University of Alberta

LOCOMOTOR RECOVERY AFTER SPINAL CORD INJURY IN RATS: BEHAVIORAL COMPENSATION, AXONAL SPROUTING, AND TREATMENT INDUCED REPAIR

by

Mark Ballermann

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ABSTRACT

Persistent sensorimotor impairments after mammalian spinal cord injury (SCI) are partly due to axons' inability to regenerate. In rats with SCI, overground and skilled walking behaviors provide post-injury functional readouts. Moderate spontaneous recovery of walking following incomplete SCI occurs in rats and patients, and interventions promoting this recovery may form future treatments. This thesis examined behavioral and anatomical correlates of recovery of walking in rats after incomplete SCI, and treatments to improve post-injury walking. Movements performed by rats recovering walking after dorsal SCI were examined using kinematics and electromyography. I found that rats with visibly normal post-injury walking use altered muscle activation patterns, including increases in fore limb extensor activity, altered recruitment of a back muscle inserting into the pelvis, and elevated hind limb postures during stance. In skilled walking, rats crossing horizontal ladders use haptic cues from the forelimb to locate rungs. Compared to overground walking, rats increased hind limb flexion during swing to reach the bar the fore limb had contacted immediately before. Fore limb sensation impairments from dorsal column injuries resulted in hind limb placement on adjacent bars and more falls between bars. Post-injury walking changes may underlie walking recovery in rats, and inspire clinical interventions to improve mobility by mimicking analogous changes in patients.

Next, I investigated some nervous system changes that likely contribute to post-injury walking recovery. The reticulospinal tract (RtST) activates neuronal networks that generate walking in rats. I found that after lateral hemisection of the thoracic spinal cord, increased projections of the spared RtST into the lumbar grey matter accompanied weight-bearing stepping recovery. Finally, two pharmacological treatments were given to separate groups of rats to examine if either could improve post-injury walking (as previously shown), and whether additional improvements in walking occurred alongside RtST projection changes. An inhibitor of ROCK and a molecule blocking known components of myelin inhibition of axon regeneration were given intrathecally. These treatments did not improve walking or

affect the RtST.

Although treatment-induced recovery was not found, my thesis shows rats with SCI developing alternative walking patterns and altered connectivity of the RtST, which could improve walking after SCI.

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LIST OF ABBREVIATIONS

- Amb – Nucleus Ambiguus
- ABC – avidin-biotin-peroxidase detection
- BBB – open field locomotor score developed by Basso, Beattie and Breshnahan (Basso et al., 1995)
- BDA – biotin dextran amine
- CNS – central nervous system
- CPG – central pattern generator
- CSPG – Chondroitin sulfate proteoglycan
- CST – corticospinal tract
- DAB – diaminobenzidine
- EMG – electromyography
- GEF – guanine exchange factor
- Gi – gigantocellularis nucleus of the medial reticular formation
- GiV – ventral gigantocellularis of medullary reticular formation
- IOD – inferior olive - dorsal nucleus
- IOM – inferior olive - medial nucleus
- IOPr – inferior olive - principal nucleus
- L – lumbar spinal cord level

- LED – light emitting diode
- LONG – longissimus muscle
- LPGi – lateral Paragigantocellularis
- MAG – myelin associated glycoprotein
- MRF – medial reticular formation
- NgR Fc – soluble Nogo receptor ectoplasmic domain linked to IgG, also known as NgR(310)ecto-Fc from (Li et al., 2004)
- OMgp – Oligodendrocyte Myelin associated glycoprotein
- RhoA – small guanine exchange factor regulating actin cytoskeleton formation of stress fibers and growth cone collapse
- ROCK – RhoA-associated kinase
- RtST – reticulospinal tract
- RST – rubrospinal tract
- RVL – rostral ventrolateral thalamic nucleus
- SCI – spinal cord injury
- SD – standard deviation
- T – thoracic spinal cord level
- TA – tibialis anterior muscle
- TBST – tris-buffered saline with 0.5% Triton X-100
- TRI – triceps brachii muscle

- VL – vastus lateralis muscle
- VLF – ventro-lateral funiculi
- Y27632 – ROCK inhibitor

CHAPTER 1

APPROACHES TO INDUCE LOCOMOTOR RECOVERY AFTER SPINAL CORD INJURY IN THE RAT

1.1 SPINAL CORD INJURY IN MAMMALS

The mammalian spinal cord is a caudal outgrowth of the brain that communicates most sensory and motor information between the brain and the periphery. Damage to this structure, termed spinal cord injury (SCI), results in a loss of sensation and motor control below the level of the lesion. Recovery is limited because severed axons of the mammalian central nervous system (CNS) generally do not regenerate (reviewed in Schwab and Bartholdi, 1996), but some recovery occurs spontaneously in cases of partial injury. There have been some attempts to ameliorate the symptoms of SCI, and build on the partial recovery. Treatments to reduce the impact of SCI-related disabilities are greatly needed, in part because SCI patients tend to be young and retain a normal life expectancy. Over patients' lifetimes unmitigated disabilities presently consume a great deal of scarce health care resources. Perhaps more importantly, SCI-associated disabilities greatly impact quality of life for both patients and their families. As such there is a great need for treatments that can lessen the impact of SCI-associated disability.

One group of approaches to mitigate the effects of SCI in patients includes reducing the amount of neural tissue lost following an injury. The prevention of neural tissue damage occurring in the hours, days, and weeks after the initial injury (secondary damage) is a promising approach, but is outside the scope of this thesis and the reader is directed to more suitable reviews of this topic (Hagg and Oudega, 2006; Hausmann, 2003). A second group of general approaches to overcome disconnections resulting from SCI has been directed at inducing severed spinal tracts to regenerate and form functional connections with their denervated targets, either directly or indirectly. As our understanding of the spinal cord increases both the limitations and potential of this approach have become more clear.

Since ancient times it has been noted that the prospects for recovery following SCI were dim (reviewed in Goodrich, 2004). Early observations led investigators to believe that CNS axons were incapable of regeneration, until the findings of Aguayo and colleagues ignited the field of spinal cord repair when severed central sensory axons were found to grow

into a peripheral nerve graft (Aguayo et al., 1981; David and Aguayo, 1981). This result showed that CNS axons were capable of regenerating, if given a suitable environment to grow into. Later studies by Schwab and colleagues had found that the CNS environment, and in particular molecules associated with the myelin sheath (myelin associated growth inhibitors) are one group of factors preventing axonal regeneration (Caroni et al., 1988; Caroni and Schwab, 1988). The multiple factors preventing mature CNS neurons from regenerating severed axons can be divided into environmental barriers outside the neuron, and cell-intrinsic properties inside the neuron. The environmental barriers are introduced in the next section.

1.2 CNS ENVIRONMENT BARRIERS TO AXONAL REGENERATION

The inhibitory nature of the injured CNS environment against axonal regeneration has become better understood in the past decade. Part of this inhibition is the result of the formation of a glial scar at the injury site, where biochemical and physical barriers form against regenerating axons (reviewed in Busch and Silver, 2007). Other components of CNS inhibition are expressed throughout the CNS (reviewed in Qiu et al., 2000). Several inhibitory signaling molecules are associated with the myelin sheath surrounding the axon, and damage to these myelin sheaths occurring during SCI can result in axons being exposed to myelin associated growth inhibitors. These myelin-associated growth inhibitors can effect regenerative failure through different receptors and intracellular pathways in the axon. The expanding number of known extracellular and intracellular inhibitory pathways have led investigators to devise approaches to counter the varied components of CNS inhibition with the aim of generating novel treatments for SCI. These approaches include using cellular grafts of various types to provide a favorable growth environment for axons (Ramon-Cueto et al., 2000; Pearse et al., 2004), modifying the extracellular matrix with enzymes to remove inhibitory cues (Bradbury et al., 2002), and blocking myelin

associated growth inhibitors with specific antibodies (e. g. Schnell and Schwab, 1993; Liebscher et al., 2005) or pharmcological antagonists (Wang et al., 2006). Blocking myelin associated growth inhibitors can be accomplished via several approaches. One approach involves a receptor expressed by neurons that recognizes 3 myelin associated growth inhibitors (i. e., Nogo, Omgp and MAG), the NgR receptor (reviewed in McGee and Strittmatter, 2003; Figure 1.1). A recent approach to block this inhibition has involved the use of a soluble portion of the NgR receptor known as NgR(310)ecto-Fc (NgR Fc; Li et al., 2004). This soluble portion of the NgR receptor acts as an antagonist to the myelin associated growth inhibitors (Nogo, MAG, and Omgp), in contrast to the previously characterized NEP1-40 antagonist which only blocks the interaction between NgR and the Nogo-66 domain of the Nogo protein (GrandPre et al., 2002).

Although using pharmacological and enzymatic manipulations to modify the CNS environment has met with some success in coaxing axons to regenerate, the anatomical evidence reported thus far has shown that out of the total number of severed axons, only a small (¡1%) percentage show evidence of regeneration (Steward et al., 2003). These fibers regenerate for only a limited distance, to date no farther than several millimeters. Some insights regarding the mechanisms behind regeneration failure can be derived based on the results of these studies. In the case of descending tracts, serotonergic fibers appear most likely to sprout or regenerate, whereas corticospinal tract fibers are fairly unlikely to sprout spontaneously after injury (Inman and Steward, 2003), or regenerate in response to the administration of treatments (Fouad et al., 2005). As different descending tracts sample the same CNS environment cues, it is likely that properties intrinsic to the descending neurons account for the different cellular responses following CNS environment modification.

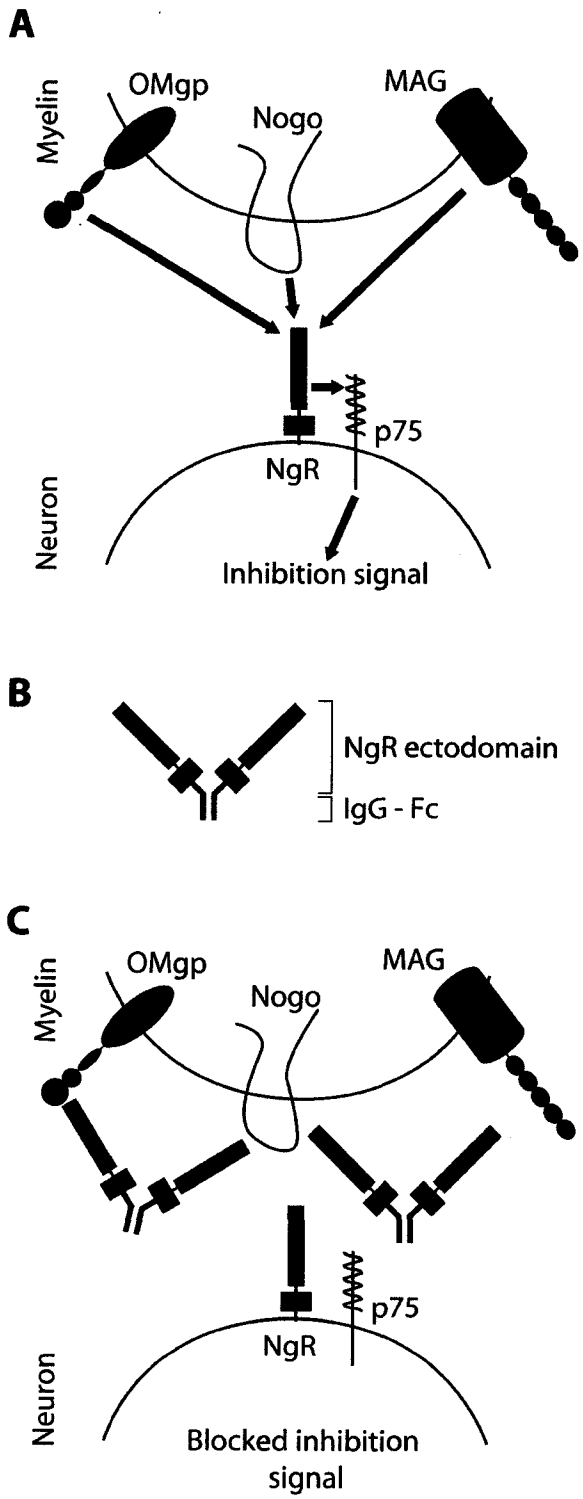

Figure 1.1: NgR Fc action – Schematics of three molecules (Nogo, OMgp, and MAG) expressed on myelin that mediate CNS-myelin growth inhibition are shown in (A). These all act on the NgR-p75 receptor complex which is expressed by neurons (bottom of panel A) and transduces the inhibitory signal. The NgR Fc molecule consists of the ectodomain of NgR (labeled rectangular boxes) fused to the Fc portion of the IgG antibody (angled lines below labeled IgG-Fc; B). This molecule should block any ectoplasmic molecule that binds to NgR, including Nogo, OMgp, and MAG (C), which results in a disrupted inhibition signal.

1.3 CELL INTRINSIC FACTORS PREVENTING SPINAL REGENERATION

In vitro, and *in vivo* adult CNS neurons are more restricted in their tendency to grow and extend neurites compared to developing neurons and axotomized neurons of the peripheral nervous system (PNS) (Fenrich and Gordon, 2004). Developing and PNS neurons will respond to injury with an increase in their expression of a group of genes and their associated proteins whose expression is highly correlated with axonal regeneration. These regeneration associated genes (RAGs) include cJun, and GAP43 and their expression becomes downregulated as axon growth is completed. The restricted degree to which adult CNS neurons will regenerate, even when given a permissive environment may partly be the result of a lack of increase the expression of RAGs. One group of approaches to improve axonal regeneration utilizes extracellular signaling molecules to put neurons into a more growth friendly state, in part by increasing the expression of RAGs. This can be accomplished via the addition of neurotrophic factors into the CNS (reviewed in Plunet et al., 2002). Neurotrophic factors include the neurotrophins: nerve growth factor, brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4/5). Also, epidermal growth factor, fibroblast growth factors, glial derived neurotrophic factor, platelet derived growth factor, ciliary neurotrophic factor and insulin-like growth factor have neurotrophic activity (reviewed in Lewin and Barde, 1996). Neurotrophins such as NT-4/5 and BDNF can also improve survival and reduce atrophy of rat rubrospinal neurons following injury (Kobayashi et al., 1997). One possible limitation of neurotrophins as a clinical treatment for SCI is that they need time to effect axonal regeneration, via second messengers such as cyclic AMP (cAMP) and transcriptional regulation. Consistent with this, it has been found that addition of BDNF or GDNF to dorsal root ganglion cultures simultaneously with exposure to myelin associated inhibitors was insufficient to block inhibition, but exposing the neurons to these neurotrophins overnight before plating them with the inhibitors could abolish inhibition of axonal regeneration (Cai et al., 1999).

The neurotrophins bring about their effects through a group of signaling pathways that have been well studied. One component of these intracellular signaling pathways is cyclic AMP (cAMP). A second approach to improve the chances of regeneration of adult CNS neurons involves increasing cAMP levels. These manipulations include the addition of a cell-permeable form of cAMP known as dibutryl-cAMP (db-cAMP) or the addition of a phosphodiesterase (enzyme responsible for breaking down cAMP) inhibitor known as rolipram. Manipulations affecting cAMP have been shown to increase neurite outgrowth on normally inhibitory substrates *in vitro* (Cai et al., 1999; Cai et al., 2001; Gao et al., 2003) and *in vivo* (Neumann et al., 2002; Lu et al., 2004), with a shorter priming period needed than neurotrophins. Interestingly, young neurons have higher levels of cAMP at developmental stages when they are insensitive to myelin associated inhibition, than older neurons (Cai et al., 2001). To an extent higher endogenous levels of cAMP in developing neurons would increase the likelihood of neuronal survival up to the point when axons elongation is complete, and the developed neuron becomes dependent on target derived factors for survival. Manipulations of cAMP levels can result in the growth cones (the chemosensitive tips of extending axons) of older neurons becoming insensitive to inhibitory cues in the cellular environment as well (Ming et al., 1997; Cai et al., 2001; Nishiyama et al., 2003). This leads to a third approach, where investigators can add drugs to the spinal environment to render growth cones insensitive to inhibitory environmental cues.

One group of molecular switches known as Rho-guanine exchange factors control growth cone extension and collapse by acting on actin polymerization. The extending growth cone relies on a balance of activities between several antagonistic Rho-guanine exchange factors: Rac, Cdc42 and RhoA (Figure 1.2). These antagonistic signaling factors regulate the formation of different portions of the growth cone. Activation of Cdc42 or Rac1 results in increased polymerization and growth cone extension (Hu et al., 2001), whereas activation of the RhoA kinase or its downstream target ROCK leads to growth

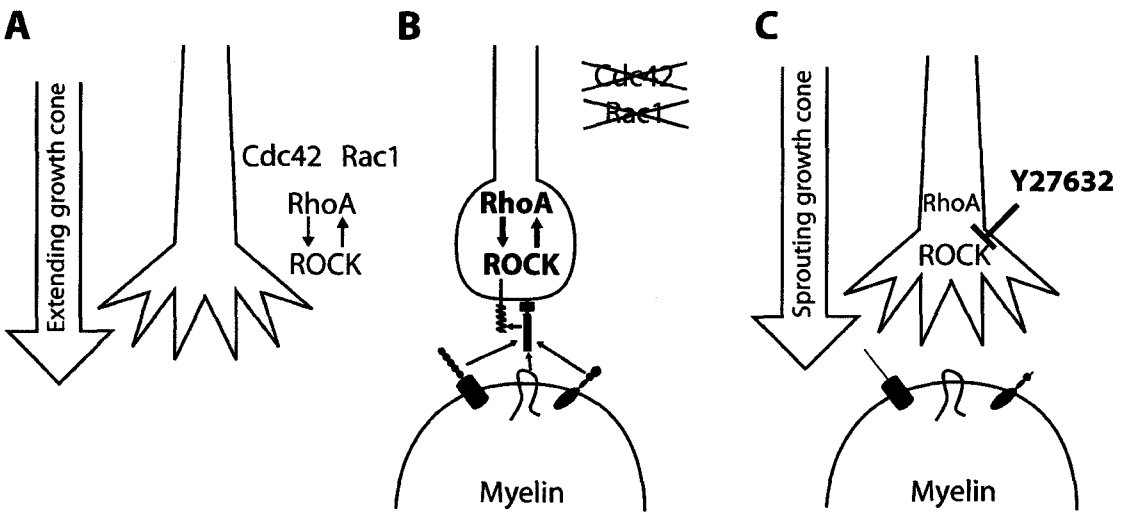

Figure 1.2: Y27632 action – A schematic of an extending growth cone from the tip of an axon during development is shown in (A). During normal extension the activities of the Rho family of guanine exchange factors Cdc42, Rac1 and RhoA are in balance (normal typeface). ROCK represents the downstream target of RhoA. Upon encountering myelin, the activity of RhoA/ROCK becomes greatly increased and Cdc42/Rac1 becomes reduced. The sum of activities of these molecules on actin polymerization results in growth cone collapse, as depicted by the presence of a retraction bulb in (B). When Y27632 is added, ROCK becomes inhibited leaving the possibility for the growth cone to become insensitive to CNS/myelin inhibition, and regeneration and/or sprouting to occur.

cone collapse. Direct manipulation of the molecular pathways affecting growth cone dynamics reportedly increases sprouting of both severed and intact spinal tracts following SCI (Dergham et al., 2002). One approach involves blocking RhoA or ROCK via the application of Y27632 which has resulted in some functional recovery following different incomplete SCI models (Dergham et al., 2002; Sung et al., 2003; Chan et al., 2005; Chan et al., 2007).

In all, the many mechanisms blocking CNS axonal regeneration show that repairing an injured spinal cord will require a combination of treatments to allow axonal regeneration to occur. Although the obstacles to regeneration are clearly a hindrance in the injured CNS, the fact that they have arisen through evolutionary processes, and have been broadly conserved across many mammalian species, suggests that they have a critical role in nervous system function.

1.4 CNS REGENERATION AND PLASTICITY

The various mechanisms preventing axonal regeneration after SCI are conserved across all mammals. The fact that many species share features making their CNS inhibitory to axonal regeneration after injury might lead one to conclude that they serve some function under intense evolutionary selection pressure. Alternatively, as injuries sufficiently severe to damage the CNS are usually lethal, it may be the result of a lack of selection pressure to maintain the regenerative capabilities observed in invertebrates and some lower vertebrates (Yin and Selzer, 1983). Given the varied nature of the obstacles against axonal regeneration, it seems more likely that there exists a positive selection pressure to limit the regenerative capacity of the CNS. It is certain however, that these obstacles will need to be overcome to induce regeneration and consequent functional repair in animal models or patients. A possible source of this selection pressure could have to do with nervous system development. The developing nervous system passes through "critical periods", during which the establishment of new functional connections and the synaptic

pruning of unnecessary connections can take place (Hubel and Wiesel, 1970). During these "critical periods" in development, the nervous system has a greater capacity to form new connections which come to underlie important functions, such as vision, in adulthood. In mammals, the "critical period" comes before the organism needs to survive on its own. In adulthood, however, the connections formed during development come to underlie functions that are likely to be critical to the survival of the adult organism. It may be that the mechanisms preventing regeneration stabilize the nervous system in adulthood, maintaining connections needed to last long periods of time, and in the process, limit regeneration in a way that is beneficial to intact organisms. In general, the process of forming connections, and strengthening or weakening connections such that the nervous system can acquire new functions is termed plasticity.

This arrangement introduces complexity for researchers testing potential new treatments for SCI. Whether the approaches to treat SCI are aimed at making the CNS environment less hostile to growth or affecting neuronal responses to that environment, most have the capacity to alter CNS circuitry to some extent. Treatments administered at an injury site with the intention of overcoming the obstacles against regeneration, could destabilize the CNS by inducing changes at areas away from the injury site, and result in positive or negative changes in CNS function. For example, treatment-induced sprouting of sensory afferents onto ascending spinothalamic neurons in lamina II of the spinal cord could result in neuropathic pain as a side effect (reviewed in Finnerup and Jensen, 2004). Plasticity-enhancing treatments could also improve functional recovery in patients with partial injuries by rerouting motor commands around damaged tissue by using spared (uninjured) fibers (Fouad et al., 2001; Bareyre et al., 2004). Treatments can manipulate nervous system plasticity using spared fibers to improve functional outcome, or improve the chances of transmitting commands through a lesion site via axonal regeneration (Figure 1.3). Both treatment-related mechanisms have the potential to contribute to improvements in functional outcome after SCI, in animal models and in patients. The use of accurate

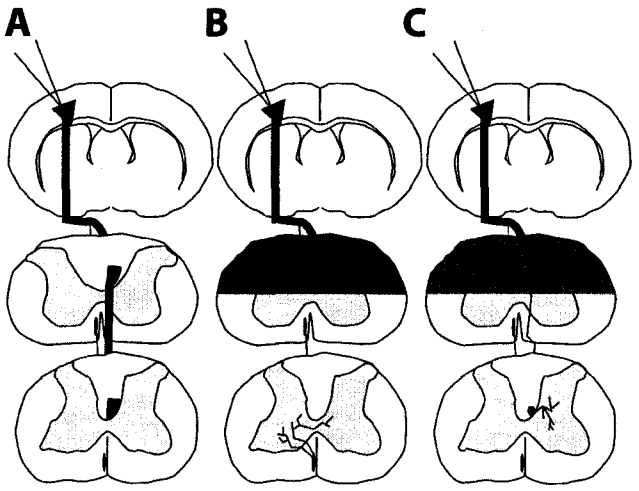

Figure 1.3: Anatomical plasticity of spared and severed CST fibers – Anatomical plasticity of spared and severed CST fibers – The normal pattern of CST innervation to the cervical spinal cord is shown in A. The CST cell bodies are represented by a triangle at the level of the cortex, which send projections down the spinal cord in the form of the crossed dorsal CST (thick line) and the uncrossed ventral CST (thin line). The middle panel represents C3 and lower panel represents C5. For simplicity, the lateral CST is not shown. After a dorsal hemisection injury at the level of C3 in B, spared ventral fibers may sprout and increase their innervation caudal to injury. After treatment, severed axons may also regenerate (thin twisted line) through the lesion site (shown in dark grey) to reinnervate the grey matter at C5.

assessments of spinal function in animal models should improve the likelihood of finding clinically useful treatments. Rats are frequently used as animal models of SCI to test pharmacological treatments and investigate the nervous system response to injury and treatment. Walking assessments are frequently used as readouts of spinal cord function after SCI.

1.5 WALKING AS A READOUT OF SPINAL CORD FUNCTION IN RATS

Animal models provide the capability to test different treatments intended to repair the spinal cord and find the degree to which functional recovery occurs after SCI. A commonly used readout of spinal cord function in animal models (e. g., rats) is provided by locomotor behaviors, including walking. Locomotor behaviors are commonly used in studies of SCI as they can be easily scored and are repeatedly and spontaneously performed by rats and other species. To understand how spontaneous recovery of walking occurs in the rat model of SCI, it is important to understand how the behavior is generated by the intact CNS (reviewed in (Kiehn, 2006)). The muscles of the legs are activated in the appropriate sequence and to the appropriate degree by motoneurons in the spinal cord. This output pattern of the legs is called the locomotor pattern. Hind limb motoneurons are activated by neuronal networks within the gray matter of the lumbar spinal cord, known as central pattern generators (CPGs; Figure 1.4). The CPG integrates descending input and sensory information to activate motoneurons appropriately to generate the locomotor pattern. The locomotor pattern is thought to be governed by the sequential activation and inhibition of mutually inhibitory groups of neurons termed half-centers. The mutual inhibition acts between half centers governing extensor motoneurons (i. e., extensor half-centers) and flexor half-centers on each side of the spinal cord. The extensor and flexor half-centers on each side also sequentially inhibit their counterpart on the contralateral side. CPG activation is governed by many factors, including sensory input and descending signals

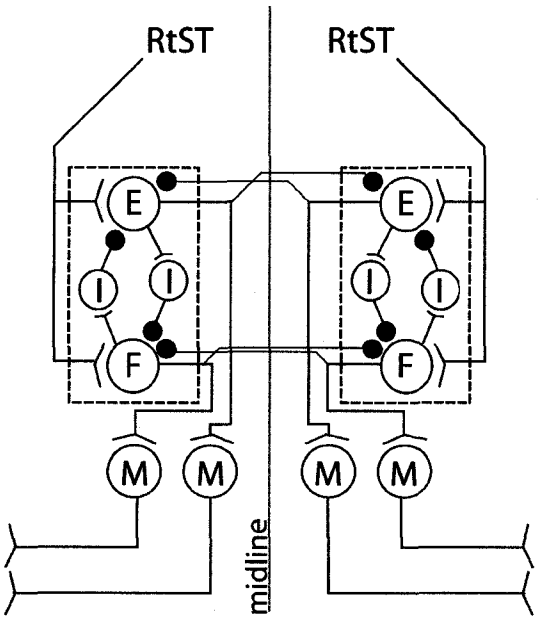

Figure 1.4: Schematic of CPG organization – Circles represent the different groups of central neurons in the lumbar spinal cord that control hind limb movements during locomotion. Inhibitory connections are represented by filled circles at the end of each line originating from a half-center.The dashed boxes contain the components of the CPG on each side. The "E" and "F" labeled circles represent the extensor and flexor half-centers respectively. These half-centers inhibit each other via inhibitory interneurons (labeled with "I") and stimulate their respective motoneurons (labeled with "M") which in turn innervate and activate the appropriate hind limb muscle. The extensor and flexor half centers also innervate their contralateral counterpart in a reciprocal manner. The CPG is activated by tonic input from the RtST (reticulospinal tract).

carried by the reticulospinal tract (RtST).

The RtST is a prominent relay between the brainstem and the CPG, and is known to play an important role in the initiation of locomotion by providing tonic stimulation to the CPG (Orlovsky, 1970; Noga et al., 1988; Noga et al., 1991; Mori et al., 1992; Jordan, 1998). The RtST is a heterogeneous tract with cell bodies in dispersed nuclei in the reticular formation of the brainstem (Jones and Yang, 1985). The RtST consists of noradrenergic, glutamatergic, GABAergic, and serotonergic fibers, which mainly project ipsilaterally, but also has some projections running contralaterally, in the ventral, ventro-lateral and dorso-lateral funiculi (Jones and Yang, 1985). There are several lines of research examining the role of the reticulospinal tract in locomotion (Orlovsky, 1970; Mori et al., 1992), including findings showing that spinal cord lesions involving the RtST abolish the initiation of stepping (Steeves and Jordan, 1980; Noga et al., 1991). Given that RtST projections are needed for the initiation of stepping movements, observing locomotor behaviors should allow the determination of whether functional RtST connections have been restored after SCI. In injury models where partial injuries are employed, the degree of ventral tract (and RtST) sparing correlates well with walking performance and open-field walking scores (Loy et al., 2002; Schucht et al., 2002).

1.6 SPONTANEOUS RECOVERY OF LOCOMOTOR FUNCTION

Partial SCI models have the advantages of simpler animal care, and better prospects for locomotor recovery, while allowing investigators to determine the roles of the different spinal tracts. Both humans and animals have been suggested to perform behavioral tasks using abnormal compensatory movements in order to counteract a motor deficit following CNS damage including SCI (McKenna and Whishaw, 1999; Webb and Muir, 2002; Webb and Muir, 2003; Grasso et al., 2004). These movements may result when the full palette of movements is not available due to enduring deficits. The precise neural correlates of such compensatory movements will likely prove difficult to elucidate, but a necessary

step in examining how the nervous system recovers locomotion is to describe compensatory movements adopted by rats with SCI (Kaegi et al., 2002). It may be that changes in muscle activation patterns during locomotion can allow a spinal cord injured rat to walk. It has been hypothesized that changes in the timing of muscle activation may serve to stiffen the limb and improve weight supported stepping (Kaegi et al., 2002). Additionally, recovery of weight supported stepping following neonatal spinal cord injury is associated with the formation of an enlarged cortical motor representation of axial muscles (Giszter et al., 1998). One possible explanation for the enlarged cortical representation of the back is that back extensors take on a new role during weight supported stepping, but this has never been explicitly examined. Another change in walking following spinal cord injury is alterations in weight support as measured by ground reaction forces. Rats tend to shift their weight away from limbs affected by spinal cord injury toward limbs that are unaffected (Webb and Muir, 2002; Webb and Muir, 2003). Other compensatory movements related to these likely exist that may be revealed by comparing detailed kinematic and electromyographic recordings from pre- and post- injury conditions.

Surprisingly, the movements used in performing overground walking have not been extensively quantified. Rats were tested using kinematic and electromyographic recordings of walking after injuries ablating portions of the dorsal spinal cord, including the main portion of the corticospinal tract (i. e., an important descending tract involved in voluntary control of fine movement). Pre- and post-injury conditions were compared in chapter 2.

1.7 HORIZONTAL LADDER WALKING TASK IMPROVES ASSESSMENT OF DESCENDING CONTROL OF THE CPG

Treatment effects on the CNS can result in behaviors more similar to that of rats with an intact CNS which can be interpreted as improved performance, but this interpretation of improved function can be complicated by several factors. Open field walking is frequently scored using a locomotor score designed by Basso and colleagues known as the BBB

score (Basso et al., 1995). Rats recovering from SCI tend to have their BBB scores clustered around certain threshold values (Figure 1.5). The first threshold is at 8, where rhythmic hind limb movements can be performed, but plantar stepping is not achieved. At a BBB score of 14, rats walk with consistent fore limb / hind limb coordination and weight support, but higher scores are prevented by a lack of parallel paw placement and toe clearance. Additionally, in models where the fore limbs remain unaffected following SCI, the hind limbs can be pulled over the surface, cutaneous and proprioceptive receptors can become activated as a result. Both of these types of input are capable of activating the CPG and inducing stepping movements. Spinal networks below the level of an injury are capable of becoming more sensitive to input with (Fouad et al., 2005) or without (Thompson et al., 1992) exposure to plasticity-enhancing treatments. Consistent with this, the authors of the BBB open field walking score found that rats with complete spinal cord transections frequently retain some hind limb movement, with group means of 3.3 and a standard deviation of 2.1 (Basso et al., 1996). A score of 3 on the BBB scale represents 'extensive movements of two hind limb joints' (Basso et al., 1995). Changes within the spinal networks that control stepping movements may make it difficult to unambiguously determine the extent to which descending input has been restored after a treatment. This presents a confound in that some improvements in overground walking outcome can occur independently of reconnection of descending input through or around an injury site. One example of improved walking that does not depend on reconnection is shown by one study where the addition of embryonic raphe cells into the rat spinal cord at T11 below complete injuries at T8 resulted in activation of the walking pattern (Ribotta et al., 2000). To address this shortcoming, open-field walking assessments are frequently complemented by the use of horizontal ladder walking.

The horizontal ladder task requires the precise placement of paws onto the variably spaced rungs. Precise paw placement requires both descending input from the CST as well as the RtST input for walking movements. When one or both types of input are

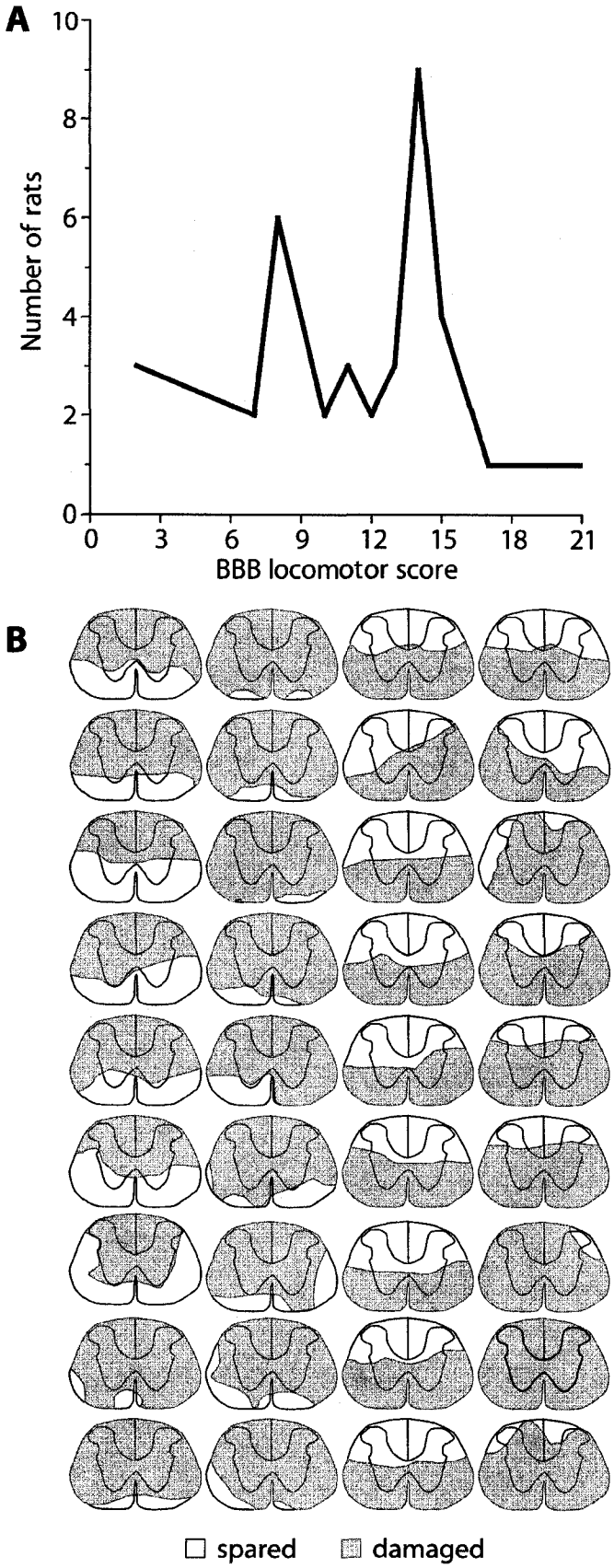

Figure 1.5: Clustering of rats' BBB scores after random thoracic injuries – Thresholds in the BBB score are indicated by the large numbers of rats at two thresholds of the score (A). The injury extents are indicated by shaded areas over the drawings of spinal cord cross sections (B). Adapted from Schucht et al. (2001).

interrupted by SCI, rats' paws will fall in between the rungs during the task, and these are frequently scored as errors. Error counts can be used without careful analysis of the walking behavior to provide a rapid method of quantifying the degree of walking recovery in the rat, but careful analysis of post injury walking patterns may reveal aspects of movements under the control of the CST that would otherwise be undetectable. Careful kinematic and EMG analysis can reveal strategies used by the rats to reduce their errors when injury related disruption of normal descending input makes normal control and correction of hind limb movements unlikely. By applying the gathered information about how rats with SCI perform horizontal ladder walking, we can improve the degree of accuracy and precision of this assessment to better determine the degree of spared and/or recovered descending control over the CPG. The detailed assessment of walking movements performed by intact rats and rats with SCI is shown in chapter 3. Careful assessment of skilled walking should provide a better approach to examine the extent to which functionally meaningful repair of the spinal cord can be achieved after an injury. The likelihood of finding a treatment with clinical value should be improved if the mechanisms behind walking recovery are understood. The neural basis of skilled and unskilled walking recovery after an injury depends on multiple factors, some of which are at least somewhat understood. It is important to understand the many causes of recovery to possibly utilize them in improving walking after SCI.

1.8 NEURAL BASES OF POST-SCI WALKING RECOVERY IN RATS

Depending on the severity of an SCI, there is an initial period of severe locomotor dysfunction followed by some degree of spontaneous recovery of function. In the first days following the injury, recovery depends on the reestablishment of transmission in spared axonal pathways, which becomes transiently blocked during the phase of spinal shock (Holaday and Faden, 1983; Hiersemenzel et al., 2000). In the first weeks following

injury, a second phase of functional recovery is attributed to the remyelination of spared axons (Jeffery and Blakemore, 1997). Following these beneficial changes, synaptic rearrangements and changes in the activity level of spared spinal axons may account for further functional improvements. Such changes in nervous system connectivity and function are frequently referred to as plasticity. To date, these plastic changes have not been well studied, although they may present a promising avenue for the improvement of post-injury spinal cord function. Treatments intended to induce regeneration of severed axons through an injury site have been shown to affect connections between CNS nuclei away from the injury site (Z'Graggen et al., 2000). Furthermore, new spinal circuits are spontaneously formed after incomplete SCI. One example of such circuitry forms between the corticospinal tract and propriospinal interneurons (Bareyre et al., 2004; Vavrek et al., 2006). Although this example of plasticity is interesting, it is unlikely to be important in the recovery of overground walking, as the RtST is most important in driving the activation of the CPG networks controlling walking. The importance of spared RtST fibers in the spontaneous recovery of walking has been repeatedly shown, as improvements in post-lesion locomotor function are greatest when there is substantial sparing of the ventro-lateral funiculus (VLF) in various animal models, the location where the RtST runs (Little et al., 1988; Gorska et al., 1996; Brustein and Rossignol, 1998; Schucht et al., 2002). To begin to address the mechanisms underlying spontaneous post-SCI improvements in locomotor function, a specific lesion model is needed where there is significant motor recovery, and the RtST projection pattern can be specifically assessed.

1.9 INJURY INDUCED CHANGES TO RTST PROJECTIONS

Although the RtST provides the tonic input that triggers the CPG, little is known about how its projections change following SCI and SCI-related treatments. To date, most studies examining central neuronal regeneration have examined the anatomically well-defined (CST) or rubro-spinal (RST) tracts. In the rat, these tracts run as fairly distinct bundles

in the dorsal half of the spinal cord and can be easily lesioned, as well as histologically examined. This stands in stark contrast to the RtST, where fibers run diffusely through the ventral white matter, intermingled with varied descending tracts including the RST, vestibulospinal, and tectospinal, as well as ascending spinocerebellar, and spinothalamic tracts. This anatomical arrangement of the RtST makes specific lesions impossible, and histological examination more difficult than is the case with the CST or RST. A lateral hemisection injury approach where the RtST is spared on one side provides an opportunity to study possible changes in the RtST, despite the fact that many other tracts are also injured. In the rat lateral thoracic hemisection model, large post-lesion behavioral deficits initially exist that partially spontaneously recover between 2 and 6 weeks post-lesion (Harris et al., 1994; Kaegi et al., 2002). This recovery can be abolished with either a second commissural lesion to the lumbar spinal cord, or by making a transverse lesion on the spared side at the level of the original injury (Harris et al., 1994). Commissural lesions to the lumbar spinal cord do not significantly affect locomotion in the otherwise intact rat, so it seems likely that a strengthening of crossed fibers occurs following incomplete spinal cord injury to compensate for the loss of innervation on the injured side (Figure 1.6). It has been found that this recovery is accompanied by increases in synapsin expression, a protein associated with the formation of new synapses (Gulino et al., 2006). The crossed fibers responsible for this recovery have not been described, but based on what is known about the neural control of locomotion, it is possible to hypothesize that RtST projection changes are involved. This hypothesis, that RtST projections change as a rat recovers walking following a lateral hemisection injury is addressed in chapter 4.

1.10 TREATMENT INDUCED CHANGES IN RTST PROJECTIONS

Although many different experimental interventions have been explored with the goal of improving recovery in models of SCI, the extent to which RtST projections change

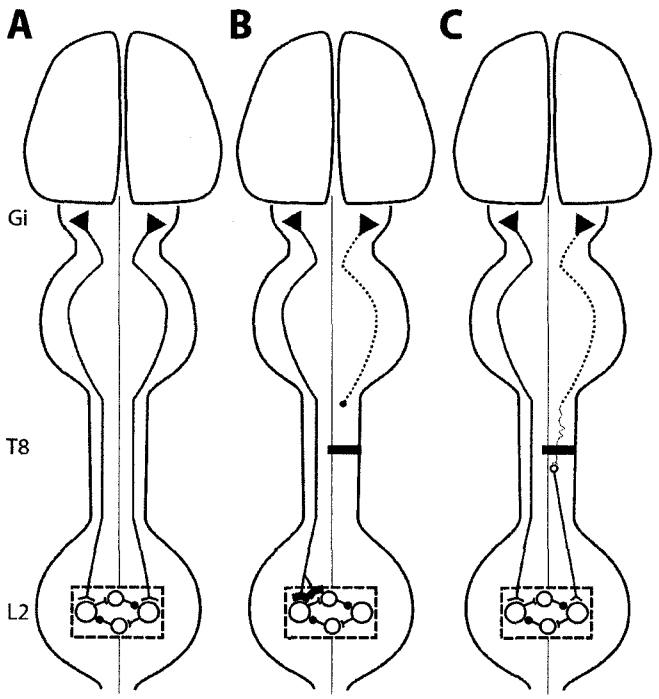

Figure 1.6: Regeneration and plasticity of RtST fibers – Schematics of the CNS are depicted with the cortex at the top and caudal spinal cord at the bottom. The Gigantocellular portion of the medial reticular formation in the medulla is represented by Gi, where the cell bodies of the RtST reside. In the intact CNS (A) the mostly uncrossed RtST sends fibers down each side of the spinal cord to drive the CPGs (neuronal networks in the dashed box), which in turn generates the walking pattern in rats. The RtST is shown by the triangular cell bodies sending projections caudally to the CPG. After lateral hemisection injury at the level of T8 (B) there can be strengthened connections from spared RtST fibers' onto the CPG opposite the injury. This is denoted by additional bold synaptic connections onto the CPG. Alternatively, axonal regeneration (C) of severed fibers on the injured could travel through the injury site to form connections onto spinal networks that could in turn connect with the CPG. These regenerated fibers are depicted by the smaller, twisted axon passing into the injury site. Not shown, is the possibility that regenerated fibers could connect with their original targets, as regeneration in the mammalian spinal cord has not been this successful to date.

in response to treatment is not known. One approach involves modifying the inhibitory CNS environment via the neutralization of myelin associated growth inhibitory factors. The neutralization of myelin growth inhibitors can support axonal growth and sprouting in the CST (Fournier et al., 2002), in parallel with some locomotor recovery (Bregman et al., 1995; GrandPre et al., 2002; Li et al., 2004). A second approach involves altering how growth cones interpret inhibitory signals in the CNS environment by inhibiting the RhoA pathway and its downstream inhibitor ROCK (Dergham et al., 2002). These two approaches represent two points where the molecular pathways for CNS myelin inhibition converge. As such, these approaches appear to be two of the most promising current candidate treatments to induce regeneration. The extent to which they may promote regeneration in CST projections has been explored previously, but it has not been shown to what extent these treatments affect plasticity in the RtST. If the treatments are able to improve walking in rats, they may have their effects on spared RtST fibers. To investigate a probable causal relationship between locomotor recovery and the application of this protein, the projection pattern of the RtST will be examined following the addition of NgR(310)ecto-Fc using the protocol of (Li et al., 2004). If the blockade of myelin associated growth inhibition improves locomotion via RtST sprouting, it will provide a critical clue to improving functional outcome after incomplete SCI. Y27632 has had some positive effects on reaching ability following a cervical injury, but the degree of improvement in locomotor function following incomplete SCI has not been well-studied. To investigate the extent to which Y27632 may affect RtST projections following a lateral hemisection injury in the thoracic spinal cord, RtST projections will be examined following Y27632 administration. The experiments utilizing intrathecal Y27632 and NgR Fc are detailed in chapter 5.

The mechanisms behind spontaneous and treatment induced functional recovery are not completely understood. By demonstrating plasticity in a tract that directly influences walking, the main readout of spinal cord function, it should lead to a better understanding

of how functional recovery occurs in general. A detailed description of the movements rats make when walking following SCI is needed to understand how recovery occurs when using open-field scores such as the BBB locomotor score. In chapter 2, compensatory movements made by rats walking with incomplete spinal cord injuries will be examined.

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CHAPTER 2

ADAPTATIONS IN THE WALKING PATTERN OF SPINAL CORD INJURED RATS

Adapted from Ballermann M, Tse ADY, Misiaszek J, Fouad K J Neurotrauma. 2006 Jun;23(6):897-907.

2.1 INTRODUCTION

A common animal model used to evaluate potential therapies designed to repair the injured spinal cord is the rat (Horner and Gage, 2000; Edgerton and Roy, 2002). Evaluation of potential treatments has often depended on post-lesion walking performance, as rats will spontaneously perform this behaviour and the interpretation of a rhythmic movement pattern such as walking appears straightforward. A frequently used approach to create statistically analyzable numeric data from observed behaviours is the use of scoring systems. In the case of locomotion in spinal cord injured rats a commonly used measure is the BBB open-field locomotor score (Basso et al., 1995), that was originally designed to evaluate the outcome in rats with thoracic contusion injuries. Other commonly used assessments of post-lesion walking include narrow beam walking, footprint analysis, and walking on a horizontal ladder among others (Kunkel-Bagden et al., 1993; Muir and Webb, 2000; Metz et al., 2000).

To date, mechanisms by which treatments designed to repair the injured spinal cord improve walking in rats with spinal cord injury (SCI) have been unclear (reviewed in Fouad and Pearson, 2004). A much desired mechanism would be that severed descending axons regenerate to reconnect with their targets, but actual mechanisms of recovery following incomplete SCI are unlikely to be this straightforward. Other mechanisms such as anatomical rearrangements in axons above and below the lesion may allow descending signals to bypass the lesion and contribute to the recovery (Raineteau and Schwab, 2001; Weidner et al., 2001; Bareyre et al., 2004). Furthermore, locomotion is orchestrated in part by neuronal networks – central pattern generators (CPGs) - within the spinal cord that can be triggered by descending tonic signals or afferent input (Grillner and Wallen, 1985; Mori et al., 1992). Adaptations in these networks most likely contribute to locomotor recovery following SCI (reviewed in (Rossignol et al., 2004)). For example CPGs and motoneurons were reported to become more excitable following spinal cord injury thus allowing reduced input, or somatic stimulation to induce stepping movements (Li et al.,

2004; de Leon et al., 1999; Basso et al., 1996).

Adaptive changes in the walking pattern of rats with SCI present a particular challenge when interpreting treatment-induced improvements in locomotion. Evaluation of post-SCI behaviour in rats has shown the adoption of qualitatively different movement patterns to locomote (Giszter et al., 1998; Kaegi et al., 2002; Webb and Muir, 2002; Webb and Muir, 2003), suggesting that compensatory strategies may represent a method to recover locomotor function when enduring CNS deficits make the complete palette of normal movements unavailable as has been described in cats (Brustein and Rossignol, 1998). Currently locomotor rating scales generally compare post-lesion walking improvements to the intact walking pattern. This makes visual observable adjustments in the walking style (e.g., foot rotation) a limitation and only visually normal walking receives maximum achievable scores. It is however unclear whether treatment induced recovery of locomotion will promote the recovery of normal locomotion or promote compensatory changes and therefore an alternative walking style. Thus, there is a need to examine and define possible compensatory strategies for locomotor recovery following a spinal cord injury. The present study uses kinematics and EMG recordings to determine how thoracic spinal cord injuries change walking style and the orchestration of muscle activity. As it is expected that the severity of the injury will influence post injury walking style, we observed the outcome across intentionally varying injury severities. In order to narrow the spectrum of post injury locomotion styles, we focused on animals that were capable of walking by supporting their weight during locomotion (i.e., BBB score >12).

Our kinematic and electromyographic results show significantly different locomotor patterns amongst rats that recovered weight supported stepping following spinal cord injury, confirming the adaptability of the locomotor system as recently reviewed in cats (Rossignol et al., 2004).

2.2 METHODS

2.2.1 Animals

Experiments were conducted using 12 adult (i.e., 3 months old) female Lewis rats (180-200g), in which EMG electrodes were surgically implanted (see below). All rats were kept at a 12:12 h light dark cycle with water and food provided *ad libitum*. This study was approved by the local animal welfare committee, and complies with the guidelines of the Canadian Animal Care Council.

Rats were handled for 2 weeks, familiarized with the open-field arena, and were trained to walk on an elevated flat wooden surface (i.e., runway) 1 m long by 5 cm wide.

2.2.2 Electromyographic-electrode implantation

Teflon-insulated multi-stranded stainless steel wires (Cooner Wire, AS 631, USA) were used as bipolar EMG electrodes soldered to a ten pin connector (5-row CLE, Samtec, New Albany, IN). Wires were attached to 30 gauge hypodermic needles for implantation into muscles, as described previously (Merkler et al., 2001; Kaegi et al., 2002). All surgical procedures were carried out using Dormicum (Midazolam, 6mg/kg, s.c., Roche, Switzerland)/Hypnorm (Fentanyl, 4mg/kg, s.c., Janssen-Cilag, Belgium) anaesthesia, with buprenorphine (0.05 mg/kg, s.c. Abbott, Mississauga, ON) injected immediately following surgery and again 8 hours post-operatively for 16 hours of analgesia. Wires from the connector were inserted into a single cutaneous incision in the back, and the connector was sutured to the surrounding skin. The electrode wires were drawn subcutaneously using a metal tube toward their respective implantation sites. EMG electrodes were implanted in the right Triceps brachii, right side of lateral Longissimus (LONG) intermediate portion – see (Gramsbergen et al., 1999), left and right Tibialis Anterior (TA), and the right Vastus Lateralis (VL). Earlier studies that compared kinematic recordings in rats and mice with and without implantation ensured that the implanted electrodes do not interfere with the locomotor pattern (Pearson et al., 2005; Kaegi et al., 2002).

2.2.3 *Functional testing*

Before SCI, baseline EMG and kinematic data were collected from rats walking on a runway. Rats were tested on 3 consecutive days prior to SCI. Individual rats took differing amounts of time before their scores on the BBB open-field locomotor scale plateaued (Basso et al., 1995) following SCI resulted in some animals being tested at different time points post injury. See Table 2.1 for time points of testing. Walking speed was compared before and after injury conditions.

Electromyography: During runway walking, EMG signals were amplified by a factor of 200 (Cyberamp, Axon Instruments, Union City, CA), digitized at a sampling rate of 1 kHz (Digidata, Axon Instruments; Figure 2.1), and low-pass filtered at 300 Hz. No high pass filter was applied. For amplitude comparisons before and after injury the recodings were also rectified. Sequences where three or more steps were completed within 2 sec were used for analysis to ensure regular walking sequences were compared. This time window was chosen as step cycles in a bout of walking in unlesioned rats normally last between 200-400 ms. A minimum of 20 step cycles per animal (for each condition) were used for EMG analysis. The burst amplitude, burst duration, and time to the start of the next cycle were determined for each trace. F-tests were used to decide whether the relationship between the amplitude of Triceps EMG bursts and stepping frequency changed following SCI in each rat (Prism, GraphPad Software, San Diego, CA). Step cycle duration, activity of longissimus, overlap between TA and VL were compared between pre- and post injury conditions in the same rat.

Kinematic analysis: Rats walked on a runway to ensure locomotion in a straight line. The animals were filmed from the lateral aspect while walking using a digital video camera filming 60 fields/sec, shutter speed set to 1/500 sec (Optura 100-MC, Canon, Lake Success, NY). EMG data were collected simultaneously. Synchronization between the EMG and video records was obtained using a light emitting diode (LED) placed within view of the camera. The LED was manually triggered during walking sequences and the voltage pulses

Rat	Final BBB Score	Days Recovery	Lesion/ Day	Walking Speed (cm/s)		Step Duration (ms)		Iliac Crest Height During Stance (cm)		Mean Joint Angle During Stance (°)		TA/VL Separation: (+) Overlap: (-) (ms)		Triceps activation/ step frequency		Longissimus Activity (mV)	
				Mn	SD	Mn	SD	Mn	SD	Mn	SD	Mn	SD	Slope	r^2	Mn	SD
1	13	13	Pre	39	5.6	347	49	**5.7**	**0.24**	77	**2.5**	**-8**	**16**	15.9	0.31	14	10.6
			Post	55	13	373	85	**6.7**	**0.32**	**103**	**7.3**	**77**	**74**	13.3	0.11	11	7.6
2	13	9	Pre	32	7.4	402	35	6.7	0.25	84	4.2	**-80**	**71**	25.6	0.28	15	9.7
			Post	30	5.1	410	60	6.9	0.38	92	5.1	**-13**	**41**	32.3	0.09	11	9.5
3	14	17	Pre	**67**	**9.2**	**262**	**25**	6.7	0.25	98	3.2	n/a	n/a	**22.0**	**0.46**	n/a	n/a
			Post	**35**	**16**	**408**	**91**	5.5	0.34	98	24.5	n/a	n/a	**33.9**	**0.20**	n/a	n/a
4	16	13	Pre	**84**	**16**	**210**	**20**	**6.9**	**0.72**	**77**	**4.0**	n/a	n/a	n/a	n/a	34	26.1
			Post	**68**	**8.4**	**275**	**31**	**7.9**	**0.47**	**103**	**9.8**	n/a	n/a	n/a	n/a	29	33.4
5	16	15	Pre	56	6.2	277	22	**5.9**	**0.37**	**80**	**3.2**	n/a	n/a	n/a	n/a	n/a	n/a
			Post	57	18	279	39	**6.9**	**0.36**	**97**	**11.3**	n/a	n/a	n/a	n/a	n/a	n/a
6	17	17	Pre	45	7.3	365	57	**5.9**	**033**	**75**	**6.0**	9	16	**24.3**	**0.35**	**16**	**14.7**
			Post	52	9.4	321	55	**7.3**	**0.52**	**99**	**6.6**	9	32	**29.6**	**0.07**	**22**	**7.0**
7	18	13	Pre	83	8.5	219	20	6.1	0.41	**71**	**4.0**	**4**	**17**	**54.2**	**0.41**	**23**	**11.9**
			Post	78	23	238	69	6.6	0.46	**93**	**4.5**	**46**	**31**	**87.5**	**0.33**	**45**	**27.8**
8	21	22	Pre	59	22	285	72	6.5	0.69	83	7.0	n/a	n/a	**18.9**	**0.91**	**1**	**4.0**
			Post	63	24	280	69	6.6	0.76	87	6.1	n/a	n/a	**24.5**	**0.36**	**11**	**6.8**
9	21	13	Pre	55	8.0	280	20	6.3	0.56	77	8.1	52	23	**29.1**	**0.29**	**7**	**8.1**
			Post	58	12	244	27	6.5	0.30	87	7.6	31	30	**46.0**	**0.32**	**15**	**12.5**
10	21	14	Pre	**52**	**22**	**323**	**69**	6.6	0.17	83	5.0	**-4**	**31**	**18.7**	**0.65**	**26**	**12.2**
			Post	**66**	**16**	**281**	**61**	6.0	0.48	74	6.3	**19**	**27**	**28.7**	**0.43**	**37**	**16.7**
11	n/a	n/a	1st Day	**58**	**11**	**261**	**22**	7.0	0.76	77	2.7	-16	20	1.3	0.45	14	10.9
			2nd Day	**75**	**19**	**211**	**34**	6.9	0.61	73	3.5	16	20	1.4	0.14	11	5.6
12	n/a	n/a	1st Day	**64**	**16**	**275**	**14**	6.5	0.20	79	4.6	39	9	25.1	0.62	9	4.5
			2nd Day	**74**	**19**	**250**	**26**	6.3	0.26	77	5.0	50	24	30.4	0.11	12	6.0
13	n/a	n/a	1st Day	**46**	**9.5**	**314**	**36**	6.1	0.64	76	3.8	14	23	n/a	n/a	13	7.4
			2nd Day	**79**	**11**	**223**	**29**	6.2	0.32	73	5.7	19	24	n/a	n/a	9	3.9
14	n/a	n/a	1st Day	35	13	342	65	6.3	0.37	84	5.1	n/a	n/a	4.6	0.48	n/a	n/a
			2nd Day	34	15	390	90	6.5	0.27	89	6.9	n/a	n/a	5.1	0.47	n/a	n/a
15	n/a	n/a	1st Day	45	11	309	58	6.4	0.36	81	4.3	45	30	20.3	0.02	9	6.9
			2nd Day	55	22	283	46	6.3	0.59	83	7.9	28	28	22.6	0.45	9	7.4

Table 2.1: Rat walking measures compared between pre and post injury conditions – Significant difference in measures as found with Mann-Whitney U tests ($p<0.05$) are in boldface.

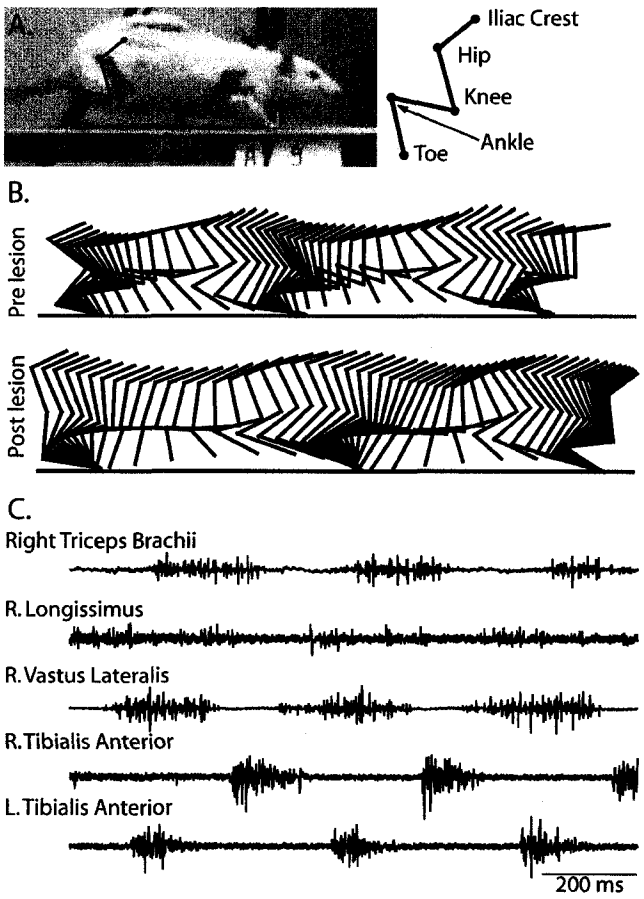

Figure 2.1: Kinematic and EMG recording of runway walking in rats – A. Frame from video showing a rat walking on elevated runway, and the spatial model used for kinematic analysis. B. Stick figures of two step cycles for pre- and post- lesion walking from rat 2 (BBB=13) post injury. C. Typical EMG recordings (from rat 2 recorded 9 days post-injury) of different muscles gathered during runway walking.

were recorded with the EMG signals. A computer based system (Motus, Peak Performance Technologies, Centennial, CO) was used to identify the x and y coordinates of markers placed at the iliac crest, hip, ankle, and toe (Figure 2.1). Customized software written in LabView (National Instruments, Austin, TX) calculated the location of the knee joint based on fixed distances corresponding to the lengths of the femur and tibia.

Coordinates were acquired and analysed for their kinematic properties in a minimum of 5 walking step cycles per rat. Measures taken included the temporal duration of the entire step cycle, the time spent in swing and stance phases, the stride length, height of each point above the runway, and joint angles. These measures were taken for the full step cycle, as well as being broken down for the stance and swing phases. Stance was defined as the portion of the step cycle where the hind limb was on the ground. As spinal cord lesions were intentionally varied to explore the strategies available to walking rats after SCI, group comparisons were not used. Strategies adopted by individual rats were identified by comparing pre and post-injury data in the same rat.

2.2.4 Spinal Cord Lesions

Rats were subjected to incomplete spinal cord injury. The T8 vertebra was dissected through an incision in the back. A laminectomy of the dorsal half of the segment was performed and a customized micro-blade created from a breakable blade was used to cut the dorsal portion of the spinal cord. Bilateral dorsal transections of varying depths (1-1.5mm) were inflicted. The lesions were intentionally varied to examine the changes in walking from injuries with different severities. Five rats (designated 11 though 15) did not receive spinal cord lesions and were used as controls.

Following SCI, rats were evaluated daily in the open-field for walking using the BBB criteria (Basso et al., 1995). Once BBB scores had stabilized (i.e. remained the same in each rat, for at least 3 days) EMG and kinematic analysis of runway walking was performed. Two days following the stabilization of BBB scores, EMG and kinematic data were gathered and compared against pre-lesion values. The number of days following

injury before runway testing was completed varied between 9 and 22 days (Table 2.1).

2.2.5 Statistical Analysis

Comparisons were made between pre- and post-injury steps within the same animal using unpaired t-tests (Mann-Whitney U tests). Significant changes in measures within each individual rat are shown in the individual data panels in each figure. To examine effects due to walking speed, Pearson correlations were calculated.

2.2.6 Histology

Once runway and open-field testing was completed, rats were given an overdose of Euthanyl (Bimeda-MTC, Cambridge, ON), and were intracardially perfused with 0.9% heparinized saline followed by 4% formalin. Segments containing the lesion were dissected and post-fixed in 4% formalin, cryo-protected in 30% sucrose, then frozen and sectioned using a cryostat. Cross sections of 50 μm through the spinal cord lesion site were counter-stained with cresyl violet and reconstructions of the largest extent of the lesion were composed from adjacent sections. Percentage of spared white matter in each rat was calculated using area measurements from the reconstructions (Scion Image, Frederick, MD).

2.3 RESULTS

Depending on the severity of their spinal cord lesion it took the rats a minimum of 8 and a maximum of 22 days following their injury to plateau in their locomotor performance as assessed by the BBB score. During runway walking, neither average walking speed nor step cycle duration significantly changed following SCI. When individual rats' post injury walking speed as well as step cycle duration was compared to their pre-injury values, 3 out of 10 injured rats and 3 of 5 controls changed their step cycle duration and walking speed during the course of the experiment; changes are reported in Table 2.1.

Reconstructions from cross-section of the maximum lesion extent from each rat are

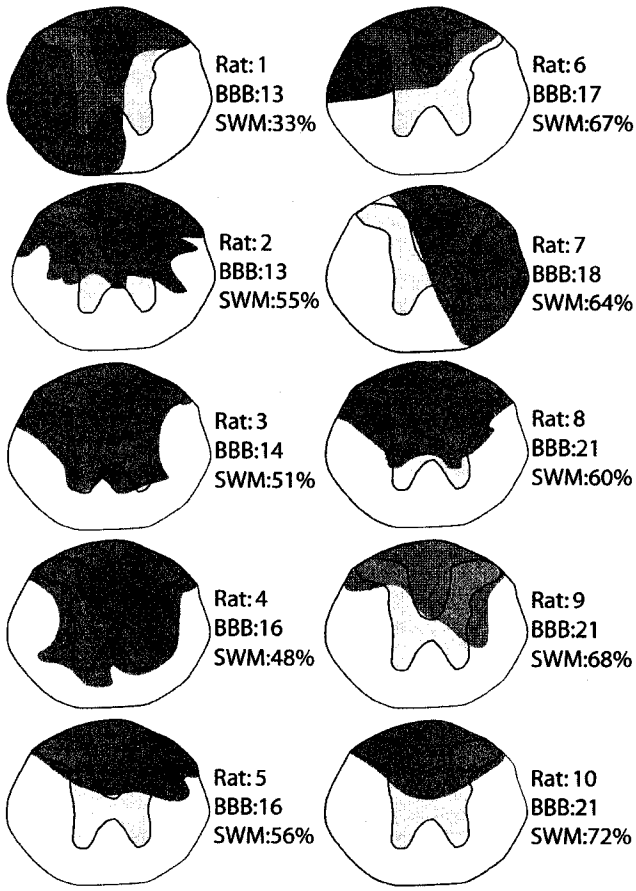

Figure 2.2: Analysis of lesion extent – Reconstructions of the maximal extent of the lesion are shown for individual rats. Rat numbers, BBB scores, and total spared white matter (SWM) are given next to each drawing. Shaded areas represent lesioned areas.

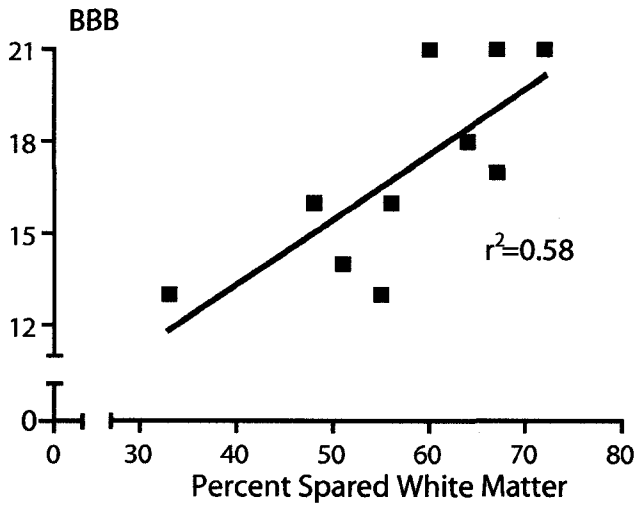

Figure 2.3: Correlation of BBB open field walking score with spared white matter – Endpoint BBB scores of individual rats are plotted against spared white matter percentage.

shown in Figure 2.2 . Lesions consistently damaged dorsal portions of the spinal cord, including the dorsal columns (containing ascending sensory tracts as well as the main portion of the corticospinal tract) and dorsal horns. The lateral funiculi (containing rubrospinal tract fibres) were damaged in all but one (Rat 10) of the rats. Varying amounts of white matter in the ventral and lateral funiculus were spared containing among others reticulo- and vestibulo- spinal tract fibers (Schucht et al., 2002). As we described earlier (Schucht et al., 2002), the amount of spared white matter was significantly correlated with the final open-field BBB score (Figure 2.3).

Both to verify the consistency of EMG recordings and kinematic measures, and to determine the extent to which rats with intact spinal cords change their walking pattern during the course of the experiment, data in control rats were compared between 2 and 14 days post-implantation.

2.3.1 Increases in hind limb extension during stance

The hind limb posture during the stance phase of walking was determined using joint angles and the height of joints above the walking surface. Trajectories for the iliac crest, hip, knee, ankle and toe through a representative pre- and post lesion step cycle are shown in Figure 2.4 A. We found that in 4 out of 10 rats the height of the iliac crest during the stance phase had significantly increased (Figure 2.4B, Table 2.1, Height of Iliac Crest during stance). This increase in hind limb extension was the result of varying combinations of increases of all joint angles. Changes in the mean of the three joint angles during mid-stance (maximal weight support) significantly increased in 5 out of 10 rats (Figure 2.4C, Table 2.1, Mean Joint angle during stance). Note that in rats with maximal BBB score (21) no changes in the hind limb extension were found. Furthermore, in rats where hind limb extension increased post-injury, iliac crest height was significantly correlated with walking speed only pre-injury (r^2 =0.43). This correlation was no longer significant in the post-injury condition (r^2 =0.05). The mean spared white matter of rats showing increased hind limb extension during stance was 71.1% (SD=18.2%).

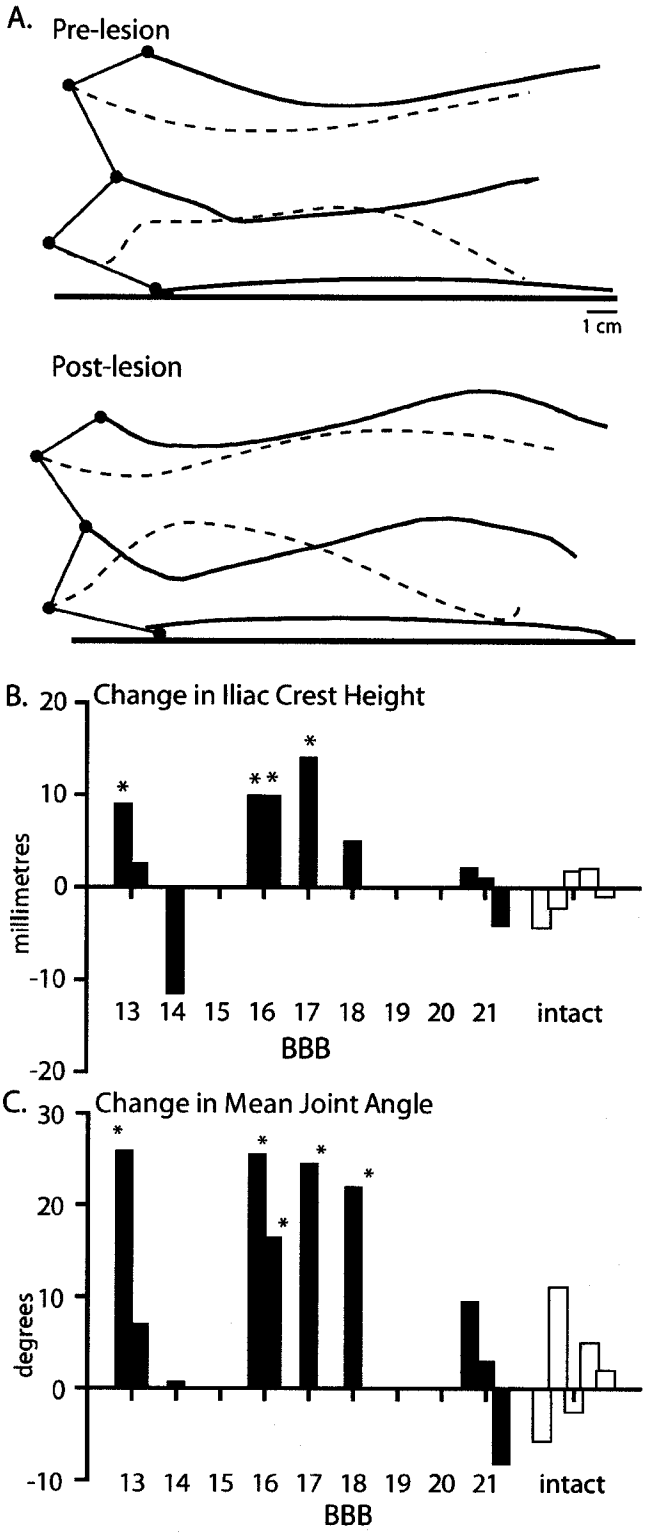

Figure 2.4: Analysis of extended stance posture – A. Trajectories of joints through a step cycle before (above) and after (below) SCI in rat 7. The joint trajectories are shown by solid (iliac crest, knee, toe) or dashed lines (hip, ankle). B. Change from pre to post-lesion values of iliac crest height above the walking surface during stance in individual rats. C. Change in the mean of hindlimb joint angles during stance in individual rats indicating degree of hind limb extension. Rats are sorted according to endpoint BBB score. Data from control rats (11 to 15) were compared between separate days. Significant changes from pre to post injury conditions for each rat are shown. (* – $p<0.05$)

2.3.2 *Muscle timing patterns vary among rats with SCI*

To analyze the relation of activity in the TA and VL muscles this analysis necessitates that both recordings were functional during the entire course of the experiment. Generally, TA (an ankle flexor) is active during the swing phase, whereas VL (a knee extensor) is active during the end of the swing (when the leg starts to extend) and during the stance phase. Earlier studies reported an increased overlap in activity of these two antagonistic muscles following SCI (Kaegi et al., 2002).

The amount of time separating the active periods of the two muscles was determined from the EMG recordings in 5 injured and 4 control rats (Figure 2.5 A-B, Table 2.1, TA/VL Separation). Significant decreases but no increase in the overlap between TA and VL were found in 4 out of the 5 lesioned rats. In rats where the timing of TA and VL activation was significantly changed the mean spared white matter was 69.2% (SD=21.7%).

2.3.3 *Fore limb extensors increase activity*

Fore limb extensor muscles may increase their activity to compensate for reduced hind limb propulsion in injured rats. We found that the activity of the Triceps brachii muscle (a fore limb extensor) increased in post-lesion walking. The average amplitude of the EMG signal from the triceps muscle was measured in pre- and post-lesion step cycles (Figure 2.6 A). As the amplitude is varied by the step cycle duration we plotted stepping frequency in relation to the EMG amplitude (a representative example from rat 10 is shown in Figure 2.6B). Statistical comparisons of the relationship between stepping frequency and EMG amplitude in single rats was performed using F-tests. Individual changes in the slope of the regression curve are indicated in Figure 2.6C and shown in detail in Table 2.1 in the column labelled 'Triceps activation'. In unlesioned rats we found a relation between stepping frequency and EMG amplitude that did not change between successive testing days. In contrast in 6 out of 8 lesioned rats triceps EMGs recordings showed a significant increase independent of stepping frequency. Note that the two rats with the lowest BBB

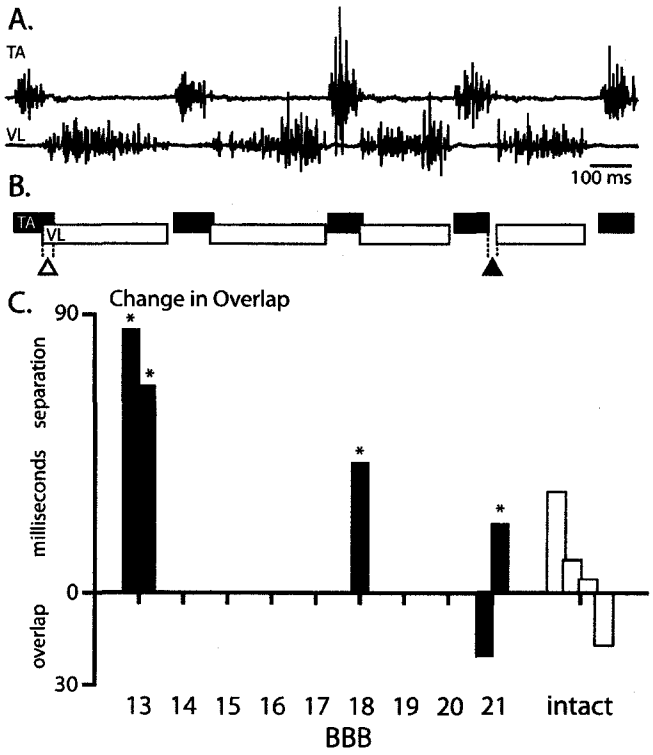

Figure 2.5: Step cycle timing – A. EMG recordings of Tibialis Anterior (TA) and Vastus Lateralis (VL) from a pre-injury EMG recording. B. Active periods of each muscle are shown by the solid bar for TA, and the empty bar for VL. The empty arrowhead at left shows overlap between the active periods of TA and VL. The solid arrowhead at right shows separation between the active periods of TA and VL. C. Change in separation between active periods is shown for individual rats. Significant changes from pre to post injury conditions for each rat are shown. (* – $p<0.05$) Increases in separation are indicated by positive values; increased overlap is indicated by negative values.

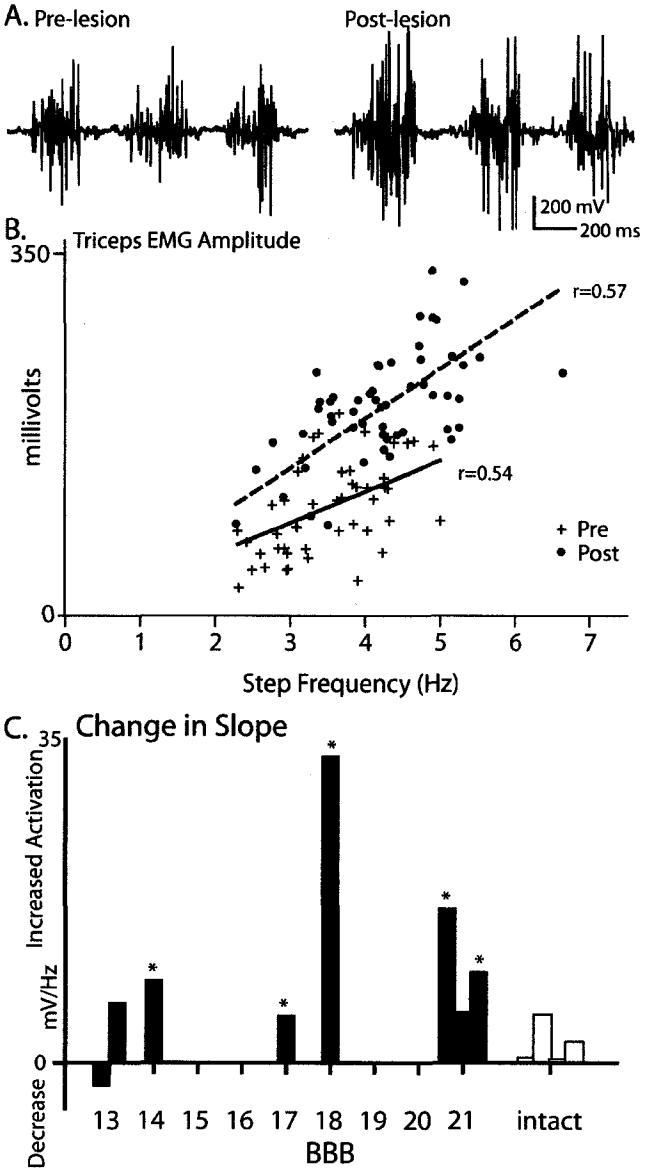

Figure 2.6: Triceps activity during walking – . EMG recordings from triceps muscle. B. Amplitude of rectified pre and post lesion recordings during firing plotted against stepping frequency for rat 10. Lines represent linear regressions showing changes in relationship between pre and post-lesion data. C. Changes in slopes of the linear regressions are plotted for individual rats. Significant changes from pre to post injury conditions for each rat are shown. (* – $p<0.05$)

score did not show changes in triceps activity. The mean spared white matter of rats with increased triceps EMG amplitude was 80.4% (SD=13.2%).

2.3.4 Post-injury increases in Longissimus longus activity

Pre-lesion EMG recordings in the middle portion of the LONG muscle showed no obvious relationship between stepping and its activity (Figure 2.7 A). Qualitative examination of post-lesion EMG traces temporally normalized to the onsets of TA bursts showed an increase between 60% and 80% of the step cycle (Figure 2.7B). Bins of the step cycle between 60% and 80% were averaged together for statistical analysis. The portion of the step cycle analyzed corresponds to the indicated stick figures in Figure 2.7C. Significant changes in the activity of LONG at this point of the step cycle were found in 5 out of 8 rats with SCI but not in controls (Figure 2.7D and Table 2.1, column 'Longissimus activation'). It is noteworthy that these changes are significant in rats with a BBB score of 21 but were not found in rats with a score below 17. The mean spared white matter of rats where there were significant post-injury increases in LONG activity recorded here was 82.7% (SD=13.3%).

2.4 DISCUSSION

The present study shows the importance of using quantitative measures of behavioral recovery rather than qualitative visual scores only. Qualitative visual scores as the commonly used BBB score {Basso, 1995 #67} can provide a rapid screen which can be valuable in experimental approaches especially in evaluating candidate pharmacological interventions where large animal numbers are needed. We find changes in kinematic and EMG measures in rats with dorsal spinal cord injuries, and even in rats that appear to have recovered visually normal walking. These changes include more extended hind limbs during stance phase, altered timing of hind limb muscle activity, increased fore limb extensor activity, and increased activity of a back extensor muscle.

Weight support represents a major challenge in rats with SCI. Increases in the body

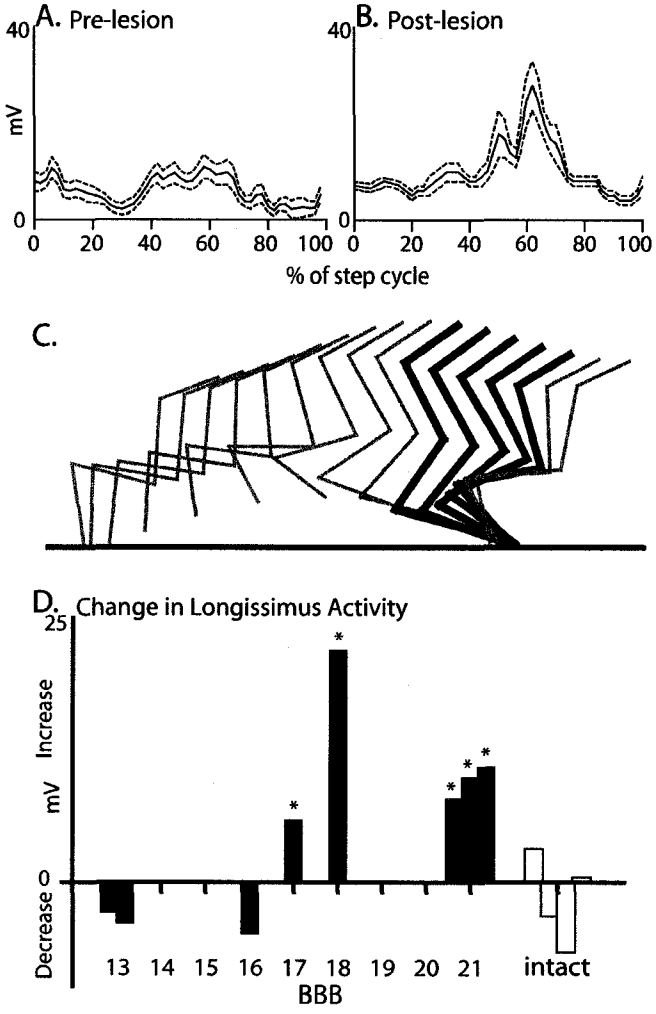

Figure 2.7: Longissimus (LONG) activity during walking – Rectified EMG recordings of LONG muscle during walking were averaged across steps shown for a rat with a BBB score of 21 and temporally normalized to onsets of TA bursts. Pre-lesion (A) and post-lesion (B) EMG data are shown. C. The phase of the step cycle corresponding to the change in EMG activity (B) is shown by the bold stick figures. D. Changes in Longissimus activity between 60% and 80% of the step cycle are plotted for each rat. Significant changes from pre to post injury conditions for each rat are shown. (* – $p<0.05$)

elevation during the stance phase of walking, as well as the extension of stance joint angles suggest that rats use a more upright, columnar posture after SCI. Consistent with data shown here, rats with SCI may adopt this postural adaptation to enhance weight support in a manner analogous to larger mammal species reducing bone and muscle stresses during walking (Biewener, 1989). The adoption of a more upright walking posture allows the animals to align their limbs more closely with ground reaction forces. An upright walking posture reduces the torques applied about the joints and in turn reduces the forces applied by the muscles to support weight. Small animals such as rats may benefit from a more crouched posture in that it allows better manoeuvrability, which is beneficial in evading predators. Following SCI, however, rats may switch postures, sacrificing agility to reduce the force necessary to support their weight. This postural adjustment may be reflected in subtle changes to the timing of muscle activation in the EMG record that were not detected in this study. It is likely that muscles besides those recorded from were involved in this posture shift. Alternative explanations exist for an extended hind limb posture during stance. This posture may be a consequence of post-injury increases in excitability of spinal networks and/or motoneurons (Bennett et al., 2004; Thompson et al., 1992). Excitability increases could preferentially increase the activity of extensor muscles as they may be governed to a larger extent by afferent feedback (Dietz and Duysens, 2000). If force feedback effects from muscle afferents were increased it may result in a more extended hind limb. Alternatively, a forward shift in injured rats' centers of gravity could result in unloading of the hind limb and an extended posture. This could also partly explain the increase in triceps brachii activity.

It has been previously suggested that rats may change the timing of muscle activity during walking such that antagonist pairs contract simultaneously, possibly to stiffen the limb and thus reduce the effort needed for weight support (Kaegi et al., 2002). In our study, the amount of temporal overlap between TA and VL muscles was increased in only 1 out of 6 rats. Most rats with apparent open field walking deficits increased the temporal separation

between TA and VL. A possible explanation for the contradictory results in comparison to Kaegi et al. (2002), is the difference in lesion size, as their study examined the effects of larger lesions than those used in our study. It is likely that rats change their muscle activation timings depending on many factors including the extent and localization of the lesion, and effects of post-lesion activity on anatomical plasticity.

Hind limbs provide the bulk of the propulsive forces required for walking in the intact rat (Webb and Muir, 2002), whereas propulsion in rats with complete spinal cord injury is provided exclusively by the fore limbs. It appears intuitive that rats with partial SCI use their fore limbs to partly compensate for deficits in hind limb propulsion. We accounted for effects due to stepping frequency (Hof et al., 2002), and found that the amplitude of the EMG signal from the fore limb extensor Triceps brachii increased. A comparable finding has also been previously noted in cats (Brustein and Rossignol, 1998). This suggests that, as in cats, rats increase the use of their fore limbs to apply propulsive forces when hind limb function is compromised. An alternative explanation for these findings could be that the fore limb is extended similarly to the hind limb, which could increase stepping frequency in the fore limb, and partially account for the increases seen here, however this was not confirmed. We used kinematic examination of fore limb shoulder height in two rats with significant changes in the activity of Triceps, and found no changes in the fore limb stance posture (data not shown). Qualitative examination of the other rats walking suggests that they also do not increase the extension of the fore limb during stance. A second alternative explanation for these results is that the EMG electrodes may have moved and/or become encapsulated within the muscle, resulting in the quality (i.e., the signal to noise ratio) of the recordings changing over time. In the controls and in our previous studies, however it has been shown that burst amplitudes remain stable over many days of recording (Kaegi et al., 2002).

Past studies have raised the possibility that spinal cord injured rats with hind limb weight supported walking may use axial muscles to provide balance, steering control and

propulsive forces to move the pelvis and assist hind limb stepping (Giszter et al., 1998). Dorsal muscles of the back with attachments to the pelvis would likely be involved in such a strategy, some of which have been shown to be rhythmically active during walking in rats (Gramsbergen et al., 1999), and humans (Thorstensson et al., 1982). Specifically, in adult rats, recordings from rostral and caudal portions of lateral LONG show rhythmic activity, but recordings from the intermediate portion of the muscle showed tonic, but not rhythmic activity (Gramsbergen et al., 1999). In our study EMG recordings showed no obvious relationship between stepping and back activity, in agreement with previous findings (Gramsbergen et al., 1999). However, in 5 spinal cord injured rats we find rhythmic activity shown by an increase of EMG activity during the onset of the stance phase of the ipsilateral hind limb. This supports the idea that axial muscles become recruited to assist in leg movements. Other examples of increased axial muscle activity have been implicated in other spinal cord injury related studies. Spinal cord injured humans were reported to recruit axial muscles when walking to compensate for deficits in moving more distal body parts, possibly resulting in the generation of kinematically equivalent movements, from different underlying motor patterns in humans (Giszter et al., 1998; Grasso et al., 2004).

Although open field scores of walking compare hind limb activity of injured animals to walking in intact animals, previous research, as well as our findings, shows that recovery of walking following spinal cord injury does not necessarily follow a course toward the pattern of intact animals. For example, Webb and Muir (2002) showed asymmetrical patterns of ground reaction forces during walking in response to thoracic hemisection. Unilateral dorsal column lesions alone or in conjunction with rubrospinal tract lesions also induce rats to shift their weight away from the limb ipsilateral to the injury (Webb and Muir, 2003). Similar principles have been demonstrated in a rat model of reaching, where cervical lesions of the dorsal column induce rats to use different movements to retrieve pellets, possibly as compensation for a loss of haptic input (McKenna and Whishaw, 1999;

Ballermann et al., 2001). We show three changes in the rat walking pattern that may play a role in recovery. Changes in the measures observed here appear to be specific to rats with spinal cord injury as similar EMG and kinematic measures of rats with intact spinal cords remained consistent over time. Inevitable variations between individual lesions in rats result in varied adaptations in the post-injury walking pattern, thus emphasizing the need for quantitative measures in assessing recovery following SCI as have been suggested previously (Metz et al., 2000; Anderson et al., 2005; Koopmans et al., 2005).

The presented results demonstrate that while visually based locomotor scales can provide rapid assessment of recovery, spinal cord injured rats develop alternative locomotor patterns that cannot be discriminated without more quantitative tests. Kinematic analysis of rat walking (especially at frame rates over 30Hz) provides the opportunity to identify post-injury deficits undetectable with the naked eye. In addition, when both kinematic and electromyographic data are recorded simultaneously, insight into alternative patterns of recovery can be detected, often giving insight into mechanisms. Results reporting adaptive changes in the walking pattern not only promote the use of precise outcome measures in assessing recovery after spinal cord injury, but also encourage the exploration of new avenues in rehabilitation following SCI promoting the adaptive capabilities of the nervous system.

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CHAPTER 3

TASK SPECIFIC ADAPTATIONS IN RAT LOCOMOTION: RUNWAY VERSUS HORIZONTAL LADDER

Adapted from Bolton DAE, Tse ADY, Ballermann M, Misiaszek J, Fouad K, (2006) Behav Brain Res. 168(2):272-9.

Authorship note: I was involved in the data collection, data analysis, writing, and figure preparation of this chapter.

3.1 PREFACE

In chapter 2, changes in the walking pattern were examined using kinematic and EMG measures following dorsal SCI. The main findings included a more extended hind limb posture during mid-stance in rats with consistent weight-supported stepping in open-field walking scored with the BBB walking scale. Another finding was an increase in the amplitude of EMG activity from a forelimb extensor, triceps brachii, which is consistent with an increased role for the fore limb in propulsion during walking. This change was consistent across rats at all levels of performance on the BBB walking score. Finally, increased activity of a back extensor during early stance of the ipsilateral hind limb suggests that more proximal musculature, which is innervated by spinal levels rostral to injury, may become more active during walking after a dorsal SCI. Rats with visually normal walking measured by BBB scores showed this phenomenon suggesting that this may be one change in the orchestration of muscle activity in response to persistent loss of the normal palette of movements following SCI. Altogether, these muscle activity patterns and kinematic changes following dorsal SCI are likely a source of improved walking performance on visual walking scores such as the BBB walking scale, which is frequently interpreted as post-SCI recovery of function.

Walking scores such as the BBB score are designed to screen for pharmacological treatments that can render clinically relevant improvements in function following incomplete SCI. While treatment-induced improvements in overground walking performance in rats represent good first steps in finding clinically relevant treatments for SCI, the mechanisms frequently remain unexplored. Improvements in overground walking are unlikely to be sufficient to prove that functional connections through an injury site are restored by a treatment. One example of this can be seen in the observations published in the first papers describing the BBB walking score (Basso et al., 1996), where BBB scores of rats with complete thoracic transection injuries rise to group means of 3.3 by 6 weeks post-injury, with some individuals scoring as high as 6. A BBB score of 3 represents

extensive movement of 2 hind limb joints, whereas a BBB score of 6 represents extensive movements of 2 hind limb joints and slight movement of the third joint. Rats frequently recover hind limb movements such that they will receive scores of 3 and higher on the BBB score following complete thoracic transection injuries. Descending spinal tracts are unlikely to significantly influence walking in that case. Thus, changes in spinal networks caudal to the injury site influencing the motoneurons innervating the hind limb are almost certainly involved in the recovery of hind limb movement observed after complete injury.

Due to the confounding factors at work in improvements in open-field walking, a more demanding walking task, the horizontal ladder frequently complements open-field walking scores in assessing walking after SCI (Metz et al., 2000). In this task, rats traverse a ladder of irregularly spaced metal bars. Rats with intact spinal cords usually place their paws precisely on the bars, making few slips in between. A paw slipping between the bars results in an error being scored. Rats change their walking pattern from that used during overground walking, to accurately place their paws on the bars while crossing the horizontal ladder. In this chapter, the altered walking patterns of rats with intact spinal cords were examined using both kinematic and EMG analysis. Understanding how rats change their walking pattern on the horizontal ladder provides a baseline to examine how rats further alter their walking pattern following damage to the dorsal portion of the CST, an important tract in precise control of limb movements (Whishaw et al., 1993). In this chapter, the movements made by rats following ablation of the dorsal portion of the CST at T8 were compared to rat with intact spinal cords.

Due to the more demanding nature of the horizontal ladder task when compared to open-field walking, improvements in functional outcome are more likely to be the result of restored descending input from the CST. The movements made by rats crossing the horizontal ladder following CST damage show several important features that would likely lead to reduced error counts that could be interpreted as improved functional outcome. In order to be able to make a strong case that improved performance on the horizontal

ladder is the result of restored descending connections, it would help to minimize the effect of potential compensatory movements that could confound the interpretation of reduced numbers of errors. In this way, the goals of this chapter are both 1) to improve the specificity of horizontal ladder assessment with respect to restoration of voluntary control over hind limb movements, and 2) learn more about how the movements made by rats with incomplete SCI can contribute to improved performance on the horizontal ladder in general and lead to functional recovery.

3.2 INTRODUCTION

Walking in quadrupeds is characterized by a rhythmic alternation of flexor and extensor muscles of the limbs. This pattern is orchestrated by neuronal networks within the spinal cord collectively referred to as central pattern generators (CPGs) (Cazalets et al., 1995; Kjaerulff and Kiehn, 1996) that are activated by tonic input from the reticulospinal tract (Jordan, 1998; Mori et al., 1992). The existence of spinal CPGs and the diffusely projecting reticulospinal tract fibers throughout the ventral and lateral funiculus of the spinal cord can explain why stepping movements can still be elicited after severe but incomplete spinal cord injuries in animal models (Little et al., 1988; Loy et al., 2002; Steeves and Jordan, 1980; Schucht et al., 2002). Rats are often used to model spinal cord injury (SCI) and although the walking pattern is coordinated by CPGs in the lower spinal cord (and thus not necessarily reflecting the integrity of experimentally injured spinal tracts) hind limb function during walking is a common functional readout. To quantify the walking performance several approaches have been employed including visual rating scales of open field locomotion (Basso et al., 1995), or other more specific measures including footprint analysis (de Medinaceli et al., 1982; Kunkel-Bagden et al., 1993; Metz et al., 2000), kinematic analysis and electromyographic recordings (Merkler et al., 2001). These approaches however focus on stereotyped walking on a flat surface. One drawback of using overground walking outcome measures after SCI originates

from spinal circuitry dictating the basic walking pattern. Improvements in overground walking after SCI maybe due to restored descending input or due to local changes in spinal neurons. An alternative approach in locomotor assessment is to study skilled walking as it relies upon the additional contribution of pathways such as the cortico- (CST) and rubro-spinal tract (RST). Lesions to these pathways result in pronounced and permanent deficits when environmental challenges are imposed (Metz and Whishaw, 2002; Webb and Muir, 2003). Their relatively distinct projection pattern makes RST and CST popular choices for studies of SCI, and the evaluation of skilled walking allows for a greater connection between the function and integrity of these tracts.

While skilled walking can be assessed using error counts without a detailed evaluation of the movement itself (Schucht et al., 2002; Onifer et al., 2005; Houweling et al., 1998), analyzing the walking pattern in greater depth gives the opportunity to observe otherwise undetectable subtle features and adjustments possibly controlled by input from the CST and RST. For example, adjustments following various spinal cord lesions have been revealed in the walking pattern of rats as expressed through ground reaction forces (Webb and Muir, 2003; Muir and Whishaw, 1999) or electromyographic recordings during rat treadmill locomotion (Kaegi et al., 2002). Metz and Whishaw (2002) used video analysis to provide a qualitative assessment of locomotion on a horizontal ladder; however quantitative measures such as comparison of joint angles have not been reported. These measures along with electromyography can possibly provide insight into fine motor control during walking and may offer a link between functional performance and the integrity of descending tracts responsible for skilled locomotion (i.e., CST and RST).

The present study examines healthy rats walking over a flat runway or a horizontal ladder while EMG activity and kinematic data were recorded to profile both forms of locomotion in detail and identify key variables of distinction. Subsequently, the strategies of ladder walking in healthy rats and rats with a dorsal column lesion were compared.

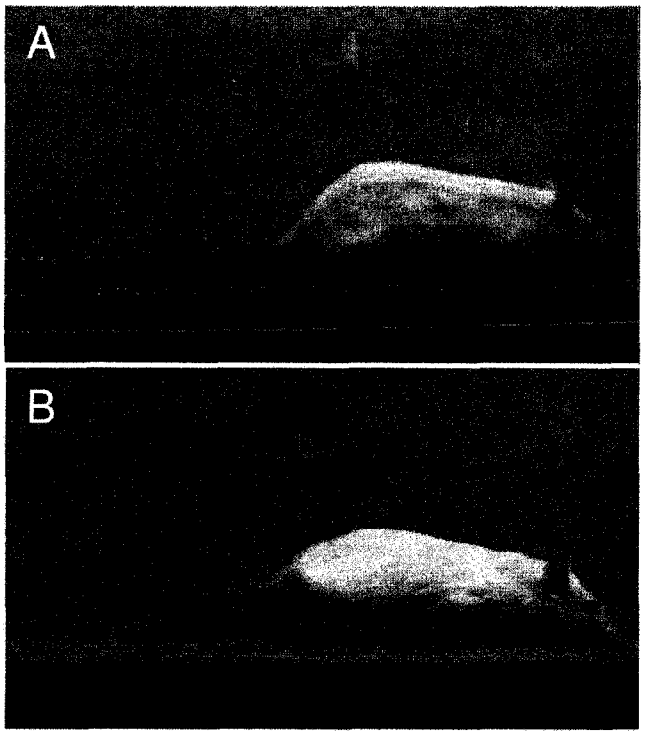

Figure 3.1: Testing apparatus – A 5 cm wide horizontal ladder with bars randomly spaced between 1.5 and 4 cm (A), and a 5 cm wide wooden runway with a smooth surface (B).

3.3 METHODS

Experiments were conducted using 10 adult female Lewis rats (180–200 g; five with and five without spinal cord lesion) in which EMG electrodes were surgically implanted (see below). All rats were kept at a 12:12 h light dark cycle with water and food provided ad libitum. This study was conducted in accordance with the Canadian Council on Animal Care guidelines and policies with approval from the Health Sciences Animal Policy and Welfare Committee for the University of Alberta. Rats were trained to walk on the horizontal ladder, as described previously (Kunkel-Bagden et al., 1993; Metz et al., 2000; Metz and Whishaw, 2002). The animals walked on a 1 m long by 5 cm wide ladder of metal bars with spaces between the bars varying in width between 1.5 and 4 cm (Figure 3.1 A). To provide a comparison with a stereotypical walking pattern rats were also trained to walk on an elevated flat wooden surface (i.e., runway) 1 m long by 5 cm wide (Figure 3.1B).

While walking on the ladder, rats were filmed from the lateral aspect using a digital video camera filming 30 frames/s (separated into 60 fields/s), shutter speed set to 1/500 s. EMG data were collected simultaneously. Synchronization between the EMG and video records was obtained using a light emitting diode (LED) placed within view of the camera. The LED was triggered during walking sequences and the simultaneous voltage pulses were recorded with along with the EMG signals.

3.3.1 Electromyography

All surgical procedures were carried out using Dormicum (Midazolam ,6 mg/kg, s.c., Roche, Switzerland)/Hypnorm (Fentanyl , 4 mg/kg, s.c., Janssen Cilag, Belgium) anaesthesia, with buprenorphine (0.05 mg/kg, s.c. Abbott, Mississauga, ON) given for 24 h postoperatively for pain control. Teflon-insulated multi-stranded stainless steel wires (Cooner Wire, AS 631, USA) were used as bipolar EMG electrodes soldered to a ten-pin connector (5-row CLE, Samtec, New Albany, IN). Wires were attached to 30 gauge hypodermic needles for implantation into muscles, as described previously (Merkler et

al., 2001). The EMG wires were passed subcutaneously to a connector sutured onto the back. EMG electrodes were implanted in the right Triceps brachii (TRI-R), right side of lateral Longissimus (LONG-R) intermediate portion – see (Gramsbergen et al., 1999), right and left Tibialis Anterior (TA-R and TA-L), and the right Vastus Lateralis (VL-R). During ladder and runway walking, EMG signals were amplified (Cyberamp, Axon Instruments, Union City, CA) and digitized (Digidata, Axon Instruments). EMG signals from each muscle were recorded at a sampling rate of 1 kHz, amplified by a factor of 200, rectified and low-pass filtered at 200 Hz. Sequences where three or more steps were completed within 2 s were used for analysis to ensure that regular walking sequences were compared. A minimum of 10 step cycles per animal, for each condition were used for analysis.

EMG traces were analyzed with customized software using Labview (National Instruments, USA). A step cycle was taken as the onset of one TA-R burst to the subsequent burst. Time intervals were determined for TA-R and VL-R muscle activity within a step cycle. TA-L was used only to aid in the visual assessment of step alternation. TA-R and VL-R duration was plotted relative to step cycle duration for each animal separately (based on the TA-R step cycle). The average amplitude of the EMG signal was also determined for TA-R and VL-R . This value represents the average EMG activity for each muscle over a given step cycle. All average amplitude values were normalized relative to the average runway walking values. A speed-matched subgroup of the runway walking data was determined for average amplitude to account for possible speed-related influences. This subgroup consisted of selected runway stepping sequences which fell within ± 1 standard deviation of the average step cycle duration on the horizontal ladder.

3.3.2 Procedures in rats with spinal cord injury

In five rats dorsal column lesions were performed during the same surgery as the EMG implantation. Therefore, a laminectomy was made at thoracic level (T8) followed by a transection of the dorsal funiculus with irridectomy scissors. After surgery the animals were kept on a thermostatically regulated heating pad until completely awake. Analgesics

were administered as described above. Ringer solution (4 ml) was given subcutaneously daily for 2 days following surgery.

Following all experimental procedures, rats were given an overdose of Euthanyl intraperitoneally (Bimeda-MTC, Cambridge, ON), intracardially perfused with 0.9% heparinized saline followed by 4% formalin. Segments containing the lesion were dissected and post-fixed in 4% formalin, cryo-protected in 30% sucrose, then frozen and sectioned using a cryostat. Cross sections of 50 μm through the spinal cord lesion site were counter-stained with cresyl violet. The completeness of the dorsal column lesion was confirmed under light microscopy.

Since EMG electrodes were implanted at the same time as the lesion was performed, comparisons of EMG data pre- versus post-lesion within animals could not be performed. Instead, post-lesion EMG data of ladder walking was pooled for all animals and compared with pooled pre-lesion data of the other group of rats that did not receive a lesion.

3.3.3 Video analysis

During all testing sessions animals were filmed from the lateral aspect. Ink markers were put on the iliac crest, greater trochanter (hip joint), lateral malleolus (ankle joint) and the fifth metatarsophalangeal joint on the right hind limb of each animal. Due to loose skin about the knee joint, the position of this joint was extrapolated through hip and ankle joint measurements and by using predetermined lengths of the femur and tibia using the software Peak Motus (ViconPeak, Colorado). Joint angles were calculated from five steps for both ladder and runway walking from each animal. The analyzed steps were chosen from steady and rhythmic walking sequences. Comparisons included joint angles for ankle, knee, and hip joints, in addition to the angle created by the pelvis relative to the horizontal plane. For the purpose of comparison step cycles were divided into four points; (a) toe-off, (b) mid-swing, (c) touch-down, and (d) mid-stance. Thus, each joint angle was compared between horizontal ladder and runway walking at each of these four points.

Stride length of the fore limbs were analyzed from 10 steps of each animal during

runway walking and ladder crossing in rats with and without spinal cord lesion.

3.3.4 Statistical analysis

Paired t-tests were used to compare joint angles at each of the four points in the step cycle and for comparisons with average amplitude. Regression analysis was used to evaluate muscle activity duration in TA-R and VL-R relative to the step cycle duration. Furthermore regression lines between ladder and runway walking trials were compared using F-tests on residual variances, slope and elevation (or adjusted means). Significance was set at $p < 0.05$. The distribution of hind limb placements on different rungs between lesioned and unlesioned rats was compared using the Chi-square test. Average values are presented with standard deviation (S.D.).

3.4 RESULTS

3.4.1 General observations

Rats generally cross the 1 m long horizontal ladder slower than the same distance on a flat runway. Based on EMG analysis (which has a higher time resolution than the kinematic analysis) the average step cycle duration (out of 10 steps of each rat; ±S.D.) was 537 ms ± 57 when walking on the ladder and 329 ms ± 85 when walking on the runway. The average stride length out of 15 steps/animal was 12.4 cm ± 0.4 during runway walking, which was significantly longer than in rats walking on the horizontal ladder (10.5 cm ± 0.3). To exclude changes in the analysis that were dependent on the stepping frequency, a frequency matched subgroup of runway walking trials was created. Trials with step cycle durations between 480 and 594 ms were chosen, thus representing ±1S.D. around the average stepping frequency for walking on the horizontal ladder. This selection window eliminated the data of one rat (only from the frequency matched subgroup), therefore 10 step cycles of four rats were included in that group. The resulting stepping frequency matched subgroup presented an average step cycle duration of 524 ms ± 9.

During regular (runway) walking the hind limb is normally placed directly behind the

location occupied by the ipsilateral fore limb. When walking on the horizontal ladder rats increase the stride length of their hind limbs to place them on the rung occupied by the ipsilateral fore limb in rapid succession (as described by Metz and Whishaw (2002). We found that if the hind limb did not land on this rung, a mistake (i.e., footfall/slip) typically occurred (data not shown). Fore limb placement generally appears more exploratory as a foot is positioned frequently on one rung and then repositioned onto another rung further ahead prior to the commitment of body weight. This results in frequent uncoupling of the fore and hind limbs.

3.4.2 Joint angle comparisons

Stick figure representations of the hind limb during ladder and runway walking are presented as group averages in Figure 3.2 A for each of the analyzed points (i.e., touch-down, mid-stance, lift-off, and mid-swing). To allow for the extended stride length of the hind limbs during stepping on the horizontal ladder, adaptations in joint angles (as compared to runway walking) have to occur. Kinematic analysis from video recordings at the four positions of the step cycle revealed two major differences to walking on the runway. We found a significant increase in the pelvic rotation relative to horizontal during touch down when comparing walking on the horizontal ladder (45 $\pm$ 4.4, average $\pm$ S.D.) versus runway walking (32 $\pm$ 6.1; see Figure 3.2B). This change in posture (pelvic rotation) can qualitatively be confirmed when comparing the pictures in Figure 3.2.

The second significant change in the joint angles was found in the hip during mid swing (Figure 4.2A), which decreased in all the animals during ladder walking (80 $\pm$ 6.9) as compared to runway walking (98 $\pm$ 6.6). No further statistically significant differences were found among joint angle comparisons at any of the four analyzed positions of the step cycle.

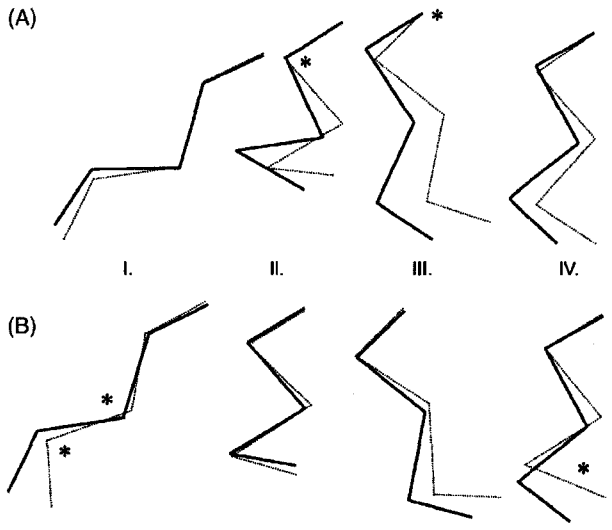

Figure 3.2: Stick figures from kinematics recordings comparing hind limb posture at four points of the step cycle – Points of the step cycle analyzed include (I) toe-off, (II) mid-swing, (III) touch-down, and (IV) mid-stance. (A) Shows the recording from one intact rat during runway (solid line) and ladder walking (dotted line) and (B) illustrates a comparison of the group average of lesioned rats (dotted line) to unlesioned rats (solid line). Asterisks beside the joints indicate statistically significant differences.

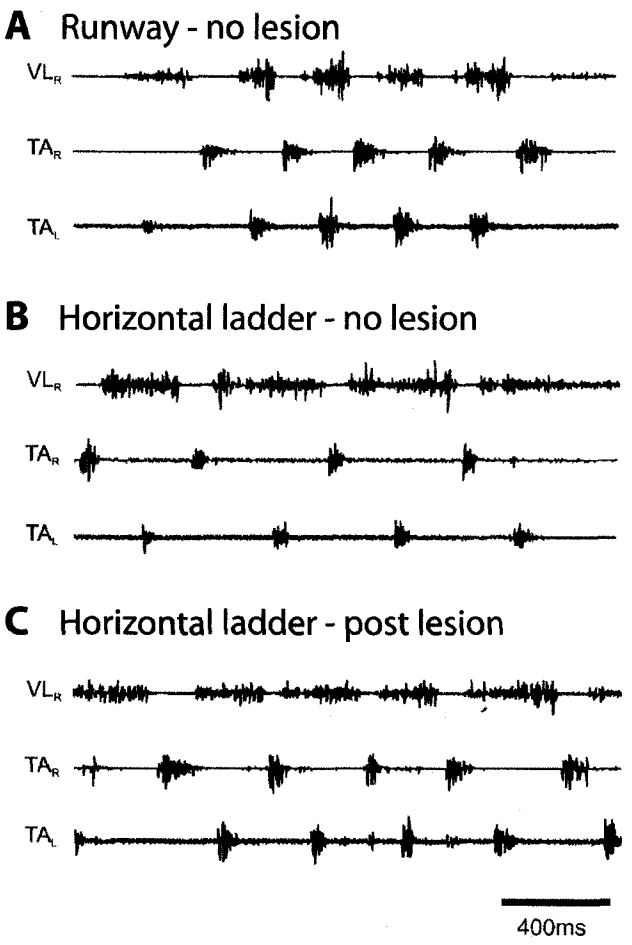

Figure 3.3: The basic pattern of EMG activity during rhythmic walking on a runway in the Tibialis Anterior muscle (TA) and Vastus Lateralis (VL) – (A) is comparable to that during walking on the horizontal ladder. (B) Differences seen in burst duration depend on the stepping frequency and are not due to adaptations during ladder crossing. Also following ablation of the dorsal funiculus the EMG pattern remains stable during ladder walking (C).

3.4.3 Electromyographic (EMG) analysis

Muscle activity during both ladder and runway walking was recorded bilaterally in Tibialis anterior (TA) an ankle flexor, unilaterally in Vastus lateralis (VL) a knee extensor, in Longissimus (LONG) a back extensor, and in Triceps brachii (TRI) an elbow extensor. Representative examples of EMG recordings are presented in Figure 3.3 .

Figure 3.4 shows that the activity pattern of a flexor muscle (TA) but not an extensor (VL) is regulated in a task dependent manner. As the step cycle duration increases for both walking conditions an increase in duration of the TA burst is noted. For a given step cycle duration the TA burst duration is lower while walking over the horizontal ladder. This decrease was significant in 3 out of 5 animals tested. For VL burst duration a clear relationship with step cycle duration was noted, however, no consistent difference was found between runway walking and walking on the horizontal ladder.

The average EMG amplitudes were compared between the frequency-matched runway trials and the ladder walking with the results normalized to the average runway data (Figure 3.5). When comparing the matched trials to average runway walking, we found that reduced stepping frequency (of the matched trials) resulted in a significant reduction in VL activity (0.74 ± 0.09; $p = 0.001$). Walking on the horizontal ladder resulted in a further but non-significant reduction in VL activity when compared with the frequency-matched group (0.63 ± 0.09). Thus changes in the stepping frequency but not the different task (walking on the ladder) may account for the differences in VL EMG amplitude. Amplitudes of TA bursts did not change in a step cycle frequency dependent manner (0.95 ± 0.17 in the frequency matched group) nor did they change during ladder walking (0.95 ± 0.19).

Based on the postural adaptations noted during walking on the horizontal ladder (see above), adaptations of axial muscle activity were expected. To examine this, we recorded from the intermediate portion of Longissimus (LONG), which is a back extensor inserting into the pelvis. Since this muscle was active throughout the entire step cycle and produced no apparent phasic activity pattern, burst duration could not be evaluated in the same

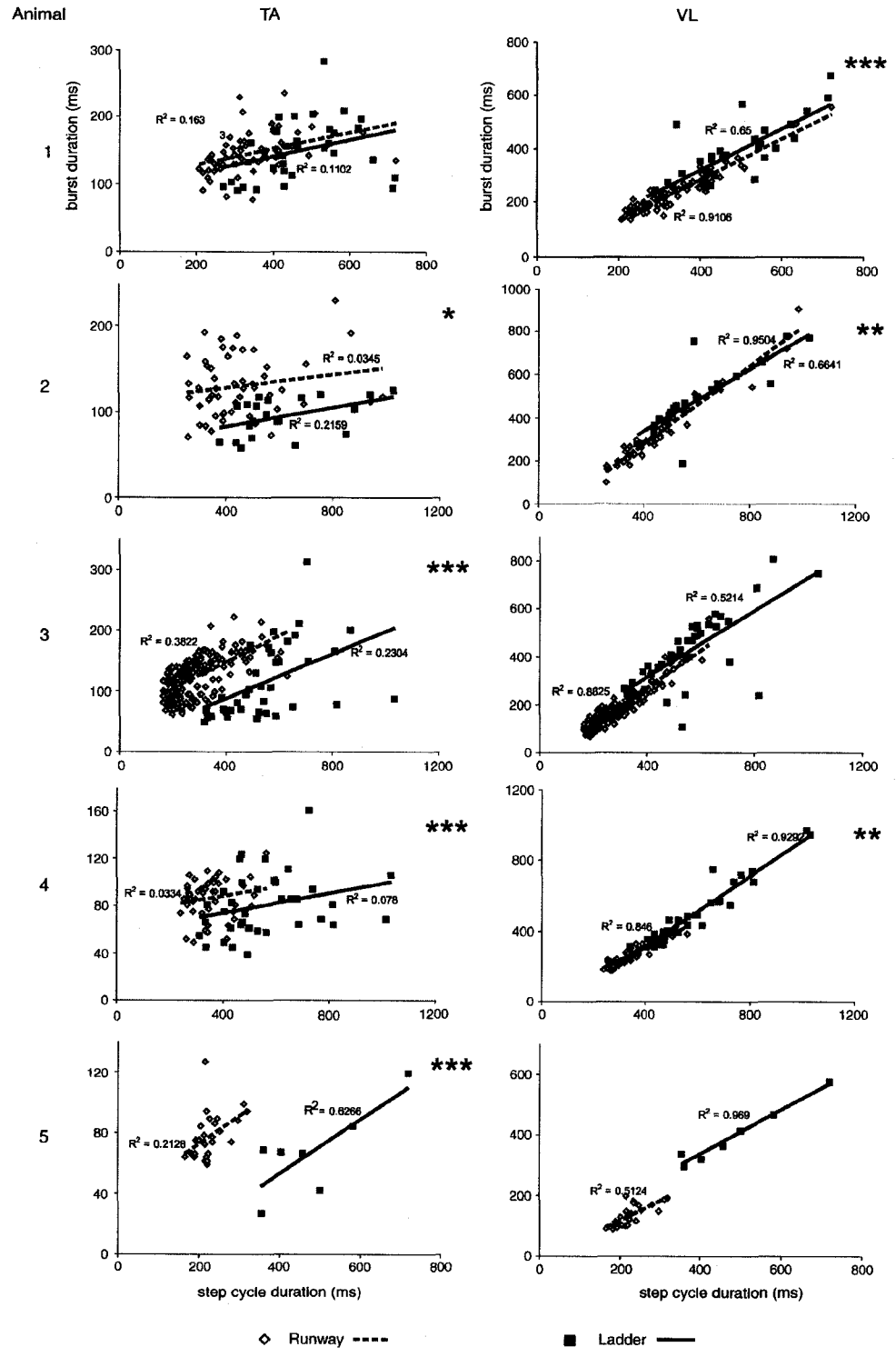

Figure 3.4: The activity pattern of a flexor muscle (TA) but not an extensor (VL) is regulated in a task dependent manner – Right TA and VL burst duration are plotted relative to the step cycle duration (based on right TA). Significant differences are reported for comparisons for homogeneity of variance*, slope**, and adjusted means***.

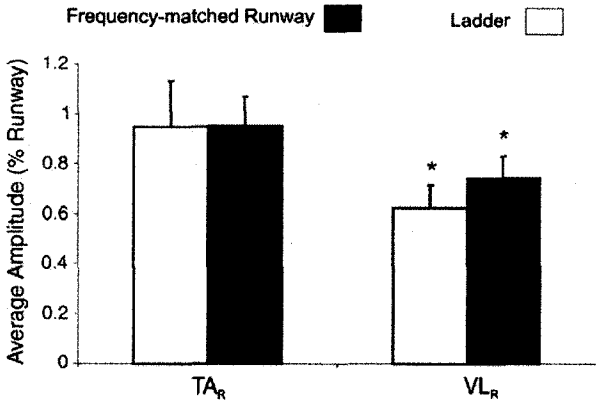

Figure 3.5: Averaged and normalized (to the runway average) EMG amplitudes for VL and TA muscles in the frequency matched group and when crossing the horizontal ladder – VL activity is significantly reduced in both groups (*) with no statistical difference between matched group and ladder walking. Error bars indicate standard deviation.

manner as for TA and VL. Average amplitude of LONG was analyzed over the entire step cycle to compare between walking conditions (i.e., the average amplitude corresponding to the interval between consecutive right side TA bursts). Values were normalized relative to the runway average resulting in a 10% reduction, which does not constitute a significant difference. This lack of difference between ladder and runway walking made a further comparison to the frequency- matched group unnecessary.

To characterize the behavior of the fore limbs in relation to the locomotor rhythm of the hind limbs, we recorded from a fore limb extensor muscle, triceps brachii (TRI). We found that the TRI burst duration increased significantly from average runway walking (194 ms $\pm$ 52) to speed matched ladder walking (288 ms $\pm$ 33). However, due to the absence of consistent TRI activity pattern and/or the presence of multiple bursts in a single step cycle during walking on the horizontal ladder, a quantitative comparison between grid walking and speed-matched runway walking was not conducted. Qualitative observations showed that TRI activity during runway walking demonstrated a consistent activity pattern, with one burst per step cycle in a time-locked relation to the TA burst. Initiation of this burst slightly preceded the TA burst and then TRI remained active during most of the same TA burst. For the grid walk this bursting was highly variable. Often multiple bursts occurred within a single step cycle. During periods of visibly "regular" walking over the ladder, a runway-like pattern was noted for the TRI activity when compared with the TA-based step cycle. This pattern however was not an exact comparison as it was often changed in a variety of ways including multiple bursts due to fore limb repositioning, and the distinction of fore limb propulsion and braking. This frequently resulted in an uncoupling of the fore and the hind limbs.

3.4.4 Effects of CST lesions on ladder walking

Histological analysis showed that in all five lesioned rats the dorsal funiculus (containing the main portion of the CST) was completely ablated (Figure 3.6), with variable degree of local gray matter damage. In one of these rats the dorsolateral funiculus

Figure 3.6: Lesion site – Cross section through a representative lesion site at the spinal segment T8, showing the complete ablation of the dorsal funiculus.

was also unilaterally lesioned, likely ablating parts of the RST.

As described by Muir and Whishaw (1999), these animals have no obvious deficits in overground locomotion, however errors (defined as slips from a rung or missing of a rung) are observed when crossing the horizontal ladder. On average (n = 30 steps/rat) we counted mistakes every 4 ± 1.8 steps in lesioned rats, and every 21.4 ± 4.1 steps in unlesioned rats. Importantly we found that in rats with lesions of the dorsal funiculus the foot placement strategy for the hind limbs had changed significantly. In contrast to unlesioned rats where the hind limb generally follows the fore limb position, lesioned rats stepped in a much more random manner, sometimes on the same bar as the fore limb, other times on the one in front or the one behind. This adaptation in paw placement was found to be significant in 4 out of 5 lesioned rats and is illustrated for one animal in Figure 3.7 before and after a lesion. The "0" indicates the rung where the forelimb has been positioned, "-1" the rung before, and "+1" the rung after. Another statistically significant change in the walking strategy of the rats with spinal cord injury was a reduction in stride length of the fore limbs (and therefore of the hind limbs). The average space between the chosen rungs was 10.5 cm ± 0.3 in healthy rats compared to 9.3 cm ± 0.3 in rats with dorsal column lesions.

Due to the increased variability of hind paw placing following injury we limited the kinematic analysis to steps where the hind limb stepped on the same rung as the fore limb did and no mistake occurred during that and the preceding step cycle. The resulting stick figures (Figure 3.2B) illustrate the observed changes and the asterisks indicate significant changes in the specified joint angle. Probably due to shorter stride length flexion is initiated with less leg extension when compared to unlesioned rats. This is reflected by a significant reduction in the knee and ankle angle (88.5 ± 8.1 and 95 ± 13.7, respectively) when compared to unlesioned animals (107 ± 12.6 and 128 ± 7.4). The only other significant change in joint angles was found during mid stance, where the ankle angle was reduced in lesioned rats as compared to unlesioned ones (47.75 ± 11.6 and 82.65 ± 8.1, respectively). Comparable differences in joint angles were not found during runway walking in rats with

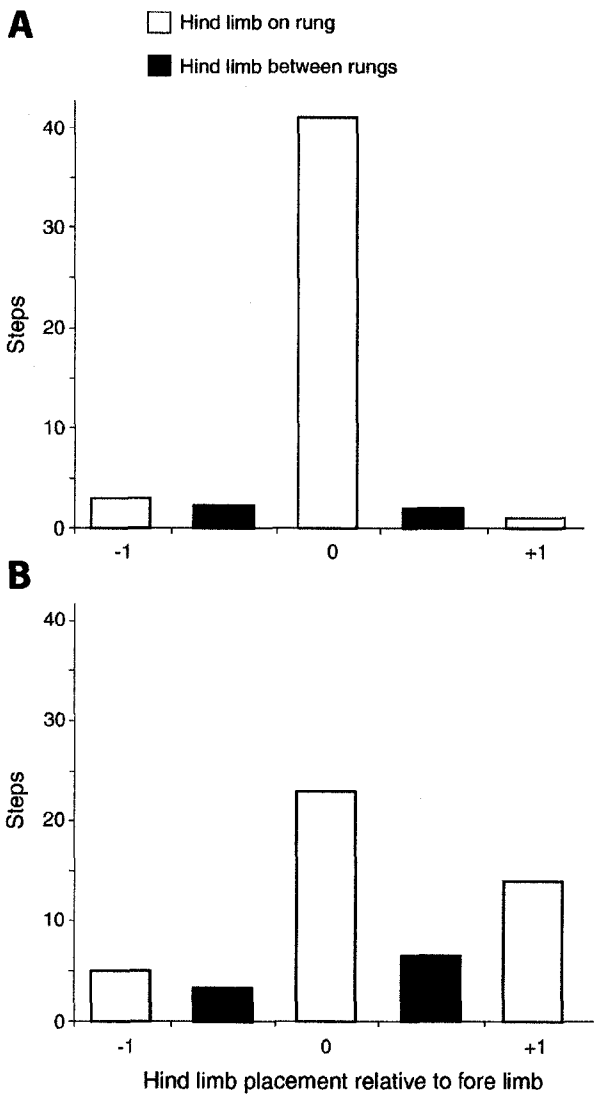

Figure 3.7: Placement of the hind limb in relation to the fore limb in a rat walking on the horizontal ladder task before (A) and after (B) lesion of the dorsal column – Zero indicates the rung where the fore limb has been placed, +1 or -1 are representing the rung in front or behind. Following lesion the distribution changes significantly. Note that in a traditional error count the number of steps placed on -1 or +1 would not be considered errors.

comparable lesions (Ballermann et al., 2006).

When comparing the electromyographic recordings between spinal cord injured and uninjured rats only steps without mistakes were evaluated (Figure 3.3). Overall the recordings looked very similar and only subtle changes were noted. Step cycle duration for lesioned rats crossing the horizontal ladder was 518 ms ± 125 with no significant difference to rats without lesion. While crossing the horizontal ladder, burst duration of VL demonstrated a similar positive slope relationship with increasing step cycle duration for rats with and without injury. In contrast, the relationship between TA burst duration and step cycle duration revealed a difference between injured and uninjured rats. In the uninjured animals, TA burst duration increased slightly, but significantly as step cycle duration increased. In post lesion rats this relationship disappeared (Figure 3.4).

3.5 DISCUSSION

The present study compared walking in adult rats under two conditions representing different levels of motor complexity: walking on a smooth surface coordinated mainly by neuronal circuits within the spinal cord (Kiehn and Kjaerulff, 1998) and walking on a horizontal ladder, a task that necessitates additional descending input of CST and RST (Metz and Whishaw, 2002; Muir and Whishaw, 1999). In a second experiment descending input was reduced by a lesion of the dorsal column, thereby ablating the major projection of the CST.

An important aspect of the interpretation of the presented results was the reduced stepping frequency of the uninjured rats when crossing the ladder. As the stride length during runway walking was also longer than during ladder crossing, rats on the runway walked faster. The importance of speed matching (in this study based on stepping frequency) the results becomes obvious as walking on the runway with reduced speed often produced similar results to those noted for crossing the ladder. We found only a few changes in the gait pattern of the hind limbs that were independent of stepping frequency,

including a different foot placing pattern and altered activity in an ankle flexor (TA). The minor changes in the hind limb gait pattern might be explained by the moving strategy of the animals, as the increased challenge imposed by the horizontal ladder is placed on the fore limbs. The fore limbs perform an exploratory task, whereas the hind limbs appear to perform a less demanding task by following the position of the fore limbs. Only when the fore limb position is established will the ipsilateral hind limb step to the same rung as per observations by Metz and Whishaw (2002). According to this strategy the only observed changes in the walking and muscle activity pattern found in hind limb kinematics are reflecting the extended reach of the hind limbs to land on the same rung where the fore limb had been placed. This was accomplished with an exaggerated forward reach of the hind limb facilitated by pelvic rotation and increased ankle flexion during the swing phase.

In contrast to the hind limb activity during ladder walking, the fore limbs produced a less consistent pattern, where TRI-R bursts were often indistinct and not time-locked to the TA burst, therefore making it difficult to identify start and stop points. Furthermore, in the trials where bursts were clearly distinguished, multiple bursts over a given cycle were frequently noted in all the animals. Also, in these cases the relative onsets for these bursts varied greatly. TRI-R activity therefore deviates from stereotypical runway walking in a manner qualitatively different from the influence of diminished walking speed. This change in the activity pattern of the triceps might reflect a different walking strategy, involving increased tactile feedback. The idea of tactile sampling in the fore limb during locomotion on regular surfaces has been introduced by Clarke (Clarke, 1995). His study proposed the concept of an early "soft-contact phase" for the initial portion of stance. The implication of this phase is to allow a tactile sampling of the terrain prior to committing to a stance strategy. Our results suggest that such tactile sampling becomes increasingly important under an unstable walking condition to achieve accurate paw placement onto the rungs of the ladder. This provides a stable platform for weight bearing and for placing the hind limbs. Such tactile sampling is likely reflected in the multiple TRI bursts often observed

during ladder walking.

A key premise underlying the current study is that adjustments to the stereotypical walking pattern during skilled locomotion rely upon descending contributions to modify activity in the spinal central pattern generating network for locomotion. Stereotypical walking can be initiated via spinal pathways projecting through the entire lateral and ventral spinal cord, therefore blurring the relation between recovery of walking and anatomical integrity (Schucht et al., 2002). This stands in contrast to the focal projection of pathways involved in fine motor control (e.g., CST and RST) where motor function more closely relates to anatomical integrity. We confirmed the results from earlier studies that rats with CST lesions produce more errors when crossing the ladder (Muir and Whishaw, 1999; Metz and Whishaw, 2002). An important finding of the present study is that lesioned rats choose a different strategy to cross the horizontal ladder: stride length was reduced and hind limb placement occurred on rungs besides those chosen by the fore limb, thereby reducing the error counts (if errors are considered falls between rungs only). Few changes were found in the kinematics and electromyographic analysis following CST lesion. Most importantly, we found an increased yield in the ankle during mid-stance (Figure 3.2), which could be interpreted as a deficit in weight support or as a mechanism to lower the centre of gravity in order to improve balance. Since rats with comparable lesions do not show deficits in weight support during walking on a flat surface (Muir and Whishaw, 1999), the increased challenge on balance during ladder crossing appears to be a more likely explanation. Another finding was the uncoupling of TA burst duration with stepping frequency. This can however most likely be explained with the higher variability in the stepping pattern (and thus also in TA burst durations) found after injury.

A ramification of the changes in stepping strategy on the horizontal ladder (i.e., hind limbs strictly follow the position of the fore limb) in terms of testing application is to ensure that rung spacing is wide enough (>3 cm) such that the consequence of not placing the hind limb on the rung chosen by the fore limb will force an error in foot placement

(i.e., footfall). Alternatively, one could consider steps on rungs other than the one where the fore limb has been placed as errors. This analysis will only be possible by using video recordings. A second issue is that during ladder crossing, the hind limb relies on the successful placement of the fore limb, and therefore the performance of the fore limb should to be taken into account when evaluating hind limb mistakes. Fore limb stepping can be perturbed resulting in a lack of weight support or even a foot slip by an earlier misstep of a hind limb. This will then enhance the possibility of a subsequent error in the next hind limb step. Therefore, another approach to improve the precision of error counts in animals crossing a horizontal ladder could be to not count the number of errors on a certain distance (or number of rungs), but rather assess the maximal number of subsequent steps without error.

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CHAPTER 4

SPONTANEOUS LOCOMOTOR RECOVERY IN SPINAL CORD INJURED RATS IS ACCOMPANIED BY ANATOMICAL PLASTICITY OF RETICULOSPINAL FIBERS

Adapted from Ballermann M, Fouad K, (2006) Eur J Neurosci. 23:1988-96.

4.1 PREFACE

Chapters 2 and 3 have investigated the changes in overground and skilled walking that occur following incomplete thoracic SCI. In chapter 3, overground walking was compared to movements made while walking on the horizontal ladder task. Rats with intact nervous systems adjust their gait from landing with their hind limb behind the landing point of the fore limb in overground walking to landing precisely in the same location as the ipsilateral fore limb during skilled walking. Rats exaggerated the flexion of the hind limb during swing to accomplish this limb placement. When rats with injuries of the dorsal columns, including the CST, were compared to rats with intact nervous systems, more errors were observed, but in addition the rats adjusted their hind limb trajectory in such a way as to increase the chances of landing on the bar ahead or behind the one their ipsilateral fore limb had contacted. This would likely result in error counts that would underestimate the degree of impairment in a rat with SCI. Furthermore, the results in chapter 3 point to an important role for fore limb hapsis (sense of touch) in rat fore paws in locating and placing the limbs on the rungs while completing the horizontal ladder task. The experiments to this point have investigated movements used by intact rats and rats with SCI to walk in an overground fashion as well as in the horizontal ladder task.

Based on what is known about the neural control of walking, we can investigate anatomical changes that correlate with and are likely to underlie functional recovery. Most anatomical investigations examine the CST or RST for technical reasons: they can be more specifically injured and quantitative observations of tracing studies are simpler than that of the reticulospinal tract (RtST), an important tract that activates the networks of the lumbar spinal cord to produce the walking pattern. In order to study this tract, lateral hemisection injury at the level of T8 were used, These injuries result in large degrees of impairment to the ipsilateral hind limb initially but this recovers over the weeks following injury. Previously it has been shown that recovery depends on the spared fibers as well as crossed connections as a second lesion to either substrate can abolish the recovery (Little et al.,

1988). Additionally, after recovery following lateral hemisection injury in cats, stimulation of the spared RtST results in a strengthened output from the contralateral ventral roots below the injury (Eidelberg et al., 1986). Changes in the reticulospinal projection pattern following injury may be important following lateral hemisection injuries and improve recovery of walking. Anatomical plasticity in spared descending tracts can represent a means by which the CNS can reroute descending commands around an injury site, via new spinal circuitry. In this chapter, we investigated the question of how do lateral hemisection injuries in rats thoracic spinal cord result in RtST projection pattern changes?

4.2 INTRODUCTION

Many studies on SCI in animal models have focused on the cortico- (CST) or rubro-spinal tracts, with locomotor assessments providing functional readout (e. g. Liebscher et al., 2005). These tracts run as somewhat distinct bundles in the dorsal spinal cord and thus can be relatively easily ablated and/or histologically examined. Although these tracts play major roles in controlling fine motor movements (Whishaw et al., 1998), their contribution to locomotor function is limited (Steeves and Jordan, 1980; Muir and Whishaw, 1999). An important tract in eliciting locomotion is the reticulospinal tract (RtST). Its role in eliciting activity in spinal networks that coordinate rhythmic stepping movements (central pattern generators, CPGs) has been demonstrated in electrophysiological (Orlovsky, 1970; Shefchyk et al., 1984; Jordan, 1998; Mori et al., 1998) and lesion studies (Steeves and Jordan, 1980; Little et al., 1988; Schucht et al., 2002). The RtST is a heterogeneous tract originating from dispersed nuclei in the reticular formation. The RtST descends mostly in the ipsilateral dorso- and ventro-lateral funiculi (VLF; Jones and Yang, 1985). Anatomical studies have demonstrated that following SCI, sparing of only a small unilateral portion of the VLF is sufficient for spontaneous locomotor recovery (Little et al., 1988; Harris et al., 1994; Gorska et al., 1996; Brustein and Rossignol, 1998; Schucht et al., 2002). In rats, this includes recovery of the hind limb on the completely ablated side of the

spinal cord, which occurs within 2-3 weeks. Considering the role of the RtST in triggering locomotion and the delayed recovery, we hypothesized that sprouting of the RtST with collaterals crossing the mid line is involved in promoting this recovery.

A goal of this study is to examine changes in the projection pattern of spared RtST fibers after lateral hemisection injuries of the thoracic spinal cord (Figure 4.1 A). We examined such changes in rats with minor recovery 7 days after injury (short recovery group) and in those that recovered to a higher degree over 42 days (long recovery group). An anterograde tracer was injected into the left reticular formation and the lateral hemisection injury at T8 was carried out on the right side of the spinal cord (Figure 4.1B). We examined RtST collaterals and gray matter branches at L2 as hind limb movements are generated by pattern generating networks located in the lumbar enlargement (Kiehn and Butt, 2003).

Another possibility is that the recovery after thoracic hemisection lesion may be promoted by rerouting of severed RtST fibers rostral to the injury, in a manner comparable to the rerouting that has been described in the CST (Bareyre et al., 2004). The cervical enlargement contains pattern generating networks for the fore limbs, which are connected to the lumbar pattern generating networks via both crossed and uncrossed long propriospinal interneurons (Stokke et al., 2002; Bannatyne et al., 2003). These interneurons may represent an intermediate target for severed RtST fibers. To support this idea, we examined whether severed RtST axons increase their collaterals into the gray matter above the injury level. We used left-sided lesions with left-sided tracer injections (Figure 4.1C), and RtST collaterals were quantified at cervical level (Figure 4.1D).

4.3 METHODS

4.3.1 Animals

The experiments were approved by the University of Alberta animal care committee and conducted in accordance with rules set by the Canadian Council of Animal Care. All rats were kept at a 12:12 hrs light/dark cycle and received water and food *ad libitum*. Animal

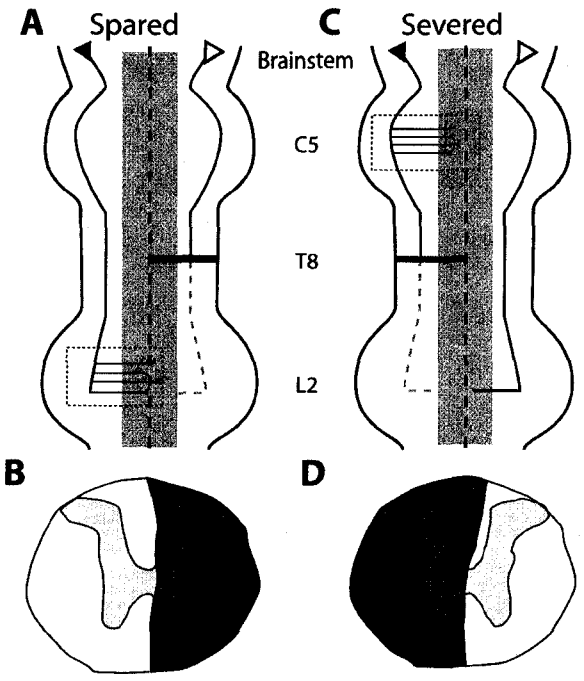

Figure 4.1: Schematic of experimental design showing rat spinal cords and brainstems with RtST fibers, analysis sites and lesion extent – Black triangles on the left represent BDA labeled giant reticular neurons, with the lines below representing RtST axons descending into the spinal cord. A: In the spared fibers experiment, the lesion was performed at T8 on the right side (thick black line) contralateral to the traced fibers, and spared fibers were quantified at the level of L2 (dashed box). B: A reconstruction of a representative right sided lesion is shown. C: In the severed fibers experiment, the lesion was made on left side ipsilateral to the traced fibers, and injured fibers were quantified at the level of C5. D: A reconstruction of a representative left sided lesion is shown.

handling began 14 days prior to the SCI surgery. Experiments examining sprouting of spared fibers at lumbar level were performed using 12 adult female Lewis Rats (165-180 g; Charles River, Wilmington, MA). Experiments examining sprouting of lesioned fibers rostral to the lesion were performed using 9 adult female Lewis Rats. Additionally, the RtST was traced and quantified in 4 rats that did not receive lesions.

4.3.2 Spinal Cord Lesions

All operations were performed under Hypnorm (Janssen Pharmaceutics, Beerse, Belgium; 120 μl per 200 g body weight) and Midazolam (Sabex, Boucherville, QC, Canada; 0.75 mg in 150 μl/200 g body weight. 750 μl total volume diluted with H_2O.). Eye lubricant (Tears naturale, Alcon Canada, Inc, Mississauga, ON) was applied to protect the eyes from dehydration.

Rats received laminectomies of the dorsal half of the eighth thoracic vertebra. This was followed by a lateral hemisection of either the right (to study sprouting of spared fibers; n=12; Figure 4.1A) or left side (to study sprouting of lesioned fibers; n=9; Figure 4.1C) using a customized blade. Following lesion, the dorsal back musculature was sutured and the skin closed with surgical clips.

After surgery the animals were kept on a thermostatically regulated heating pad until completely awake. The analgesic Buprenex (Buprenorphine; Reckitt & Colman, Richmond, VA) was administered subcutaneously (0.03 mg/kg) immediately after operation, and every eight hours for 74 hrs. Warm Ringer's solution (4 ml) was given subcutaneously daily for the first week and at later stages if animals showed signs of dehydration.

4.3.3 Behavioral testing

BBB locomotor score: Open field locomotion was evaluated by using the BBB locomotor scale (Basso et al., 1995). The rats were placed into a 30 x 80 x 130 cm transparent Plexiglas box with a smooth surface and were observed by 2 investigators for

a 4 min period. Testing was performed weekly over the recovery period (7 days after SCI for short recovery group, 42 days for long recovery group).

Horizontal ladder: The animals walked on a 1 m long horizontal runway of metal bars elevated 30 cm from the ground in weekly testing sessions. A defined 20-bar sector was chosen for analysis. To prevent habituation to a fixed bar distance, the bars in this sector were placed irregularly (1-3 cm spacing) and were changed in every testing session. The rat's performance was monitored using a digital video camera (Canon, Optura 100, 60 fields/s) and analysis was performed by counting the number of errors in foot placing.

4.3.4 Anterograde tracing of reticulospinal projections

Reticulospinal projections were compared between groups of animals with a short recovery period (7 days after injury) and in a group allowed to recover over 42 days. Since the tracer needs 14 days to reach the lumbar spinal cord, anterograde tracing was performed at different time points, either 7 days before the lesion (in the short recovery group; spared fibers experiment n=5; lesioned fibers experiment n=4) or 28 days after lesion (spared fibers experiment n=7; lesioned fibers experiment n=5). Based on preliminary experiments (data not shown), 7 days is not sufficient time for the tracer to travel past the lesion site therefore in the experiment examining spared fibers, any tracer found caudal to the lesion can be attributed to these fibers.

The RtST was traced via iontophoretic injection of the anterograde tracer biotin dextran amine (BDA; 10,000 MW, Invitrogen, Eugene, OR), into the gigantocellular division of the medullary reticular formation. The injection coordinates were 0.8 mm lateral to mid-line, 2.8 mm caudal to lambda, and 8.2 mm ventral to dura. The injection was performed using a BDA filled glass capillary with a tip diameter of approximately 70 μm and a current of 3.5 μA applied in 5 sec on, 5 sec off cycles for 10 min. The animals were euthanized 14 days after tracing using Pentobarbital (Euthanyl, Bimedia-MTC, Cambridge, ON; 300mg/kg, i.p.) and perfused transcardially with a Ringer solution containing 100,000 IU/l heparin, followed by fixation with 4% formalin and 5% sucrose in 0.1 M phosphate buffer.

The spinal cords were removed, postfixed overnight in 4% formalin/5% sucrose, and then transferred to a 30% sucrose solution for 3 d. The spinal cords were embedded with Tissue Teck (Sakura Finetek USA Inc, Torrance, CA) and frozen at -40°C. Sagittal sections of 25 μm from the cervical and lumbar enlargements were taken on a cryostat. Cross sections of 25 μm were taken through the lesion site and the brainstem injection site, and were processed as described below.

Staining was performed as described previously (Herzog and Brosamle, 1997). The slides were washed 3 times for 30 minutes in a 50 mM Tris buffered saline, pH 8.0, containing 0.5% Triton X-100 (TBST). Afterwards the slides were incubated over night with an avidin-biotin-peroxidase complex (Herzog and Brosamle, 1997) in TBST (ABC elite, Vector Labs, Burlingame, CA) according to the instructions of the manufacturer. Subsequently the diaminobenzidine (DAB) reaction was performed using the Vector DAB kit (Vector Labs, Burlingame, CA). The reaction was monitored and stopped by washing in water. To counterstain the slides, they were immersed for 3 minutes in 0.1% cresyl violet. The slides were dehydrated stepwise through alcohol, cleared in xylene, and cover-slipped with Permount (Fisher Scientific, Nepean, ON).

4.3.5 Quantification of reticulospinal projections

Analysis focused on three parameters: i) the location of RtST axons within the white matter, ii) the density of RtST collaterals entering from white into gray matter, and iii) the density of RTST branches within gray matter laminae.

Due to the large anatomical area where RtST axons descend and project within the gray matter, a density measurement was used to quantify and compare projection patterns. Even-numbered sections (i.e., every second section) throughout the spinal cord were photographed using a brightfield microscope (Figure 4.2 A; Zeiss Canada, Toronto, ON). The density of axons, collaterals, and gray matter branches was assessed by counting the numbers of labeled fibers in white matter, at the gray-white matter interface, and within the gray matter respectively. Counts were performed within 30 squares of a 50 μm grid

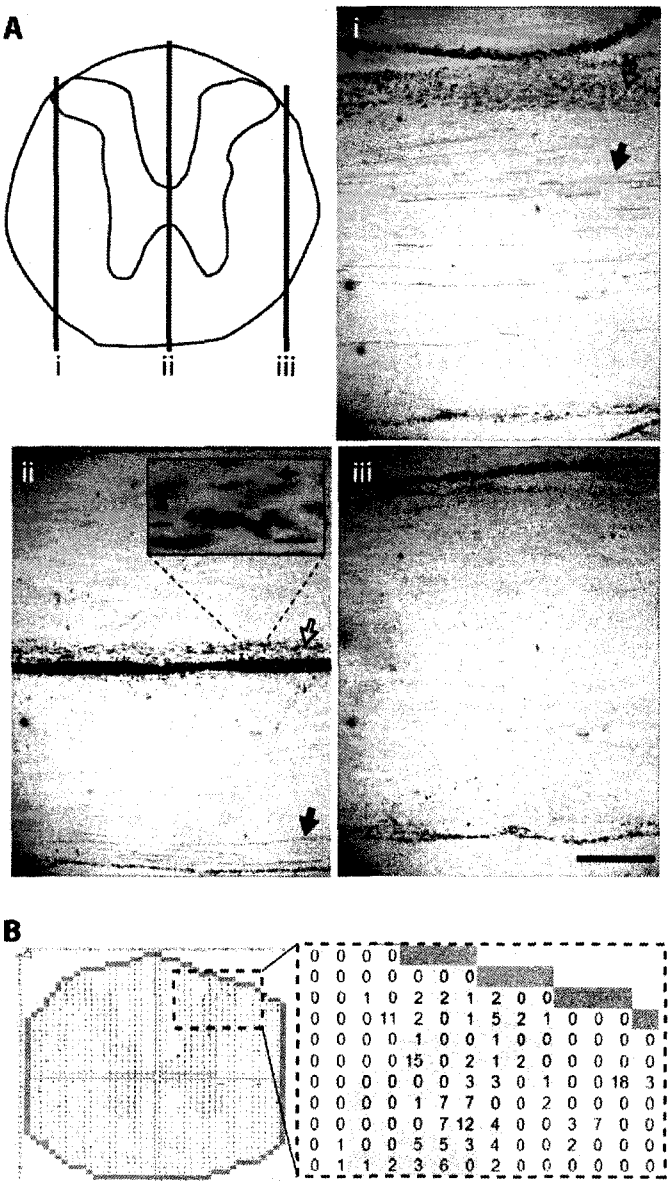

Figure 4.2: Fiber density quantification – A: Micrographs of sagittal sections taken from T8. Planes of section are shown marked by i, ii, and iii, with i being taken from the injected side of the spinal cord. Hollow arrows indicate gray matter. Solid arrows indicate labeled fibers in panels i and ii. Scale bar = 400 μm. Dorsal is up. Inset shows higher magnification of fibers passing by the central canal (through the plane of section) in the intermediate gray matter. Scale bar = 25 μm. B: Numbers of fibers within 50 μm squares of a superimposed grid were counted and entered into a spreadsheet with gray and white matter information preserved, to form a cross-section representation of fiber density. Dashed box represents magnified area of an Excel spreadsheet with fiber density entered.

overlaid on the sagittal sections. Thus, the rostro-caudal distance analyzed was 1500 μm. The average value per square for each horizontal row of the grid was transferred into a spreadsheet representing a cross section (Figure 4.2B).

For each parameter (axons, collaterals, branches in gray matter) specific rostral-caudal levels were chosen for analysis: *i)* Location of RtST axons were analyzed in unlesioned rats at C5, T9 and L2. Axons ran in the plane of the section and were therefore easily discerned and counted. *ii)* RtST collaterals entered from white into gray matter, in many different angles relative to the plane of section, thus short fragments of the sectioned collaterals were visible. These fragments were counted in squares within 50 μm of the interface between gray and white matter at the level of C5 when severed RtST fibers were analyzed and at the level of L2 when spared RtST fibers were analyzed. *iii)* branches within the gray matter run in many angles relative to the plane of section, and sectioned fragments were counted at the level of L2 in rats where spared RtST fibers were studied. This analysis was not performed at cervical levels as no increases in collateral sprouting of lesioned RtST fibers were found.

To correct for inter-animal variations in the numbers of labeled axons, fiber densities (fiber counts per 50 μm square) were normalized (i.e., divided) by the numbers of labeled fibers counted on cross sections rostral to the analysis site (C1 for cervical analysis, T8 for thoracic and lumbar analysis).

As we found an increase in collateral density of the long recovery group we performed an additional analysis, where we also normalized gray matter fiber density to the RtST collateral density. This allowed us to discern whether changes in gray matter fiber density were due to increased collateral sprouting alone.

4.3.6 Lesion Site

Cross sections of 25 μm through the spinal cord lesion site were counter-stained with cresyl violet and the largest extent of each lesion was reconstructed from adjacent sections, and from these reconstructions spared white matter measurements were performed (Scion

Image, NIH, Bethesda, MD).

4.3.7 Tracing Injection Site

Cross sections of 25 μm were taken from the injection sites in the brainstem and were processed to visualize the BDA as described above and counter-stained with cresyl violet. The precision of the injection site was confirmed by the presence of labeled cells in the gigantocellular division of the reticular formation. The tracer spread was determined from photomicrographs of the injection site and calibrated using a scale bar taken at the same magnification.

4.3.8 Statistics

Normalized reticulospinal fiber density in the spinal cords from the intact, short and long recovery groups were compared using non-parametric Kruskal-Wallis tests, as they do not rely on the assumption that fiber density values are normally distributed (GraphPad, Prism software version 4.01, San Diego, CA www.graphpad.com). Where significant differences were found, groups were compared in a pairwise manner using Mann-Whitney U tests. Comparisons between behavioral observations taken from day 7 and day 42 in the long recovery groups were done with paired t-tests (Wilcoxon signed rank). Correlations were performed using Spearman r-tests. Significance was set at p=0.05.

4.4 RESULTS

4.4.1 Injection site analysis

In all animals, tracer injection sites were verified by the micrographic examination of cross-sections in the ventral gigantocellularis nucleus (GiV; Figure 4.3 A). Cross-sections were compared to representations of the ventral brainstem nuclei taken from Paxinos et al. {, 1998 #149}, which include the GiV shown in Figure 4.3B. Animals where the center of the tracer injection site was outside the boundaries of the GiV, or did not show any labeled cells, were excluded from the study. In all remaining animals, large cell bodies

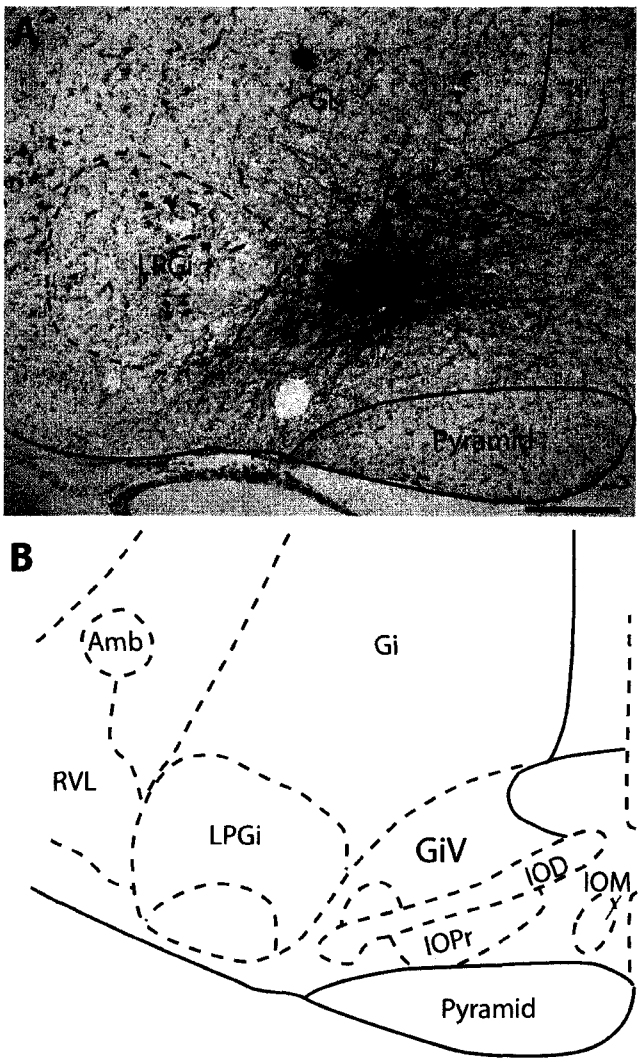

Figure 4.3: BDA injections labeling the ventral gigantocellular division of the medial reticular formation – A: Photomicrograph of transverse brainstem section stained for BDA labeled cells at the level of the medulla as shown in B. The midline is at the right edge of the photomicrograph. Structures bordering the GiV are labeled. Scale bar: 400 μm. B: Diagram of cross-section of ventral brainstem nuclei surrounding the ventral gigantocellular division (GiV). Gi: Gigantocellularis, Amb: Nucleus Ambiguus, LPGi: Lateral Paragigantocellularis, RVL: rostral ventrolateral thalamic nucleus, IOD: inferior olive - dorsal nucleus, IOPr: inferior olive - principal nucleus IOM: inferior olive - medial nucleus (Adapted from Paxinos and Watson, 1998.

(profiles of 75-100 μm in diameter) were labeled in the left GiV, at the level of the medulla. Sections through the pontine reticular formation showed no labeling. The mean spread of labeled cell bodies was 573 μm (standard deviation (SD)=272 μm) and was not different between the intact, short or long recovery SCI rats. Labeled projections arising from the GiV extended into the spinal cord in the lateral, ventrolateral, and ventral funiculi.

4.4.2 Reticulospinal projections of intact rats

The RtST projections of axons, collaterals, and gray matter branches were visualized after unilateral, left-sided tracer injections in four intact rats. Then, the number of axons at two levels of the spinal cord was counted. At the level of C1, the mean number of labeled axons was 290 (SD=84), and at the level of T8, the mean number of labeled axons was 160 (SD=56). Representative locations of fiber densities at the levels of C5, T8 and L2 in an intact rat are shown in Figure 4.4 . RtST axons descend in the lateral, ventrolateral and ventral funiculi of the ipsilateral spinal cord. Collaterals project into the gray matter and arborize to form gray matter branches. There is a small percentage of fibers on the contralateral side at the level of C5 (approximately 15% of the total number of axons) which cross at some point above C5. The numbers of labeled crossed axons are similar in the thoracic spinal cord with 15% at T8.

4.4.3 Experiments in injured rats: Lesion sites

In the 2 lesion experiments, the side of injury was varied to examine the projection pattern changes in spared or severed RtST fibers. Specifically, in one group of rats, lesions were inflicted on the right side to investigate the projections of spared fiber tracts below the injury. In the other group lesions were inflicted on the left side to investigate changes in the projections of severed fiber tracts above the injury. Histological examination of adjacent cross sections revealed that the lesions interrupted the spinal cord tracts on either the right (Figure 4.1B) or left side of the mid-line in all injured rats (Figure 4.1D). The overall mean percentage of spared white matter was 49.5%, with a standard deviation of 9.8%.

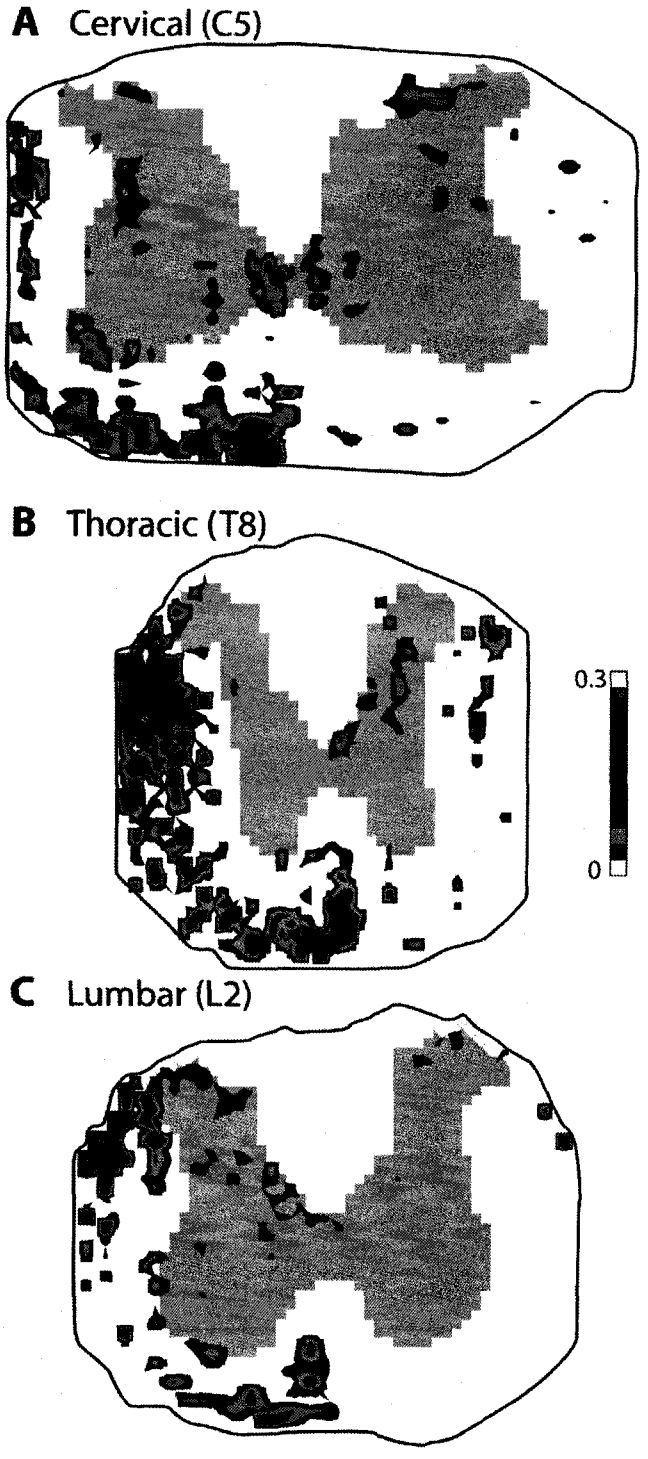

Figure 4.4: RtST fiber density in intact rats at spinal levels C5, T8, and L2 – Surface plots of fiber density for C5, T8, and L2 are shown in A, B, and C respectively. Fiber density is represented by different colors with no fibers represented as transparent (gray/white matter visible)

Percentages of spared white matter did not significantly differ between the short and long recovery groups, nor did they differ between rat groups with left- or right-sided lesions.

4.4.4 Spontaneous locomotor recovery following SCI

Following SCI, open-field locomotion was scored weekly using the BBB open-field locomotor scale {Basso, 1995 #67}. All lesioned rats showed large initial impairments in hind limb function. At 7 days post-injury, the short-recovery group had average BBB scores of 10.5 (standard deviation=3.1). The long-recovery group had average BBB scores of 12.0 (SD=1.9; Figure 4.5 A) that, at 7 days post-injury, did not significantly differ from the short recovery group. The long recovery group continued to gradually recover over the next 35 days and reached a plateau at a group mean score of 15 (SD=2.3). At 42 days post-lesion the BBB scores were significantly different from the short recovery group ($p<0.0001$). Within the long recovery group, there was a statistically significant improvement between mean BBB scores from day 7 and day 42 ($p<0.0005$)

Rats were also trained pre-injury to traverse a horizontal ladder, where slips between the rungs were scored as errors (Figure 4.5B). The mean number of errors made while traversing 20 bars before injury was less than 1 for both groups (mean=0.76). Both groups showed large initial impairments in traversing the horizontal ladder, with mean numbers of errors of 8 to 10 at 7 days post-injury. The rats in the 42 day survival group gradually improved their performance with errors decreasing to a group mean of 6 errors per 20 bars traversed (SD=2.4). Within the long recovery group there was a statistically significant reduction in the number of errors between day 7 and day 42 ($p<0.001$).

4.4.5 Projection changes of the reticulospinal tract following spontaneous recovery

Projections of spared RtST fibers at lumbar level and projections of injured fibers above the lesion (at cervical level) were compared between the intact, the short-recovery and the long-recovery groups. In both scenarios (spared or injured) the RtST was traced with BDA

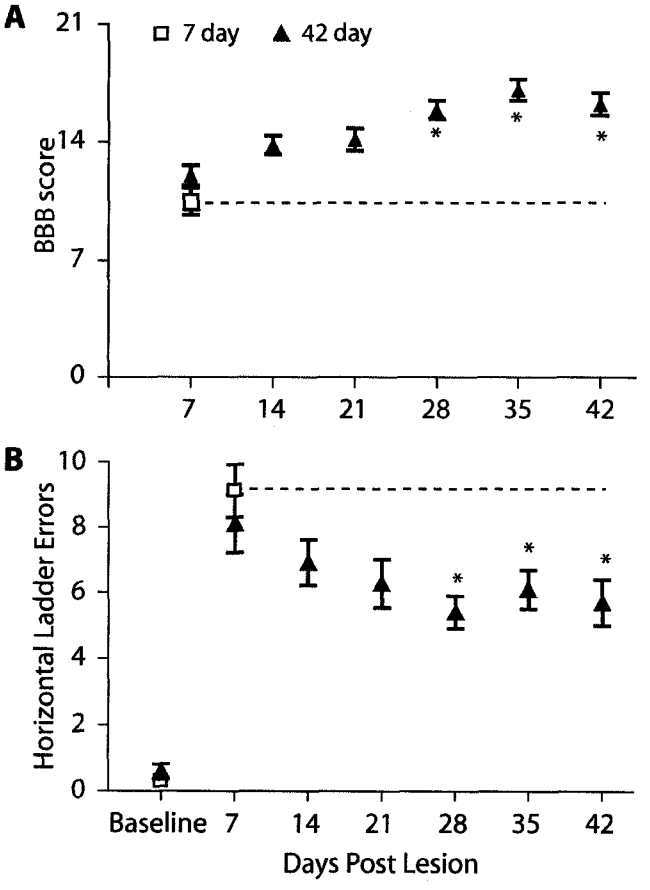

Figure 4.5: Functional recovery following lateral thoracic hemisection of the spinal cord – A: Open field scoring of locomotion (BBB score) applied to the impaired limb (ipsilateral to lesion). B: Numbers of errors committed by rats traversing 20 bars on the horizontal ladder following lesion. Values represent means ± SEM. Asterisks indicate significant changes as compared to values from day 7 ($p<0.05$).

injections on the left-side.

Spared RtST projections were examined at L2 in rats with right hemisections, below the level of the injury. We found collaterals passing from the lateral and ventral funiculi into the gray matter in all the groups (Figure 4.6 A). Following injury and 7 days recovery there was no significant increase in the density of RtST collaterals passing from the lateral funiculus into the dorsal horn and intermediate gray matter (204% of intact values ± 20% S.E.M.; Figure 4.6B). Following 42 days recovery, however, there were significant increases in the density of the dorsal RtST collaterals relative to both the intact group and short recovery group (550% of intact values ± 192% S.E.M.; Figure 4.6B). When the entire gray-white matter interface was compared, the increase in collateral density was not statistically significant (Figure 4.6C).

The location of RtST branches within the gray matter was determined using fiber density calculations in the gray matter as performed above. When normalized to the number of traced fibers at C1, sparse RtST projections were found within the intermediate gray matter in intact rats (Figure 4.7 A). Following an injury of the contralateral thoracic spinal cord and 7 days recovery, there were statistically insignificant increases in fiber density in the dorsal horn (264% of intact values ± 50% S.E.M.) and intermediate gray matter on the injected side (177% of intact values ± 41% S.E.M.; Figure 4.7A&B). In the long recovery group, there were statistically significant increases in fiber density above that of intact and rats and those with short recovery in the dorsal horn on the injected (left) side (1095% of intact values ± 315% S.E.M.; Figure 4.7B). In the intermediate gray matter on the injected (left) side there was a significant increase in branches above that of the intact group only (399% of intact values ± 138% S.E.M.; Figure 4.7B). There were no significant changes in the fiber density in the ventral horn or in the intermediate gray matter on the side contralateral (right) to the injection.

When gray matter RtST fiber density was normalized to the collaterals density, there were still significant increases in RtST fiber density within the dorsal horn (606% of intact

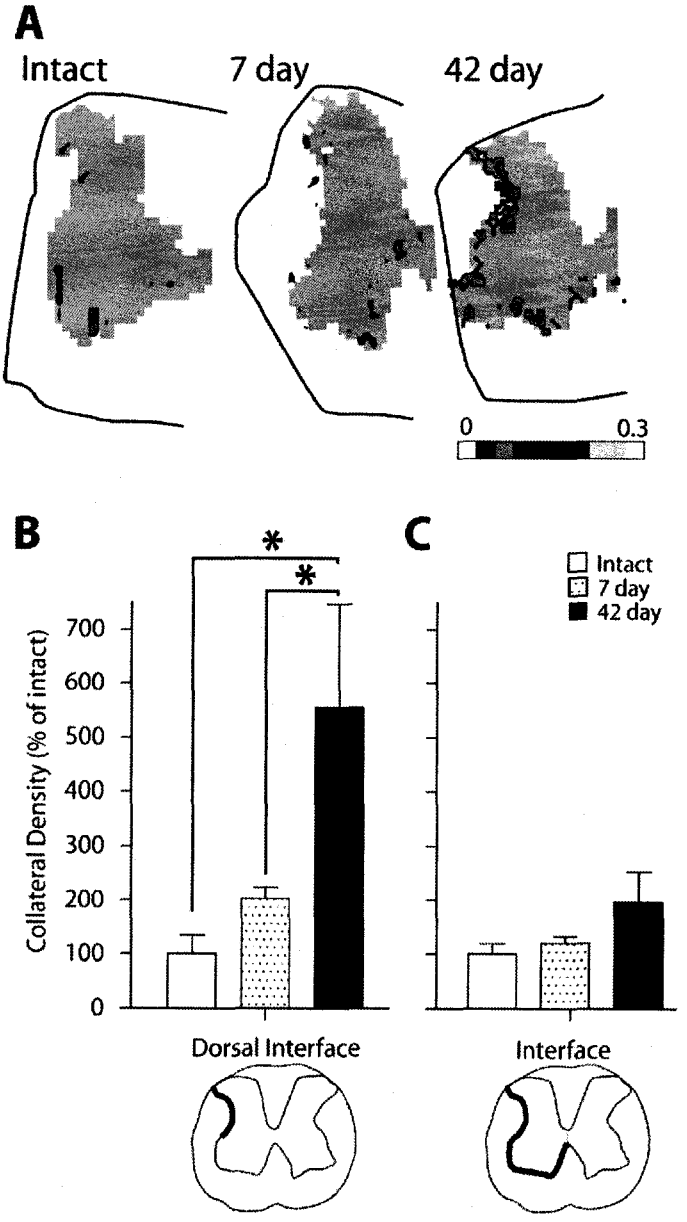

Figure 4.6: Collateral density of spared RtST fibers at the level of L2 at 7 and 42 days post-injury at the gray-white matter interface – A: Normalized collateral density for rats at 7 and 42 days post-injury on cross-sectional representations of scored areas of spinal cord. Brighter colors represent higher reticulospinal fiber density. B: Comparison of collateral density normalized to values in intact animals at the dorsal portion of the gray-white interface left. C: Comparison of collateral density normalized to values in intact animals at the entire gray-white interface of the left side. *=$p<0.05$. Values represent group means ± SEM.

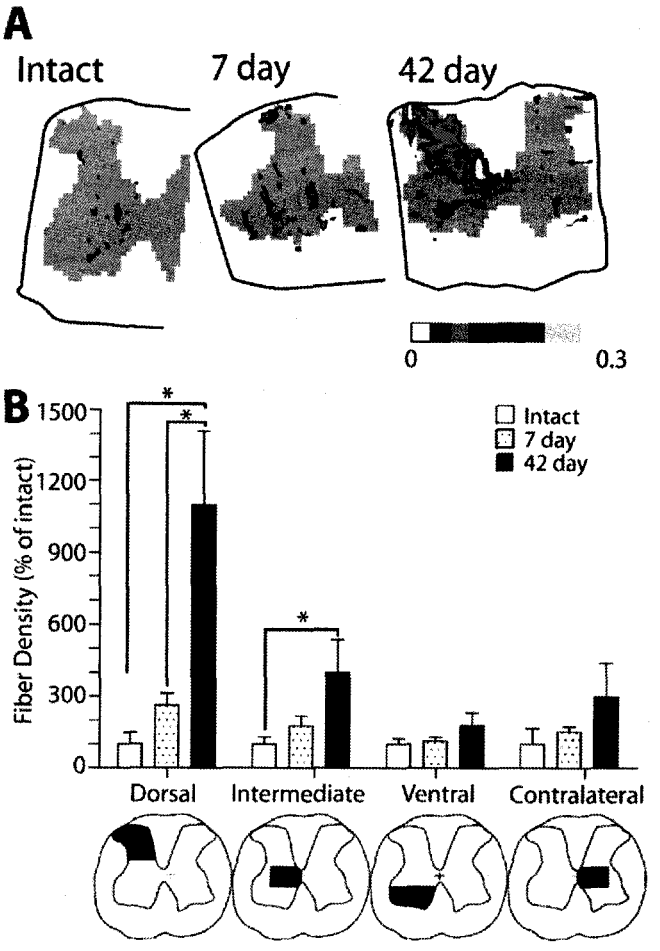

Figure 4.7: RtST projections in the gray matter of intact rats and following recovery after thoracic hemisection of the spinal cord – A: Normalized fiber density for intact rats, and rats at 7 and 42 days post-injury. Brighter colors represent higher RtST fiber density. B: Fiber density normalized to values in intact animals for each scored area of the gray matter, depicted at bottom. *=$p<0.05$. Values represent group means ± SEM.

values ± 112% S.E.M.; Figure 4.7B) and intermediate gray matter (232% of intact values ± 50% S.E.M.; Figure 4.7B). These findings indicate that the branching within the gray matter increased above that which would be explained by increased collaterals alone.

Correlational analysis between the collateral density at the dorsal horn and rats' final open-field BBB scores was used to determine whether increases in fiber density were associated with improved walking. We found a significant correlation in that rats with the largest degree of recovery in open-field locomotion also had the greatest increases in RtST collateral density ($r^2 = 0.65$, $p < 0.05$; Figure 4.8). Similar correlations between RtST density within the dorsal horn ($r^2 = 0.32$) or intermediate gray matter ($r^2 = 0.38$) and locomotor BBB scores were not significant.

In the second set of experiments we investigated changes in the projections of severed RtST fiber at the level of C5 above the injury. At this level, rats in all groups had diffuse collaterals crossing throughout the gray-white matter interface and at the intermediate gray matter (Figure 4.9 A). When normalized collateral density was compared, it did not significantly differ in the gray-white matter interface, nor did it differ in the dorsal portion of the interface between gray and white matter (Figure 4.9B). Branches within the intermediate gray matter were also compared and did not significantly differ between the groups.

4.5 DISCUSSION

In this study, we compared the projection pattern of the RtST in uninjured rats, and rats with thoracic spinal cord hemisection after a short recovery (i.e., 7 days), or after a long recovery (i.e., 42 days). The RtST projections from the gigantocellular division of the medulla in uninjured rats were similar to those described by Jones and Yang (1985), in that projections from the white matter enter the gray matter and terminate within the intermediate lamina (i.e., lamina 10). Additional projections were found in the ventral horn (i.e., laminae 7 and 8) as well as ventral portions of the dorsal horn (laminae 4

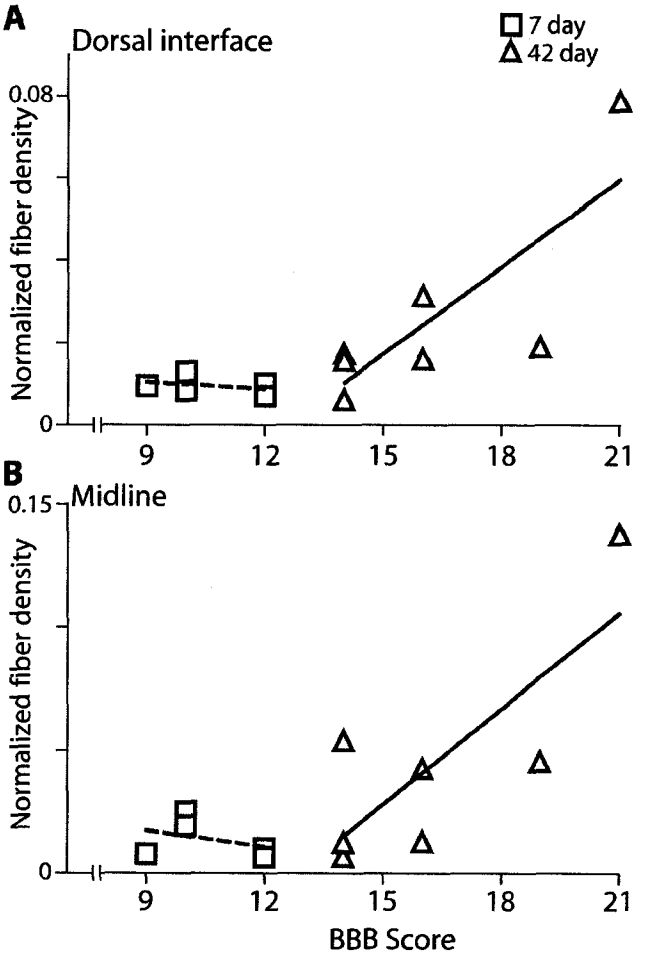

Figure 4.8: Correlation between RtST collateral fiber density and locomotor recovery – A: Normalized RtST fiber density and B: midline RtST fiber density at the dorsal interface for rats at 7 days post-injury (squares) and 42 days post-injury (triangles) plotted against final BBB scores.

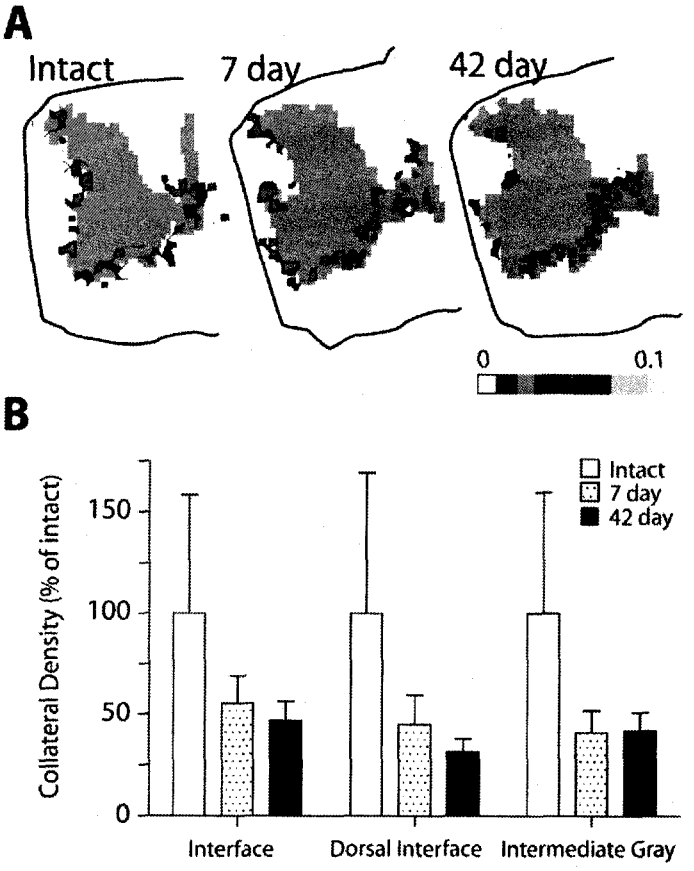

Figure 4.9: Projections of severed RtST fibers at C5 on 7 and 42 days post-injury at the gray-white matter interface, dorsal portion of the gray-white matter interface and in the intermediate gray matter – A: Normalized fiber density for rats at 7 and 42 days post-injury. Brighter colors represent higher RtST fiber density. Note that the range of the color scale has been reduced in this figure. B: Mean normalized fiber density of each scored area. Values represent group means ± SEM.

and 5). Importantly, we found that the RtST projection pattern changes following SCI. Specifically after the long recovery period, spared RtST fibers sprouted below the injury (at L2). We observed increased collateral density at the gray-white matter interface adjacent to the dorsal horn and increased fiber density within the ipsilateral dorsal horn and the intermediate gray matter. We also found that the degree of the observed changes in RtST collateral density was correlated with improved locomotor function. In contrast to anatomical changes of spared RtST fibers caudal to the lesion we did not find changes in the projection of injured fibers rostral to the lesion, comparable to what has been reported for the CST (Bareyre et al., 2004).

Although injured CNS axons are unable to regenerate, a moderate degree of spontaneous recovery is commonly observed in patients and animal models of SCI (reviewed in Fouad and Pearson, 2004). Understanding the mechanisms of this recovery may provide the basis for new approaches to further promote functional recovery. Several mechanisms possibly contributing to spontaneous recovery have already been described. One mechanism that may account for the first phase of this recovery is the abatement of spinal shock (reviewed in Ditunno et al., 2004). Later phases of recovery may be the result of remyelination of spared axons (reviewed in Blight, 1993), and adaptations in spared components of the CNS, termed plasticity (reviewed in Fouad and Pearson, 2004). Plasticity has been observed rostral and caudal to the injury site as in changes in the cortical maps of patients and animals models (Brustein and Rossignol, 1998), excitability changes in the central pattern generating network for locomotion in the lumbar spinal cord (Edgerton et al., 1992; reviewed in Edgerton and Roy, 2002), changes in motoneuron properties (reviewed in Heckmann et al., 2005), and sprouting of spared and/or injured CST fibers in rats, (Fouad et al., 2001; Weidner et al., 2001; Bareyre et al., 2004). Analogous to sprouting of spared CST fibers (Weidner et al., 2001) we found projection pattern changes in RtST fibers. The functional role of the RtST (reviewed in Mori et al., 1992; Mori et al., 1998; Jordan, 1998; Orlovsky, 1970;

Shefchyk et al., 1984), and the linked time course between its plasticity and locomotor recovery suggest a causal relationship. This is supported by our finding that the changes in RtST projections of individual rats correlate with their locomotor recovery. This causal relationship raises the question of how unilateral RtST fibers can generate alternating activity in both hind legs. This question can be resolved if one considers that the RtST is thought to initiate stepping movements by unpatterned activity (Orlovsky, 1970; Drew et al., 1986). This activity excites central pattern generating networks (CPGs) within the spinal cord that coordinate rhythmic and alternating leg movements (reviewed in Butt et al., 2002). As the excitatory drive of the RtST is unpatterned, strengthened RtST connections on the spared side may be sufficient to promote recovery in our lesion model, if the RtST fibers are capable of transmitting their signal to the CPGs of the lesioned side. Although we did not find an increase in RtST fibers crossing the midline at lumbar level themselves, RtST fibers could innervate commissural interneurons. This is supported by the finding that the recovery found after hemisection can be abolished by a lesion of commissural fibers in the lumbar cord (Harris et al., 1994). Additionally, we found that the sprouted fibers enter the lumbar intermediate lamina (i.e., lamina 10), where commissural interneurons that are part of the CPG are located (Stokke et al., 2002; Bannatyne et al., 2003).

In our study we found considerable variability in the degree to which the rats recovered and the anatomical changes of the RtST. As these two variables were correlated we would like to suggest that increased RtST sprouting promotes locomotor recovery. This consequently raises the question of the source of variability in sprouting. One factor involved in this variability could be the inter-animal differences in locomotor activity in the post-lesion period. This idea is based on findings of activity dependent increases in neurotrophin levels and their receptors in rat spinal cord following injury (Ying et al., 2003), which can influence sprouting (Gallo and Letourneau, 1998; Schnell et al., 1994). Additionally, various reports have shown that post-SCI exercise improves recovery

of sensory function (Hutchinson et al., 2004), regeneration of sensory fibers (Molteni et al., 2004), and increased levels of molecular markers associated with anatomical rearrangements, including the expression of neurotrophins such as BDNF (Gomez-Pinilla et al., 2002). As RtST fibers have been shown to express TrkB receptors (King et al., 1999), it seems likely that activity may affect both anatomical plasticity, and the associated locomotor recovery. Understanding why some animals exhibited increased anatomical plasticity and improved locomotor recovery may provide important clues to future treatment approaches for SCI.

In conclusion, our findings suggest that like the corticospinal tract (Bareyre et al., 2004; Fouad et al., 2001; Weidner et al., 2001), the RtST is capable of anatomical rearrangements that possibly promote locomotor recovery following spinal cord injury. Harnessing this property may provide treatments to induce functional recovery following spinal cord injury in the future.

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CHAPTER 5

SPINAL INJURY INDUCED ANATOMICAL RETICULOSPINAL PLASTICITY IS NOT ENHANCED BY INTRATHECAL RHO-ASSOCIATED KINASE INHIBITOR Y27632 OR A SOLUBLE NOGO-66 RECEPTOR

5.1 PREFACE

In chapter 4, I investigated changes in RtST projections occurring in response to lateral hemisection injury. A logical next step will be to find out whether this plasticity is affected by different pharmacological treatments that may induce regenerative sprouting of either severed or spared tracts. Regenerative sprouting could be an important mechanism by which treatments that improve recovery of walking after SCI have their effects on the CNS. Treatments introduced in this chapter have been shown to have some beneficial effects on walking recovery in rats after incomplete thoracic SCI along with inducing sprouting of the CST (Fournier et al., 2003; Li et al., 2004; Ji et al., 2005; Wang et al., 2006). The two treatments act on respective molecules thought to play roles in the failure of CNS regeneration mediated by different pathways. One treatment acts by blocking a receptor (i. e. NgR) that recognizes three molecules (i. e. Nogo, MAG, and Omgp) involved in myelin associated inhibition of axonal regeneration and sprouting (Figure 1.1). The NgR receptor represents a 'chokepoint' within the myelin inhibition pathways in that treatments that blocks its function, should completely address myelin associated inhibition of axonal regeneration. The second treatment increases sprouting and regeneration by blocking the molecular pathway in the growth cone that effects growth cone collapse (i. e. RhoA and its downstream target ROCK). The treatment consists of the intrathecal administration of a small-molecule inhibitor of ROCK, known as Y27632, which can prevent growth cone collapse and thus improve the chances of regenerative sprouting. Growth cone collapse represents a final common step in the inhibition of axonal regeneration and could increase sprouting of spared RtST fiber in such a way that it could contribute to anatomical sprouting of the spared RtST. The anatomical basis for the recovery of walking remains largely unknown. Some of the investigations utilizing these two treatments have shown sprouting of severed CST fibers approaching the injury site. It has been observed that spared ventral CST fibers are capable of sprouting in the caudal spinal cord below the level of injury, and this sprouting can be augmented by the administration of the two treatments

introduced in this chapter. Various treatments intended to induce axonal regeneration can result in sprouting at sites away from the injury site in severed (Z'Graggen et al., 1998; Fouad et al., 2001) or spared (Thallmair et al., 1998; Weidner et al., 2001) fibers. This can result in new anatomical pathways to carry descending commands to the CPG, and a possible mechanism for treatment induced improvements of walking recovery after incomplete injuries. In this chapter, we used the drug administration protocols from two studies using the treatments NgR Fc (Li et al., 2004) and Y27632 (Chan et al., 2005) to investigate if they affect the projection pattern of the RtST alongside the previously documented recovery of walking.

5.2 INTRODUCTION

One of the major goals of SCI research is aimed at inducing the injured spinal cord to improve its function, either by inducing regeneration of severed fiber tracts, or by using and enhancing the capability of the spinal cord to form new connections to reroute signals around an injury site. To date, many studies on SCI in animal models have focused on the cortico- (CST) or rubro-spinal tracts, with locomotor assessments providing functional readout (e.g., Liebscher et al., 2005). These tracts run as somewhat distinct bundles in the dorsal spinal cord and thus can be relatively easily ablated and/or histologically examined. Although these tracts play major roles in controlling fine motor movements (Whishaw et al., 1998), their contribution to locomotor function is limited (Steeves and Jordan, 1980). An important tract in eliciting locomotion is the reticulospinal tract (RtST). Its role in eliciting activity in spinal networks that coordinate rhythmic stepping movements (central pattern generators, CPGs) has been demonstrated in electrophysiological (Orlovsky, 1970; Mori et al., 1998; Jordan, 1998; Shefchyk et al., 1984) and lesion studies (Steeves and Jordan, 1980; Schucht et al., 2002; Little et al., 1988). Anatomical studies have demonstrated that following SCI, sparing of only a small unilateral portion of the VLF is sufficient for spontaneous locomotor recovery (Little et al., 1988; Harris et al., 1994;

Gorska et al., 1996; Brustein and Rossignol, 1998; Schucht et al., 2002). In rats, this includes recovery of the hind limb on the completely ablated side of the spinal cord, which occurs within 2-3 weeks. Alongside this recovery, spared RtST fibers contralateral to a thoracic lateral hemisection injury sprout at the level of L2, and likely via excitatory interneurons, strengthen the descending drive to the CPG (Ballermann and Fouad, 2006). It is possible that this form of anatomical plasticity could be responsible for the spontaneous recovery of hind limb motor function in the rat model. It is not known, however to what degree the reticulospinal tract responds to regenerative and/or plasticity inducing treatments.

Many studies have shown varying degrees of improved post-SCI recovery with different treatments but an understanding of the mechanisms behind the improved recovery remains elusive. It is likely that treatments intended to induce axonal regeneration (i. e., regrowth of axons from their severed tip at the injury site) have effects on anatomical plasticity (such as sprouting of axon collaterals) elsewhere in the CNS (reviewed in Bradbury and McMahon, 2006). As overground locomotion is a common readout of spinal cord function, treatment induced functional recovery could act via anatomical plasticity of the RtST. Previous research has shown that growth cone extension and collapse is the result of the activation of a cascade of signaling molecules including the Rho GTPases (Dergham et al., 2002). RhoA induces growth cone collapse and acts via a downstream effector ROCK (reviewed in (Mueller et al., 2005). Inhibition of ROCK has resulted in increased plasticity of the CST and some improvement of stepping in the mouse model of SCI (Dergham et al., 2002). A second line of research has investigated myelin associated growth inhibition (reviewed in Qiu et al., 2000. Myelin associated growth inhibition is mediated by Nogo, MAG, and OMgp proteins, which all exert effects via the NgR receptor (reviewed in McGee and Strittmatter, 2003). Different approaches have been devised to show the influence of this receptor on regeneration failure in the CNS, including antagonistic peptides (Li and Strittmatter, 2003), and functional deletions in genetically modified mice (Cafferty

and Strittmatter, 2006). One of the most promising approaches uses an IgG-Fc fragment combined with an externally facing domain of the NgR protein, which binds to and blocks the function of any myelin associated growth inhibitors acting through NgR (NgR Fc; Ji et al., 2005; Li et al., 2004; Wang et al., 2006). Administration of NgR Fc has resulted in increased regenerative responses both in *in vitro* and *in vivo* models (Ji et al., 2005; Li et al., 2004; Wang et al., 2006).

In the present study, Y27632 and NgR Fc were given via osmotic mini pump to attempt to ascertain their effects on the projection pattern of spared reticulospinal fibers, as well as to determine their effects on severed reticulospinal fibers. In separate groups of rats the CST was also traced after Y27632 was given to act as a positive control.

5.3 METHODS

5.3.1 Animals

The experiments were approved by the University of Alberta animal care committee and conducted in accordance with rules set by the Canadian Council of Animal Care. All rats were kept at a 12:12 hrs light/dark cycle and received water and food ad libitum. Animal handling began 14 days prior to the SCI surgery. Rats were trained to walk in the horizontal ladder task during this time. Experiments were performed using a total of 40 adult female Lewis Rats (165-180 g; Charles River, Wilmington, MA).

5.3.2 Lesion surgeries and drug delivery

All operations were performed under Hypnorm (Janssen Pharmaceutics, Beerse, Belgium; 120 μl per 200 g body weight) and Midazolam (Sabex, Boucherville, QC, Canada; 0.75 mg in 150 μl/200 g body weight. 750 μl total volume diluted with H2O.) anesthetic. Eye lubricant (Tears naturale, Alcon Canada, Inc, Mississauga, ON) was applied to protect the eyes from dehydration.

Rats received laminectomies of the dorsal half of the eighth thoracic vertebra. This was followed by a lateral hemisection of the right side of the spinal cord using a customized

blade. Following lesion, osmotic mini pumps (0.5 μl/hour, Alzet 2002, Durect Corp., Cupertino, CA) containing either sterile saline (n=13), 20 μM Y27632 (n=16) dissolved in sterile saline were implanted. The remaining rats received 1.2 mg of NgR Fc kindly provided by Dr. Daniel Lee, dissolved in sterile saline and applied to the spinal cord with a larger type of osmotic mini pump (2.5 μl/hour, Alzet 2ML4, Durect Corp. n=11) A sub-dural catheter connected to the mini-pump was fixed in place with tissue glue to musculature adjacent to the vertebra. The dorsal back musculature was sutured and the skin closed with surgical clips.

After surgery the animals were kept on a thermostatically regulated heating pad until completely awake. The analgesic Buprenex (Buprenorphine; Reckitt & Colman, Richmond, VA) was administered subcutaneously (0.03 mg/kg body weight) immediately after operation, and every eight hours for 74 hrs. Warm Ringer's solution (4 ml) was given subcutaneously daily for the first week and at later stages if animals showed signs of dehydration.

5.3.3 Behavioral testing

BBB locomotor score: Open field locomotion was evaluated by using the BBB locomotor scale (Basso et al., 1995). The rats were placed into a 30 x 80 x 130 cm transparent Plexiglas box with a smooth surface and were observed by 2 investigators for a 4 min period. Testing was performed weekly over the recovery period (56 days).

Horizontal ladder: The animals walked on a 1 m long horizontal runway of metal bars elevated 30 cm from the ground in weekly testing sessions. A defined 15-bar sector was chosen for analysis. To prevent habituation to a fixed bar distance, the bars in this sector were placed irregularly (2-5 cm spacing) and were changed in every testing session. The rat's performance was monitored using a digital video camera (JVC-GV50, 60 fields/s). An error was scored if any limb fell through the plane of the bars.

5.3.4 Anterograde tracing of corticospinal and reticulospinal projections

The RtST was traced via iontophoretic injection of the anterograde tracer biotin dextran amine (BDA; 10,000 MW, Invitrogen, Eugene, OR), into the gigantocellular division of the medullary reticular formation on both sides. The rats receiving RtST tracer injections had saline pumps (n=5), Y27632 pumps (n=8), and NgR Fc pumps (n=11). The injection coordinates were 0.8 mm lateral to mid-line, 2.8 mm caudal to lambda, and 8.2 mm ventral to dura. The injection was performed using a glass capillary filled with 10% BDA. The tip diameter was approximately 70 mm and a current of 3.5 mA applied in 5 sec on, 5 sec off cycles for 10 min. In a separate group of rats (received saline pumps n=8; received Y27632 pumps n=8), the CST was traced via pressure injection of BDA in a separate group of rats into the hind limb motor cortex on the side contralateral to the lesion. BDA was injected at 4 sites using a hamilton syringe, centered around 2 mm caudal to bregma, 2 mm left of the midline and 1.5 mm below the dura.

The animals were euthanized 14 days after tracing using Pentobarbital (Euthanyl, Bimedia-MTC, Cambridge, ON; 300 mg/kg, i.p.) and perfused transcardially with a Ringer solution containing 100,000 IU/l heparin, followed by fixation with 4% formalin and 5% sucrose in 0.1 M phosphate buffer. The spinal cords were removed, postfixed overnight in 4% formalin/5% sucrose, and then transferred to a 30% sucrose solution for 3 d. The spinal cords were embedded with Tissue Tek (Sakura Finetek USA Inc, Torrance, CA) and frozen at -40°C. Horizontal sections of 25 μm were taken on a cryostat from the thoracic spinal cord through the lesion site and the lumbar enlargement. Cross sections of 25 μm were taken from above the lesion site (T4) and the brainstem injection site, and were processed as described below. Staining was performed as described previously (Herzog and Brosamle, 1997). The slides were washed 3 times for 30 minutes in a 50 mM Tris buffered saline, pH 8.0, containing 0.5% Triton X-100 (TBST). Afterwards the slides were incubated over night with an avidin-biotin-peroxidase complex (Herzog and Brosamle, 1997) in TBST (ABC elite, Vector Labs, Burlingame, CA) according to the instructions of the manufacturer.

Subsequently the diaminobenzidine (DAB) reaction was performed using the Vector DAB kit (Vector Labs, Burlingame, CA). The reaction was monitored and stopped by washing in water. To counterstain the slides, they were immersed for 3 minutes in 0.1% cresyl violet. The slides were dehydrated stepwise through alcohol, cleared in xylene, and cover-slipped with Permount (Fisher Scientific, Nepean, ON).

5.3.5 Density quantification of reticulospinal projections

Spared reticulospinal projections sprout below the level of the lateral hemisection injury (at L2) alongside spontaneous behavioral recovery (Figure 5.1 A). Analysis focused on two parameters: i) the density of RtST collaterals entering from white into grey matter, and ii) the density of RtST branches within grey matter laminae. Due to the large anatomical area where RtST axons descend and project within the grey matter, density measurements were used to quantify and compare projection patterns, which have been described previously (Ballermann and Fouad, 2006). Even-numbered sections (i.e., every second section) throughout the spinal cord were photographed using a brightfield microscope (Figure 5.2 A; Zeiss Canada, Toronto, ON). The density of collaterals, and grey matter branches was assessed by counting the numbers of labeled fibers within 50 μm of the grey-white matter interface, and within the grey matter respectively. Counts were performed within 30 squares of a 50 μm grid overlaid on the sagittal sections. Thus, the rostro-caudal distance analyzed was 1500 μm. The average value per square for each horizontal row of the grid was transferred into a spreadsheet representing a cross section (Figure 5.2B).

For each parameter (collaterals and branches in grey matter) specific rostral-caudal levels were chosen for analysis: i) Location of RtST collaterals crossing the boundary from white into grey matter were analyzed at the level of L2. The locations of CST collaterals were analyzed at the level of T7, just rostral to the level of the injury (Figure 5.1B). RtST collaterals entered from white into grey matter, in many different angles relative to the plane of section, thus short fragments of the sectioned collaterals were visible. These fragments were counted in squares within 50 μm of the interface between grey and white matter at the

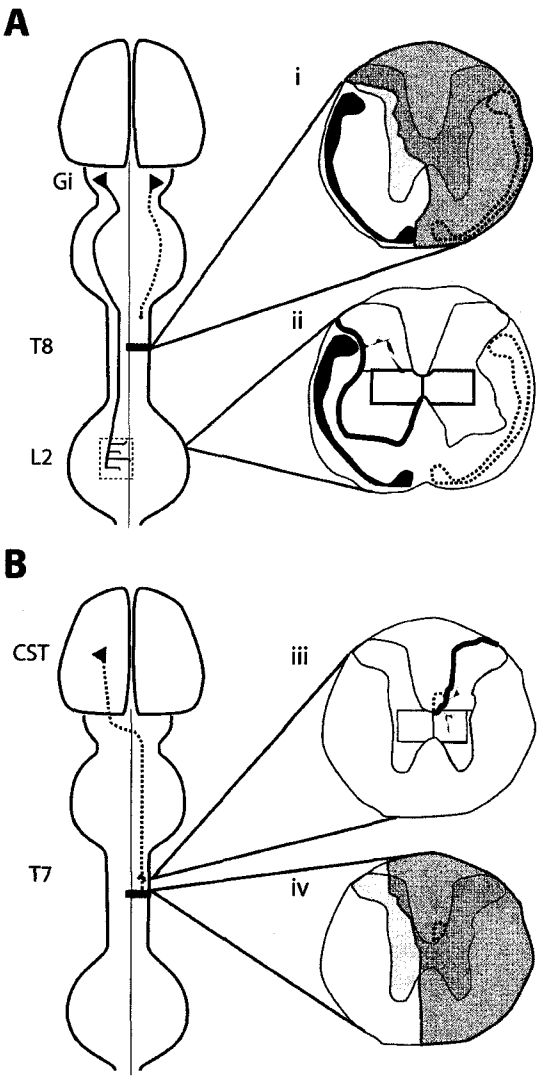

Figure 5.1: Schematic of experimental design showing lesion locations and extent, and anatomical RtST and CST fiber densitometry – A. A schematic of the brain and spinal cord showing cortices at the top, and spinal cord (with cervical and lumbar enlargements) at the bottom, where bilateral anterograde tracings were performed on the RtST (black triangles; Gi: gigantocellularis division of the medial reticular formation). Lesions were performed on the right side at T8 (thick black line). Spared fibers are denoted by solid lines originating from the Gi cell bodies. Severed fibers are denoted by dotted lines. i. Representative maximal lesion extent is shown on a T8 cross-section drawing with the spared (left) and severed (right) RtSTs labeled. ii. The areas compared using densitometry are shown on an L2 cross-section drawing. Reticulospinal fiber density was quantified in the area of the grey-white matter interface (thick black line). Grey matter branching was analyzed in the dorsal horn, intermediate grey matter, ventral horn, and contralateral grey matter (outlined over the L2 grey matter).B. Unilateral anterograde CST tracing were used to examine sprouting of severed fibers above the level of the injury, at T7. iii. Similar areas were used for fiber densitometry, after the lesion was performed caudally, at T8, where the maximal extent of a representative lesion is shown in (iv).

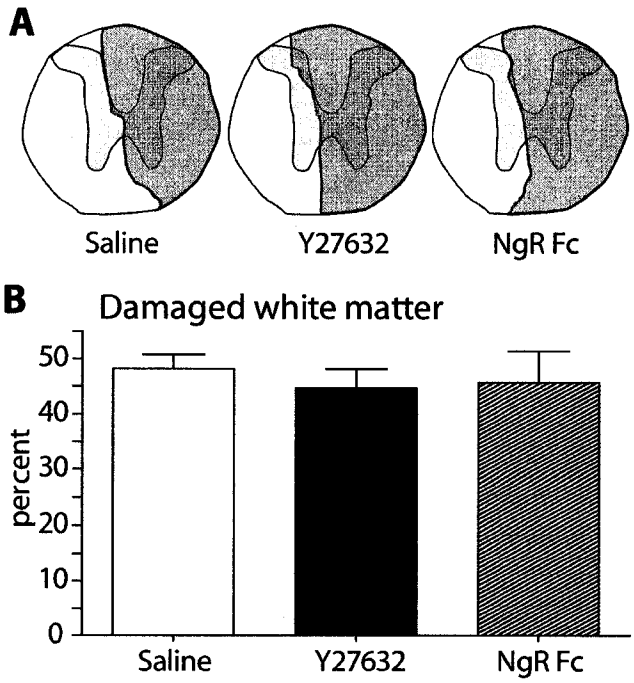

Figure 5.2: Analysis of lesion extent – Reconstructions of the maximal extent of the lesion in cross-sectional view are shown from representative individual rats from each group (A. Saline B. Y27632 C. NgR Fc). Shaded areas represent lesioned areas.

level of L2. ii) Branches within the grey matter run in many angles relative to the plane of section, and sectioned fragments were counted at the level of L2 in rats where spared RtST fibers were studied and T7 in rats where severed CST fibers were compared. Grey matter branch density was broken down into density in the dorsal horn, intermediate lamina and ventral horn on the traced sides, and the intermediate grey matter on the contralateral side.

To correct for inter-animal variations in the numbers of labeled axons, fiber densities (fiber counts per 50 μm square) were normalized (i.e., divided) by the numbers of labeled fibers counted on cross sections at T4, rostral to the analysis sites. Results were then normalized to the mean density for each area from rats that received saline mini-pumps.

5.3.6 Lesion site

Horizontal sections of 25 μm through the spinal cord lesion site were counter-stained with cresyl violet and the largest extent of each lesion was reconstructed from adjacent sections, and from these reconstructions spared white matter measurements were performed (Scion Image, NIH, Bethesda, MD). Additionally the largest extent in the rostral-caudal dimension for each animal was also recorded to see if lesion spread was different between the different treatment groups.

5.3.7 Axonal dieback quantification

In the horizontal sections through the injury site labeled fibers could be visualized. The distance between the lesion epicenter and the most caudal severed labeled fiber was compared between the different treatment groups. The rostral-caudal extent of the lesion site was not different among groups (see results). The distance was compared between injured CST and RtST fibers as well.

5.3.8 Statistics

Normalized reticulospinal fiber density in the spinal cords from the intact, short and long recovery groups were compared using non-parametric Kruskal-Wallis tests, as they do not rely on the assumption that fiber density values are normally distributed (GraphPad,

Prism software version 4.01, San Diego, CA www.graphpad.com). Where significant differences were found, groups were compared in a pairwise manner using Mann-Whitney U tests.

5.4 RESULTS

5.4.1 Lesion site analysis

Lesion sites were examined in all animals to verify that the right side of the spinal cord was completely ablated. Horizontal sections through the lesion site were photographed at the level of the lesion epicenter. Measurements were taken of the lesion and grey and white matter and this was compiled into a cross sectional representation of the maximum lesion extent. Lesions were not significantly different in the three groups of rats (Figure 5.2). The mean percentage of spared white matter in the saline controls, Y27632 and NgR Fc treated rats were 48.2%, 44.7% and 45.7% respectively. Standard deviation in the three groups were 11.1%, 13.5% and 14.1%. Lesion dimensions were also compared in the rostral-caudal dimension as well. Lesion extent at the maximal length in the plane of section in the saline controls, Y27632 and NgR Fc treated rats was 1623 μm, 1516 μm, and 1837 μm respectively. Standard deviation in the three groups were 324 μm, 278 μm, and 628 μm. There was no significant difference in the rostral-caudal dimension either (p = n.s.). The right lateral and ventrolateral tracts carry the major portion of the reticulospinal tract and were completely ablated in all rats included in the study. In all rats where the corticospinal tract was traced, lesions included the entire dorsal portion of the CST.

5.4.2 Reticulospinal tract and corticospinal tract dieback

Anterograde tracing of CST fibers was performed in animal groups receiving saline and Y27632. Injections in these animals were performed on the left side only, such that projections of severed fibers on the right side and responses of these fibers to treatment with Y27632 could be compared. Bilateral anterograde tracings of RtST fibers were performed to compare the effects of NgR Fc and Y27632 on both severed and spared projections. The

severed fibers in both CST and RtST traced animals did not appear to regenerate in any case. CST fibers, with retraction bulbs, were found rostral to the lesion epicenter. The location of the most caudal severed fiber, (i. e., closest to lesion epicenter) was determined for each rat, and the distance between this fiber and the lesion epicenter was recorded (Figure 5.3). The mean distance of CST axon dieback in saline treated rats was 1.48 mm (S. D. = 0.32 mm). RtST fibers on the injured side were found at a significantly larger distance (mean = 4.07 mm; S. D. = 0.97 mm) rostral to the lesion epicenter. The addition of Y27632 in CST traced rats (mean = 1.31 mm; S. D. = 0.25 mm), and Y27632 (mean = 3.94 mm; S. D. = 1.85 mm) or NgR Fc (mean = 5.27 mm; S. D. = 0.68 mm) in RtST traced rats did not have any significant effects on axon dieback.

5.4.3 Spared lumbar RtST projection patterns

Spared RtST projections were also examined using anterograde tracing contralateral to the lateral hemisection lesion (Figure 5.4 A). At the level of L2, collateral projections passing through the grey-white matter interface were quantified using fiber density measures. The number of fibers per unit of volume in rats given Y27632 and NgR Fc treatments were normalized to those of rats receiving saline pumps. There were slight, but not statistically significant increases in fiber density when Y27632 was applied at the lesion site (Figure 5.4B). There were no significant changes when collateral fiber density was broken down into the areas dorsal and ventral to the central canal. When fiber density was quantified in the grey matter (specifically, the dorsal and ventral horns on the spared side and intermediate grey matter on both sides), there were no statistically significant changes between saline and treated rats in any area (Figure 5.4C).

5.4.4 Severed thoracic CST projections

Severed traced CST projections were examined at the level of T7, using similar density measures to those employed in the quantification of the spared RtST at L2. In this case, corticospinal fiber density in Y27632 treated rats significantly increased the density of CST

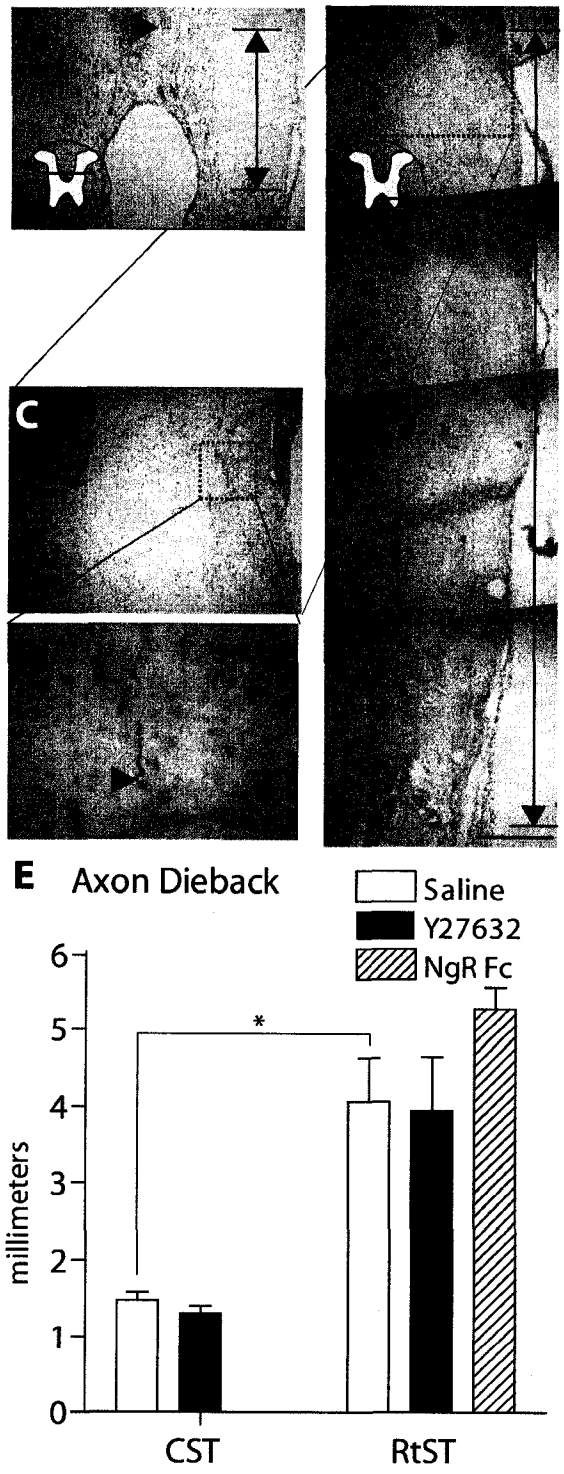

Figure 5.3: Comparison of axon dieback in CST and RtST fibers after lesion and treatment – A. A photomicrograph of a horizontal section at the level depicted in the spinal cord cross-section drawing shows traced CST fibers (indicated by the arrowhead at the top, rostral) retracted from a lesion cyst at the bottom (caudal). The distance between the closest traced axon and the lesion epicenter is shown by the double headed arrow and hash marks. Scale bar = 250 μm. B. A horizontal section is depicted with traced RtST fibers (arrowhead) and the distance retracted from the injury site at the bottom (caudal). Scale bar = 500 μm C. A higher magnification view of the dotted box shown in B. Scale bar. 100 μm D. A higher magnification view of the dotted box shown in C, where severed labeled RtST fibers have retracted. Scale bar = 25 μm E. Group comparisons of the distance between the lesion epicentre and the closest severed labeled axon. Bars indicate means +/- S. E. M.

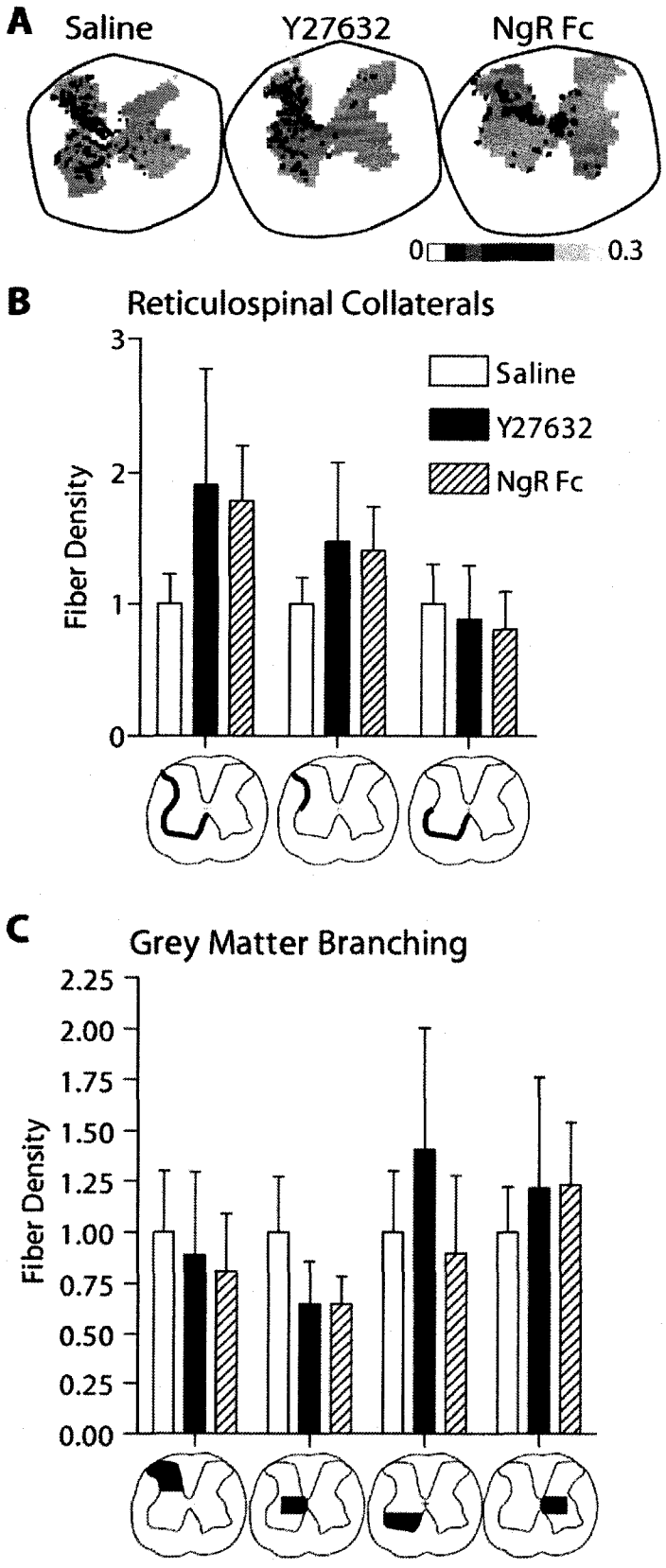

Figure 5.4: Fiber densitometry of spared RtST fibers (below the level of the injury) – A. Surface plots are shown of normalized RtST fiber density at the level of L2 taken from rats with representative mean density values. B. Reticulospinal density passing through the grey-white matter interface is shown for each of the groups. Fiber density values are normalized to saline controls. Bars indicate means +/- S. E. M. Density values obtained from the entire grey-white matter interface (left; see cross-section drawing at below graph), dorsal portion of the grey-white matter interface (middle) and ventral portion of the interface (right).

collaterals passing between the grey and white matter adjacent to the dorsal horn and the intermediate grey matter (Figure 5.5). Additionally there were statistically significant increases in the amount of grey matter branching in the dorsal horn and the intermediate grey matter on the right side ipsilateral to lesion. There were no statistically significant changes in the ventral horn or the intermediate grey matter on the side opposite injury.

5.4.5 Locomotor recovery

All groups of rats were evaluated weekly following SCI using an open-field walking score (Figure 5.6 A) and the horizontal ladder task (Figure 5.6B). At 7 days post-injury all rats showed similar performance on both tasks with mean BBB scores of 10.5 (Saline), 10.6 (Y27632), and 11.3 (NgR Fc). These scores represent occasional to frequent weight supported stepping with no fore limb - hind limb coordination. In the horizontal ladder test, total errors were counted in a defined 15 bar section of the test. The mean number of errors committed at this stage was 14.2 in the saline treated rats, 13.2 in the Y27632 treated rats, and 15.0 in the NgR Fc treated rats. Over the recovery period all three groups showed significant improvement to mean BBB scores of 14.4 (Saline and Y27632), and 15.0 (NgR Fc). The mean number of errors committed in the horizontal ladder task by each group dropped to 9.3 in the saline group, 9.9 in the Y27632 group and 10.2 in the NgR Fc treated group. There were no statistically significant differences between the groups at any time post-injury.

5.5 DISCUSSION

In this study, the projection patterns of the RtST and CST were compared following a lateral hemisection injury and treatment with a ROCK-inhibitor, Y27632 or a function-blocking antibody against the NgR receptor. An important finding was that severed RtST fibers showed marked dieback ipsilateral to the lesion when compared to the degree of dieback observed in CST fibers. Such dieback may suggest that the cells of the RtST originating from the gigantocellular division of the medial reticular formation may be more

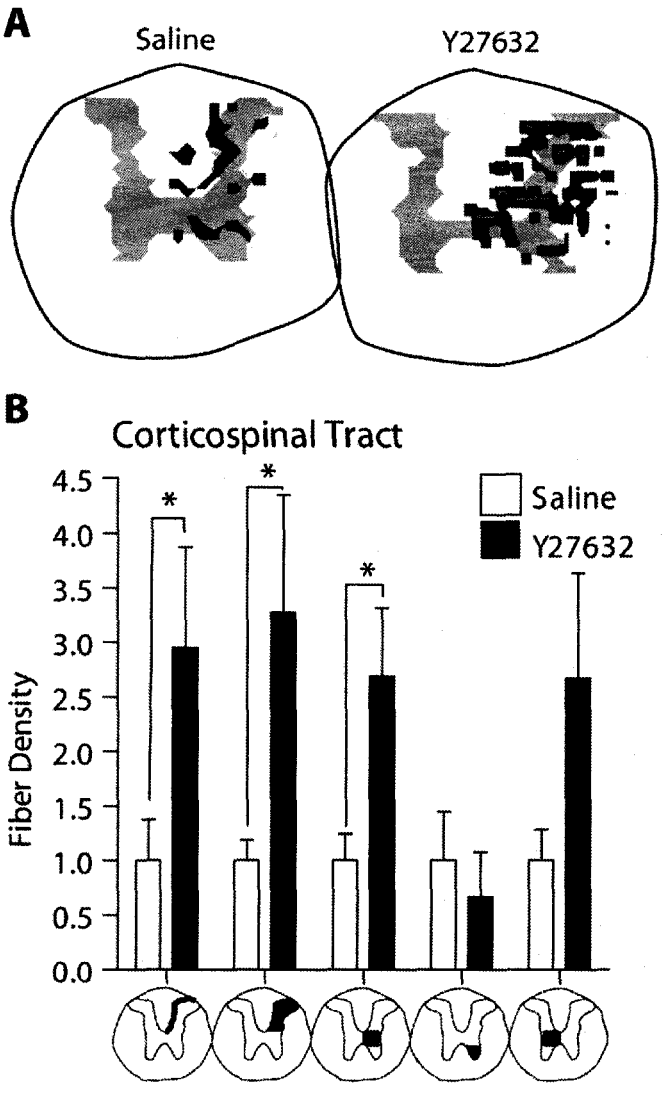

Figure 5.5: Fiber densitometry of severed CST fibers at T7 (above the level of the injury) – White bars represent saline controls, black bars represent rats treated with Y27632. Values are normalized to saline controls. Bars indicate means +/- S. E. M. * = $p<.05$

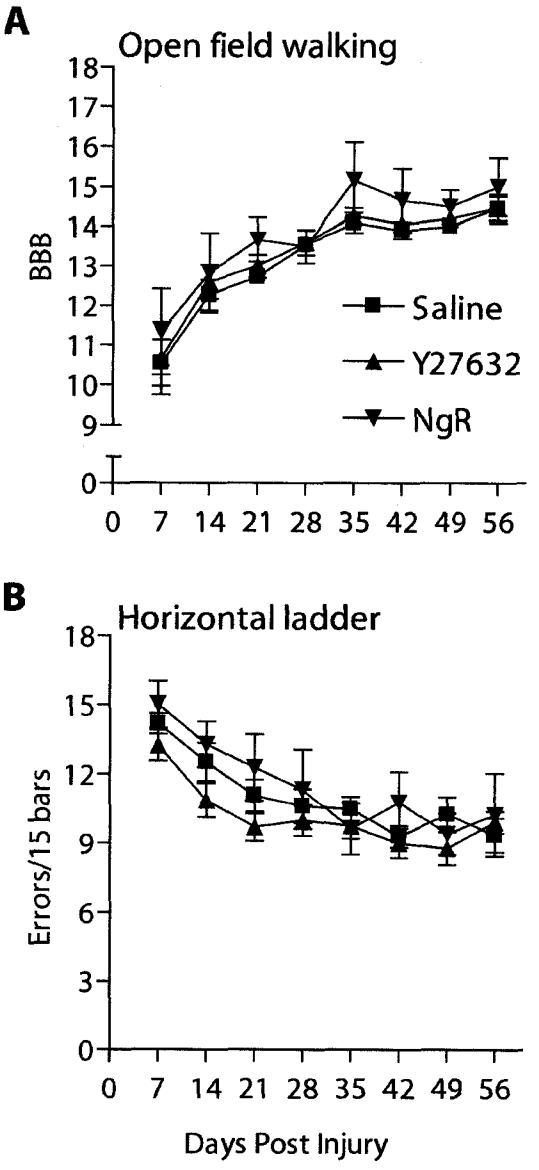

Figure 5.6: Time course of locomotor recovery – A. Open field walking scores obtained in the 8 weeks following SCI, using the BBB scale. B. Error counts obtained by rats traversing the horizontal ladder task following SCI. Points indicate means +/- S. E. M.

sensitive to traumatic injury than CST fibers. Addition of Y27632 at a dose that increased sprouting of severed CST fibers above the injury did not result in the regeneration of severed RtST fibers. The RtST also did not regenerate after administration of NgR Fc. After both these treatments were given, RtST axon dieback was unmitigated. Additionally, as spared fibers mediate the bulk of the spontaneous walking recovery observed after lateral thoracic hemisection injury in rats (Harris et al., 1994), we sought to determine whether the addition of Y27632 or NgR Fc could effect additional sprouting of spared RtST fibers (i. e. above that observed in untreated rats), as both Y27632 and NgR Fc have been shown to increase CST sprouting and/or regeneration alongside functional recovery (Li et al., 2004; Wang et al., 2006; Dergham et al., 2002; Chan et al., 2005). We found no significant increases in RtST fiber density, at the grey-white matter interface or in the grey matter in response to treatment. However, previous findings showing increased sprouting of severed CST fibers in response to Y27632 treatment were confirmed by our results, demonstrating both the biological activity of the Y27632 compound, and that the delivery of the drugs was successful in our experimental setting. Treatment-induced improvements in overground walking and horizontal ladder walking were not found in the present study following Y27632 or NgR Fc administration. The lack of improved walking recovery after the administration of these treatments is compared to other studies' results below.

A novel comparison made in this study is the difference in dieback between severed RtST fibers and severed CST fibers. The distance between the most caudal visible fiber and the lesion epicenter were compared. Lesion size in the rostrocaudal dimension was unchanged between the groups with the different treatments, as well as different tracer injections. The distance between the end of the most caudal severed labeled fiber and the lesion epicenter was much smaller in rats with CST tracings than in RtST tracings. One possible reason for this is that the injury to the RtST is closer to the cell body than it is for the CST. In the case of the rubrospinal tract (RST), injuries that are closer to the cell body result in more pronounced increases in the expression of regeneration associated

genes such as GAP-43 (Fernandes et al., 1999). Injuries closer to the cell body can result in more robust regeneration responses of RST axons, assuming that the cell does not undergo apoptosis. In RtST axons, other studies have shown that RtST axons can grow into, but not out of, neurotrophin 4/5 expressing Schwann cell grafts after dorsal thoracic hemisection where CST axons did not (Blesch et al., 2004). Additionally, after a cervical contusion injury, implantation of Schwann cells allowed regenerative sprouting of the RtST, but not the CST into a Schwann cell graft at the lesion site (Schaal et al., 2007). These findings suggest that the RtST is more capable of axonal regeneration than the CST after SCI given the correct treatment. Findings from the present research may mean that without treatment, the different populations of the RtST may retract farther than the CST, but a reason for this is not known.

The addition of Y27632 to the spinal cord effectively induced sprouting of severed CST fibers, but did not improve the recovery of walking after a thoracic hemisection injury. Further, the anatomical findings presented here did not show increased regeneration of the severed RtST, nor increased sprouting of spared RtST fibers below the injury. Other investigators have shown the effects of Y27632 administered intrathecally following different types of spinal cord injury with somewhat varying behavioral and anatomical results, depending on the species, lesion, and behavior measures used. The first, and most promising report of walking improvement due to Y27632 administration following SCI (specifically thoracic dorsal transection injury) was shown in mice (Dergham et al., 2002). In this study, a modified BBB score was used that ignored toe clearance, and the investigators found statistically significant treatment related improvements at 2 days post-injury. This rapid time course of recovery would tend to indicate that Y27632 had neuroprotective effects, or had altered spared descending or CPG connections caudal to injury. Although this study showed anatomical evidence of regenerative sprouting of CST fibers approaching the injury site in the white matter, a later study from the same group showed that Y27632 administration also reduced apoptosis (Dubreuil et al., 2003),

suggesting a more likely mechanism for the rapid recovery observed in Dergham et al (2002). In this case, further careful experimentation revealed that the regenerative sprouting observed alongside behavior recovery was probably not responsible for the recovery. This highlights the need to perform detailed lesion analysis to rule out possible treatment induced neuroprotective effects when establishing a case for walking recovery related to regeneration. Later studies investigating Y27632 treatment following SCI utilized rats, with smaller effects showing walking recovery after SCI. After a dorsal transection injury, Fournier et al. reported an acceleration in the recovery of walking, where a small effect was found at 14 days post-injury, but no difference was seen at later time-points (Fournier et al., 2003). Using a T9-T10 contusion injury, Sung et al. (2003) found no improvement after Y27632 was given. Subsequent studies have shown a possible reason for why Y27632 has varying effects on spinal cord function. Y27632 administration also affects astrocytes in the spinal cord, and results in increases in chondroitin sulfate proteoglycan expression, as well as expression of GFAP in the spinal cord (Chan et al., 2007). Thus, beneficial effects upon axonal sprouting regeneration may be offset by an increase in the density of the astrocytic scar, as well as the expression of molecular inhibitors of axonal sprouting and regeneration. Furthermore, lower concentrations of Y27632 can be harmful to functional recovery after incomplete injuries at cervical levels (Chan et al., 2005). Chan et al., (2005) used a transection of the dorsal columns at a cervical level, and found that Y27632 administered at a low dose (2 mM) was detrimental to horizontal ladder accuracy, and walking examined by footprint analysis. At a higher 20 mM dosage, reduced errors were observed on horizontal ladder walking (Chan et al., 2005). Treatment-induced plasticity may depend on a certain dose of Y27632 to achieve axonal regeneration and/or sprouting in the RtST in the face of increased scar density and CSPG expression. The addition of Y27632 at the lesion site may have been too far rostral to cause the spared RtST fibers to undergo a meaningful degree of sprouting. Furthermore, due to the RtST dieback described here (means of approximately 4 mm), the treatment solutions may have been administered

too far caudally to promote regenerative changes in severed RtST fibers. Future studies will likely address this shortcoming.

The second treatment tested was the NgR Fc molecule. We did not find significant improvements in walking performance after a lateral hemisection injury due to NgR Fc treatment. The anatomical findings did not show regeneration of the severed RtST after treatment with NgR Fc nor did the spared RtST show increases in sprouting into the grey matter at lumbar levels. Other investigators' findings showing recovery of overground walking using the NgR Fc compound after incomplete SCI have been more positive, with some anatomical findings of apparently regenerating CST fibers either approaching or passing through the lesion site (Li et al., 2004; Ji et al., 2005; Wang et al., 2006). We utilized the drug protocol from Li et al. (2004) to attempt to induce similar improvements in walking recovery after a lateral hemisection injury, but were unable to find comparable recovery to their results with a slightly different lateral hemisection lesion model. In Wang et al., (2006) NgR Fc was given in two different experiments after a thoracic contusion injury. In their first experiment the route of administration was intrathecal and resulted in an improvement in BBB scores from 9 to 10 (Wang et al., 2006). In the second experiment the rats received NgR Fc via intraventricular catheters which resulted in a BBB score increase from 6 to 9 (Wang et al., 2006). Alongside this functional recovery, more traced CST fibers were visualized approaching the injury site, but none were described entering or passing through the lesion site. Some ectopic treatment-related CST projections were found in the dorsal horn 15 mm above the level of the injury. The other two studies from Strittmatter and colleagues employed dorsal hemisection injuries at T6-T7 (Li et al., 2004; Ji et al., 2005). In one study the experiment was carried out three separate times, with endpoint BBB scores of the treated group always coming out 3 points higher than the control group, with the control group being 12 in the first experiment, 10 in the second, and 9 in the third (Li et al., 2004). Given that the BBB scale has thresholds at the scores of 8 and 14 at which rats BBB scores will tend to cluster around after SCI (Schucht et al., 2002),

such findings are somewhat puzzling. Anatomical data where the dorsal portion of the CST is severed and labeled CST fibers are visualized caudal to injury are also described (Li et al., 2004). Li et al. (2004) suggested that regeneration of the dorsal portion of the CST into the caudal spinal cord did occur, although the authors could not exclude the possibility that ventral or lateral CST fibers are responsible for the increased numbers of fibers seen caudal to injury (Li et al., 2004).

In general, although each of these treatments act at key points in the pathways responsible for the inhibition of axonal regeneration where they should have a great effect on axonal regeneration and walking recovery, the Y27632 and NgR Fc provide very limited benefit to recovery of overground and skilled walking after lateral hemisection injury in rats. Consistent with this lack of improved walking performance due to these treatments, treatment induced changes in the RtST were not found.

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CHAPTER 6

INTERPRETATION OF FINDINGS AND RELEVANCE TO THE STUDY OF PLASTICITY AFTER INCOMPLETE SPINAL CORD INJURY

6.1 WALKING PATTERN CHANGES ASSOCIATED WITH RECOVERY OF HIND LIMB STEPPING AFTER INCOMPLETE SCI

In the previous chapters, potential mechanisms underlying recovery of walking following incomplete SCI in rats were shown and discussed. Additionally, potential treatments were administered to examine their effects on anatomical plasticity, regeneration, and recovery of skilled and overground walking in rats. In chapter 2, changes in the locomotor pattern during overground walking were examined before and after incomplete dorsal thoracic transection injuries. The main findings of this chapter included increased hind limb extension during stance in rats that attained "normal" (i. e. based on visual scoring) weight supported stepping. Rats walking with extended hind limbs in stance were able to consistently support their weight with the hind limbs, but their BBB scores were limited by other aspects of their walking. This behavior may represent a strategy involved in reducing the effort required for hind limb weight support. A second strategy was found when muscle activity in the fore limb was examined. Increases of triceps brachii muscle amplitude were found when effects due to walking speed were accounted for. This may represent increased involvement of the fore limbs in propulsion. Finally an increase in longissimus firing immediately before stance in the ipsilateral hind limb was observed in rats with visibly normal walking (i.e. maximal scores on the BBB open-field score). In attaining walking that appears normal to observers, rats may use muscles innervated by levels above the injury, but that attach onto the pelvic girdle, where most attached muscles are innervated by spinal levels below the injury. This suggests that back muscles innervated by spinal roots above the injury level may take on an expanded role during walking. This mechanism is consistent with previous observations of functional recovery in rats given complete transections of the thoracic spinal cord as neonates. After the addition of fetal spinal tissue, recovery of walking was accompanied by an expanded

representation of back musculature in the rats' primary motor cortices (Giszter et al., 1998). The changes in the pattern of muscle activation during walking in rats with SCI, and the movements measured using kinematics may represent adaptive strategies that improve walking performance during overground locomotion.

6.2 HORIZONTAL LADDER ASSESSMENT OF SKILLED WALKING MOVEMENTS AND THEIR RECOVERY AFTER SCI

In chapter 3, locomotor pattern changes were examined between overground and skilled walking conditions, and following lesions of the dorsal column that ablated the dorsal component of the CST. Skilled walking provides a useful complement to overground walking assessments as they may be under a greater degree of supraspinal voluntary control than is the case with overground walking (Metz et al., 2000; Metz and Whishaw, 2002). Rats with intact spinal cords place their paws accurately on the rungs of the horizontal ladder task while crossing, whereas the paws of rats with SCI will frequently fall between the rungs of the ladder. These falls are typically scored as errors. During the days and weeks following an injury, rats commit many errors initially, but improve their walking at later points. Reductions in errors are usually interpreted as recovered function. This may be the case, but changes in the walking pattern while crossing the horizontal ladder may confound this interpretation. Detailed descriptions of the movements made by rats while completing the horizontal ladder task compared to overground walking have yielded clues as to how rats improve their performance on this task. Additionally, the data presented in chapter 3 are consistent with rats' use of fore paw haptic (sense of touch) cues to place the hind paw on the bar. Injuries that result in impairment of fore paw sensation may result in an exaggeratedly high number of errors being committed.

Rats completing the horizontal ladder task alter their walking to place their hind limbs precisely on the bars the fore limbs land on, which may be the result of rats using fore limb hapsis to identify the location of bars. The movements of rats with intact spinal cords were

initially compared between overground and skilled walking conditions. When rats walk on an elevated runway (i. e., overground walking condition) their hind limb tends to land behind the ipsilateral fore limb. The proximal movements involved in advancing the hind limb to land in the same location as the fore limb were examined using kinematic analysis. Increased flexion was observed at the hip, knee, and ankle to advance the hind limb during the swing phase of walking and accomplish the placement of the hind limb onto the same bar the fore limb contacted. An additional observation supporting the interpretation of rats using their sense of touch (i. e., hapsis and haptic cues) to walk on the horizontal ladder task comes from the bursting pattern of the fore limb extensor triceps brachii. EMG recordings frequently showed a multiple bursting pattern, which has been implicated in a "soft contact phase" of stepping (Clarke, 1995). The first burst occurs at initial contact while the rat gathers haptic cues about the bar they touch before the rat proceeds to weight bearing stance on that fore limb. Taken together, these observations suggest that rats utilize haptic cues in identifying the location of bars for the hind limb to step on. This leads to two major issues in interpreting error counts gathered from rats walking on the horizontal ladder task. One is that errors made by the fore limb are likely to lead to subsequent errors in the hind limb and increase the numbers of errors in a way that does not reflect the state of the rats spinal cord. Secondly, deficits in fore limb sensory function as would result from cervical models of SCI are especially likely to inflate the number of errors rats will commit while walking on the horizontal ladder. Although the singular use of simple error counts would show the degree of voluntary control related to spinal function after an injury, additional tests would certainly help in determining whether increased (or reduced) error counts are related to impaired (or recovered) fore paw sensation. Additional sensory tests could include sticky paper removal (Schallert and Whishaw, 1985), von Frey hair testing (Christensen et al., 1996), plantar heater testing (Christensen et al., 1996), or more time-consuming analysis such as haptic discrimination (Ballermann et al., 2001). The number of errors made by the fore limb is very low when thoracic injuries are used, such as in chapters

4 and 5 (unpublished observations). In this case, simple error counts should provide an accurate estimation of spinal function.

6.3 RETICULOSPINAL SPROUTING ALONGSIDE SPONTANEOUS POST-SCI WALKING RECOVERY

In chapter 4, anatomical correlates of overground locomotor recovery were examined. The RtST provides tonic activation of the neuronal networks of the lumbar spinal cord that in turn, generate the pattern of muscle activation involved in walking. To examine the extent to which RtST projection patterns change following SCI, an anterograde tracer was injected into the medial reticular formation, and the spinal projections were quantified. The RtST projections of rats with intact spinal cords were compared to the RtST of rats 7 days following lateral hemisection injury at T8 and rats 42 days following T8 lateral hemisection injuries. Changes in the severed RtST above the level of the injury were not found. Traced RtST fibers opposite the lateral hemisection injury employed in this chapter were spared and increased their collateral density entering the grey matter at the level of L2. The projections entered the dorsal horn and intermediate grey matter, (an area where excitatory commissural interneurons reside, see Bannatyne et al., 2003), but did not cross the midline. The increase in projections entering the grey matter, dorsal horn and intermediate grey matter correlated well with the degree of walking recovery observed with the BBB open-field scale. The RtST projection pattern changes are consistent with strengthened connections between the RtST and the neurons of the CPG on the side opposite the hemisection injury, but the improvements in the BBB score were the result of improved stepping of the hind limb ipsilateral to the hemisection injury. The unpatterned nature of the RtST activation to the CPG means that the bilateral walking recovery could be driving by CPG sprouting on the spared side. The bilateral nature of the walking recovery implies that the axonal sprouting of the RtST documented here is not solely responsible for the recovery. There are excitatory crossed interneurons that would likely

be able to activate the CPG on the injured side (Bannatyne et al., 2003). It has also been observed that reduced glycinergic inhibition between the two sides of the spinal cord is also important for training-induced recovery of walking following incomplete SCI (de Leon et al., 1999). Finally, the motoneurons controlling the muscles of the hind limbs become more excitable following SCI and loss of descending input (Bennett et al., 2004; Li et al., 2004b). The SCI-related increase in motoneuron excitability can improve walking, but also hinder walking in some cases when spasticity results (Bennett et al., 2004). Another possible mechanism that could underlie both the recovery, and the anatomical plasticity is the amount of home-cage walking activity. Higher levels of activity that result when rats are given access to running wheels in their home-cage result in higher expression of markers associated with synaptic plasticity including BDNF and synapsin (Gomez-Pinilla et al., 2002). Thus individual differences in home-cage activity could underlie both better recovery and increase axonal sprouting of the RtST. There was some variability in the degree of locomotor recovery, which correlated well with the amount of reticulospinal tract sprouting.

6.4 ROLE OF TREATMENTS IN ENHANCING ANATOMICAL PLASTICITY AND RECOVERY OF WALKING

In chapter 5, potential treatments thought to increase sprouting, axonal regeneration, and recovery of walking were tested on rats following lateral thoracic hemisection injuries, to examine if pharmacological treatments act by increasing the axonal sprouting of the RtST that was observed in chapter 4. The treatments included the NgR Fc molecule designed to block myelin associated inhibitors acting via the NgR molecule and Y27632, an inhibitor of ROCK (one member of the pathway involved in growth cone collapse). Both treatments have been found to increase axonal sprouting in the CST (Li et al., 2004a; Dergham et al., 2002; Chan et al., 2005), but severed RtST fibers did not appear to respond to treatment with NgR Fc or Y27632. Severed RtST fibers retracted from

the injury site to a greater distance than was seen with the CST. The degree of RtST dieback was similar in the control and NgR Fc and Y27632 treated groups suggesting that there was no treatment-induced regenerative sprouting of the fibers approaching the injury site. When Y27632 was administered in rats where the CST fibers were later traced, it was found that the severed fibers did not grow toward the injury site, but rather sprouted into the adjacent grey matter, entering the dorsal horn and intermediate grey matter on the side contralateral to tracer injection. When spared RtST fibers were examined, and compared between rats given saline and either NgR Fc or Y27632, there were no significant increases in fiber density entering or within the grey matter at the level of L2, which is the level where statistically significant changes had been observed in chapter 4. Rats did not show an improved degree of walking recovery after SCI when given NgR Fc or Y27632 treatments and tested with the BBB open-field score and horizontal ladder task. Taken together these findings confirm the biological activity of the Y27632 and that the delivery to the spinal cord was effective. These data suggest that these two treatments do not have a detectable effect on the RtST sprouting phenomenon described in chapter 4, nor do they improve walking after incomplete SCI. As discussed in chapter 5, studies using Y27632 in rats have not shown large improvements in walking after dorsal overhemisection injuries to the thoracic spinal cord (Dergham et al., 2002; Sung et al., 2003; Fournier et al., 2003).

In contrast to the lack of observed overground walking recovery found after treatment with Y27632, previous studies from Strittmatter and colleagues have shown robust improvements in overground walking after dorsal overhemisection injuries resulting from treatment with NgR Fc (Wang et al., 2006; Li et al., 2004a; Ji et al., 2005). Additionally, these studies showed increases in sprouting of dorsal and ventral CST axons approaching the lesion site (Wang et al., 2006), or growing past the injury site and into the caudal spinal cord (Li et al., 2004a). Given that the CST has a limited role in overground walking (Steeves and Jordan, 1980; Muir and Whishaw, 1999), we investigated whether there were

changes in the projections of the RtST in rats given NgR Fc after a lateral hemisection injury, as changes in the projections of this tract are more likely to be causally responsible for improvements in overground walking. The protocols used to administer the drug were taken from Li et al., (2004a). In Li et al., (2004a), improved overground walking was apparent by 28 days after injury. In chapter 5, there were no apparent changes in overground walking performance throughout the 56 day post-injury survival time. In Li et al., (2004a), experiments where NgR Fc was given were repeated three times, where rats treated with NgR BBB scores improved by approximately 3 points by 28 days after injury (Li et al., 2004a). In these three experiments, the mean BBB scores at the end of each experiment ranged from 9 to 12 in saline treated rats, and 12 to 15 in NgR Fc treated rats (Li et al., 2004a). The effects in two of those experiments straddled a major threshold of the score (i. e. BBB=14; see introduction: Figure 1.5) where rats attain consistent weight supported stepping, but other impairments prevent higher scores. The consistency of a 3 point effect in this case is somewhat puzzling. There is a paucity of published data on the effects of the NgR Fc treatment in animal models after SCI, aside from those published by Strittmatter and colleagues. Their findings have been universally positive in rats with dorsal hemisection injuries at T6-7 (Ji et al., 2005; Li et al., 2004a; Wang et al., 2006). Although a lateral hemisection injury approach may be somewhat different especially in terms of the populations of RtST fibers spared that are likely to play a role in the recovery of walking, the uniformity of the findings from this group are puzzling. The findings in chapter 5 would suggest that these treatments in their current form have limited value in terms of improving walking function in rats. Consequently, these findings do not support the translation of these two therapies into clinical trials.

6.5 INTERPRETATION & APPLICATIONS

6.5.1 Injury induced changes involved in walking recovery after SCI

The findings in this thesis show some of the mechanisms involved in recovery after incomplete thoracic SCI. These mechanisms include changes in skilled and overground walking and adaptations in the RtST, an important descending system for walking. The extent to which these mechanisms generalize across different animal models would be one indicator of how likely it is they will lead to a valuable treatment capable of invoking meaningful improvements in functional outcome in patients, or repairing the spinal cord. The mechanisms underlying recovery of motor function may include reconnection or re-routing of descending commands to spinal networks below the level of the injury. Given the role of the RtST in providing the activation for walking movements, it is likely involved in the formation of new spinal circuitry that can underlie recovered function. Compensatory adjustments to the skilled and overground walking patterns in rats are of critical importance in appraising whether treatments shown to improve functional outcome in rats are likely to have benefit when moved into clinical trials. Additionally, compensatory adjustments to walking patterns in rats can also form the basis of new treatment approaches in patients. For example, in patients with incomplete thoracic SCI, walking could be improved by generating leg movements using a swinging motion, acting via the pelvic girdle, and initiated by the back muscles. Some back muscles' innervation may be above the injury site and thus would have preserved function. Thus, some compensatory movements documented in rats could be applied in human patients and used in the field of physical therapy. In general, this may represent a more promising approach to restoring lost function after SCI, to use alternative movements to substitute for those movements lost to enduring deficits after SCI.

One of the challenges of using walking as a readout of spinal cord function is presented by the complexity of the physiology and anatomy of its neural and biomechanical control. Some improvements in overground walking outcome can occur independently

of reconnection of tracts through or around an injury site. One example of improved walking that does not depend on reconnection is shown by one study where the addition of embryonic raphe cells into the rat spinal cord at T11 below complete injuries at T8 resulted in activation of the walking pattern (Ribotta et al., 2000). The cells innervated L1 and L2, and added serotonin which improved walking greatly compared to rats with complete spinal cord injury (Ribotta et al., 2000). Improved overground walking performance is not always the result of improved connections to and from the brain. The use of a skilled walking task such as the horizontal ladder test can allow better assessment of the integrity of spinal tracts related to voluntary control of hind limb movements, but there are still aspects how rats perform this task to be considered before attempting to assess whether repair of the spinal cord has occurred. An additional issue to consider in the study of walking, is that the projections of the RtST (the descending system responsible for activating walking CPGs) are more challenging to quantify, and due to its diffuse nature among many other spinal tracts, impossible to specifically injure. Understanding how spinal cord injuries change the CNS substrates that control walking, including the RtST, interneurons and motoneurons in the spinal cord is an important first step to understand how treatments can induce recovery of overground and skilled walking. The changes in the neural systems that generate the walking pattern, in addition to the anatomical arrangement of the hind limb (musculature, bone structure, sensory structures, and their innervation) are likely involved in the compensatory movements described in chapters 2 and 3. The changes in CNS connectivity as well as walking behaviors occurring after SCI are likely important in determining how best to bring about recovery with pharmacological or other interventions.

6.5.2 Treatment-induced changes involved in walking recovery after SCI

Although the molecular pathways involved in the inhibition of axonal regeneration of the CNS have been at least partly elucidated, the ways the CNS responds to potential treatments are only beginning to become apparent. The pathways of CNS inhibition converge at a mediator of growth cone collapse known as ROCK. Growth cone collapse

can be blocked via the administration of Y27632, a specific ROCK inhibitor, which has been shown to increase sprouting of neurons grown *in vitro*. Y27632 also increases sprouting in CST axons in mice and rats with incomplete SCI (Dergham et al., 2002; Fournier et al., 2003; Chan et al., 2005). When ROCK inhibitor is administered intrathecally after incomplete SCI, the sprouting effects are also accompanied by decreases in apoptotic cell death (Dubreuil et al., 2003), leading to smaller lesions and in the case of Dergham et al. (2002), improved functional recovery. Small increases in white matter survival likely account for the improved functional recovery observed in Dergham et al., (2002) and might preclude the involvement of rerouted descending commands passing around the lesion, or axonal regeneration. One can speculate about the mechanisms behind this sparing. One possibility is that Y27632 has unknown unspecific effect on the apoptotic cell death pathways. A different, more specific inhibitor acting on the ROCK pathway could resolve this confound. A second possibility, is that Rho and/or ROCK themselves act on apoptotic pathways. Dubreuil et al. (2003), discuss this possibility extensively. A third possibility is that Y27632 induces increased sprouting of severed fiber tracts near the injury site which results in increased local synaptic connections and better retrograde transport of target derived survival factors such as the neurotrophins. If that were the case, any treatment that improves sprouting would also improve sparing to some degree, but the fact that subsequent investigations largely did not replicate the improved functional recovery (Fournier et al., 2003; Sung et al., 2003), suggests that the recovery observed in Dergham et al., was more likely due to the initial variability in the injuries received by the mice (2002). The walking scores achieved by the treated mice from 1 day post-injury by Dergham et al. (2002) appear to support that interpretation. Continued investigation of how ROCK inhibition after incomplete SCI changes the CNS has led to a possible explanation as to why large-scale regenerative changes are not obvious. In particular, findings from the Tetzlaff and colleagues may account for why the use of Y27632 inhibition does not lead to large-scale improvements in axonal regeneration through lesion sites (Chan et al.,

2007). In rats given dorsal column injuries at cervical level, Chan et al., (2007) found that Y27632 treatment resulted in increased expression of markers related to scar formation including activated astrocytes and chondroitin sulfate proteoglycans (CSPG). CSPGs are expressed throughout the CNS extracellular matrix, and inhibit axonal regeneration. Chan et al. (2007), found that GFAP and neurocan were more highly expressed at the injury scar as Y27632 dosage increased. Scar formation is a complex process regulated in part by the post-injury inflammatory response. Interleukin 1 beta (IL1β) is strongly expressed shortly after SCI (Herx and Yong, 2001) and activates astrocytes via the deactivation of RhoA/ROCK in astrocyte cultures (Figure 6.1 A; John et al., 2004), which increases scar formation early after an injury. Extended treatment with Y27632 results in reduced ROCK activity in astrocytes (John et al., 2004). Additional astrocyte stellation can result in a more dense astrocytic scar and more expression of CSPG, thus offsetting the benefits of inhibiting ROCK in neurons (Figure 6.1B, (John et al., 2004).

Another possible reason why axonal sprouting and regeneration was not observed in the reticulospinal fibers after Y27632 treatment could be that the distance between the sites where projections were quantified and where the drugs were given was too great, and the effective dosages at those sites were too low to result in sprouting and regeneration. The beneficial effects of Y27632 on functional outcome and axonal sprouting in rats with SCI are highly dose dependent (Chan et al., 2005). The administration technique employed in chapter 5 involved the use of osmotic mini pumps with catheters attached that supplied Y27632 and NgR Fc compounds to the lesion site. Spared RtST projections at the level of L2 are approximately 25 mm from the lesion site, whereas in severed RtST fibers, the dieback phenomenon observed resulted in retraction bulbs at distances of 4 mm rostral to the lesion epicenter. Thus the drug administration may have been at once too far rostral to evoke sprouting in the spared fibers and too far caudal to evoke sprouting in the severed fibers. Future experiments will likely address this possibility by using catheters applied directly to L2, or slightly rostral to the injury site.

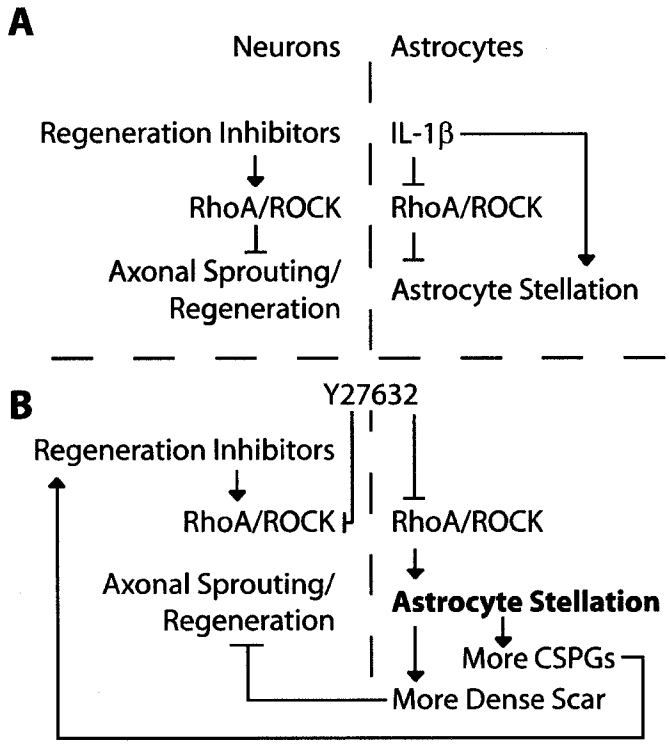

Figure 6.1: Effects of Y27632 in neurons and astrocytes following SCI – A. RhoA and its downstream effector ROCK are active in both neuronal (left side of dashed line) and astrocyte populations (right side) after injury. RhoA/ROCK activation is involved in the inhibition of sprouting and regeneration of CNS fiber tract. Early after injury IL-1β is expressed as part of normal scar formation. This results in a reduction of RhoA/ROCK activity, and increased astrocyte stellation. B. During treatment with Y27632, inhibition of RhoA/ROCK can result in increased scar formation and CSPG expression, limiting the beneficial effects of Y27632 in neurons.

6.5.3 *Reaching behavior as readout of spinal cord function*

The neural control of walking presents some challenges to investigators utilizing it as a readout of spinal cord functions, in that its descending system the RtST is anatomically diffuse making the study of its anatomy more difficult than some other spinal tracts. Challenges involved in studying walking after SCI and the RtST has led to various SCI researchers utilizing skilled reaching models to assess functional recovery and anatomical plasticity after SCI (reviewed in Webb and Muir, 2005). These studies enjoy the advantage of somewhat simpler anatomical examination of the descending tract controlling fine digit movements, the CST. Additionally, electrophysiological examinations can be carried out on the CST using intracortical micro-stimulation (Fouad et al., 2001). This can be contrasted with electrophysiological studies of the RtST and one of its primary inputs, the mesencephalic locomotor region . To study RtST output via attain stable recordings of field potentials in the spinal cord, inhibitory influences must be removed via decerebration (e. g., (Noga et al., 1991), which technically complicates the experiment by requiring intubation and paralysis. Finally, the activation of motoneurons involved in reaching relies on more direct control from the CST. Motoneurons involved in walking become activated more indirectly via tonic activation from the RtST acting through the interneurons composing the half-centers making up the CPG in the spinal cord. Neural changes within the motoneurons after SCI are factors in recovery in both cases, but the changes in the CPG networks and their inputs also need to be considered when interpreting the quantifications of overground walking (open-field scores such as the BBB), and skilled walking (error counts in the horizontal ladder). Success in reaching can be objectively scored and provide a assessment of reconnection through or around an injury site. This does not, however, mean that the study of reaching and its anatomical counterpart the CST represents a panacea for animal studies of SCI. First, skilled reaching also requires a period of training that is more labor intensive than is required for either skilled or overground walking assessments. Additionally, the use of fore limb testing requires that cervical injury models

be employed. Depending on the precise level and severity of cervical injuries, animal care can become very onerous if fore limb impairments are sufficiently severe that they impair feeding. Complete injuries, frequently employed to preclude spared fibers from supporting functional recovery after SCI, are out of the question in this case due to loss of respiratory function. Skilled reaching testing also requires that the rats be food deprived during some part of the study. Food deprivation itself has been documented to lead to changes in expression of proteins related to CNS plasticity including BDNF (Duan et al., 2001; Lee et al., 2002), and the formation of neural cells in the dentate gyrus (Lee et al., 2000). Further, the degree of axonal regeneration through an injury site and recovery of reaching accuracy are not free of confounding variables. Specific and complete spinal injuries to the rat CST are not currently possible. At the level of the closed medulla the dorsal portion of the CST crosses over, whereas the ventral and lateral portions do not. As each division of the CST runs in a different spinal funiculus, this makes it impossible to damage them all in the spinal cord without collateral injury to adjacent white matter tracts. Additionally, injuries that spare one or more of the CST divisions can lead to anatomical changes in spared fibers, much like the ones shown in chapter 4. For example, one well-cited study has found that ventral CST fibers can take over the control of reaching when the larger dorsal CST is ablated (Weidner et al., 2001), but a subsequent study from Piecharka et al. (2005) did not support this conclusion. These two approaches are not identical, as Piecharka et al. (2005) used a hemi-pyramidotomy injury in the brain stem, whereas Weidner et al. (2001) used a dorsal column SCI. Both approaches ablate the dorsal portion of the SCI and spare the ventral CST fibers, however. Whether ventral CST fibers support functional recovery in rats with dorsal CST injuries is an important question to resolve as treatments acting on myelin associated growth inhibitor have been frequently tested in rat models with dorsal transections or contusions that spare ventral and/or lateral CST fibers (e. g. (Zheng et al., 2005). If skilled reaching is to be used as a measure of the extent to which reaching control is restored by axonal regeneration of the dorsal CST, the contribution of the

ventral CST sprouting both without and with plasticity-enhancing treatments needs to be addressed. The ventral CST represents one alternative pathway for descending commands related to skilled reaching, but there is also a second descending pathway that needs to be considered. Descending CST fibers pass by the red nucleus, the site of the cell bodies of the RST. One study using unilateral pyramidotomies in neonatal rats found that crossed corticorubral and corticopontine projections were greatly increased, which resulted in the severed CST establishing a cortico-rubro-spinal projection that substituted for the damaged direct CST pathway (Figure 6.2 A; Z'Graggen et al., 2000. The importance of rerouted connections to the spinal cord is highlighted by a second study from this group using the IN-1 antibody following unilateral pyramidotomies in adult rats (Z'Graggen et al., 1998). Here, improvements in skilled reaching performance related to IN-1 treatment were paralleled by strengthened crossed CST connections to the red nucleus and basilar pontine nuclei, in addition to changes in the contralateral spared CST sprouting below the injury (Figure 6.2B, Thallmair et al., 1998. These findings highlight CNS plasticity away from lesion sites as a potential mechanism behind functional recovery as well as a potential confound in assessing the degree to which axonal regeneration through lesion sites is responsible for reaching recovery as well as walking recovery in rats with SCI.

Whether the forms of CNS plasticity represent a possible avenue to advance clinical treatment of SCI in patients is a separate question. The findings presented here show how the anatomy and function of the rat CNS responds to injury, and is important for interpreting studies investigating recovery of walking in rats with SCI. The varied approaches to overcome the numerous barriers against regeneration have the potential to improve outcome by affecting plasticity at sites away from the injury site. Perhaps more pertinent to the present findings, however, is the potential for treatments administered to the CNS that should enhance anatomical plasticity in neurons may also result in changes in other cell types that can limit the treatments' benefits. Future studies may investigate this possibility. Currently, however, the use of Y27632 and NgR Fc in clinical trials as treatments for SCI,

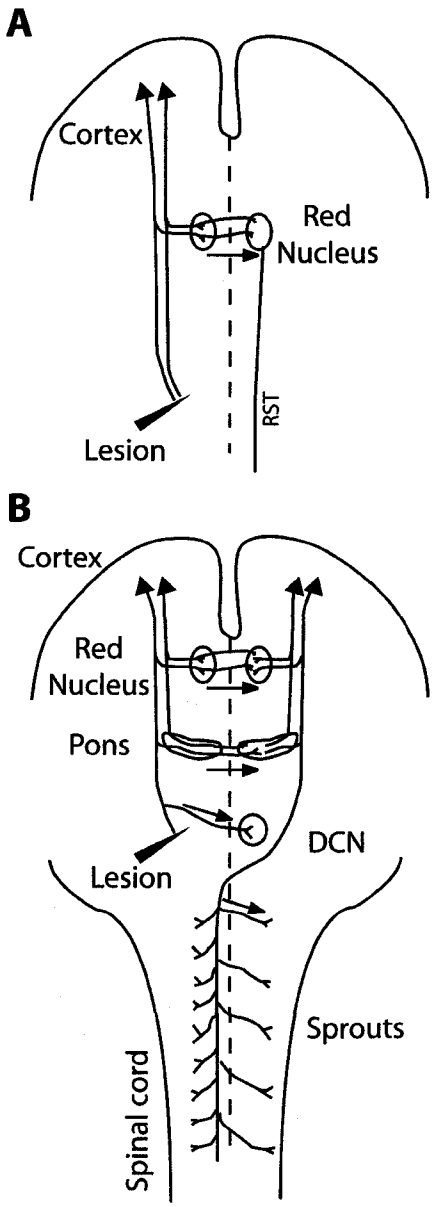

Figure 6.2: Cortico-rubro-spinal connections after pyramidotomy in neonates and IN-1 treatment – A schematic of descending connections from cortex at top to spinal cord at bottom is depicted. A. After a unilateral pyramidotomy (black triangle) in neonatal rats severed cortical fibers sprout increased collaterals across the midline (dashed line) to the contralateral red nucleus (arrows), forming a new pathway to the red nucleus on the uninjured side. B. After IN-1 treatment in adult rats with a unilateral pyramidotomy similar crossed connections are seen in the brain to the red nucleus, basilar pontine nucleus (Pons), and cuneate and gracilus nuclei (DCN), as well as sprouting of the spared CST across the midline.

would seem quite premature. Future studies will hopefully lead to better understanding of the nature of CNS inhibition of axonal regeneration and axonal plasticity. A sound understanding of CNS inhibition should lead to better chance of successful treatments being designed. The alternative involves moving investigations of the current treatment into higher species and into clinical trials. Although there is some argument to be made about the degree to which the rodent and primate nervous systems differ (Courtine et al., 2007), there seems to be little evidence to warrant any belief that current treatments will have better effects on patients or in primate SCI models.

6.6 FUTURE EXPERIMENTS

6.6.1 Changes in commissural lumbar interneuron projections

There are many questions remaining to be addressed about both spontaneous and treatment induced functional recovery, and what changes occur within the RtST that may support the recovery of walking in rats with SCI. As the walking recovery is bilateral, but the changes observed in RtST projections were largely unilateral, it seems likely that there are changes within the CPG interneurons in the lumbar cord. One possibility is that during the spontaneous recovery period, interneurons below the injury site form strengthened excitatory connections with lumbar motoneurons on the injured side. One way to address this possibility, would involve the injection of anterograde tracers such as biotinylated dextran-amine (BDA) into the ventral grey matter on the same side as the injury. The number and locations of labeled axons within the grey matter could be compared between rats given 7 days to recover after lateral hemisection injury and rats given 6 weeks, similar to the experimental design in chapter 4. If strengthened excitatory connections are involved in this recovery, one should see an increase in the density of BDA labeled projections on the contralateral side. The neurotransmitter identity of the neurons undergoing the changes could also be determined with double immunohistochemistry for BDA and vesicular glutamate transporter 1 and 2. Projections originating from cells

labeled for both BDA and vesicular glutamate transporter 1 or 2 would likely have more elaborate branching on the side below the injury. Alternatively there may be a reduction in the strength of connections from crossed inhibitory neurons. Similarly, this could be determined with double immunohistochemistry for BDA and either glutamic acid decarboxylase (marks GABAergic neurons), or glycine transporter 2 (marks glycinergic neurons). If changes in the projections of inhibitory neurons were involved, one would expect density of projections orginating from doubly labeled cells in the grey matter opposite the injection site. These changes could account for the bilateral nature of the walking recovery documented in chapter 4. Understanding how interneuron projections change after recovery from lateral hemisection injury would provide a the groundwork for investigating how treatments that improve walking affect interneuron connections in the lumbar spinal cord.

6.6.2 Testing pharmacological treatments to enhance walking recovery

The experiments in chapter 5 used an inhibitor of the ROCK kinase, and an antagonist of molecules associated with myelin associated inhibition of regeneration to attempt to induce sprouting of the RtST. Slight changes to the method used in administering these treatments might result in significant improvements in walking recovery as well as regeneration of severed RtST fibers and sprouting of spared fibers. One change could be to use a different catheter placement, and administer the drug either slightly rostral to the injury site, or to the lumbar spinal cord. Both of these sites could be given drug simultaneously through the use of a junction in the catheter to apply the drug solutions to both site simultaneously.

One possible reason why Y27632 did not have as large an effect as hoped was that it appears to increase scar formation by acting within astrocytes at the lesion site. One way to attempt to address this would be to administer chondroitinase enzymes to digest the scar tissue at the lesion site to reduce the amount of chondroitin sulfate proteoglycans, a major source of inhibition at the scar site. This could be provided in addition to the Y27632 drug to examine whether RtST projections would then be induced to sprout or regenerate. An

alternative approach would be to attempt to increase the activity of ROCK in astrocytes, with the goal of decreasing scar formation at the injury, while simultaneously decreasing ROCK activity in neurons. This potentially could be accomplished by administering a DNA virus such as an adenovirus with an constitutively active form of ROCK (John et al., 2004), whose expression would be controlled by an astrocyte specific promoter (Casper et al., 2007). This type of approach should improve sprouting of the RtST while reducing the degree of Y27632-related scar formation. If this is the case, it may also result in improved recovery of walking performance after lateral hemisection injury.

Two somewhat related treatment approaches that may increase the degree of RtST sprouting include the addition of cAMP or BDNF. RtST axons express the tyrosine kinase B (TrkB) neurotrophin receptor (King et al., 1999). The neurotrophin molecular pathways are known to have important roles during development involved in cell survival and neurite extension (reviewed in Lewin and Barde, 1996). It may be that the extracellular addition of BDNF or drugs acting like BDNF to the lumbar spinal cord may induce sprouting of RtST axons. Alternatively BDNF could be added at the cell body of reticulospinal neurons in the medial reticular formation to induce the expression of regeneration associated genes that may be important in inducing axonal sprouting. This approach may lead to improvement in recovery of walking. A related approach to the use of neurotrophins such as BDNF and BDNF-related agonists is manipulations that increase cellular levels of cAMP. BDNF and the TrkB receptor have their effects on neurons by increasing cAMP levels (Cai et al., 1999). This increase can be accomplished through the addition of db-cAMP, a membrane soluble analogue of cAMP. Combined with the addition of rolipram, an inhibitor of phosphodiesterase an enzyme that breaks down cAMP, spinal levels of cAMP can be raised above those normally found in adult neurons (Pearse et al., 2004). Raising cAMP levels in neurons has been shown to improve neurite elongation *in vitro* (Cai et al., 2001), as well as in combination with the use of Schwann cell grafts, improve recovery of walking in rats with contusion spinal cord injuries (Pearse et al., 2004). By increasing cAMP at

the injury site, severed RtST fibers may also be induced to regenerate. A second approach would be to add rolipram at the lumbar spinal cord to investigate whether spared RtST fibers could be induced to sprout and form new connections into the lumbar grey matter. If these treatments are capable of inducing RtST sprouting or regeneration, they may also result in improved walking function.

6.6.3 Effects of walking training on RtST projections

Another group of treatment interventions alter CNS connections and improve various functions by using training regimes. These alter CNS activity with the expectation that this will result in altered connectivity between spinal centers, resulting in improved function. Training in humans attempts to approximate the movements made during walking, usually on a treadmill with physical assistance rendered by a trainer if needed (Thomas and Gorassini, 2005). Walking movements are considered to provide the sensory stimulation to the spinal cord to alter the pattern of connections in the spinal cord (reviewed in Harkema, 2001. In animal models treatments can include voluntary exercise (such as wheel running) or treadmill training. In some studies, these have been found to improve standing (de Leon et al., 1998) or stepping (de Leon et al., 1999; Ichiyama et al., 2005) in rats and cats with SCI, depending on the specific training protocol used. Additionally, early intervention following SCI can result in better functional outcomes than delayed intervention (Norrie et al., 2005). Studies of exercise in rats frequently use wheel running as a model for exercise (Gomez-Pinilla et al., 2002) or treadmill training (Gomez-Pinilla et al., 2001) to investigate how locomotor related activity can change the CNS. One recent approach that has been used to improve functional outcome after contusive SCI has been swim-training (Smith et al., 2006). One possible reason swim-training may provide benefits above those seen in other types of post-injury exercise training is that many hind limb swim cycles, and thus more sensory stimulation is more likely to drive changes in the CPG connectivity. The CPG networks in the rat lumbar spinal cord are thought to be involved in generating rhythmic muscle output involved in swimming (Kiehn and Kjaerulff, 1998).

It is plausible that improvements in swimming performance resulting from swim training would occur alongside changes in sprouting of spared RtST fibers in the lumbar spinal cord. The functional improvements derived from swim training do not generalize to overground walking (Smith et al., 2006). In general, the benefits derived from different forms of training seem to be limited to the type of movement performed during training, and do not generalize to functions that were not trained (Tillakaratne et al., 2002). Nonetheless, training can increase the expression of different molecular pathway components involved in CNS plasticity in the spinal cord. These components include BDNF, GAP43, and synapsin expression in the spinal cord (Gomez-Pinilla et al., 2002). Increases in BDNF expression associated with exercise seems likely to effect changes in the projection pattern of the RtST, as the RtST expresses the TrkB neurotrophin receptor (King et al., 1999).

6.6.4 Effects of electrical stimulation on RtST projections

An alternative to the training regimes involves electrical stimulation applied via chronically implanted electrodes implanted in areas of the brain where cell bodies reside to attempt to induce axonal regeneration and/or sprouting. One possible advantage to this approach is the large number of stimulation bouts that could be administered to alter connectivity of spinal circuitry involved in activity the CPG. Flexible microwires have been chronically implanted into the CNS previously (Mushahwar et al., 2000). These flexible microwires could be implanted into the MRF to depolarize reticulospinal neurons in an orthodromic fashion and induce intracellular changes that could lead to axonal sprouting. Electrical simulation has been shown to increase the expression of GAP-43, BDNF, and TrkB receptors in regenerating axotomized femoral motoneurons (Al-Majed et al., 2000). Additionally, calcium influx resulting from electrical activation may result in neurons becoming less sensitive to myelin associated inhibitory molecules, and regenerating (Hansen et al., 2001; Ming et al., 2001). Increases in intracellular calcium through L-type calcium channels leads to a group of changes including increases of neurotrophin expression (Hansen et al., 2001) that can increase axonal plasticity and

improve the chances of neuronal survival.

Stimulation of the RST to induce regeneration into a peripheral nerve has been attempted (Harvey et al., 2005). In this experiment, 1 hour and 8 hour bouts of 20 Hz antidromic stimulation were used, and the numbers of RST neurons that regenerated into a peripheral nerve graft did not appear to increase (Harvey et al., 2005). Although those findings are somewhat disappointing, different stimulation parameters may improve the degree of RST axonal regeneration. Firstly, the stimulation frequency of 20 Hz used induces sensory axons to regenerate, but other frequencies are typically used to stimulate descending tracts. For example, CST is frequently stimulated at frequencies of 330 Hz (e. g. Fouad et al., 2001). Electrical stimulation at 330 Hz applied for 2 hours per day over 2-3 weeks to the spared CST contralateral to a CST lesion has resulted in improved compensatory sprouting (Salimi and Martin, 2004). In contrast, the extent to which the RtST responds to electrical stimulation is not known. Spared and severed RtST neurons after a lateral hemisection injury may respond to repeated stimulation with new collateral sprouts or axonal regeneration. This will likely require the use of a combination treatment to neutralize the growth inhibitory effect of CNS myelin, such as the implantation of a permissive PNS graft into the injury site. Additionally, there are many stimulation parameters which can be investigated. These include the optimal frequency of stimulation, the duration of stimulation bouts, the frequency of bouts, the time the stimulation is applied relative to when the injury is given, and the length of time the stimulation is applied are all likely to impact the degree to which the RtST responds to this manipulation.

6.7 CONCLUSIONS

The findings in this thesis show mechanisms that are likely to be involved in recovery of walking after incomplete SCI, including anatomical plasticity and behavioral compensation. The generation of walking movements by the CNS is controlled by neural circuitry that includes CPGs. These CPGs can change their outputs and connectivity

in ways that likely result in improved overground and skilled walking following SCI. Additionally, anatomical changes in the spinal cord occur in ways that could result in descending commands being rerouted around partial interruptions to the RtST, the descending tract that provides the tonic excitation of the CPG networks. Neuronal sprouts from spared RtST fibers project into the region where CPG neurons are thought to reside. Treatments, including training, stimulation, and pharmacological intervention improve walking in some studies after incomplete SCI. It is not well understood how this happens in most cases. Two different treatments known to enhance axonal sprouting in other descending tracts were tested to see if they could enhance the phenomenon of axonal sprouting of RtST projections. Although the treatments tested here did not have the desired effects on walking performance in overground or skilled walking, the rerouting of descending commands around an injury site remains a likely mechanism through which locomotor improvement following SCI can occur.

Regeneration of severed axons has enjoyed much of the spotlight in terms of the expected mechanism behind recovery. Some studies making claims of treatment induced regeneration alongside functional recovery have been difficult to replicate however (Steward et al., 2006). Careful examination of the anatomical and behavior changes behind recovery of walking after SCI, such as those in this thesis, suggest that plastic changes within the CNS may be much more important for walking recovery than the regeneration of severed axons. Pharmacological, training, and stimulation treatments that exploit the plastic nature of the CNS likely have great potential to result in beneficial clinical treatments for SCI.

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