overground walkway and horizontal ladder tasks (Figure 2-8A). After injury, some cats increased the duration to weight support and others decreased

this period on overground walkway, while on horizontal ladder all cats increased this duration. The greatest average absolute changes however, were seen during overground walkway crossing. The magnitude of the changes was greater at 20 weeks post-injury on the overground walkway compared to the horizontal ladder (Figure 2-8B).

Prior to injury, maximum ankle flexion during yield just prior to weight support was similar during the overground walkway and horizontal ladder tasks (Figure 2-8C). After injury, ankle angular degrees increased in some cats while decreasing in others during crossing of the overground walkway. In contrast, all cats showed an increase in ankle angular degrees while crossing the horizontal ladder at both four and 20 weeks after injury, indicating a decrease in ankle flexion at these time points. The change from pre-injury was decreased on horizontal ladder compared to the overground walkway at 20 weeks (Figure 2-8D).

Discussion

Overall, results from this study demonstrate that there are several differences across the performance of overground walkway and horizontal ladder. Several features were different across these two tasks in the normal cat prior to injury. These features include crossing speed, stride length, stance percentage, and double support period. For some of these features, like crossing speed and double support period, SCI resulted in an amplification of these differences across tasks. The differences seen in stride length prior to injury remained similar after injury. Some of the greatest changes seen after injury occurred in the contralateral hindlimb. Prior to injury, stance percentage on horizontal ladder was longer in both hindlimbs compared to overground walkway. This effect was lost after injury in the ipsilateral hindlimb, but magnified in the contralateral

hindlimb resulting in extreme asymmetry across these two limbs. This asymmetry was amplified on the horizontal ladder task.

There also were several gait features that were similar across tasks prior to injury but became different after injury. These features include the cycle period, footfall patterns, mediolateral movement, and distal joint stability. After SCI, the cycle period became decreased in all cats on horizontal ladder but had a less drastic change on the overground walkway, with the cycle period increasing in some and decreasing in others. Footfall patterns after injury remained similar to what they were pre-injury for overground walkway, but changed drastically on the horizontal ladder. Mediolateral movement became more enhanced on the horizontal ladder after injury to a much greater extent than on the overground walkway, with a similar effect occurring for the duration to positive support in the ankle. While maximum ankle joint flexion during yield became less flexed on the horizontal ladder in all cats, it was differentially affected on overground walkway, with some cats increasing in flexion and other decreasing.

Task-Specific Spatiotemporal Gait Changes after Injury

Crossing of the horizontal ladder is accomplished more slowly than on the overground walkway. This reflects the difficulty of the tasks and the greater supraspinal contributions. In particular, ladder requires both cortico- (Metz and Whishaw, 2002; Beloozerova et al., 2010b) and rubrospinal input (Webb and Muir, 2003) which are known to play a critical role in more skillful types of locomotion. Although, there were no significant differences between the mean cycle duration across tasks, a significant decrease in stride length was seen on ladder compared to overground, indicating that on the horizontal ladder task, cats travel shorter distances in the same time period it took to travel longer distances on the overground walkway. This is most likely related to

the spacing of the ladder rungs, as they restrict the positioning of the paws, affecting stride length.

After injury, both crossing speed and cycle duration decreased during both tasks, but were more pronounced on the horizontal ladder. These enhanced decreases on horizontal ladder were accompanied by variable changes (increases and decreases) across cats in stride length on the horizontal ladder. In contrast, all cats had a decreased stride length on the overground walkway by 20 weeks. This decrease in stride length on overground walkway is a well documented affect after SCI (Barbeau et al., 1999) as well as other neural injuries (von Schroeder et al., 1995), and is typically associated with a decrease in crossing speed (Barbeau et al., 1999). The differential effects on speed and cycle duration across the two tasks most likely relates to the drastic changes in footfall patterns that occur in parallel as footfall patterns have been shown to heavily affect step length and leg propulsion (Balasubramanian et al., 2010). The novel foot fall pattern that emerges after injury on ladder facilitates increased use of the uninjured limb for support, both singly, and in double-support. Specifically, ladder crossings were characterized by significantly longer periods of support relative to swing, in addition to longer periods of double support compared to that seen during overground walking. These features would enhance balance and postural control more greatly during horizontal ladder than overground walkway performance (Hazime et al., 2012).

Trunk Support on Skilled Tasks after Injury

The enhanced mediolateral movement of the ipsilateral iliac crest particularly during stance while the contralateral hindlimb is in swing, may be related to changes in muscle activity after injury. For example, several studies have reported that active flexion of the hip during stance is responsible for increased hip and knee flexion of the

other limb in swing (Patla and Prentice, 1995). Furthermore, others have reported an increase in the activity of hip abductors and adductors activity, specifically the tensor fascia latae and adductor longus, as a way to provide additional pelvic stability and support of thigh acceleration during late stance and early swing of uninjured populations while ramp or stair walking (Gottschall et al., 2011). The rectus femoris, gluteus maximus, and contralateral obliques, which are hip and postural muscles, also have been shown to become significantly peaked during perturbed stance (Stanek et al., 2011). In combination, these results suggesting that the increase in mediolateral movement to the contralateral side may be a result of contralateral hip overcompensation. By compensating for the weakened, affected muscles of the ipsilateral limb, the contralateral limb helps to produce appropriate gait features which allow successful completion of the ladder task. Indeed, it has been reported that the musculature of the contralateral side recovers substantially quicker than the ipsilateral side after injury (Little and Halar, 1985).

Task Specific Changes in Distal Joint Positioning after Injury

Changes in ankle position after injury, particularly on the horizontal ladder, also may be related to task-specific changes in muscle activity after injury. For example, the lateral gastrocnemius, an ankle extensor and knee flexor, has been shown to have increased activity during ladder in uninjured cats just prior to the beginning of stance, and early stance (Beloozerova et al., 2010b). Additionally, the ankle plantar flexors gastrocnemius and soleus, also have been found to provide trunk support during single leg stance (Neptune et al., 2001) suggesting that prolonged activity of the ankle joint immediately after toe touchdown may be representative of a heightened need to maintain balance and postural control for target accuracy on the more challenging

ladder task. The lack of maximum ankle flexion as seen during overground crossings may not necessarily suggest normalized ankle function, but instead may be a result of increased lateral gastrocnemius, ankle extensor activity necessary for proficient ladder performance. Alternatively, the significantly greater increase in maximum ankle flexion seen on overground after toe touchdown may be a speed-related change as the cats cross overground faster than ladder. The enhanced capability for cats with a high ipsilateral hindlimb targeting percentage to maintain weight support on a ladder rung despite abnormal metatarsal paw placement compared to those with low targeting accuracy, may be related to some of the previously described changes in muscle activity. Specifically, enhanced activity of the lateral gastocnemius and soleus muscles, as described in Neptune et al., 2001, may be enhanced in these cats with higher accuracy. Furthermore, the inability to maintain weight support after abnormal paw placement in a subset of animals, suggests that these low accuracy animals may have issues with proprioception (Witchalls et al., 2012).

Conclusions

Collectively, the results from this study characterize several task-specific spatiotemporal, trunk, and distal stability features prior to injury, as well as task-specific changes to these features after injury. These findings suggest that maximization of functional recovery in incomplete SCI populations may require a strong focus on certain functional components of the hip and ankle, like increasing strength of the plantarflexors as well as the musculature of the hip, like the rectus femoris and gluteus maximus. Subsequently, the increased use of the contralateral hindlimb during successful ladder accuracy suggests an important facilitator role of this limb; BSS and other incomplete

SCI populations may strongly benefit from therapeutic strategies that focus on the contralateral limb as much as they do the more greatly affected limb.

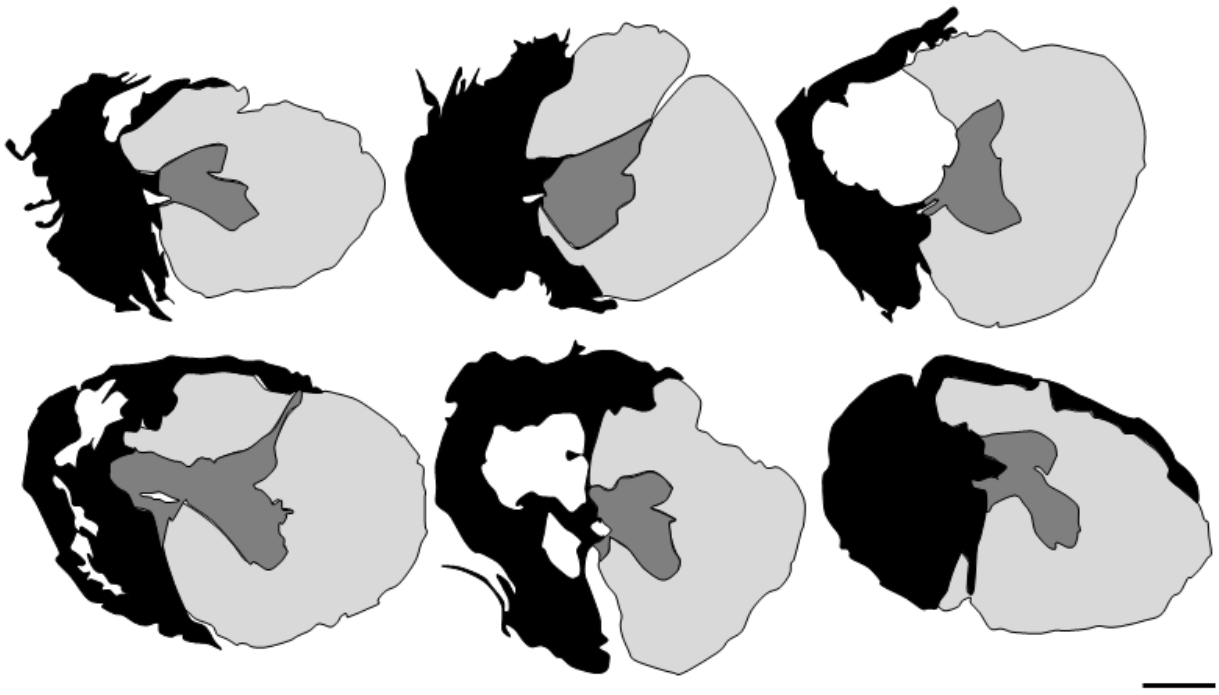


Figure 2-1. Lesion magnitudes. The amount of spared tissue at the lesion epicenter for all six cats included in the study. Scale bar is 1mm.

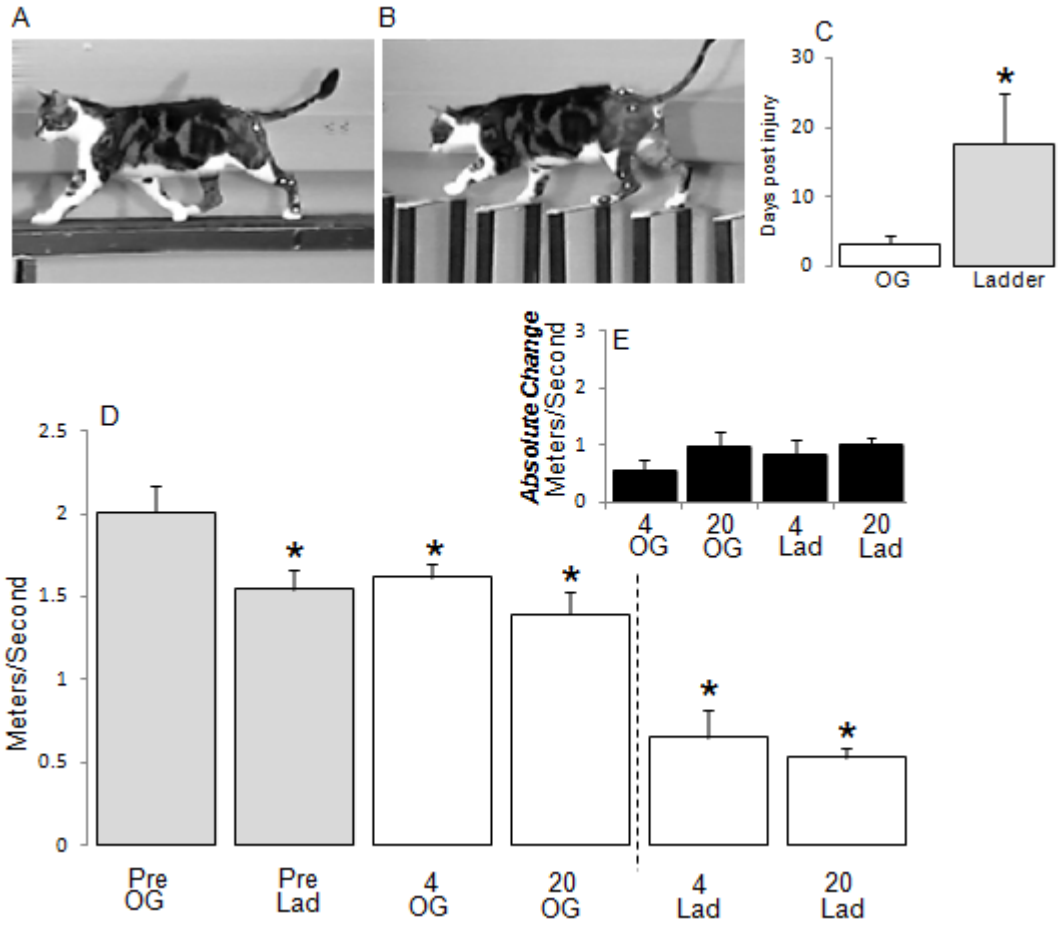
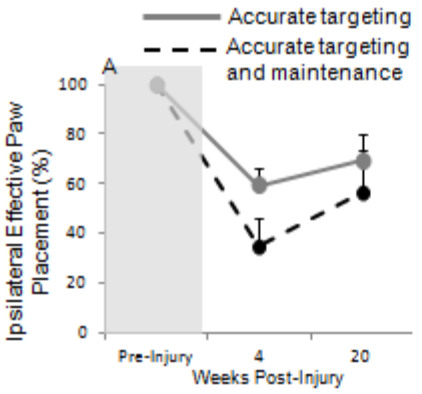


Figure 2-2. Tasks, onset of recovery, and crossing speeds. Images of the overground walkway (A) and horizontal ladder task (B), as well as the number of days required to recover these tasks after injury (C) were assessed. Pre-injury crossing speeds (D, gray bars), post-injury crossing speeds at four and 20wks after injury (D, white bars), and the absolute change in crossing speeds from pre-injury at four and 20 weeks (E) were compared ($p=<0.05$). The black bars (E) indicate when all animals had decreased values from pre-injury.



D

Midpoint	Group 1		Group 2	
	% MT	% MT success	% MT	% MT success
1SEM	100	34		
2SEM	100	15		
3SEM	36	50		
4SEM			69	94
5SEM			23	100
6SEM			58	67
Average	79	33	50	87*

E

20 wks	Group 1		Group 2	
	% MT	% MT success	% MT	% MT success
1SEM	85	13		
2SEM	45	13		
3SEM	38	89		
4SEM			73	100
5SEM			17	78
6SEM			95	70
Average	56	38	62	83

Figure 2-3. Phalangeal versus metatarsal placement onto ladder rungs. Post-injury, cats lose the ability to place and maintain their ipsilateral paw on the ladder rungs. Overtime, this shows some recovery (A). Normal, uninjured cats step onto ladder rungs with the phalangeal portion of their foot (B). However, after injury cats also used an abnormal placement approach, placing their foot onto the rung with the metatarsal (MT) portion of their foot (C). Cats were separated into groups based on their ability to accurately target and maintain their ipsilateral hindlimb on the rung (irrelevant of type of placement; phalangeal or metarsal). Group 1 consisted of cats able to accurately target and maintain ipsilateral hindlimb placement between 12-40% of the time by 20 weeks post injury. Group 2 consisted of those cats able to accurately target and maintain ipsilateral hindlimb placement between 75 to 100% of the time by 20 weeks post injury. The percentage of times cats used a metatarsal approach with their ipsilateral hindlimb when crossing the horizontal ladder (% MT) was compared across Group 1 and Group 2 cats at 4 (D) and 20 (E) weeks after injury. The percentage of times the ipsilateral hindlimb successfully remained on the rung despite a metatarsal placement (% MT Success) also was compared across these groups at both post injury time points (D,E). (*=≤0.05)

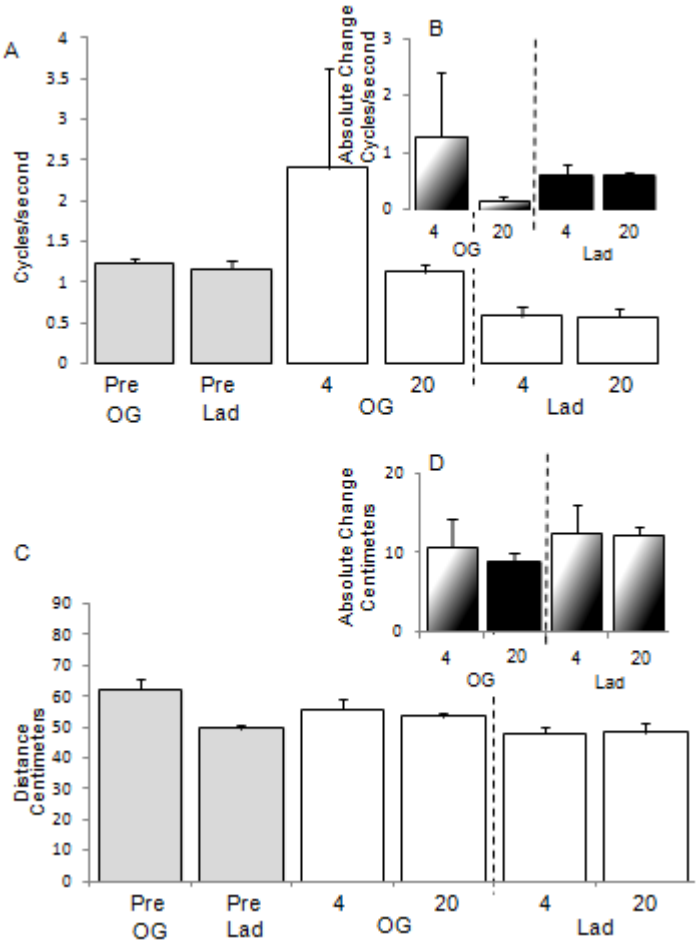


Figure 2-4. Changes in cycle duration and stride length. The average step cycle durations (A) and stride lengths (C) on overground walkway and horizontal ladder were determined prior to injury (gray bars), and at four and 20 weeks after injury (white bars). The absolute change from these pre-injury values at four and 20 weeks post injury also was determined for the cycle duration (B) and stride length (D). For absolute change graphs, black bars indicate when all animals showed decreased values from pre-injury, and bars with a black/white gradient indicate when some cats increased, and some decreased from pre-injury.

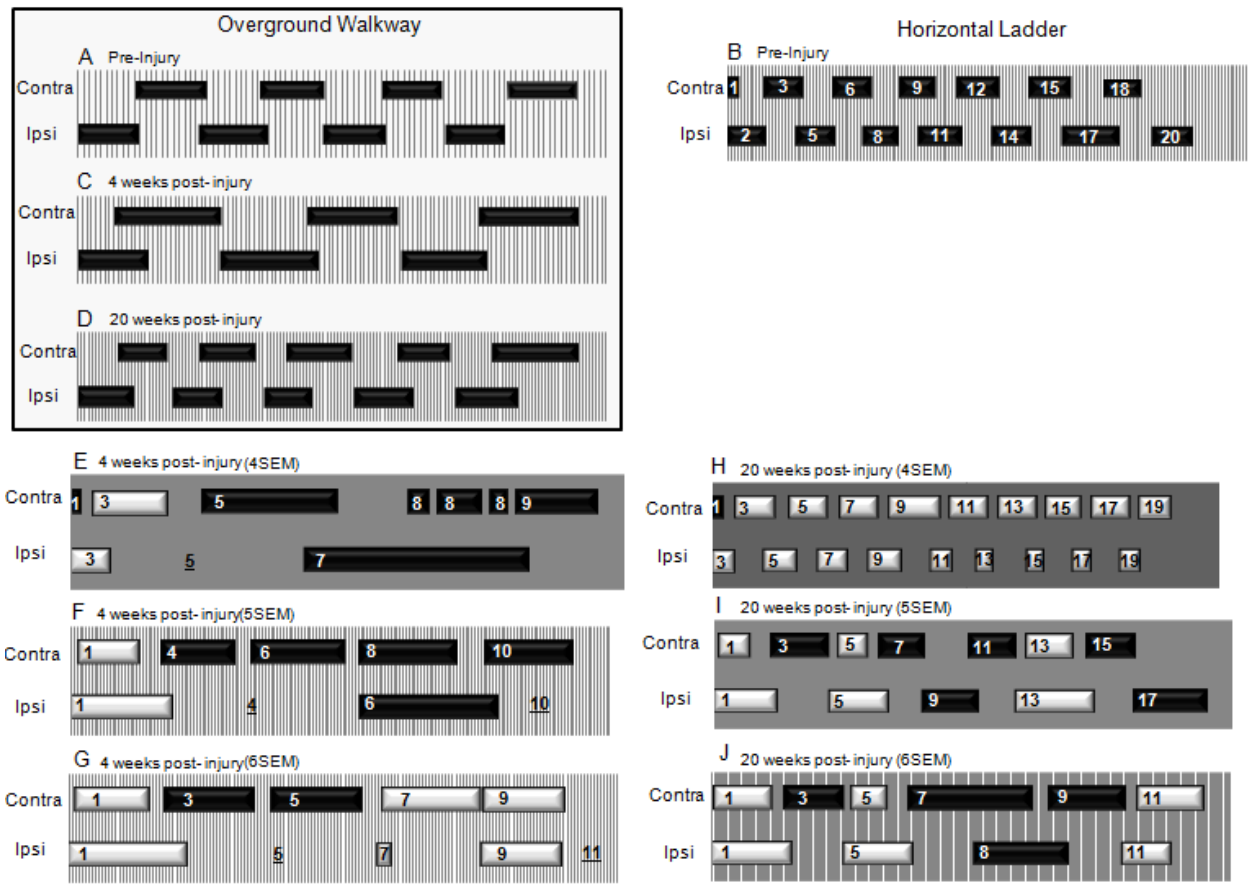


Figure 2-5. Change in hindlimb footfall patterns and hind-to-hindlimb coordination. The timing of left (ipsi) and right (contra) hindlimb foot placements on overground walkway are depicted for a representative cat at pre-injury (A), 4 weeks post-injury (C), and 20 weeks post-injury (D). Black rectangles indicate when a limb is in stance, and spaces between these denote swing. Footfall patterns for a representative cat were depicted for the horizontal ladder crossing at pre-injury (B). All cats performed similarly at this time point. The post-injury footfall patterns for each of the three cats on horizontal ladder were depicted for 4 weeks post injury (E,F,G) and 20 weeks post injury (H,I,J). Numbers inside the black rectangles for the horizontal ladder task indicate the rung that cats were standing on. Numbers outside of the black rectangles indicate a rung that the cat attempted to place their hindlimb onto, but failed. White rectangles indicate a stance period in which both hindlimbs were on the same rung simultaneously.

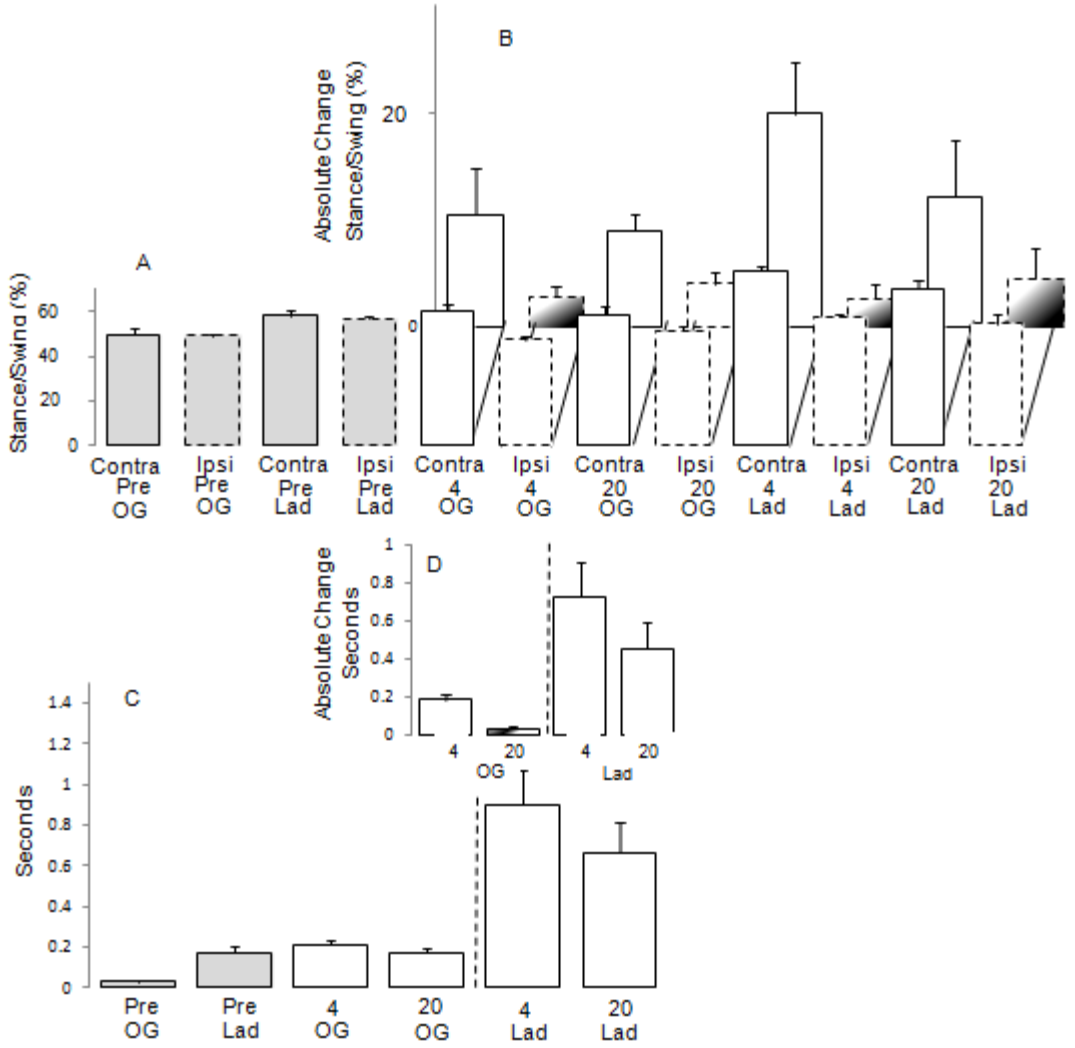


Figure 2-6. Change in hindlimb stance and double support periods. The percentage of time/step that the cat was in stance for both tasks (A) was determined for pre-injury on overground walkway and horizontal ladder for the ipsilateral (Ipsi) and contralateral (Contra) hindlimbs (G, gray bars). The same values at four weeks and 20 weeks post injury also were determined (white bars). The absolute change from pre-injury was depicted for the ipsilateral and contralateral hind limbs at four and 20 weeks after injury (B). For both tasks, the period at which the ipsilateral and contralateral hind limbs were in stance at the same time (double support period) were determined prior to injury (C, gray bars), and also four and 20 weeks after injury (C, white bars). The absolute change from pre-injury at four and 20 weeks also was determined (D). For graphs showing absolute change (B,D) white bars indicate when all animals had an increased change from pre-injury, and bars with a black/white gradient indicate when some cats increased, and some decreased from pre-injury.

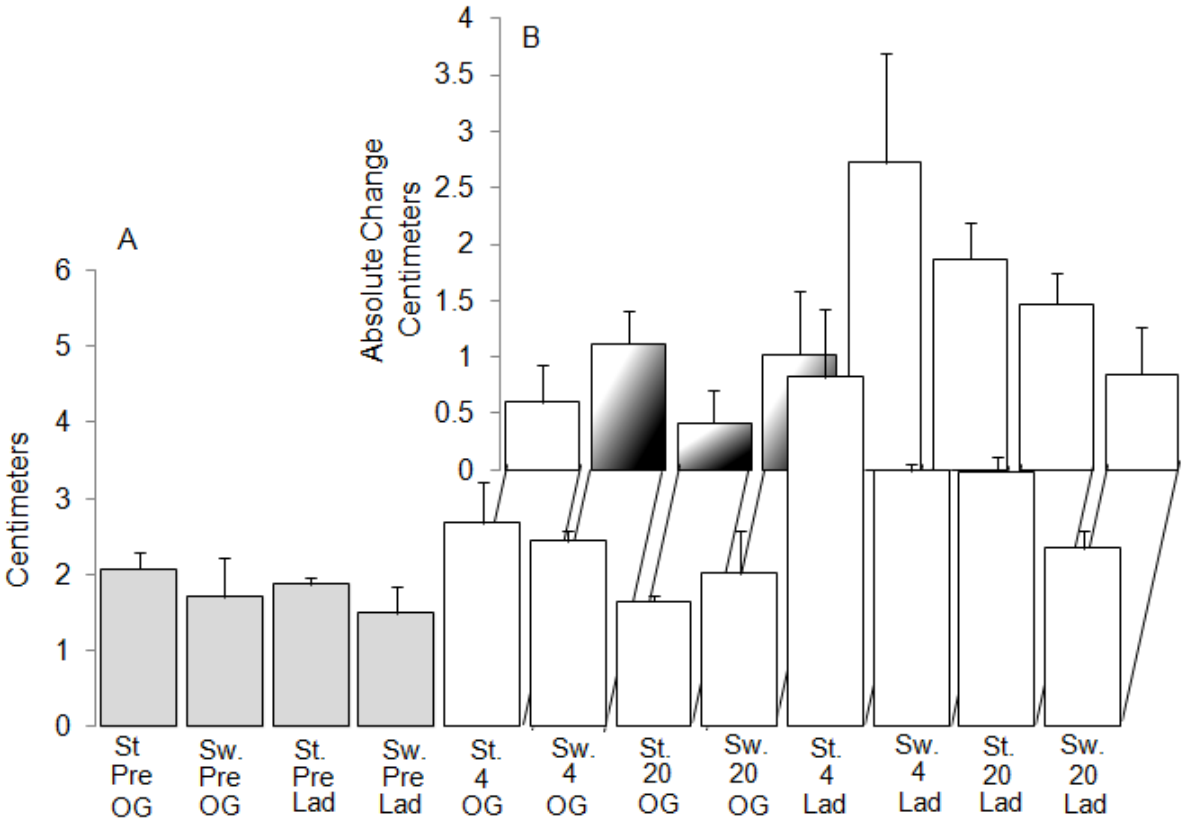


Figure 2-7. Mediolateral movement of the iliac crest. The values of iliac crest's lateral deviations during stance and swing were compared across overground and horizontal ladder performance pre-inury (A, gray bars), four, and 20 weeks after injury (A, white bars). The absolute change from pre-injury also was determined from 4 and 20 weeks after injury (B). For graphs showing absolute change, white bars indicate when all animals had an increased change from pre-injury, and bars with a black/white gradient indicate when some cats increased, and some decreased from pre-injury.

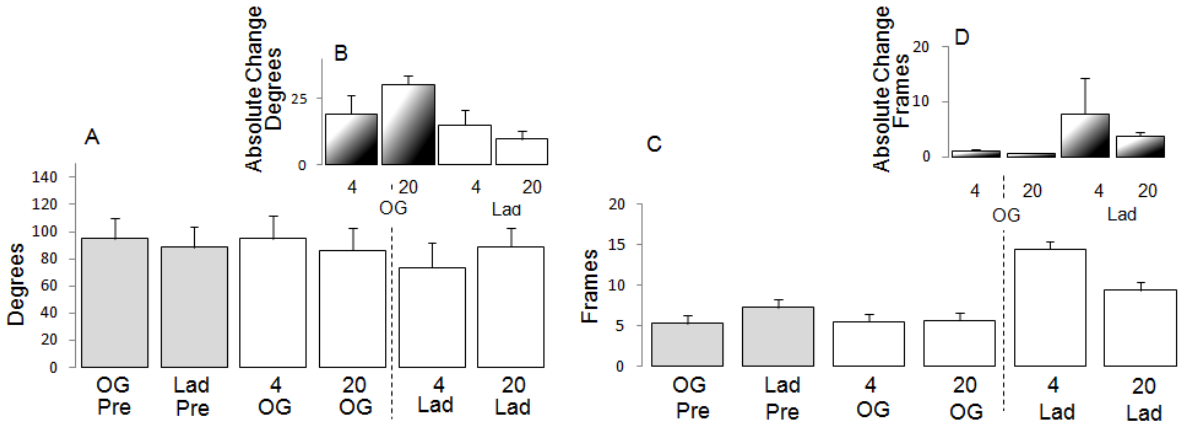


Figure 2-8. Swing to stance transition. The duration to positive support at the beginning of stance (A), and maximum ankle flexion at yield (C) were determined at pre-injury (gray bars), four, and 20 weeks after injury (white bars). The absolute change from pre-injury at four and 20 weeks after injury also was determined (B,D). For graphs describing absolute change white bars indicate when all animals had an increased change from pre-injury, and bars with a black/white gradient indicate when some cats increased, and some decreased from pre-injury.

CHAPTER 3
THE EFFECT OF DIFFERENT CHONDROITINASE ABC TREATMENT DURATIONS ON LOCOMOTOR RECOVERY AND SUPRASPINAL TRACT PLASTICITY IN A FELINE MODEL OF SCI

Introduction

Many studies (Jones et al., 2002; For review see Morgenstern et al., 2002; Jones et al., 2003a; For review see Busch and Silver, 2007; Galtrey and Fawcett, 2007), including our own (Lemons et al., 1999; Tester and Howland, 2008) have shown that after spinal cord injury (SCI) there is an upregulation of chondroitin sulfate proteoglycans (CSPGs) at the lesion site. Chondroitin sulfate glycosaminoglycans (CS-GAG), which are attached to these CSPGs, are extremely inhibitory to axonal growth and play a primary role in preventing the axonal plasticity necessary for functional recovery (Snow et al., 1990; Smith-Thomas et al., 1994; Zuo et al., 1998). Removal of these CS-GAGs using the bacterial enzyme chondroitinase abc (ch'abc) following SCI in rodent models enhances both behavioral recovery (Bradbury et al., 2002; Cafferty et al., 2008) and axonal growth (Grimpe and Silver, 2004; Barritt et al., 2006; Houle et al., 2006). The Howland laboratory has successfully translated use of ch'abc to a cat model of SCI (Jefferson et al.; Tester and Howland, 2008). These studies showed enhanced locomotor recovery and plasticity of red nucleus projections following a hemisection lesion and 4 weeks of intralesional ch'abc administration.

A multitude of studies completed in rodent models of SCI have shown beneficial effects using similar (Vavrek et al., 2007; Lee et al., 2009) and shorter ch'abc treatment durations ranging from a single injection to two weeks (Bradbury et al., 2002; Barritt et al., 2006; Cafferty et al., 2008; García-Alías et al., 2008; Iseda et al., 2008; Garcia-Alias et al., 2009). It is currently unknown how a shortened administration period will affect

locomotor recovery and anatomical plasticity in a feline model of SCI and if four weeks of treatment is necessary. Cats are a valuable translational model for ch'abc studies as they have similar pre-dominant CS-GAG lyase products after ch'abc digestion as humans (4S and 6S disaccharides), while the rat shows a single dominant (4S disaccharide) lyase product (Tester and Howland, 2008). Thus, results from this study have meaningful implications for treatment duration in regards to the advancement of this therapeutic to a clinical setting.

The upregulated temporal expression of CSPGs after injury suggests that two weeks of ch'abc expression may be sufficient for enhancing plasticity and functional recovery. In rats, peak upregulation of neurocan, brevican, and versican CSPG expression occurs at two weeks post injury, with peak neuron-glial antigen 2 (NG2) expression occurring at one week. Both neurocan and versican production then steadily decrease to only minimal expression by four weeks (Jones et al., 2002; Jones et al., 2003a). It is currently unknown whether this same trend occurs in cats, but if so, two weeks of ch'abc administration may be sufficient to enhance locomotor recovery due to the natural decrease of a substantial proportion of CS-GAGs beginning at two weeks. Furthermore, an unpublished finding from our previous work was greater recovery of some locomotor features, like accurate targeting on a horizontal ladder, by two weeks post-injury in ch'abc treated cats. This indicates that substantial plasticity has already taken place by this two week time point and that additional ch'abc administration may be unnecessary for continued functional improvements. In the current study we investigate the effects of a shorter, two week period of ch'abc

administration on rubro-and cortico-spinal plasticity, locomotor recovery, and the interaction of lesion size with treatment effects.

Materials and Methods

All animal procedures were conducted in accordance with the NIH guidelines for the care and use of experimental animals and were approved by both the Malcom Randall VA Medical Centers and University of Florida's Institutional Animal Care and Use Committees. A total of 12 cats (6 ch'abc treated, 6 control) with similar lesion magnitudes were used in this study to assess the effects of two weeks of ch'abc treatment. All were trained 5x/week on 2-3 of 5 tasks daily. Some cats were used only in the behavioral aspects of the study as they did not receive Fluorogold (FG) injections, or their injections did not meet inclusion criteria. Inclusion criteria were as follows: 1) FG labeling at the injection site must be in both the ipsilateral and contralateral dorsolateral funiculi where the rubrospinal tract (RuST) and corticospinal tract (CST) traverse 2) FG-labeled neurons in the non-axotomized red nucleus or motor cortex must be easily detectable at 20X in order to be used for these analyses.

The lesion ranking section of this study uses a sample of convenience (laboratory data bank) which incorporates 35 cats with varying lesion sizes. Only those that were trained similarly to the paradigm described for the two week treatment groups were included.

Subjects

All cats were purpose bred, SPF, spayed, adult, female cats. Spays were performed to prevent any hormonally based behavioral changes during the study (For review see Sribnick et al., 2003; Sribnick et al., 2005). Cats were placed into one of two groups: two week control (N=6) or two week Ch'abc (N=6). At the beginning of the

study, animals were trained to perform five tasks that they were consistently trained and assessed on throughout the study, similar to Tester and Howland, 2008: 30.5 cm overground runway, bipedal treadmill, horizontal ladder, peg walkway and narrow beam. Once cats were able to consistently perform the tasks, they were filmed for baseline data collection. Following injury, cats continued to be trained and were filmed performing each of the tasks regularly for five months. Cats then received intraspinal FG injections caudal to the lesion site for retrograde tract tracing. Following a 13 day survival period animals were transcardially perfused and lesion morphology, as well as axonal plasticity, assessed.

Surgical Procedures

Low thoracic spinal hemisection

A detailed description can be found in Tester and Howland 2008. Briefly, all cats received a left, T10 spinal hemisection created by iridectomy scissors. Following hemisection, an injectable port body (Solomon Scientific ©, San Antonio, TX) was glued and sutured subcutaneously to left dorsum musculature. Port tubing was positioned over the lesion site and dura sutured closed around tubing to maintain proper positioning within the lesion. Protease free ch'abc treatment (1U/200µL) (Seikagaku Corporation ©, Tokyo, Japan), or vehicle (Sterile saline or Tris-HCL) were injected immediately after port placement in order to visually confirm proper functionality of the port system. A layer of durafilm and gelfoam were then placed over the lesion followed by muscle and skin sutured closed in layers.

Chondroitinase ABC administration

Prior to use, ch'abc enzymatic activity was tested and confirmed with fluorophore-assisted carbohydrate electrophoresis (FACE) as described in (Tester et al., 2007).

Ch'abc (0.25 U in 50 µL of saline or Tris-HCL) or vehicle (Saline or Tris-HCL) injections began the day cats were injured and continued every other day for two weeks. 50 µL injections were made slowly at 0.14cc/s with a syringe pump.

Fluorogold spinal injections

Between 22 and 35 weeks following injury, nine out of the twelve cats underwent a second surgery to receive intraspinal injections of the retrograde tracer Fluorogold™ (FG) caudal to the lesion site. The details of this procedure are fully described in Jefferson et al., 2011. In brief, 0.5% FG (Fluorochrome, LLC, CO) was mixed in sterile, de-ionized water in an aseptic hood the morning of the procedure and kept on ice prior to use. The lesion site was re-exposed, and regions of the spinal cord caudal to the lesion site also were exposed for injections. The most caudal dural suture at the lesion site was located from the previous surgery, and FG injections were made ~13mm caudal to this suture. Four injection sites were made in a staggered formation using a 33 gauge Hamilton syringe. Each injection site consisted of two FG dumps of 0.25µL in each, for a total of 2µLs. The exposed spinal cord was then protectively covered with durafilm and gelfoam, the muscle and skin closed in layers. A survival period of 13 days allowed for FG to travel retrogradely to the brainstem and cortex. Post-surgical care procedures are described in depth in previous studies (Howland et al., 1995aa; 1995bb).

Behavioral Procedures

Behavioral tasks and training paradigm

Prior to surgery, cats were conditioned to perform five tasks for a food reward. Tasks range from simple to challenging: Bipedal treadmill (0.5m/s), 30.5 cm wide overground runway, horizontal ladder, peg walkway, and 5 cm wide narrow beam (for detailed

description of tasks go to Tester and Howland 2008). Cats were trained 5x/week. Specifically, each day consisted of training on bipedal treadmill as well as one of the other tasks, which were alternated equally. Within 48 hours following SCI, training continued in the same manner as previously described for 22-35 weeks after injury. However, depending on motor function, the skilled tasks were exchanged for the 30.5 cm wide overground runway during the initial week after injury.

Assessment of locomotor recovery

For qualitative assessments a daily log was kept of each animal's overall performance. Additionally, the "Onset of Recovery" ie: the number of days for cats to recover the ability to perform a task after injury, was recorded for 30.5 cm overground runway, bipedal treadmill, horizontal ladder, peg walkway, and narrow beam. For bipedal treadmill, this was the first day they took three consecutive, independent steps even if at a speed slower than the standard training speed (0.5m/s). For 30.5 cm 12" overground runway, horizontal ladder, peg walkway, and narrow beam, this referred to the first day animals were able to make three independent crossings, consecutive or non-consecutive. For quantitative assessment, animals were filmed on each of the tasks using a 3D pan and tilt system (Peak Vicon®). Filming occurred once prior to injury, as well as multiple times following SCI. For the first two months post-SCI, cats were filmed on each task every two weeks, then once a month for the remainder of the study.

Horizontal ladder, peg walkway, and narrow beam assessment: Limb accuracy

In addition to determining "Onset of Recovery" as previously described, cats were also assessed for their ipsilateral hindlimb targeting accuracy. The percentage of times cats were able to accurately place their ipsilateral hindlimb onto a rung or peg was

calculated for each crossing. The average percentage from the three best crossings were computed and used for this comparison.

Tissue Processing

Perfusions and tissue preparation

The details of this procedure are described in our previous work (Tester and Howland, 2008). In brief, at thirteen days following FG injections, cats were deeply anesthetized with an overdose of sodium pentobarbital (>40mg/kg, i.p.). Supplemented doses were given i.v. as needed to ensure that animals were properly sedated. Cats also were intravenously injected with 1cc heparin (1000U/i.v.), and 20 minutes later with 1cc of 1% sodium nitrite i.v.. Immediately after, cats were transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The spinal cord and brain were dissected, blocked into segments, and post-fixed in 30% sucrose and 4% paraformaldehyde. The lesion, injection site, rostral midbrain, and medulla were cut on a cryostat at 25µm thick sections, while the motor cortex was cut at 40µm. Sections were stored in 0.1 M phosphate buffered saline (PBS; pH 7.2, saline 0.9%) at 4º Celsius until processed.

Cresyl violet and myelin staining

This procedure is described in detail in our previous work (Tester and Howland, 2008). In brief, every 10th section of the lesion site was mounted onto colorfrost slides (Fisher Scientific©) subbed with chrom-alum and poly-L-lysine (chromium potassium sulfate and poly-L-lysine, Sigma-Aldrich®; gelatin, Fisher Scientific©). Tissue was fume fixed on the slides with 4% paraformaldehyde to enhance adherence. Tissue was first dipped in water, and then dehydrated in increasing increments of alcohol (five minutes each). After immersing in xylene (10 minutes), tissue was rehydrated in decreasing

increments of alcohol and placed in an aqueous myelin dye (Eriochrome Cyanine R; Fluka™) for 10 minutes. After washing in water, differentiation took place in 1% ammonium hydroxide (one minute). Tissue was then placed in 0.5% cresyl violet (cresyl violet with acetate, Sigma-Aldrich®) for three minutes, and washed thoroughly in water, 70% alcohol, and then differentiated in 95% alcohol with glacial acetic acid. Following this, tissue was dehydrated again in increasing increments of alcohol and then immersed in xylene. Tissue was coverslipped with DPX (Fluka™).

Assessment of injection site

Every 40th section of the injection site from animals that received FG injections was mounted on Superfrost/Plus slides (Fisher Scientific©) and coverslipped with Prolong Gold Antifade Reagent (Molecular Probes®) Sections were assessed using fluorescent microscopy to ensure that the injection site reached the dorsolateral funiculi carrying the RUST and CST for both hemispheres of the cord. Those that did not have sufficient labeling were excluded from the tract tracing portion of the study.

Immunohistochemistry

One, 25 µm section, every 200µms through the red nucleus, and every 40th section, every 640µms through the motor cortex were processed using the polyclonal anti-FG antibody (1:10,000, Fluorochrome, LLC). This procedure is described in our previous study (Jefferson et al., 2011). In brief, sections were washed in a quenching solution (30% H_2O_2 and PBS) for 30 minutes, followed by 2, 10 minutes rinses in 1% goat serum in PBS containing 0.4% Triton X-100 (1% S-PBS-T). Tissue was then blocked in 5% S-PBS-T for one hour and then incubated in anti-FG antibody overnight at room temperature. The next day, tissue was rinsed in 1% S-PBS-T and incubated in 5% biotinylated anti-rabbit secondary antibody made in goat (Vector Laboratories©) for 1

hour. After a second rinse with 1% S-PBS-T tissue was incubated in avidin, biotin, complex (ABC) method (Vector Laboratories©). Following this incubation tissue was rinsed with PBS, then reacted with 3,3'-Diaminobenzidine (DAB) brownish reaction product (Sigma-Aldrich®) for 9 minutes. The DAB reaction was stopped with a PBS rinse. Tissue was subsequently mounted onto chrom-alum and poly-L-lysine coated slides, and fume fixed in 4% paraformaldehyde for at least one hour. Tissue was then dipped in water and dehydrated in alcohol at increasing increments at 5 minutes each. Tissue then was placed in xylene for 5 minutes and coverslipped with DPX (Fluka™).

Fluorogold-labeled cell counts

FG-labeled neurons of each stained section in the red nucleus, and motor cortex were counted using a 20x microscope objective. Only those neurons with visible soma were included in the counts. The non-axotomized cell counts were separated from the axotomized cell counts. Additionally, the axotomized cell counts were taken as a percentage of the non-axotomized cell counts to control for internal differences between cats (uptake of tracer, tissue processing, etc).

Lesion ranking

The lesion epicenters from 35 cats were stained with cresyl violet and myelin dye as described above. The control group consisted of nineteen cats, the two week ch'abc group had seven cats, and the four week ch'abc group consisted of nine cats. Three individuals unfamiliar with the cats, but well versed in spinal cord morphology, ranked lesions based on the amount of total spared tissue with one being the lowest and 35 being the greatest amount of spared tissue. Individuals were asked to rank lesions that they felt were extremely similar with the same value. Each of the three individual sets of ranks were compared to ensure that rankings were relatively similar and that rankers

properly understood and performed the task. The three sets of ranks were then averaged for each lesion and correlated against the onset of each of the five tasks, as well as the percentage of accurate ipsilateral hindlimb targeting at four, eight, and 16 weeks post injury.

Statistical Analysis

Statistics were performed using Microsoft Excel's Analyse-it statistical software (Microsoft Excel®). The Kruskall Wallis test was Bonferroni corrected and used to compare accurate ipsilateral hindlimb targeting across time points. The Mann Whitney U test was used to assess group differences and the Spearman test was used to correlate lesion rank and behavior.

Results

Lesion Magnitudes

The lesions of 12 cats, six control and six ch'abc, were stained with cresyl violet and myelin dye to determine the extent of their injury. Only those with similarly hemisected lesions were included in the part of the study assessing the effects of two weeks of ch'abc treatment on anatomical and behavioral recovery. Figure 3-1 shows the range of lesion sizes for the control (Figure 3-1A) and ch'abc (Figure 3-1B) groups. In both groups there was slight sparing present within the gray matter and ventromedial white matter. Additionally, both groups had lesions that extended into the contralateral gray matter and ventromedial white matter. Overall, the minor variations across within each group was equal across groups and did should not have skewed the results.

Axonal Projections below the Lesion Site

Both the RuST and CST tracts have been implicated in controlling adaptive types of locomotion similar to the peg walkway and narrow beam (Morris et al.; Beloozerova

and Sirota, 1993; Lavoie and Drew, 2002; Pettersson et al., 2007). In a previous study we found that four weeks of ch'abc delivery enhanced RuST circuitry below the level of the lesion (Jefferson et al., 2011), however we have yet to address the effects of two weeks delivery on this same circuitry. Additionally, the effects of ch'abc delivery on CST plasticity after either two or four weeks of ch'abc delivery has yet to be determined in a feline model of SCI. Four week control and ch'abc red nucleus neuronal counts were taken from the same animals used in our previous publication, with the addition of one, four week ch'abc cat who underwent the same treatment and behavioral training protocols as others in that group (Jefferson et al., 2011). In that study, only the percentages of axotomized to non-axotomized FG-labeled neurons were reported. Here, we report absolute neuronal counts as well, due to recent findings from our lab showing enhanced neuronal counts in both the axotomized and non-axotomized red nuclei in control cats (Blum, 2010). This finding indicates that the non-axotomized red nuclei/motor cortex may undergo more plastic changes than previously thought, and should be assessed independently.

As expected, there were significantly more FG-labeled neurons in the non-axotomized, contralateral side (Figure 3-2B,E) compared to the axotomized, ipsilateral red nucleus (Figure 3-2A,E) in the two week control (Figure 3-2E; Mann-Whitney U test, $p=0.01$), two week ch'abc (Mann-Whitney U test, $p=0.05$), four week control (Mann-Whitney U test, $p=0.01$), and four week ch'abc group (Mann-Whitney U test, $p=0.01$) as a result of the hemisection. The same was true for the motor cortex (Figure 3-2C,D,E; Mann-Whitney U test, two week ch'abc, $p=0.01$; two week control, four week control, ch'abc, $p=0.05$). Absolute FG-labeled neuronal counts from the non-axotomized red

nucleus were similar across the two and four week ch'abc groups and their control counterparts. Absolute numbers from the axotomized side were significantly higher in the two week ch'abc group compared to their control counterpart (Mann-Whitney U test, p=0.03), and the four week ch'abc compared to their controls (Mann-Whitney U test, p=0.03). Similar to what was reported in our previous work, the two week ch'abc group had a significantly greater percentage of FG-labeled neurons in the axotomized as compared to the non-axotomized red nucleus compared to controls (Mann-Whitney U test, p=0.03) (Jefferson et al., 2011).

There were no significant differences in raw FG-labeled neurons or the percentage of axotomized versus non-axotomized neuronal counts in the motor cortex between the ch'abc and control groups, regardless of treatment duration (two or four weeks) (Figure 3-2E).

Rate of Recovery

As described in earlier studies completed by the lab, ipsilateral hindlimb motor function was completely disrupted during the first one to two days after injury (Tester and Howland, 2008; Jefferson et al., 2011). The contralateral hindlimb retained most of its function during this period, however there were some animals that could not fully weight support with this limb right away, but regained this ability within several days. There were no significant differences between the control and ch'abc treated animals regarding the recovery of basic locomotion. Recovery of stepping on 12" overground runway typically began between one and three days following injury, though there were several cats that took longer (Figure 3-3A). Typically, cats began stepping primarily with their contralateral hindlimb, while their ipsilateral hindlimb flexed and extended in a manner reminiscent of stepping, but with an extremely muted range of motion around

the joints. While the contralateral hindlimb of most cats was capable of full weight support, the ipsilateral hindlimb could only support a small proportion of their weight. Surprisingly, recovery of bipedal treadmill stepping, which is controlled primarily by intraspinal circuitry, took significantly longer to recover compared to 12" overground stepping, which involves more voluntary components (Figure 3-3A; Mann-Whitney U test, control, p=0.01; ch'abc, p=0.004).

Recovery of the skilled locomotor tasks: horizontal ladder, peg walkway, and narrow beam, took longer than basic locomotion as they require multiple supraspinally controlled locomotor features, including but not limited to balance, interlimb coordination, and accurate limb targeting (Figure 3-3B) (Whishaw et al., 1998; Metz and Whishaw, 2002). In our previous studies, we found that four weeks of ch'abc treatment significantly increased the rate of recovery on each skilled task (Tester and Howland, 2008). Here, we find that a shortened ch'abc delivery period does not have as robust of an effect. Horizontal ladder performance is the easiest of the skilled tasks to perform and was recovered significantly quicker than the peg walkway (Figure 3-3B; Mann-Whitney, U test, control, p=0.01; ch'abc, p=0.004) and narrow beam tasks (Figure 3-3B; Mann-Whitney U test, control, p=0.01; ch'abc, p=0.001), occurring between the 2nd and 3rd week after injury. Cats treated with 2 weeks of ch'abc treatment were able to recover this task (horizontal ladder) significantly quicker than controls (Figure 3-3B; Mann-Whitney U test, p=0.03).

The time frames for recovery of peg walkway and narrow beam were substantially more variable in the ch'abc group compared to controls. In the control group, cats were able to recover peg walkway between 16 and 55 days post injury, and narrow beam

between 26 and 71 days post injury (Figure 3-3C). However, in the ch'abc group there was high variability across cats. While four out of the six cats recovered peg walkway between 15 and 18 days after injury, two animals were never able to recover this task (Figure 3-3D). Onset of narrow beam recovery in this two week ch'abc group also was more variable across cats compared to the control groups, ranging from 16 to 113 days after injury (3-3C,D). Despite these differences in variability across treatment groups, there were no significant differences in their average rate of recovery for these two tasks. However, the removal of these outliers led to a significant different when comparing task onset of peg walkway, but not narrow beam (Figure 3-3E, Mann-Whitney U test, $p=0.02$).

Ipsilateral Hindlimb Accuracy

Limb accuracy of the ipsilateral hindlimb is particularly difficult for animals to recover following injury, and in fact many fail to do so on the more skilled tasks like peg walkway. In previous studies done by the lab it was showed that four weeks of ch'abc administration resulted in a significantly greater ability for cats to accurately target with their ipsilateral hindlimb onto a peg compared to controls (Tester and Howland, 2008; Jefferson et al., 2011). Here, we see that a shortened two week delivery of Ch'abc does not significantly enhance limb accuracy over controls.

Horizontal ladder

Pre-injury, all cats were able to accurately place with their ipsilateral hindlimb onto a ladder rung 100% of the time, however after injury all animals lost this ability for at least several days (Figure 3-4A). By two weeks post injury, several cats had recovered this locomotor feature, but the majority of these animals were in the ch'abc group (four cats), while only one animal in the control group had regained ipsilateral hindlimb

accuracy. Hence, the ch'abc group showed a trend towards significantly greater percentage of ipsilateral hindlimb accuracy compared to controls at two weeks after injury (Figure 3-4A; Mann-Whitney U test, p=0.07). This ch'abc enhancement of accuracy was transient and by four weeks the control animals were performing equal to the ch'abc group. Both groups had substantial recovery of ipsilateral hindlimb accuracy by eight weeks and were targeting significantly more than at two weeks post injury (Kruskal-Wallis test, control, p=0.01; ch'abc, p=0.03). This trend continued at 16 (Kruskal-Wallis test, control, p=0.02; ch'abc, p=0.003), and 20 weeks after injury (Kruskal-Wallis test, control, p=0.01; ch'abc, p=0.0008).

Narrow beam

Prior to injury, all cats were able to place their ipsilateral hindlimb onto the beam 100% of the time. After injury, recovery of ipsilateral hindlimb accuracy on the narrow beam was an all-or-nothing skill (Figure 3-4B). The majority of animals in both control and ch'abc groups struggled with recovering this feature for the first eight weeks after injury, with none recovering by two weeks and only one becoming successful at four weeks (ch'abc treated). Once animals did recover ipsilateral hindlimb accuracy the majority of them had a 100% success rate. By eight weeks post injury the control group was accurately placing their ipsilateral hindlimb significantly more than what they were at two weeks (Figure 3-4A; Kruskal-Wallis test, p=0.04). This trend continued at 16 (Kruskal-Wallis test, p=0.005) and 20 weeks after injury (Kruskal-Wallis test, p=0.004). Accuracy of the ch'abc group was not significantly greater than two weeks until 20 weeks after injury (Kruskal-Wallis test, p=0.05), indicating that this ch'abc group did not perform as well as controls on this task.

Peg walkway

Similar to the horizontal ladder and narrow beam, all cats were able to accurately place their ipsilateral hindlimb onto pegs 100% of the time prior to injury (Figure 3-4C). However, for the first two weeks after injury, ipsilateral hindlimb accuracy was completely lost. By four weeks post injury, there was one cat from the control and ch'abc group that recovered this feature. Interestingly, the ch'abc cat was accurately targeting substantially more than the control cat at 100% as opposed to 11%, respectively. Over the course of the study only two other cats from each group recovered ipsilateral hindlimb accuracy on peg walkway, suggesting the extreme difficulty of this task. Additionally, accuracy percentage remained low in both groups, with neither significantly improving from two weeks post injury at any time point in the study. Only one animal within the ch'abc group was able to reach 100% accuracy, while the other cats ranged from 7 to 23% ipsilateral hindlimb accuracy (Figure 3-4C).

The Relationship between Spared Tissue and Functional Recovery

While most of the 35 lesions obtained from the laboratory data bank were variations on a hemisection, there were a few that were notably larger with only ~25% of cross-sectional sparing, or notably smaller with ~75% of cross-sectional sparing. Furthermore, the effect of ch'abc administration on the relationship between lesion size and locomotor recovery has yet to be determined. In the current study, unbiased individuals ranked the amount of total spared tissue present at the lesion epicenter from all 35 cats: Control (19), two week ch'abc (seven), four week ch'abc (nine). An average rank was determined for each cat based on the ranks assigned by the three individuals. Lesion sizes ranged from very over-hemisected with sparing in only a quadrant of the spinal cord crossection (Figure 3-5A), to very under-hemisected with sparing in 3/4ths of

the spinal cord crossection (Figure 3-5C). Those that were ranked as having a medial amount of sparing were near perfect hemisections (Figure 3-5B). Average ranks for each animal were correlated with onset of task recovery, as well as accurate ipsilateral hindlimb placement on the horizontal ladder, peg walkway, and narrow beam.

Onset of task recovery versus lesion size

There was a significant correlation in the control animals between spared tissue rank and recovery onset of overground walkway (Figure 3-5D; Spearman test, p=0.0001), horizontal ladder (Spearman test, p=0.001), peg walkway (Spearman test, p=004), and narrow beam (Spearman test, p=0.0001). The only lack of a relationship was on bipedal treadmill (Figure 3-5). The two week ch'abc treated animals had a less striking relationship in that their lesion rankings did not significantly correlate with recovery onset of overground, or peg walkway. However, the recovery of horizontal ladder (Spearman test, p=0.03), and narrow beam (Spearman test, p=0.0004) did significantly correlate with spared tissue rank. The onset of overground walkway was close to significantly correlating with a p-value of 0.06. In the four week ch'abc group there were no significant correlations between lesion size and recovery onset on either of the tasks except for bipedal treadmill (Spearman test, p=0.04). This lack of a relationship between spared tissue and task onset in the four week ch'abc group only indicates that four weeks ch'abc decreases the relationship between lesion size and functional recovery. In other words, four weeks of ch'abc allows for animals with larger lesions and less spared tissue to recover tasks quicker than cats with a similarly sized lesion that were treated either with two weeks, or no ch'abc. This is validated when looking at the individual values for each animal, using the onset of narrow beam as an example (Figure 3-5E). Here, the onset and spared tissue rank values show that the 4

week ch'abc treated animals with less spared tissue were able to recover narrow beam much quicker than the control and two week ch'abc treated cats with similarly low levels of sparing. This effect occurs in all assessed tasks.

Ipsilateral hindlimb accurate targeting versus lesion size

The amount of spared tissue in both the control and two week ch'abc treated groups either significantly correlated or had a p value of <0.1, with ipsilateral hindlimb accurate targeting at four weeks post injury on horizontal ladder (Figure 3-6A; Spearman test, control,p=0.0004; 2 week ch'abc, p=04), peg walkway (Spearman test, control, p=0.01; 2 week ch'abc, p=0.0719), and narrow beam (Spearman test, control, p=0.01; 2 week ch'abc, p=0.07) (Figure 3-6A). The same is true at eight weeks post injury for horizontal ladder (Spearman test, control, p=0.0927; 2 week ch'abc, p=0.01), peg walkway (Spearman test, two week ch'abc, p=0.0150), and narrow beam (Spearman test, control, p=0.0004; two week ch'abc, p=0.0031). The one exception to this was the control group at eight weeks post injury on peg walkway. In direct contrast, there was no relationship between lesion size and accurate targeting on either of the tasks for the four week ch'abc group. Interestingly, by 16 weeks post injury the relationship seen between lesion size and accurate targeting was no longer present on horizontal ladder or peg walkway in the control and two week ch'abc group, though it was still present in narrow beam (Spearman test, control, p=0.0624; 2 week ch'abc, p=0.01). At 16 weeks post injury there was still no relationship between the two in the four week ch'abc group. These results further suggest the finding that ch'abc decreases the relationship between lesion size and locomotor recovery. Similar to what was seen in the onset of task recovery, the individual values of each animal, using accurate ipsilateral hindlimb targeting on horizontal ladder at four weeks as an example,

indicates that four week ch'abc treated animals with less tissue sparing (larger lesions) recover accurate targeting better than control and two week ch'abc treated animals with similar amounts of spared tissue (Figure 3-6B).

Discussion

Summary of Results

In the current study we found that there was a significant enhancement in axonal growth within the RuST, but not within the CST after both two and four weeks of ch'abc administration. Interestingly, although two weeks of ch'abc administration led to enhanced rubrospinal tract growth below the lesion similar to four week treated animals (Jefferson et al., 2011), they did not share a similar enhancement of skilled locomotor recovery. In a larger group of animals with more diverse lesion magnitudes, it was determined that four weeks, but not two weeks of ch'abc treatment leads to enhanced functional recovery in larger lesions.

Supraspinal Connectivity below the Lesion

Multiple ch'abc studies attribute locomotor recovery after SCI to the regeneration and sprouting of the CST (Bradbury et al., 2002; Barritt et al., 2006; Garcia-Alias et al., 2009). However, the current study did not find enhanced CST connectivity below the level of the lesion based on our retrograde tracing results in both the two week ch'abc cats, as well as the functionally enhanced four week ch'abc treated animals. These results suggest one of two things. First, ch'abc does not enhance plasticity within this particular pathway. In fact, multiple studies have reported a failure in significant CST axonal growth despite seeing growth in other systems like the RuST (Richardson et al., 1984b; Houle and Ye, 1999; Decherchi and Gauthier, 2000; Plunet et al., 2002). More likely though, ch'abc treatment enhances CST axonal sprouting proximal to the lesion

site, but this sprouting does not enhance the number of motor cortex neurons with connections below the level of the lesion. Alternatively, CST plasticity may be occurring in more rostral regions of the spinal cord like the cervical cord, as shown in Bareyre et al., 2004. Sprouting in those regions would not be detected by low thoracic FG injections.

Multiple ch'abc studies utilizing anterograde tracing have reported enhanced CST sprouting proximal to the lesion site (Bradbury et al., 2002; Barritt et al., 2006; Iseda et al., 2008; Garcia-Alias et al., 2009; Karimi-Abdolrezaee et al., 2010). The only ch'abc study to assess CST plasticity utilizing retrograde tracing, in addition to the current study, had similar findings in that there was enhanced connections below the level of the lesion in non-CST pathways (ReST, VST, and RuST) but none in the CST itself (Bai et al., 2010). This further suggests that if CST plasticity is occurring, it most likely occurs at the terminal ends or in more rostral segments of the spinal cord.

Locomotor Recovery and Supraspinal Plasticity

Previous studies performed by the Howland laboratory did not show a significantly shorter recovery period for bipedal treadmill, or 12" overground runway after hemisection and 4 weeks of ch'abc administration (Tester and Howland, 2008; Jefferson et al., 2011). In the current study, we also see this similar lack of an effect. This result was expected based on the aforementioned results as cats recover these tasks within the first two weeks after injury; a period when both ch'abc treatment groups had received the same amount of ch'abc delivery. Disparity between two week and four week locomotor recovery was most apparent on the skilled locomotor tasks. These tasks require much more adaptation and mimic the type of everyday tasks that

individuals with incomplete SCI, like Brown Sequard Syndrome, primarily struggle with (Little and Halar, 1985; Eidelberg et al., 1986).

The recovery period for horizontal ladder, peg walkway and narrow beam were similar across cats in the control group, with cats typically recovering horizontal ladder first, followed closely by peg walkway, then narrow beam. All control cats recovered these three tasks between 15 and 71 days after injury. The two week ch'abc group recovered horizontal ladder significantly quicker than the control cats after injury, however had substantially greater variability regarding recovery period for peg walkway and narrow beam. On peg walkway, four cats recovered within 15 to 18 days, while two never recovered. On narrow beam, four different cats recovered within 16 to 41 days, while two cats, different from those who failed to recover peg walkway, did not recover narrow beam until 111 and 113 days after injury. This extreme variability may be an indicator of aberrant plasticity and nonfunctional circuit formation resulting from an insufficient amount of ch'abc administration. This is further confirmed by a lack of variability seen across the four week ch'abc treated cats (Tester and Howland, 2008; Jefferson et al., 2011). Furthermore, results from retrograde tracing show significantly greater RuST connections below the level of the lesion in both the two and four week ch'abc groups compared to their control counterparts. However, the four week ch'abc group had significantly enhanced skilled locomotor recovery while the two week ch'abc group did not (Tester and Howland, 2008; Jefferson et al., 2011). It is possible that while two weeks of ch'abc administration enhances axonal growth, it does not allow for the plasticity necessary to make functionally appropriate connections capable of producing long term beneficial effects. Neuro-developmental studies have shown that

experience-based plasticity is dependent on a lack of CSPGs (Pizzorusso et al., 2002; Pizzorusso et al., 2006). After injury, the expression of some CSPGs, specifically phosphacan, NG2, brevican, and neurocan, remain upregulated for longer than two weeks after injury (Jones et al., 2002; Jones et al., 2003a). Additionally, aggrecan, which initially decreases after injury, begins to recover expression at two weeks post-injury (Lemons et al., 2001). These specific CSPGs have been shown to inhibit axonal growth (Sango et al., 2003), especially NG2 (Dou and Levine, 1994; Fidler et al., 1999; Jones et al., 2002; Ughrin et al., 2003), and aggrecan (Lemons et al., 2003). Although cleaved by ch'abc for 2 weeks, their ongoing expression may continue after the cessation of ch'abc at 2 weeks, and their presence may accumulate at the lesion site causing inhibition of axonal growth. In addition to ongoing CSPG upregulation 2 weeks after injury, there are multiple post-SCI inflammatory factors, such as microglia, reactive astrocytes and oligodendrocyte precursor cells that infiltrate the lesion site quickly after injury and continue to be present for longer than two weeks after injury (Hill et al., 2001; Velardo et al., 2004; Donnelly and Popovich, 2008). Additionally, some of the greatest changes to lesion morphology occur between two and four weeks after injury (Hill et al., 2001; Velardo et al., 2004). While 2 weeks of ch'abc may be enough to enhance plasticity, the combinatorial presence of these factors at the lesion site after cessation of ch'abc administration at 2 weeks may prevent these new sprouts from making appropriate connections.

Previous studies from the Howland laboratory have shown that both human and feline CS-GAG lyase products after ch'abc digestion predominantly consist of 4S and 6S disaccharides, while digestion of rodent CS-GAGs results in primarily 4S

disaccharide products (Tester and Howland, 2008). This species difference suggests that ch'abc degradation is required to cleave additional substrates in the cat versus rat spinal cord. This may partially explain differences seen in functional recovery across species that received ch'abc administration for the same period of time. Additionally, due to the larger size of the cat spinal cord compared to rat, axonal regeneration and sprouts must travel farther in order to reach appropriate connections. Combined results from the present study, as well as those previously published by the Howland laboratory, suggest that four weeks of ch'abc treatment results in much greater locomotor recovery compared to two weeks of administration in a cat model of spinal cord injury.

Lesion Size

Multiple studies have found that larger lesions significantly correlate with poorer functional recovery and that smaller lesions are paired with greater functional recovery (Byrnes et al.; Semler et al.; Molt et al., 1979; Norrie et al., 2005). Here, we have found a similar result in that spared tissue significantly correlates with onset of task recovery, and accurate targeting of the more greatly affected hindlimb on horizontal ladder, peg walkway, and narrow beam in control and two week ch'abc treated animals. In direct contrast, the amount of spared tissue in the four week ch'abc treated animals does not correlate with these same locomotor features. Further investigation of individual animals showed that this lack of a correlation is due to the fact that 4 week ch'abc treated animals with less spared tissue had functional recovery that surpassed those in control and 2 week ch'abc treated groups with similarly small amounts of spared tissue. Such findings indicate that ch'abc mediates profound enhancement of functional recovery even in large lesions, but that a minimum of four weeks of treatment is

necessary to produce this result. We believe the mechanism underlying this effect is greater plasticity of multiple circuitries in four week ch'abc treated animals that is beyond what was assessed by the tracing techniques used in the study. Although two weeks of ch'abc treatment results in plasticity, it is not enough to produce new functional connections that can surpass the limitations of a larger lesion.

Conclusion

The current study suggests that two weeks of ch'abc administration in a feline model of SCI is not sufficient for enhancement of skilled locomotor recovery. While it does lead to a significantly greater number of red nucleus neurons with axons below the level of the lesion, this increased growth may not make functionally relevant connections. As seen in our previous studies, four weeks of ch'abc enhances both red nucleus plasticity, as well as skilled locomotor recovery suggesting that this longer ch'abc administration period is necessary for functionally appropriate connections to be made. Although we did not see increased motor cortex neuronal connectivity below the level of the lesion using retrograde tracing for either two or four week treated groups, other studies have found increased CST sprouting after ch'abc treatment with anterograde tracing. These results suggest that there may be plasticity occurring at regions rostral to the injury, or at the terminal ends that cannot be detected using retrograde tracing. Our assessment of the spared tissue-functional recovery relationship indicates a positive correlation with more sparing leading to greater recovery and vice versa. The finding that four weeks, but not two weeks of ch'abc treatment mutes this relationship by causing substantial recovery in lesions with both high and lower amounts of spared tissue, further confirms that two weeks of ch'abc treatment is not as sufficient of a treatment period as four weeks in feline SCI. The high translatability of felines to

humans suggests that a longer treatment duration should be considered for future translationally designed, and ultimately clinical, ch'abc studies.

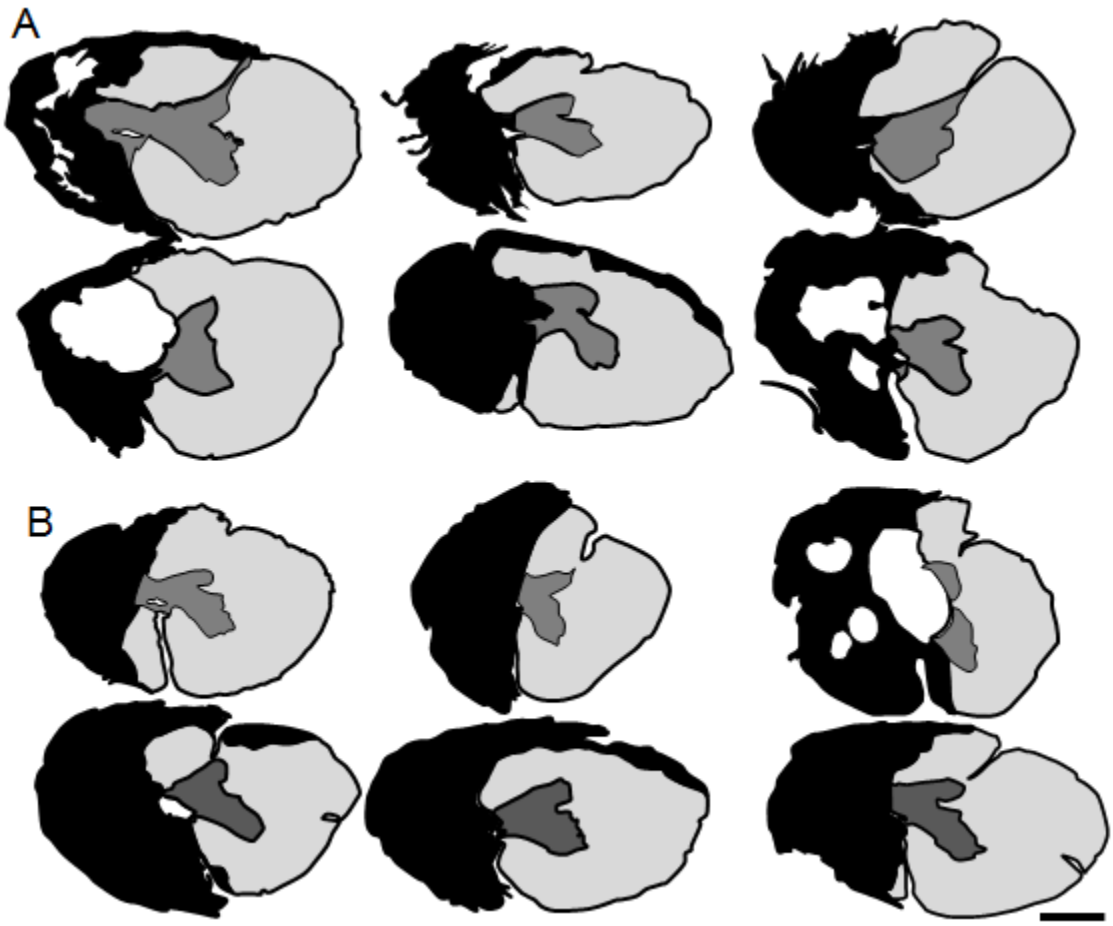
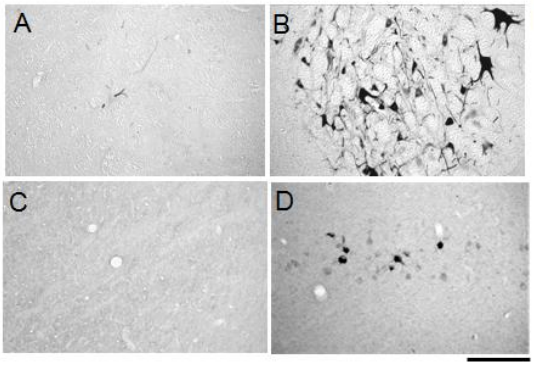


Figure 3-1. Range of spinal hemisections. Horizontal sections of lesions from each of the control (A) and chondroitinase abc treated (B) animals. The entire length of the lesion was sectioned at 25 µm and stained with cresyl violet and myelin dye. The greatest amount of damage in each of the main cord regions was determined in each section and collapsed into a single drawing. Scale bar, 1mm.



E

Rubrospinal Tract (RED NUCLEUS)	Non-Axotomized (Left)	Axotomized (Right)		Percentage (Right/Left)
2wk Control (N=4)	681 (±200)	13 (±4)	*	2 (±1)
2wk Ch'abc (N=3)	624 (±210)	**90 (±90) ***	*	**14 (±15) ***
4wk Control (N=5)	895 (±243)	69 (±48)	*	£
4wk Ch'abc (N=6)	733 (±82)	**141(± 77) ***	*	**£**
Corticospinal Tract (MOTOR CORTEX)	**Non-Axotomized (Left)**	**Axotomized (Right)**		**Percentage (Right/Left)**
2wk Control (N=3)	495 (± 323)	36 (±35)	*	6 (±6)
2wk Ch'abc (N=4)	332 (± 237)	22 (±14)	*	10 (±9)
4wk Control (N=5)	451 (±152)	21 (±12)	*	5 (±2)
4wk Ch'abc (N=4)	322 (±104)	29 (± 26)	*	10 (±10)

Figure 3-2. Supraspinal connections below the lesion. The amount of fluorogold-labeled neurons were determined for the non-axotomized and axotomized side of the red nucleus and motor cortex. Additionally, the percentage of axotomized fluorogold labeled neurons as a percentage of the non-axotomized side were calculated. *,$p<0.05$. $p=0.0628$. The axotomized red nuclei (A), and motor cortex (C), had less labeling than the non-axotomized side of the red nucleus (B) and motor cortex (D), 100 µm. Average raw values, and percentage of axotomized to non-axotomized values for the two, and four week control groups, as well as the two and four week chondroitinase abc groups were reported (E).

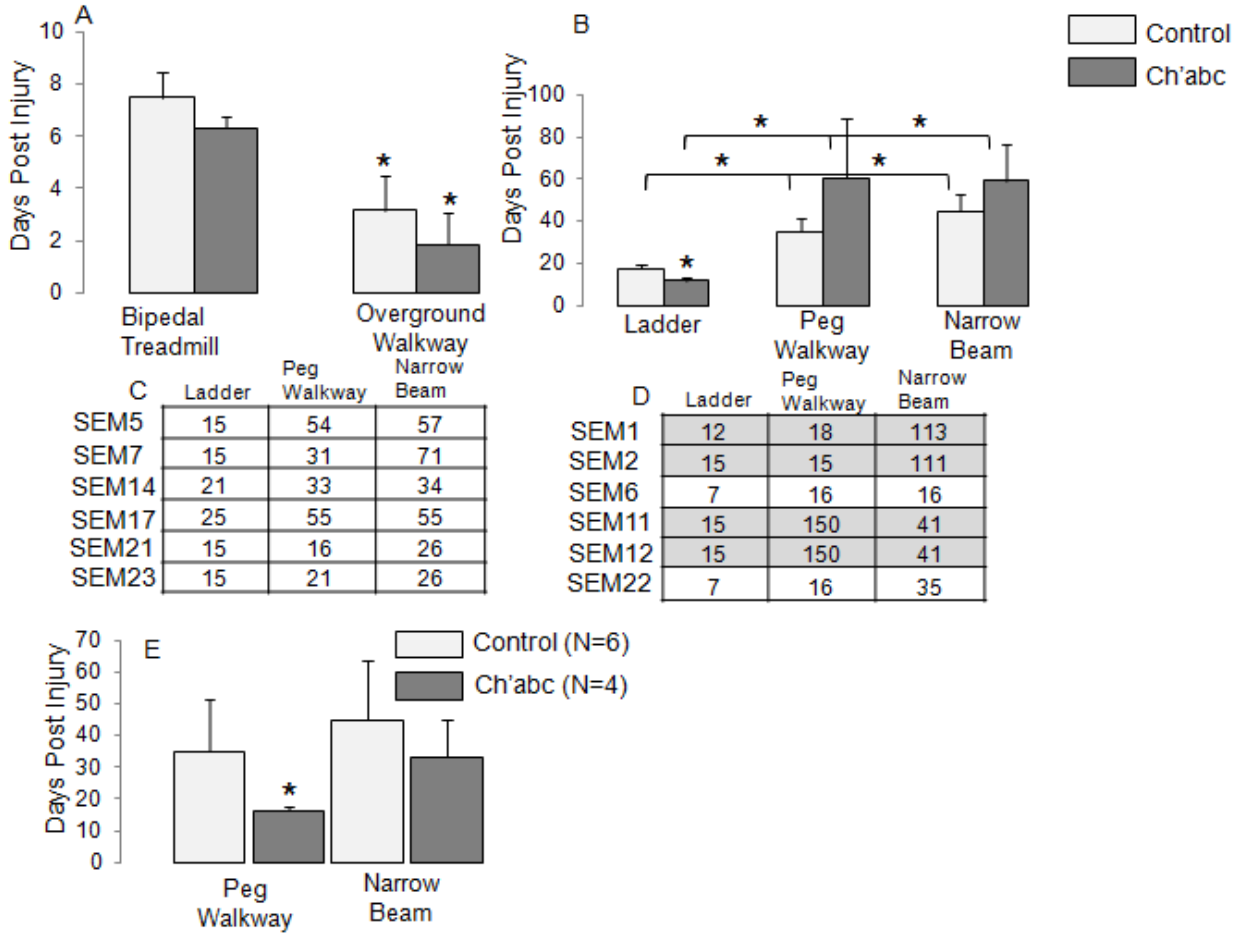


Figure 3-3. Onset of basic and skilled locomotor recovery. The number of days it took for cats to recover basic locomotor tasks: Bipedal treadmill and overground walkway (A), and skilled locomotor tasks: Horizontal ladder, peg walkway, and narrow beam (B), were compared across two week control and two week ch'abc groups. (*****, P=<0.05). The specific day of recovery after injury for each control (C) and chondroitinase abc cat (D) were listed for recovery of ladder, peg walkway, and narrow beam. Rows highlighted in gray indicate those cats that had extreme variability in their recovery period across the three skilled tasks after injury. Removal of these outliers within the ch'abc treated group led to significant group differences in the peg walkway, but not the narrow beam (E). Variation was assessed using standard error of the means.

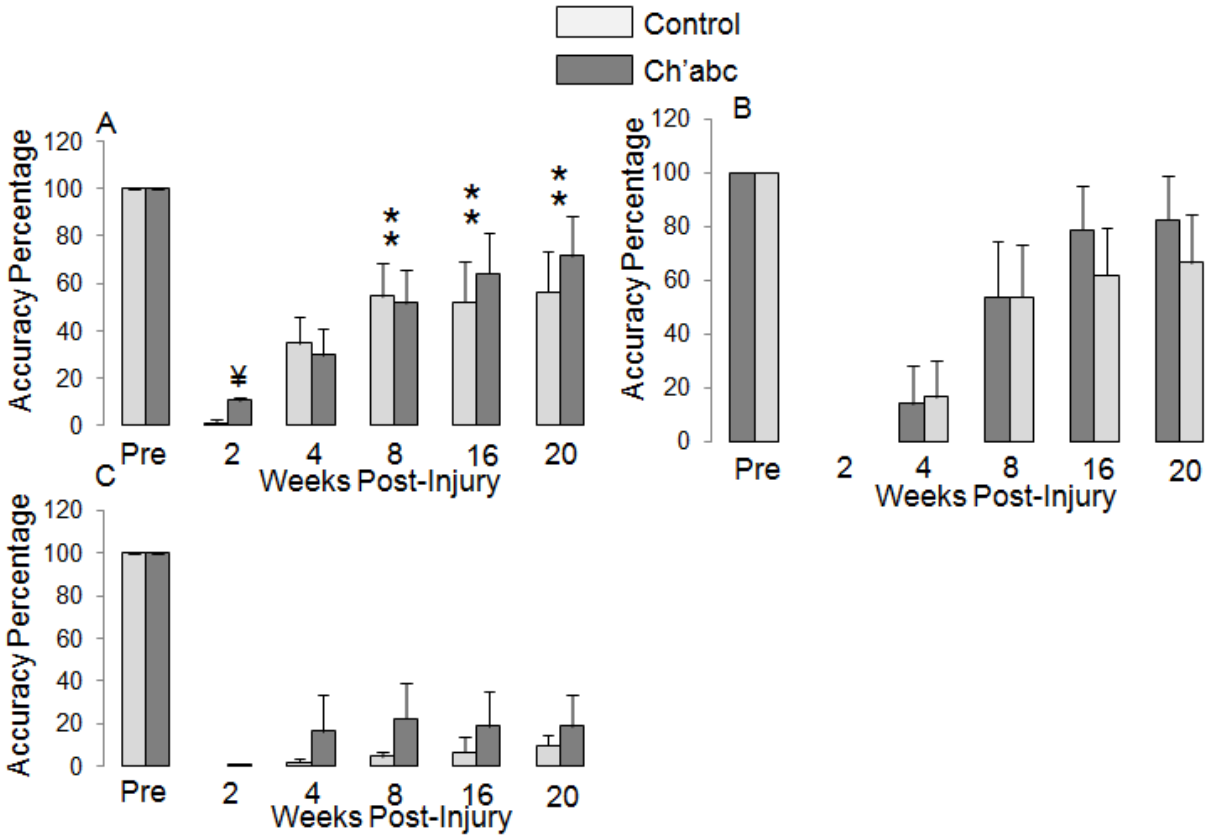


Figure 3-4. Ipsilateral hindlimb accuracy. The percentage of times cats were able to accurately target and place their ipsilateral hindlimb onto a rung, beam, or peg while crossing the horizontal ladder (A), narrow beam (B), or peg walkway (C) were determined periodically over a 20 week period post injury and compared between two week control and chondroitinase abc groups. (*p=<0.05), (¥p=0.0660). Standard error of the means are depicted in A-C.

D

Group	Onset of Overground	Onset of Bipedal	Onset of Ladder	Onset of Peg Walkway	Onset of Narrow Beam
Control (N=19)	**0.0001** *	0.1398	**0.0010** *	**0.0045** *	**0.0001** *
2wk Ch'abc (N=7)	**0.0861**	**0.0553** ¥	**0.0313** *	0.2434	**0.0004** *
4wk Ch'abc (N=9)	0.1569	**0.0378** *	0.4573	0.1573	0.4469

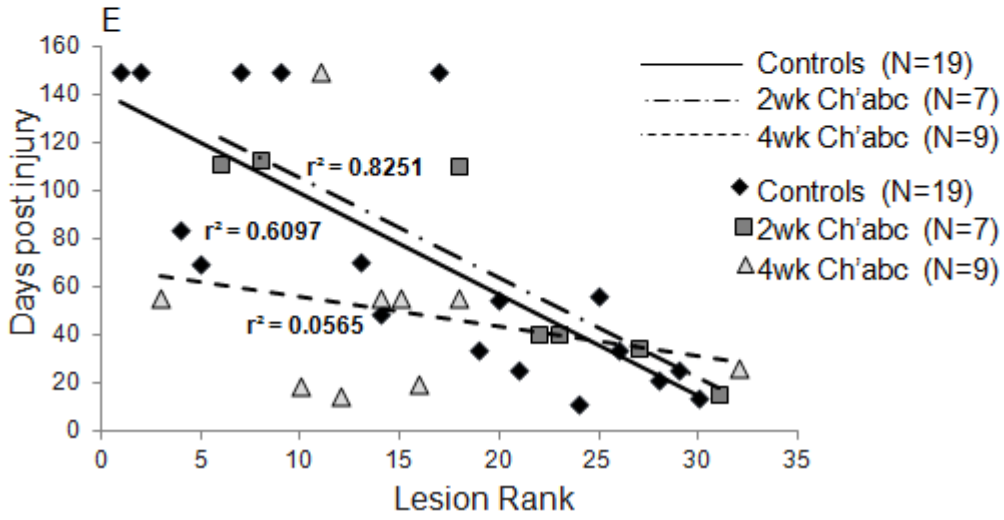


Figure 3-5. Spared tissue and task onset correlations. Lesion epicenters were stained with cresyl violet and myelin dye and then ranked from the least amount of spared tissue (rank=1) (A) to the greatest amount of spared tissue (rank=32) (C). Some lesions were ranked the same if they had extremely similar amounts of spared tissue therefore the highest ranked lesion is not equivalent to the total number of cats included in the study. The median lesion (rank=16) (B), was extremely close to a near perfect hemisection. Correlation coefficients were determined which compared lesion rank and the number of days it took for animals to recover overground walkway, bipedal treadmill, horizontal ladder, peg walkway, and narrow beam after injury (D). Many significant, or trending towards significant correlations were found in the control and two week chondroitinse abc treated groups, but none were found in the four week chondroitinase abc treated group except for on bipedal treadmill. In order to better understand these results, the values for each individual animal were plotted and compared. Here, we use recovery of narrow beam as an example (E).The trend lines, and r^2 value for each group are included. Scale bar 1mm.

A

Group	Ladder Targeting: 4wks	Ladder Targeting:8wks	Ladder Targeting:16wks
Control (N=19)	**0.0004** *	**0.0927** ¥	0.1513
2wk Ch'abc (N=7)	**0.0357** *	**0.0117** *	0.3207
4wk Ch'abc (N=9)	0.6180	0.4830	0.7418

Group	Peg Walkway Targeting:4wks	Peg Walkway Targeting :8wks	Peg Walkway Targeting:16wks
Control (N=19)	**0.0078** *	0.3303	0.3797
2wk Ch'abc (N=7)	**0.0719** ¥	**0.0150** *	0.2812
4wk Ch'abc (N=9)	0.4053	0.4483	0.5356

Group	Narrow Beam Targeting :4wks	Narrow Beam Targeting :8wks	Narrow Beam Targeting:16wks
Control (N=19)	**0.0128** *	**0.0004** *	**0.0624** ¥
2wk Ch'abc (N=7)	**0.0719** ¥	**0.0031** *	**0.0079** *
4wk Ch'abc (N=9)	**0.1084**	0.2379	0.2379

B

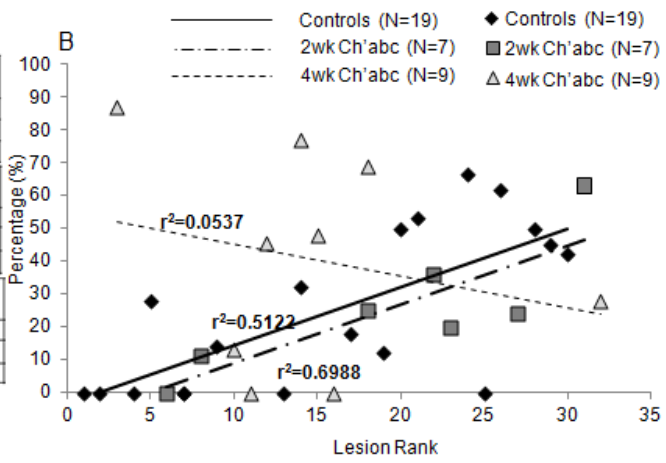


Figure 3-6. Spared tissue and accurate ipsilateral hindlimb targeting correlations. Correlation coefficients were determined which compared the lesion rank and percentage of accurate ipsilateral hindlimb targeting on horizontal ladder, peg walkway, and narrow beam (A). Similar to recovery of task onset, both the control and two week chondroitinase abc treated animals either significantly correlated, or trended towards a significant correlation with accurate targeting on all three tasks at four and eight weeks post injury. There were minimal correlations at 16 weeks post injury. In contrast, the four week chondroitinase abc treated group had no significant correlations at either of the time points. Closer analysis of this effect using the individual values of accurate targeting on ladder at four weeks as an example, shows that the four week chondroitinase abc cats with the lowest amounts of spared tissue were able to place substantially more than those animals with a similarly low amount of spared tissue (B).

CHAPTER 4
OPTIMIZATION OF FLUOROGOLD RETROGRADE TRACING

Introduction

The use of the retrograde tracer Fluorogold (FG) is a common technique for determining axonal connections in multiple different experimental paradigms performed in a variety of animal models ranging from lamprey (McClellan et al., 2006) to primate (Gimenez-Amaya and Graybiel, 1990; Hayashi et al., 2006). Tracer is placed into axons of interest and travels retrogradely to label the neuronal cell bodies of origin. There are multiple benefits to using FG in addition to its compatibility with a wide range of species. It has the rare ability to transport to the distal dendrites (Maranto, 1982; Bentivoglio and Su, 1990; Buhl and Dann, 1990; Naumann et al., 1992) and appears to label all neuronal cell populations (Zaborszky, 2006). Additionally, it has strong autofluorescent properties that make it detectable without additional processing, but its signal also can be amplified and detected using immunohistochemical (IHC) processes (Chang et al., 1990; Naumann et al., 2000; Akhavan et al., 2006).

Despite these benefits, there are several downsides to using FG which need to be minimized for tracer optimization. One prime example is the requirement of tissue damage at the injection site for tracer uptake (Schmued and Fallon, 1986). Furthermore, multiple investigators have reported that FG is cytotoxic to both motoneurons (Garrett et al., 1991; Naumann et al., 2000) and dorsal root ganglia neurons (Garrett et al., 1991) when left in vivo for several weeks. This neurotoxic feature has the potential to become a significant problem in larger species who may require longer survival periods depending upon transport distance. One way to minimize tissue damage is by decreasing FG concentration and volume (Schmued and

Fallon, 1986), however this translates to decreased autofluorescence that quenches more quickly. An anti-FG antibody is commercially available and allows for the detection of FG using conventional immunohistochemical (IHC) techniques that lead to a stable 3,3'-Diaminobenzidine (DAB) reaction product (Chang et al., 1990). This may be a promising solution for FG detection when autofluorescence is not as bright due to low FG concentration and volume, and/or permanent label is desired.

Decreasing the survival period following FG injections also will help minimize FG's cytotoxic effects. The detergent Triton X-100 (triton) has been implicated as a speed enhancing supplement for tract tracing with oregon green and fluororuby dextran amines in Possums (Fry et al., 2003). Triton is a non-ionic surfactant commonly used to permeabilize cell membranes. Since the rate of FG uptake is directly related to its ability to cross the cell membrane (Martin W, 1991), a mixture of FG and triton applied to the region of interest may enhance both tracer uptake and speed of transport which may result in minimizing cytotoxicity. Additionally, the benefits of shortening the survival period may expand into other areas specific to a study such as financial motives and time dependent issues. The objectives of the current study are to determine methods for minimizing tissue damage while maximizing FG tracing speed and detection.

Materials and Methods

The following data is based on FG tracing results from 19 cats. All procedures were conducted in accordance with NIH guidelines and were approved by both the Malcom Randall VA Medical Center's, and the University of Florida's Institutional Animal Care and Use Committees.

The injection sites of each of the four animals were processed and assessed in order to ensure adequate spread of the tracer, especially in the dorsolateral funiculi

which contains the rubrospinal (RuST) and corticospinal (CST) tracts. These tracts acted as the tracts of interest for this study as their cell bodies are located a far distance from the injection sites and we are well experienced with tracing these systems (Jefferson et al., 2011) (Refer to Chapter 3).

Subjects

All cats were purpose bred, SPF, adult, female cats (Liberty Vendors, Inc.). Animals were placed into one of five different groups: 13 day FG without triton (N=12), which was our commonly used protocol for previous tract tracing experiments (Jefferson et al.), as well as a seven day FG without triton group (N=1), seven day FG with triton group (N=2), three day FG without triton group (N=1), and a three day FG with triton group (N=3).

Surgical Procedures

Fluorogold spinal injections

Triton X-100 (2.5%, Sigma-Aldrich®) was mixed with sterile saline in a sterile setting the morning of the procedure. Fluorogold™ (0.5%, Fluorochrome, LLC) was then either mixed in sterile saline or the 2.5% Triton X-100 solution and kept on ice prior to use. The spinal cord segment T11 was exposed and injections were made in four injection sites placed in a staggered formation using a 33 gauge Hamilton syringe. Each injection site consisted of three FG injections of 0.25µL in each, for a total of 3µLs (Figure 4-1). The exposed spinal cord was then protectively covered with durafilm and gelfoam and the muscle and skin closed in layers. Our previous studies describe post-surgical procedures in detail (Howland et al., 1995b, a)

Tissue Processing: Histology and Immunohistochemistry

Perfusions

The details of this procedure are described in our previous work (Tester and Howland, 2008). In brief, at three, seven, or 13 days following FG injections, cats were deeply anesthetized with an overdose of sodium pentobarbital (>40mg/kg, I.P) and supplemented i.v. as needed to ensure that animals were properly sedated. Cats also were injected with heparin (i.v.;1000 U) and then 20 minutes later injected with sodium nitrite (I.V.;1%, 1cc). Immediately after, cats were then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The injection sites were blocked and sectioned at 25 µm, while the red nuclei and motor cortices were blocked and sectioned into 50 µm sections.

Cresyl violet and myelin staining

A full description is described in our previous work (Tester and Howland, 2008). In brief, every 12th section of the injection site was mounted onto Colorfrost slides (Fisher Scientific©) subbed with chrom-alum and poly-L-lysine (chromium potassium sulfate and poly-L-lysine, Fisher Scientific©) and fume fixed with paraformaldehyde. Tissue was first dipped in water, and then in alcohols at increasing concentrations for dehydration for five minutes. After dipping in xylene, tissue was then rehydrated in decreasing concentrations of alcohol and placed in myelin dye for 10 minutes. After washing in water, differentiation then took place in 1% ammonium hydroxide (Sigma-Aldrich©) for one minute. Tissue was then placed in 0.5% cresyl violet (Sigma-Aldrich©) for 3 minutes and washed thoroughly in water, then 70% alcohol, then differentiated in 95% alcohol with glacial acetic acid. Following this, tissue continued to be dehydrated in

increasing increments of alcohol for five minutes, and then placed in xylene. Tissue was then coverslipped with DPX (Fluka™).

Fluorogold immunohistochemistry

A full description of this procedure can be found in Jefferson et al., 2011. In brief, multiple sections of the injection sites and red nuclei were processed using the monoclonal anti-FG antibody (1:60,000, 1:10,000, respectively) (Fluorochrome, LLC). Sections were washed in a quenching solution (30% H_20_2 and phosphate buffered saline (PBS)) for 30 minutes, followed by two 10 minute rinses in 1% goat serum in PBS with 0.4% triton X-100 (1% S-PBS-T). After this, tissue was blocked in 5% S-PBS-T for one hour. Tissue was then incubated in anti-FG antibody overnight at room temperature. The following day, tissue was rinsed in 1% S-PBS-T and incubated in 5% biotinylated anti-rabbit secondary antibody made in goat for 1 hour (Vector Laboratories©). Following another rinse with 1% S-PBS-T, tissue was incubated for two hours in ABC Reagent (Vector Laboratories©). Tissue was then rinsed with PBS and reacted with 3,3'-Diaminobenzidine for 9 minutes resulting in a brownish reaction product. The DAB reaction was stopped with a PBS rinse once the reaction product was the appropriate darkness. Tissue was then mounted onto chrom-alum and poly-L-lysine coated slides and fume fixed in 4% paraformaldehyde for ~ one hour. Lastly, tissue was dehydrated in alcohols at increasing concentrations for 5 minutes, placed in xylene for five minutes, and coverslipped with DPX (Fluka™). The sections that were stained for FG immunoreactivity one and nine years ago underwent the same procedures as described above. These slides were stored at room temperature in hard cover slide boxes.

Assessment of injection site completeness and fluorogold autofluorescence

Every 12th section through the injection sites were mounted onto Superfrost/Plus slides (Fisher Scientific©) and were coverslipped using prolong gold anti-fade mounting media (Molecular Probes®). Sections were assessed using the UV cube with fluorescent microscopy. Red nucleus and motor cortex sections adjacent to those known to have heavy FG labeling based on results from the anti-FG IHC analysis described above, were processed in a similar manner in order to compare autofluoroscent and IHC based FG detection.

Results

Tissue Damage at the Injection Site

It has been previously reported that FG causes tissue necrosis at the injection site and that this damage is necessary for FG uptake and subsequent retrograde tracing (Schmued and Fallon, 1986; Divac and Mogensen, 1990). However, the extent of this necrosis, as well as its sensitivity to FG volume and concentration, have yet to be described. Here we compare the injection sites for animals that received FG injections of different concentrations, volumes, and had different survival periods after FG injections. Additionally, the effects of triton mixed in the FG solution were assessed. Cats that underwent a three (Figure 4-2C) or seven day (Figure 4-2E) survival period after injections of the same FG concentration (0.5%) and volume (3µL) had similar amounts of necrosis at the injection site. Cats that both had a 13 day survival period after receiving FG injections of either 0.5% (Figure 4-2A) or 2.5% (Figure 4-2B) had different amounts of tissue damage at the injection site, with the 2.5% FG cat having a substantially greater amount of damage. Thus, a small increase in FG concentration had drastic effects on the tissue necrosis. Increasing the volume of FG injections to the

spinal cord by just 1µL also resulted in greater damage at the injection site. Specifically, cats that received 2µLs of FG (0.5%) had some damage at the injection site but had no large regions of tissue loss (Figure 4-2A). In contrast, cats that received 3µLs of FG of the same concentration (0.5%) had several large regions of tissue loss (Figure 4-2C,E). Surprisingly, the addition of triton to the FG injections (0.5%, 3µL, three or seven day survival period; Figure 4-2D,F), which enhances cell membrane permeability, did not visibly increase the amount of tissue damage at the injection sites in cats with a similar survival period, FG concentration, and volume (Figure 4-2C,E).

Fluorogold Detection Techniques

FG's autofluorescent properties decrease following repeated exposure. Depending on the tracing parameters, autofluorescence also may not be as bright and easily detectable prior to any exposure. In the present study, FG autofluorescence in the motor cortex was extremely light in the cat following an injection of 3µLs of 0.5% FG, and a survival period of three days (Figure 4-3A). The addition of triton to the FG injections of a similar volume, concentration, and survival period did not enhance autofluorescence (Figure 4-3B). Autofluorescent FG detection also was limited in the red nucleus of the same cats despite being closer to the injection site (Figure 4-3C). Comparisons of FG detection at the injection site using autofluorescent detection versus anti-FG IHC detection showed a surprising amount of disparity across these two detection methods. Autofluorescent detection of FG at the injection site, the most FG-concentrated region of the nervous system, appeared to be strong (Figure 4-3E). However, when compared to an adjacent section with anti-FG IHC processing (Figure 4-3F), it was apparent that there was a substantial portion of FG not detected with

autofluorescence alone. The IHC processed tissue has substantially more detectable FG.

Additional benefits to using anti-FG IHC processing are that the DAB reaction products, and therefore FG detection, remain strong for years following the initial processing. Images were taken of sections immediately after processing (Figure 4-4A), one (Figure 4-4B), and nine years (Figure 4-4C) after processing. There is minimal to no fading at either of these time points.

Triton Increases Tracer Travel Time

Our past studies in the cat determined that a 13 day survival period for FG tracing from T12 to the brainstem, specifically red nucleus (Jefferson et al., 2011) as well as the motor cortex (refer to chapter 3) resulted in adequate neuronal cell body labeling. However, depending on the study being performed, a 13 day survival period may be an unrealistic or inconvenient period of time. For example, the attempt to assess the nervous system immediately following a specific event would be skewed by this 13 day survival period. Here, we assess the use of triton as a method for decreasing survival time. The amount of FG-labeled neurons in the red nucleus and motor cortex as detected using anti-FG IHC, were compared across cats that received FG-only or FG with triton after a three or seven day survival period. As used in our previous studies (Jefferson et al., 2011; Doperalski et al., in preparation) a 13 day survival period following injections of FG-only led to substantial FG-labeling in the red nucleus (Figure 4-5A) and motor cortex (Figure 4-5F). Labeling was typically darker in the red nucleus compared to the motor cortex due to the greater proximity to the injection site. A survival period of either three (Figure 4-5D) or seven days (Figure 4-5B) without triton led to some labeling of the red nucleus though was insufficient. However, those that

received FG injections mixed with triton and a survival period of three or seven days (Figure 4-5C) had more labeled neurons. Specifically, the FG-only cat (Figure 4-5B) had multiple dark FG-labeled neurons but were missing the more lightly labeled neurons as seen in the FG-triton animals (Figure 4-5C). A similar effect was seen in the three day FG-triton injected tissue which also had additional neurons labeled that were typically of a lighter shade (Figure 4-5E).

Group differences between FG neuronal labeling were more extreme in the motor cortex, which is ~2 centimeters further from the injection site compared to the red nucleus. While both the seven (Figure 4-5G) and three day (Figure 4-5I) FG-only animals had minimal labeling of just a few neurons in this region, the seven (Figure 4-5H) and three day FG-triton animals (Figure 4-5J) had robust labeling of many neurons. A seven day survival period (Figure 4-5H) led to labeling very similar to what was seen in the FG-only, 13 day survival period cats (Figure 4-5F), where-as a three day survival period (FG-triton; Figure 4-5J) led to labeling that was not as dark. However, this three day labeling was readily detectable for neuronal counting.

Overall, the results from this study show that FG is necrotic at the injection site and should be used sparingly to prevent extensive damage. Although a minimized FG concentration or volume may lead to underwhelming autofluorescence that is insufficient for precise FG detection, anti-FG IHC is capable of amplifying the FG signal to permit easy detection. Furthermore, the mixing of triton with FG injections can enhance tracer speed thus decreasing the necessary survival time for successful tracing. This shortened tracing time has the potential to benefit studies that are time dependent and/or have other rationales that require shorter tracing periods.

Discussion

Consistent with a few previous studies, this study shows extensive tissue damage at the injection site following FG injections (Schmued and Fallon, 1986; Divac and Mogensen, 1990). This study also showed that a slight change in concentration or volume greatly affected the amount of tissue damage at the injection site. A similar finding was found by Schmued and Fallon, 1986 who compared tissue damage following a larger range of concentrations (~10% versus ~3%) in the rat. In addition to tissue damage at the injection site, other studies have reported cytotoxic damage to the targeted cell bodies after FG tracing (Garrett et al., 1991; Naumann et al., 2000). It is possible that these small alterations in FG concentration and volume also may result in less damage to the cell bodies.

Although using small concentrations and volumes of FG injections minimizes tissue damage at the injection site and possibly the targeted cell bodies, we also showed that it is accompanied by decreased FG autofluorescence that is insufficient for neuronal counts or other quantitative assessments. While this lack of autofluorescence suggests that an insufficient amount of FG was present at the region of interest, we demonstrated that through anti-FG IHC amplification there is in fact abundant FG labeling. Similar results have been reported in Akhaven et al., 2006 who showed that anti-FG IHC detection led to the detection of FG in cells that no longer displayed FG-based autofluorescence. Furthermore, by comparing labeling of an adjacent section that received anti-FG IHC processing we demonstrated that in regions displaying bright autofluorescence, like the injection site itself, some FG was not being detected by autofluorescence. These findings suggest that IHC leads to optimal detection of FG compared to autofluorescence.

In large animal models like the cat, tracing often requires a relatively long survival period due to the longer distances being traced. We have shown that this survival period can be significantly decreased by mixing triton with the FG injections. This is most likely due to the increase in cell permeability as it has been reported that tracers taken up by pH trapping, like FG, are done so with greater efficiency if the cell membrane is more permeable (Martin W, 1991). Surprisingly, despite causing increased cell membrane permeability, the addition of triton to FG injections did not visibly enhance damage at the injection site.

There are multiple benefits to shortening FG tracer survival period. For example, decreasing the length of time FG is present in the nervous system will in turn decrease the amount of biochemical changes that are occurring as a response to FG's neurotoxic properties. Thus, the regions of the nervous system being investigated will be more similar to how they were prior to tracer injection. Fewer restrictions on the length of time needed for tracing also will benefit those studies that have certain time limitations or restrictions.

Collectively, the current study shows that by decreasing the concentration and volume of FG injections, as well as enhancing cell membrane permeability with triton, the amount of tissue damage at the injection site can be minimized and the tracing speed optimized. Furthermore, although these decreases in FG concentration, volume, and survival times often lead to insufficient autofluorescence, the use of anti-FG IHC processing can be used to achieve robust FG detection.

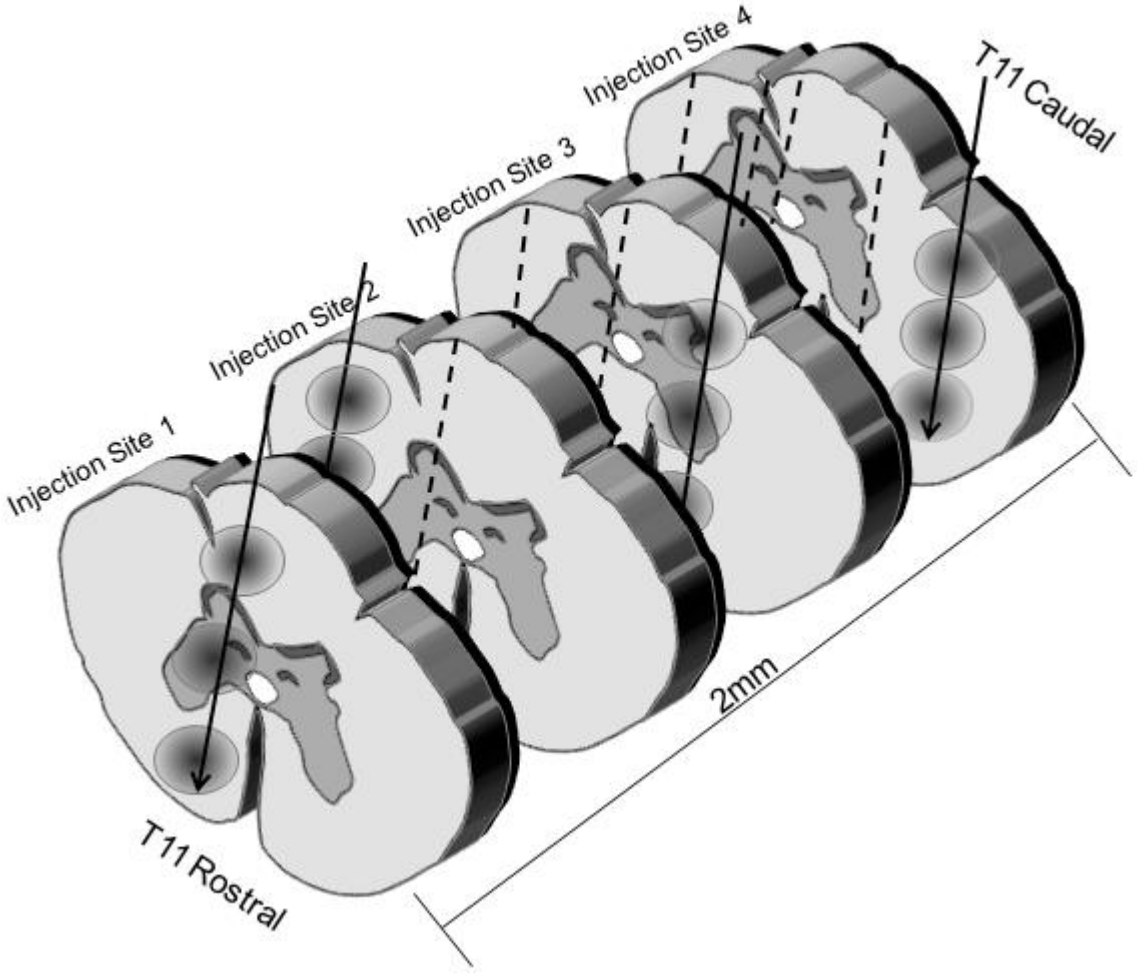


Figure 4-1. Fluorogold injection schematic. At T11 four injection sites were staggered over the span of approximately 2mm. At each injection site, three fluorogold deposits of 0.25 µL were made for a total of 3 µL.

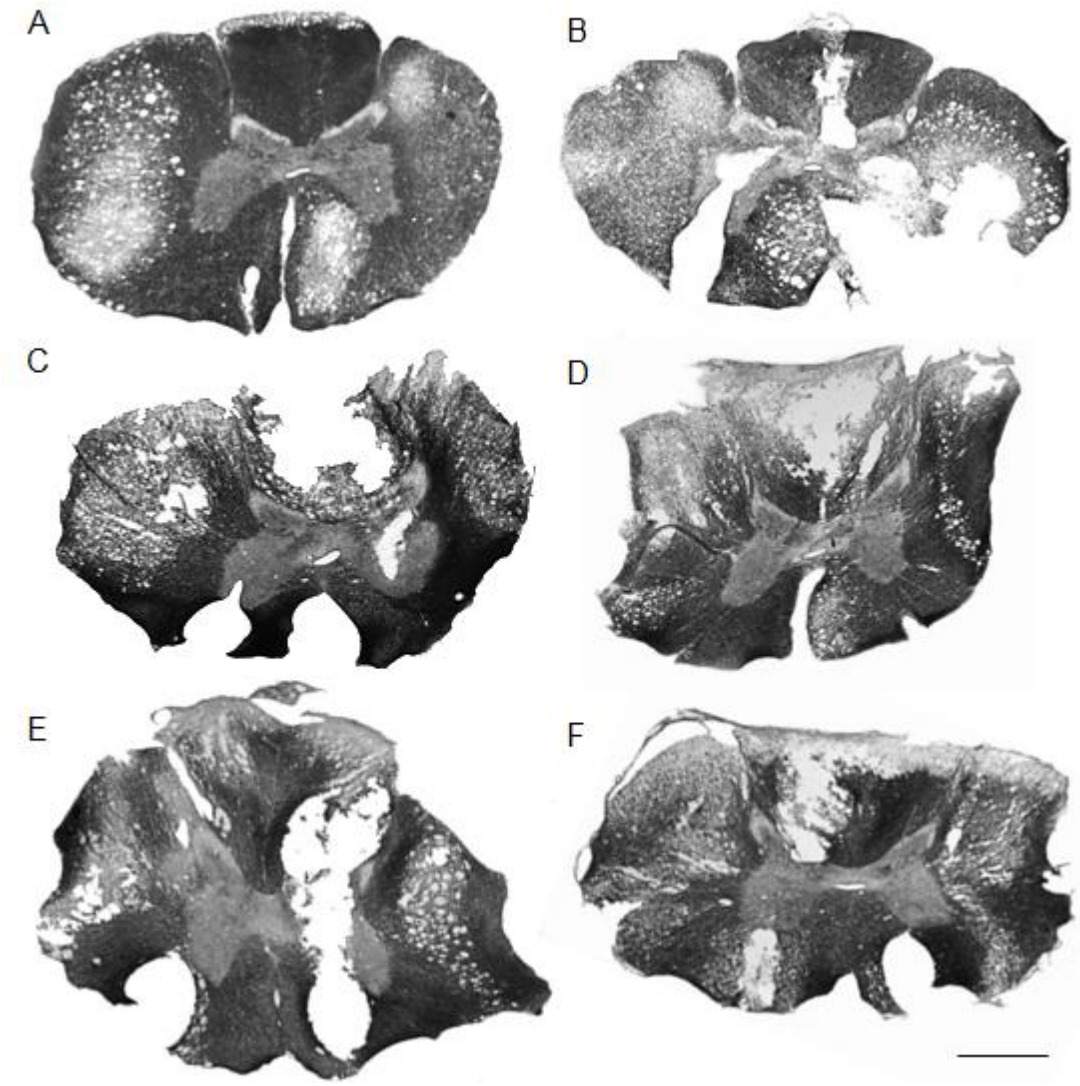


Figure 4-2. Effects of different fluorogold concentrations, volumes, and survival periods on tissue damage at the injection site. Representative sections from the injection site with the greatest amount of damage were compared across the different fluorogold injection conditions: A) 13 day survival time, 2µLs of 0.5% FG, B) 13 day survival time, 2µL of 2.5% fluorogold, C) three day survival time with 3µLs of 0.5% FG injections, E) seven day survival time with 3µL of 0.5% fluorogold injections. The effects of triton mixed with fluorogold on tissue damage also were compared in animals that received 3µL of 0.5% triton and either a three (D) or seven day survival period (F) Scale bar is 1mm.

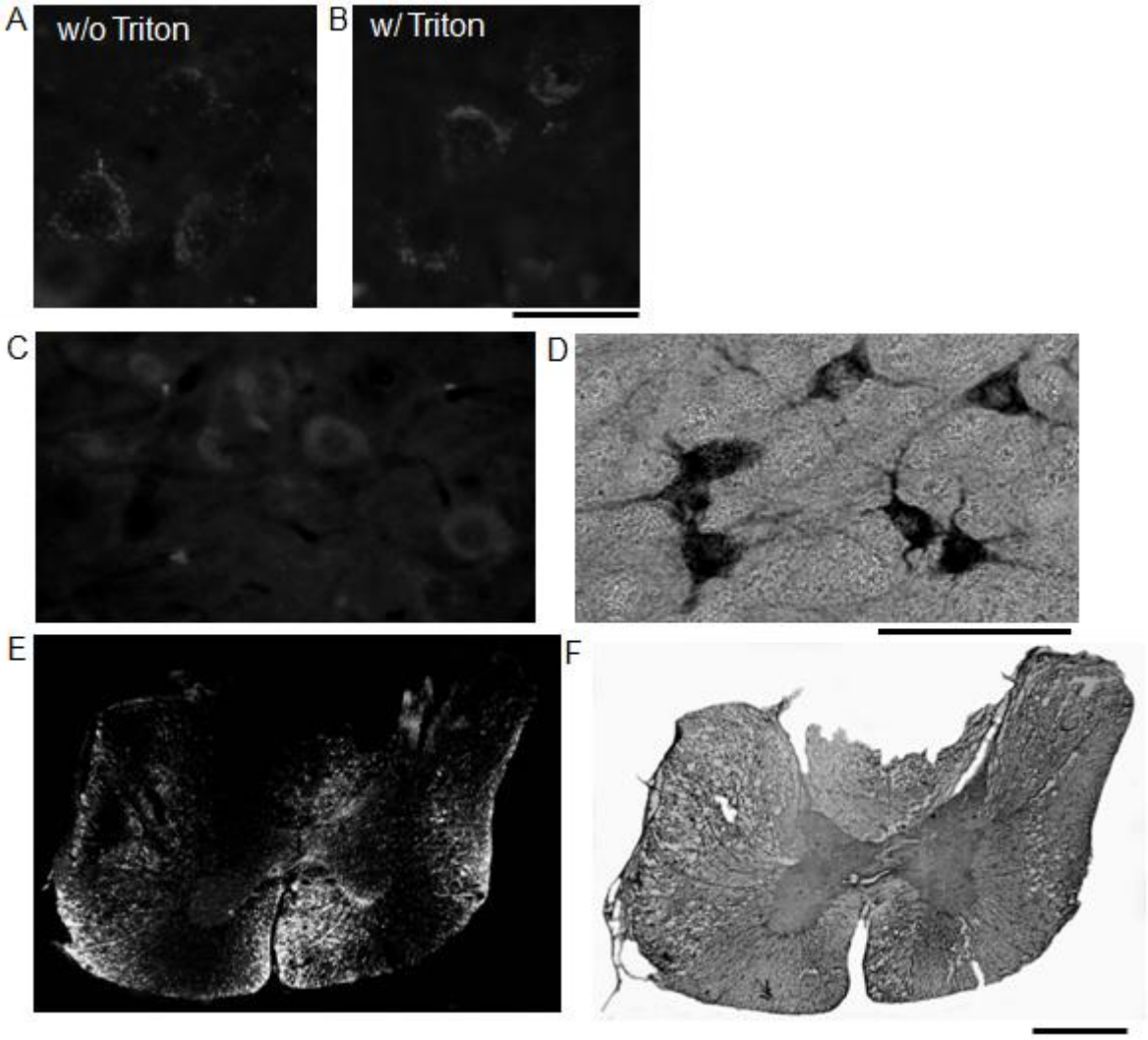


Figure 4-3. Anti-fluorogold immunohistochemical processing leads to greater fluorogold detection compared to native autofluorescent detection. Autofluorescent fluorogold (FG) detection in the motor cortex was weak following 3µL injections of 0.5% FG and a three day survival period (A). The amount of fluorescence was the same in an animal receiving the same injection parameters but with the addition of triton (B), 200µm. Autofluorescent detection was similar in the red nucleus of this same animal (C) but anti-FG IHC processing using anti-FG and DAB of an adjacent section enhanced FG detection (D), 100µm. This same effect was seen in regards to detecting the aerial spread of FG at the injection site with autofluorescence (E), and anti-FG IHC (F), 1mm.

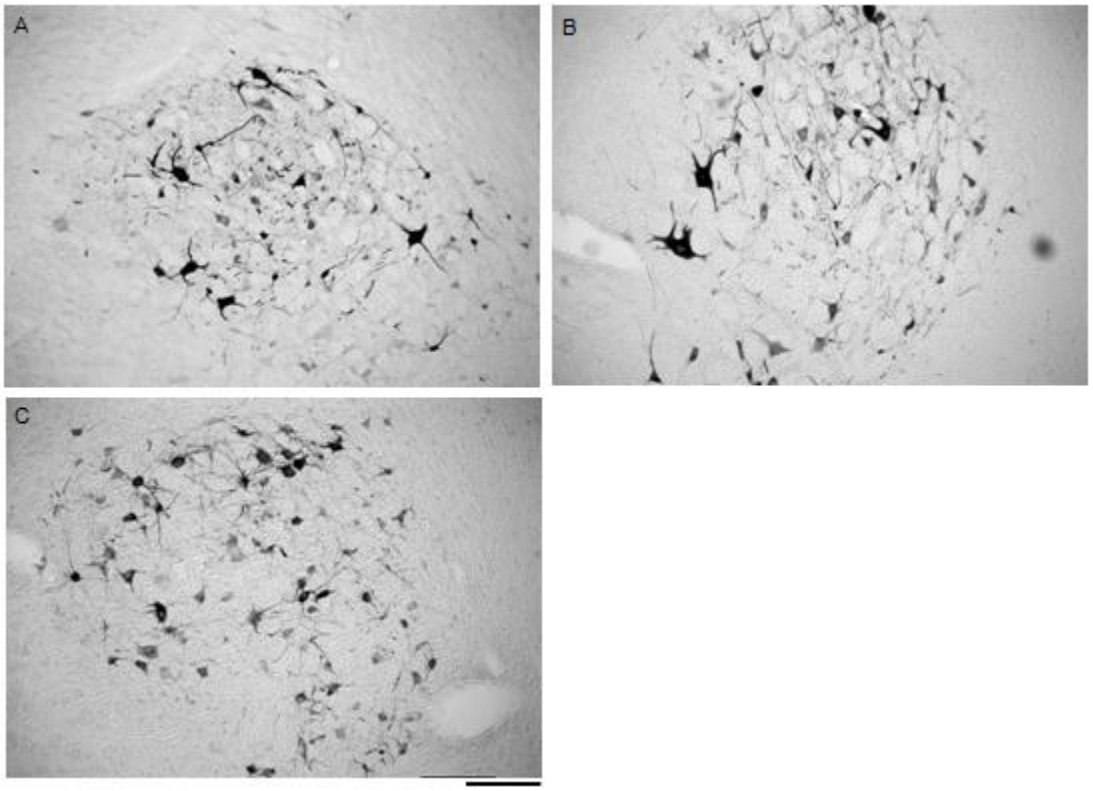


Figure 4-4. Long term fluorogold detection using anti-fluorogold immunohistochemical processing. A newly stained section of red nucleus containing immunoreactive fluorogold-labeled neurons (A) is compared to similar sections that were processed one (B), and nine years ago (C), 100µm.

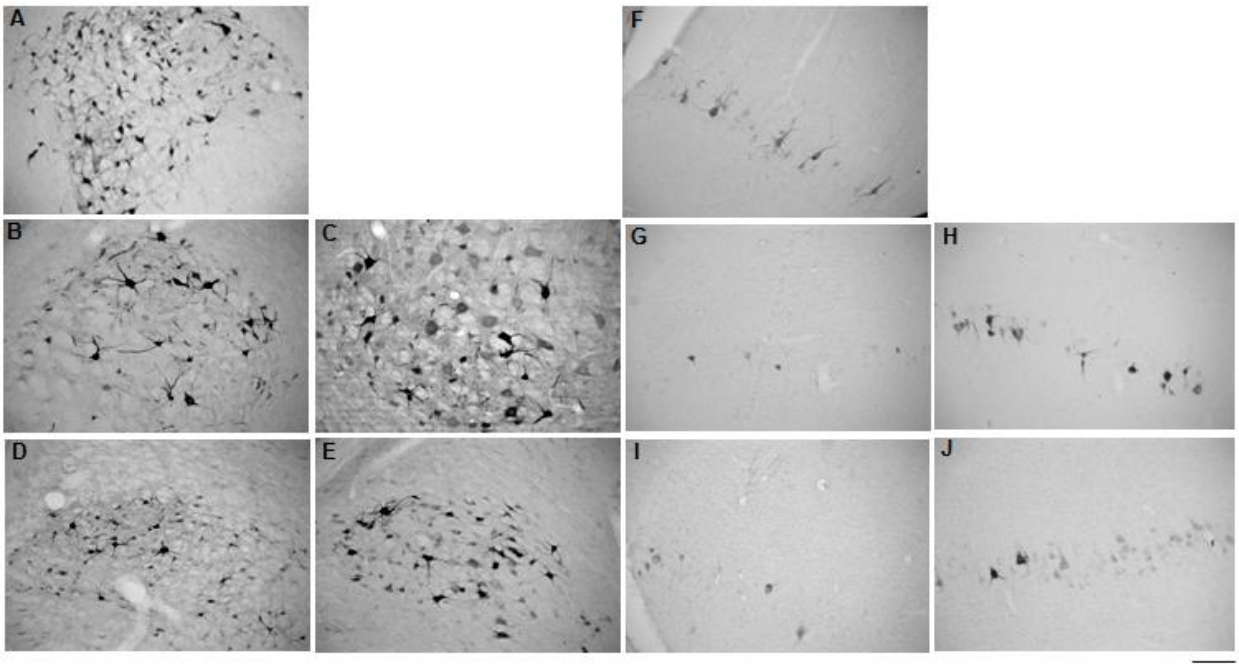


Figure 4-5. Triton enhances fluorogold tracing speed. Fluorogold-labeled neurons were compared in cats that received fluorogold without triton and had a survival time of 13, seven, or three days in the red nucleus (A,B,D) and motor cortex (F,G,I). Comparisons also were made in those that had triton mixed with their fluorogold and a survival time of seven or three days in the red nucleus (C,E) and motor cortex (H,J) 100 µm.

CHAPTER 5
SUMMARY

Defining Recovery

There is great debate amongst scientists and clinicians regarding the definition of "recovery" relative to functional changes associated with central nervous system (CNS) injury or disease. Some maintain that "recovery" has not been truly achieved unless the original functional dynamics are completely restored. Other opinions view any form of clinical improvement as being equivalent to "recovery" (Levin et al., 2009). Thus, it is critical for investigators to clearly define the definition of recovery for their specific study, as was done in Chapters 2 and 3. However, the definition employed in these studies remains controversial as they allowed for compensatory strategies supporting successful movement patterns that differed from pre-injury. The decision to use a definition of recovery that allows for compensatory strategies as opposed to the strict definition of recovery described in Levin and colleagues, is primarily due to the extensive changes in axonal circuitry that are known to occur by way of injury-induced sprouting and regeneration (Bareyre et al., 2004; Courtine et al., 2009; Blum, 2010). This novel circuitry, which underlies locomotor performance, will most likely be accompanied by novel movement patterns that differ from pre-injury. A more strict definition of recovery may be difficult to establish and not particularly applicable to individuals with neurological injuries. Using this line of thought "compensation" or "the use of new motor patterns resulting from the adaptation of remaining motor elements or substitution (Levin et al., 2009)" may be synonymous with "recovery" as new circuitry most likely will strongly incorporate and require enhanced use of spared fibers.

Advancing Chondroitinase ABC to the Clinic

As chondroitinase abc (ch'abc) continues heading towards a clinical direction the definitions for recovery in subsequent studies, as well as the functions being assessed need to be well defined and deliberately chosen. As reported in Chapter 3, models of partial spinal cord injury (SCI), similar in many respects to Brown-Sequard Syndrome (BSS), showed the greatest ch'abc-mediated functional effects on adaptive tasks like the peg walkway and were not as readily detected on more simple tasks like the overground walkway. However, ch'abc-mediated effects may be more noticeable on simple tasks in larger injury models, such as contusions. Furthermore, animals frequently employed a variety of compensatory strategies for successful completion of the more adaptive tasks that differed across ch'abc and control cats (Jefferson et al., 2011). Thus, not only is it important to carefully choose the functional assay used for the model of injury being assessed but, it also is critical to assess performance of a variety of different gait features in order to properly understand the effects of ch'abc on functional improvements after SCI. For future clinical assessments, the development of a test battery for adaptive locomotor features for the human SCI population would be beneficial and allow for quick assessment of adaptive functions as well as their improvements over time, as reported in our previous ch'abc studies (Tester and Howland, 2008; Jefferson et al., 2011).

Another major translational issue is whether the magnitude of therapeutic efficacy seen in an animal study will translate to humans. Therapeutic effects seen in small rodent models of SCI indicate promising treatment potential, but do not necessarily mean the treatment will be as effective in humans. Moving these treatments to larger animal models provides greater insight into the therapeutic potential of treatments.

Results from Chapter 3 demonstrate this issue. Despite there being multiple reports of two weeks of ch'abc administration or less leading to significant functional recovery in rodent SCI models, this treatment duration was not effective in our larger cat model of SCI, but four weeks administration resulted in significant functional benefits. It is possible that due to the larger size of humans, four weeks of ch'abc may not be sufficient for humans, but that six to eight weeks of treatment may be more optimal. It would be interesting to determine how a six week period of ch'abc administration will affect functional recovery and if greater recovery can be achieved with this longer treatment duration or, if too much ch'abc administration could be detrimental in some way. In addition to duration, optimal ch'abc concentration and treatment window also are important to assess. Although several studies have begun to look at treatment windows, the majority have used a single injection paradigm. This single dose approach, as determined in Chapter 3, is most likely not an optimal treatment duration (Yick et al., 2003; Massey et al., 2006; Iseda et al., 2008). Furthermore, determining the best mode of ch'abc administration is critical. Currently the most common mode of delivery is via intrathecal administration. However this mode has been reported to cause increased scarring and compression to the spinal cord (Jones and Tuszynski, 2001) and is not particularly feasible in humans with acute SCI. Several studies have begun to identify alternative modes of delivery such as a thermostabilized ch'abc hydrogel (Lee et al., 2009), slow ch'abc-release fibrin gel (Hyatt et al., 2010), and ch'abc lentiviral vectors (Jin et al., 2011). However, assessments of these delivery modes are limited, and it is unclear how effective they will be. The need to continue to explore clinically applicable

delivery approaches is one of the more critical variables for the future translation of this therapeutic.

Conclusions

As research continues to allow for a better understanding of the physiological responses and plastic potential of the central nervous system after injury, the development of a treatment paradigm capable of significantly enhancing functional recovery after injury appears more plausible than previously thought to be. An effective treatment paradigm will most likely require a multifaceted approach and, as depicted in Chapters 2 and 3, the therapeutic agent ch'abc and an increased focus on optimizing training/rehabilitation for simple as well as adaptive locomotor functions will be important components of this effective treatment paradigm. The optimization of tract tracing in both small and large pre-clinical animal models will aid in this treatment's development by allowing for an understanding of the treatment-mediated circuitry changes that underlie the functional changes.

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BIOGRAPHICAL SKETCH

Sarah Elizabeth Mondello was born the 2nd of three daughters during the summer of 1983, in Austin, Texas. Two years later she moved with her family to Niskayuna, NY where she spent the rest of her childhood years. Through '99-'02 Sarah attended Niskayuna High School where she grew particularly interested in clarinet performance. After graduating from high school in 2002 she attended McGill University as a music performance major and studied with the clarinetist Alain Desgagne of the Montreal Symphony Orchestra. In addition to the multiple music courses she took during her first year of study, Sarah also took several psychology courses and became increasingly more interested in that field of study. She switched her major to psychology just prior to beginning her second year of undergraduate studies.

For her remaining time at McGill, Sarah became particularly interested in the neuroscience components of her psychology studies. She spent two summers as an intern at the Biosciences division of General Electric where she was first introduced to research and studied Alzheimer's Disease. This particular experience led her to choose a career path towards neuroscience research. Following graduation from McGill in 2006, Sarah immediately began working on a neuroscience Ph.D. at the University of Florida, Interdisciplinary Program in Biomedical Sciences. She joined the lab of Dena Howland in 2007 and began her studies on plasticity and functional recovery following spinal cord injury. In August 2012 Sarah successfully defended her research and obtained her doctoral degree.