REMOTE DISRUPTION OF FUNCTION, PLASTICITY, AND LEARNING IN LOCOMOTOR NETWORKS AFTER SPINAL CORD INJURY

DISSERTATION

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ABSTRACT

Spinal cord injury (SCI) creates a diverse range of functional outcomes. Impaired locomotion may be the most noticeable and debilitating consequence. Locomotor patterns result from a dynamic interaction between sensory and motor systems in the lumbar enlargement of the spinal cord. After SCI, conflicting cellular and molecular processes initiate along the neuroaxis that may secondarily jeopardize function, plasticity, and learning within locomotor networks. Thus, we used a standardized thoracic contusion to replicate human pathology and identified behavioral, physiological, cellular, and molecular effects in rat and mouse models. Specifically, our goal was to identify kinematic and neuromotor changes during afferent-driven phases of locomotion, evaluate the role of axonal sparing on remote spinal learning, and identify mechanisms of neuroinflammation in the lumbar enlargement that may prevent locomotor plasticity after SCI.

Eccentric muscle actions require precise segmental integration of sensory and motor signals. Eccentric motor control is predominant during the yield (E2) phase of locomotion. To identify kinematic and neuromotor changes in E2, we used a mild SCI that allows almost complete functional recovery. Remaining deficits included a caudal shift in locomotor subphases that accompanied a
marked reduction in eccentric angular excursions and intralimb coordination. Underlying these metrics, we examined burst properties of various hindlimb muscles. We found distinct bursting impairments in the semitendinosus, a biarticular muscle that reflects the segmental integration of descending motor drive and afferent input from the limb. Phasic impairments during the eccentric activation of the semitendinosus improved over time and predicted the extent of recovery. Our findings suggest that maladaptive integration of spared descending and afferent signals limit central pattern generator-directed locomotion after SCI and confirm the importance of eccentric motor control in recovery.

Axonal sparing after SCI facilitates plasticity rostral and caudal to the lesion. To evaluate the role of axonal sparing on remote lumbar plasticity, we examined learning in isolated lumbar segments early (7d) and late (42d) after SCI. A proof of principle design compared rats that recovered with or without sparing. Early after SCI, spinal learning was impaired regardless of axonal substrate. Learning acquisition and maintenance was unattainable. Alternatively, axonal sparing during recovery allowed near-normal learning late after SCI. Thus, an opportunity to influence activity-based learning in locomotor networks depends on injury progression in the presence of sparing. To determine if eccentric task-specific training differentially regulates spinal learning, we delivered flat or downhill treadmill training late after recovery from incomplete SCI (34-41d). Isolated learning assessments occurred at 42d. Downhill treadmill
training improved both open field and treadmill locomotion. Similarly, central evaluations of the lumbar cord identified sustained learning in rats with downhill but not flat treadmill training. Flat treadmill training increased levels of pro-inflammatory TNFα and IL-1β in lumbar segments and worsened measures of learning and locomotion. Together, this work identifies a time dependent interaction between spared axonal systems and task-specific plasticity in locomotor networks.

Our previous work shows that microglial activation and pro-inflammatory cytokine production extend 10 segments caudal to the lesion in the lumbar enlargement and predict sensory dysfunction. To determine if remote mechanisms of neuroinflammation impede locomotor plasticity, we conducted a series of experiments in wild type and genetically engineered mice. First, we characterized the mechanism and timecourse of neuroinflammation in the lumbar enlargement after a moderate/severe thoracic SCI in C57BL/6 (WT) mice. Within 24h, resident microglia displayed an activated bushy phenotype together with increased expression of pro-gelatinase, matrix metalloproteinase (MMP)-3. By 7 and 9d, MMP-9 and TNFα reached significant elevations alongside persistent activation of resident microglia. MMP-9 activity localized around vascular endothelia in the lumbar enlargement, suggesting neurovascular reactivity at great distances from the lesion epicenter. In MMP-9 null (KO) mice, microglial activation and TNFα production in the lumbar enlargement was restored to homeostatic levels. To determine if a timecourse of locomotor plasticity exists
after SCI that depends on MMP-9-regulated pathways, we examined recovery after early (2-9d) or late (35-42d) treadmill training delivered in C57BL/6 and MMP-9 KO mice. We found that early training resulted in robust locomotor recovery in MMP-9 KO mice that was retained 4-weeks after the intervention ended. The same early intervention impaired recovery of WT mice. Despite findings of spared white matter in MMP-9 KO mice, lumbar-focused training delivered late failed to promote recovery in either KO or WT groups. With these findings, we identified a robust period of locomotor plasticity early after thoracic SCI that is blunted by remote neuroinflammation in locomotor networks.
DEDICATION

Dedicated to my amazing wife Melissa, who provides unending support; to my inspirational parents Mark and Denise, who encourage me to reach far; to Mia, who I hope will grow up to ask too many questions and never settle.
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LIST OF ABBREVIATIONS

α  alpha
β  beta
°C degrees Celsius
2D two-dimensional
ANOVA analysis of variance
BBB Basso, Beattie, Bresnahan Locomotor Rating Scale
BDNF brain-derived neurotrophic factor
BMS Basso Mouse Scale for Locomotion
BSCB blood spinal cord barrier
BSA bovine serum albumin
CNS central nervous system
CPG central pattern generator
CSPG chondroitin sulphate proteoglycans
DH downhill
dpi days post injury
dpo days post operative
dVFH dorsal von Frey hair
<table>
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<th>Definition</th>
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<tr>
<td>E1</td>
<td>initial contact</td>
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<tr>
<td>E2</td>
<td>yield</td>
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<tr>
<td>E3</td>
<td>lift off</td>
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<td>EC</td>
<td>eriochrome cyanine</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>EMG</td>
<td>electromyography</td>
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<td>ETT</td>
<td>early treadmill training</td>
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<td>F</td>
<td>peak flexion</td>
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<td>FG</td>
<td>fish gelatin</td>
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<td>FITC-gelatin</td>
<td>fluorescein isothiocyanate-conjugated gelatin</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<td>GF</td>
<td>growth factor</td>
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<td>h</td>
<td>hour</td>
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<td>HL</td>
<td>hind limb</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>Hz</td>
<td>hertz</td>
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<td>i.p.</td>
<td>intraperitoneal</td>
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<td>iba-1</td>
<td>ionized calcium-binding adapter molecule 1</td>
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<td>IH</td>
<td>infinite horizons device</td>
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<td>IL-1β</td>
<td>interleukin-1 beta</td>
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<tr>
<td>IL-6</td>
<td>interleukin-6</td>
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<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ILP</td>
<td>instrumental learning paradigm</td>
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<td>KO</td>
<td>knock out</td>
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<tr>
<td>L4</td>
<td>lumbar vertebral level 4</td>
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<tr>
<td>L5</td>
<td>lumbar vertebral level 5</td>
</tr>
<tr>
<td>LAM</td>
<td>laminectomy control</td>
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<tr>
<td>LG</td>
<td>lateral gastrocnemius muscle</td>
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<td>LTP</td>
<td>long term potentiation</td>
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<td>LTT</td>
<td>late treadmill training</td>
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<td>M</td>
<td>molar</td>
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<td>MANOVA</td>
<td>multivariate analysis of variance</td>
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<td>MMP-2</td>
<td>matrix metalloproteinase-2</td>
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<td>MMP-3</td>
<td>matrix metalloproteinase-3</td>
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<tr>
<td>MMP-9</td>
<td>matrix metalloproteinase-9</td>
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<td>NFkB</td>
<td>nuclear factor kappa B</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<td>NGS</td>
<td>normal goat serum</td>
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<td>NMDA</td>
<td>N-methyl D-aspartate</td>
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<td>NOGO-A</td>
<td>neurite outgrowth inhibitor-A</td>
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<tr>
<td>NT3/4</td>
<td>neurotrophin ¾</td>
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<td>OSU</td>
<td>The Ohio State University</td>
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<tr>
<td>PCA</td>
<td>principal component analysis</td>
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<tr>
<td>PC1</td>
<td>first principal component</td>
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<tr>
<td>PC2</td>
<td>second principal component</td>
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PB       phosphate buffer
PB       phosphate buffered saline
RIPA      Pierce lysis buffer
ROS       reactive oxygen species
Rpm       revolutions per minute
SCI       spinal cord injury
SDS       sodium dodecyl sulfate
SEM       standard error of the mean
siRNA     small interfering ribonucleic acid
ST        semitendinosus muscle
ST1       first burst of ST
ST2       second burst of ST
T2        thoracic vertebral level 2
T8        thoracic vertebral level 8
T9        thoracic vertebral level 9
TA        tibialis anterior muscle
TNFα      tumor necrosis factor alpha
TM        treadmill
TT        treadmill training
TX        transection
Tx-100    triton x-100
VFH       von Frey hair
WT        wild type
CHAPTER 1

General Introduction

Biological Basis of Exercise Treatments After Spinal Cord Injury

1.1 Introduction

Advances in biomedical research hold great promise for treating functional deficits after spinal cord injury (SCI). Currently, activity-based neurorehabilitation is the foundation of clinical intervention, whereby residual neural circuitry is activated in the hope of forming or strengthening synapses, which improve motor control. Despite intensive neurorehabilitation, deficits persist and locomotor recovery remains unattainable for most people with SCI. To change functional outcomes, the interaction between exercise-related factors and SCI sequella must be optimized.

Exercise paradigms elicit specific biophysical effects that impact CNS recovery following SCI. Therefore, it is important to determine the type of intervention and when to deliver it for different severities of SCI (Figure 1.1).
Ultimately, task-specific training will likely be effective only when delivered to a permissive cellular environment. Animal models offer the greatest advantage to identify the cellular and behavioral determinants of recovery after SCI.

1.2 Experimental SCI Models

Of the many experimental SCI models, contusion and complete transection (TX) provide important information about motor recovery and the cellular consequences of SCI (Stokes and Jakeman, 2002; Edgerton et al., 2001). The locomotor effects in these quadruped-based models appear to translate to bipedal humans. The TX model severs all grey matter and axons typically in the midthoracic region, thereby isolating the lumbar cord from brain input. Behavioral and cellular changes in the isolated cord serve as proof-of-principle of the inherent capacity of the spinal cord to learn. Methods to promote spinal cord learning likely facilitate functional recovery after less severe SCI. The contusion model offers a clinically-realistic, complex injury that reproduces many of the molecular mechanisms in human SCI (Bunge et al., 1993). The injury has a central core lesion and a peripheral rim of spared white matter. Partially-spared descending motor, ascending sensory and autonomic systems replicate the behavioral dysfunction seen clinically like paresis/paralysis, neuropathic pain and autonomic dysreflexia (Basso et al., 2002; Weaver et al., 2002; Detloff et al., 2008). Thus, contusion injury is more translationally relevant than TX for testing exercise and functional recovery.
1.3 The Role of Spinal Learning and Plasticity in Recovery after SCI

Simple Learning and Reflex Modulation.

The spinal cord has a remarkable capacity to adapt and learn, independent of supraspinal input. Using complete TX, Jim Grau showed that the isolated lumbar cord learned to avoid hindpaw shock using an instrumental learning paradigm (Grau et al., 2006). In this paradigm, hindlimb (HL) extension closes a circuit that results in shock delivery to the tibialis anterior. The isolated lumbar spinal cord adapted the resting posture of the HL by increasing flexion to minimize shock. This simple yet dramatic form of learning required protein synthesis, N-methyl D-aspartate (NMDA) receptor activation and brain-derived neurotrophic factor (BDNF) expression, whereas gamma-aminobutyric acid (GABA)-A receptor activation inhibited learning (Grau et al., 2006). Thus, sensory-driven spinal learning is capable of modulating reflex gain below the SCI and may be a fundamental component of activity-based rehabilitation.

Down-regulating segmental reflexes holds promise for reducing hyperreflexia and spasticity after SCI. Using operant conditioning, Jonathan Wolpaw showed that animals learn to reduce reflex gain even after SCI (equivalent of the stretch reflex) (Chen et al., 2010). On a cellular level, a positive shift in alpha motor neuron firing threshold was associated with more inhibitory F-type synapses and GABAergic terminals on motor neurons. Additionally, reflex
modulation improves motor function after SCI in rats. Translational work to reduce reflex gain in human SCI began recently with the expectation that leg spasticity would decrease and motor control would improve. Given that down-training in animal models requires spared descending supraspinal input to induce spinal plasticity and learning, such reflex modulation training in the clinic may be most effective after incomplete SCI (Chen et al., 2010).

**Complex Learning and Locomotion.**

Fundamental elements of stepping are controlled at the spinal cord level and represent an important target for activity-dependent learning after SCI. When the lumbar cord is isolated from supraspinal control, it is capable of complex learning, which is dependent on task-specific afferent signals. Reggie Edgerton established that the isolated spinal cord learned to stand on a stationary treadmill (TM) or step on a moving TM (Hodgson et al., 1994). However, stand- or step-training improvements did not transfer to the other task. Hence, the cord has a limited capacity for relearning multiple tasks in the absence of supraspinal input. In contrast, robust adaptive learning occurs within a task. Spinal stepping accommodates to variable environmental conditions including obstacles and treadmill speed (Edgerton et al., 2001). Cellularity, GABAergic terminals are differentially expressed in a use-dependent manner. The GABAergic response decreased with step training but increased in flexor motor pools with stand training (Tillakaratne et al., 2002). Remarkably, these cellular changes occurred after a few days of training and lasted 25 months.
Perhaps the lasting cellular localization of GABA impedes transfer of training to other tasks.

Taken together, the lumbar cord performs simple and complex learning in the absence of supraspinal input. While the breadth of skill learning appears limited when the SCI is complete, the intrinsic capabilities of the cord are sufficient to adapt to environmental demands within a given task. Inhibitory GABAergic input regulates spinal learning in a task-specific manner and may explain limitations in skill learning after SCI. Clinically, drugs which act on GABA receptors should be carefully considered as they may create a lasting barrier to activity-dependent relearning and recovery.

1.4 Training

Spinal learning and synaptic plasticity after SCI depend on intact sensory drive during task-specific training. Training changes functional outcome by pruning inhibitory synapses while strengthening synapses activated by the task. To be clinically relevant, activity-based training paradigms should induce skill learning which transfers to other tasks and environments. Interestingly, little is known about which exercise interventions and what dosing schedules will promote relearning and recovery for different SCI severities. Factors such as task specificity, training intensity and complexity are beginning to receive attention in experimental SCI.
Intensity

For locomotion, intensity of training is modulated through body weight support and stepping frequency which varies with TM speed and performance duration. High body load impairs stepping kinematics so that optimal training requires some body weight support to produce stepping of good quality and quantity (Timoszyk et al., 2005). Training sessions of 1000 steps produced better kinematics and stepping patterns than low dose sessions of 100 steps. In fact, disuse of paralyzed limbs through wheel chair use prevents motor recovery after contusive SCI in rats (Caudle et al., 2008). As multiple types of exercise promote different magnitudes of limb loading and stepping frequency, personalized training paradigms will depend greatly on task selection.

Unfortunately intensity can be excessive and induce detrimental effects. Perhaps best described after cortical injury, forced use of the impaired limb early after lesion worsens recovery and exacerbates lesion size (Kozlowski et al., 1996). In the spinal cord, we found that treadmill, standing or swimming training from 4-49 days post contusion did not exacerbate the lesion (Hutchinson et al., 2004). However, swimming at 3 days worsened a similar SCI (Smith et al., 2009).

Complexity

A fundamental tenet of skill acquisition is that the training task be sufficiently challenging to allow both success and failure. Movement errors
promote refinement toward successful motor patterns. Hence, training should encompass movement variability and trial-and-error to induce motor relearning and recovery after SCI. Clinically, two locomotor rehabilitation approaches, therapist-assisted stepping and robotic training, offer moderate or no variability, respectively. Recently, the necessity of variability for learning was shown in experimental SCI. Robotic training which provided no step-to-step variability in limb trajectory was compared to variable training in which self-initiated stepping was assisted-as-needed by a robot in rats with TX. Fixed robotic training prevented distinct, alternating EMG patterns and blocked skill learning. Thus, the opportunity for error correction is critical to locomotor recovery.

Experimentally, robust training variability occurs with environmental enrichment. The environment consists of interactive toys and structures (i.e. ramps, rope ladders, tunnels) which require a variety of motor control patterns. Continuous enrichment improves motor coordination through insulin-like growth factor (IGF)-dependent synaptic plasticity in the lumbar cord after contusion (Koopmans et al., 2006). Delivering enrichment at doses effective in animals will be difficult clinically, as 24/7 exposure to a complex environment may be unfeasible. Recent work in animal models of traumatic brain injury demonstrate effective enrichment doses at 6h, rather than 24h (Kline, 2010). The minimal effective dose for environmental enrichment in SCI must be determined before clinical feasibility is established.
Task-Specificity

Motor relearning depends on the quality of afferent input delivered to the cord during training. Training must be task-specific to engage neural circuits, produce motor patterns and regulate afferent input that closely mimic the real world task. Supportive evidence is two-fold - simple general activity does not promote skill learning; and, demanding, task-specific training improves recovery after CNS injury. In stroke and cervical SCI models, locomotion was used as general exercise training and compared to task-specific reach and grasp training in rats (Maldonado et al., 2008; Garcia-Alias et al., 2009). The stroke study used reaching and retrieving multiple pellets on a tray from various trajectories. The SCI study combined drug treatment with reaching through a grid to retrieve and shell sunflower seeds. Simple limb use failed to promote motor relearning after cortical ischemia or cervical SCI with drug treatment. Alternatively, robust recovery occurred with task-specific training. Thus, non-specific activity may not promote relearning, especially for forelimb function. More research is needed to identify the critical components of task-specific training for other tasks.

Task-specific training also benefits sensory function after SCI. We compared mechanical sensation after swim, standing and TM training in contusive SCI (Hutchinson et al., 2004). Each exercise paradigm differentially regulated mechanotransduction through the paw. After SCI, profound, lasting hypersensitivity occurred below the injury. Importantly, only TM training which provides high mechanosensory input ameliorated the allodynia-like sensory
dysfunction. This task-specific effect was associated with normalization of peripheral and central BDNF.

When to Deliver Training

Timing of exercise interventions may determine beneficial or detrimental outcomes after SCI. Intervening early after SCI may impose a greater neurotoxic risk than no intervention (Smith et al., 2009; Ichiyama et al., 2007). Increasing physical activity, heart rate and blood flow when the blood-spinal cord barrier (BSCB) is compromised, may exacerbate the existing inflammatory state. Richard Benton and David Magnuson showed that 8 minutes of swimming 3 days after SCI worsened intraparenchymal inflammation at the epicenter (Smith et al., 2009). Behaviorally, swimming kinematics were also impaired compared to a 2-week intervention. Detriments associated with early robotic step training have also been observed (Ichiyama et al., 2007). Aberrant stepping kinematics and high variability in movement trajectories occurred with early training whereas delayed training improved motor control.

1.5 Cellular Factors that Influence Training Effects

Cellular time course after SCI

Mechanical injury initiates multiple intracellular processes that create short and long-term challenges for activity-based rehabilitation. Direct damage to neuropil and blood vessels initiate inflammatory and excitotoxic cascades which
produce primary and secondary cell death along the neuroaxis (Profyris et al., 2004). Within hours, the BSCB opens and robust inflammation begins. A toxic milieu comprised of high levels of pro-inflammatory cytokines tumor necrosis factor-alpha (TNFα), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) combines with a massive release of glutamate from damaged astrocytes and peripheral afferents. The resulting excitotoxicity induces mitochondrial dysfunction within 12 hrs of injury and initiates apoptotic cascades within injured and bystander neurons and glia. Progressive mitochondrial dysfunction and phagocytosis produce oxygen free radicals which further threaten neurons and glia, especially oligodendrocytes (Sullivan et al., 2007). The injury is compounded by decreased trophic support for neurons and glia from BDNF, insulin-like growth factor (IGF) and neurotrophin 3/4 (NT3/4). Lack of trophic support combined with inflammation produces apoptosis at progressively greater distances from the epicenter. Reactive astrocytes move to the lesion border and secrete inhibitory molecules like chondroitin sulphate proteoglycans (CSPGs), creating both a physical and chemical barrier to axon regeneration.

The early inflammatory and excitotoxic events spread to spinal cord regions remote to the injury epicenter over several weeks. High levels of pro-inflammatory cytokines, activated microglia, glutamate, and apoptosis of oligodendrocytes occur in remote cervical or lumbar enlargements following contusive SCI. Chronically, low levels of BDNF, NT3/4 and IGF occur in the lumbar enlargement. Thus, inflammatory cascades, excitotoxicity, apoptotic cell
death, and glial scarring create a nonpermissive environment for synaptic
plasticity within primary sites of motor relearning (enlargements) (Profyris et al.,
2004).

*Inflammation and Learning*

Given the predominance of inflammation after SCI, surprisingly little is
known about its impact on motor relearning. In the brain, inflammation impairs
spatial learning and activity-dependent plasticity in the cortex and hippocampus.
Generally, elevated levels of pro-inflammatory cytokines (IL-1, TNFα) act as
impediments but their absence may also be deleterious (Kaneko et al., 2008).
Within the spinal cord, inflammation appears to impede learning and plasticity by
creating central sensitization, a common mechanism of neuropathic pain
(Pedersen et al., 2010; Hook et al., 2008). Moreover, elevated cytokines and
glial activation below the SCI induces profound hypersensitivity which may be
similar to neuropathic pain (Detloff et al., 2008). These robust sensory
alterations provide aberrant input and likely further disrupt motor relearning.

Exercise may effectively modulate hypersensitivity and cellular
inflammation. We showed that TM training over 7 wks ameliorates
hypersensitivity after contusive SCI (Hutchinson et al., 2004). Moreover, this
training normalized TNFα within 14 days of injury (unpublished data). Whether
exercise-induced normalization of sensation and inflammation can be used to
improve motor control remains unknown.
Influence of Exercise on Mitochondrial Dysfunction/ROS Production

Exercise, if done too intensely or under declining health, promotes oxidative stress. Given the widespread induction of oxygen free radicals (ROS) after SCI, the additional demands placed on mitochondria by exercise must be carefully examined. Activity-based interventions may induce greater mitochondrial dysfunction, jeopardizing neurons, glia, and oligodendrocytes that survive the initial mechanical damage (Sullivan et al., 2007). By precisely grading exercise treatment type, intensity, and/or intervention timing, it may be possible to reduce the risk of cell death and lesion exacerbation. An optimal exercise intervention should consider the time course of mitochondrial dysfunction and whether mitochondria are capable of responding to task specific demands of different training paradigms.

Influence of Exercise on Trophic/Growth Factors

Exercise elevates trophic and growth factors throughout the CNS which serve neurogenerative and neuroprotective functions. After SCI, widespread declines compound a destructive cellular milieu, leaving surviving neurons and glia susceptible to a host of degenerative processes. Re-establishing trophic support is critical, and weight supported motor activity represents a natural in vivo drug delivery system (Hutchinson et al., 2004). Exercise-regulated trophic factors, BDNF and IGF, promote learning, synaptic plasticity and sensory function after SCI (Hutchinson et al., 2004; Koopmans et al., 2006). However,
excessive NGF produces autonomic dysreflexia, a clinically life-threatening condition (Weaver et al., 2002). Exercise paradigms must be screened for their effects on growth factors to minimize the risk of inducing deleterious effects.

Combinatorial Interventions

Given that pharmaceutical interventions for SCI can be combined with rehabilitation, it is critical that we know how and when to deliver different exercise interventions. Surprisingly, drug treatments combined with activity-based training may compete with each other and actually worsen motor outcome (Maier et al., 2009; Garcia-Alias et al., 2009). For example, combining robotic TM training with an antibody to neutralize the myelin growth inhibitor NOGO-A impaired stepping kinematics to a greater extent than if no exercise training had been administered (Maier et al., 2009). Combining chondroitinase, which degrades CSPG, with environmental enrichment after cervical SCI produced greater motor deficits than no rehabilitation (Garcia-Alias et al., 2009). Whether task-specificity, intensity and timing can improve combinatorial treatments is unknown. Given that a NOGO inhibitor is in clinical trials in Europe, it is especially important that effective exercise paradigms be identified.

Delivering optimal task-specific training at the best time for the appropriate injury will facilitate motor relearning and recovery. Viewing exercise paradigms as a biologics intervention seems fitting given that activity-based treatments can
be potentially toxic or beneficial. Modulating the type, intensity, complexity and timing of training, may minimize risk and induce greater recovery.

The interaction between type of training, timing of the intervention, and the severity of injury within a permissive cellular environment has not been widely studied. It is at this intersection of factors that innovative personalized medical treatment emerges. Using exercise as a biological therapy to enhance the cellular environment offers a new perspective in creating state-of-the-art neurorehabilitation for SCI (Figure 1.1).

**Figure 1.1** Optimal activity-based rehabilitation emerges at the intersection of task specificity, and appropriately timed delivery of training according to the severity of the spinal cord injury.
1.6 DISSERTATION FOCUS

*Plasticity of Locomotor Networks.*

Seminal work over the past century has demonstrated abundant plasticity within locomotor networks of the spinal cord (Hodgson et al., 1994; Grau et al., 1998; Grillner and Zangger, 1979; Barbeau and Rossignol, 1987; Chen et al., 2006; Chen et al., 2010). After SCI, various forms of plasticity are evident throughout the neuroaxis. Adaptive plasticity mediates the recovery of function and is found in the immediate reorganization of the sensorimotor cortex, propriospinal bridging, and sparing-induced facilitation of locomotor networks (Aguilar et al., 2010; Fawcett, 2009; Dunlop, 2008; Basso et al., 1996). Activity-based interventions that harness adaptive plasticity have infinitely improved therapeutic opportunities. However, injury-induced plasticity is not universally beneficial. In fact, hypersensitization of nociceptive pathways, autonomic dysfunction, and hyperreflexia identify lasting mechanisms of maladaptive plasticity that may oppose motor relearning and recovery (Ferguson et al., 2012; Rabchevsky, 2006). Structural, physiological, and synaptic correlates identify complex interactions that depend on injury severity and timecourse. It becomes clear that a paucity of information exists regarding adaptive and maladaptive mechanisms within locomotor central pattern generator (CPG) networks. While the recovery of locomotion requires several plastic mechanisms for its reexpression, optimal recovery will depend on locomotor plasticity that generates adaptive and not maladaptive mechanisms.
Adaptive and maladaptive neuroplasticity result from differential cellular and molecular processes regulated by matrix metalloproteinases. For instance, synaptic remodeling and receptor insertion are critically dependent on MMP-proteolysis (Ethell and Ethell, 2007). Following a select lesion to the entorhinal cortex, distant expression of pro-gelatinase MMP-3 occurs in the dentate gyrus of the hippocampus to facilitate adaptive synaptogenesis (Falo et al., 2006; Kim et al., 2005a). Deletion of MMP-3 or the gelatinase MMP-9 prevents long-term potentiation (LTP) and impedes hippocampal-dependent learning (Nagy et al., 2006). However, the role of MMP members in CNS trauma is highly contradictory. MMPs are potent regulators of inflammatory processes that result in maladaptive plasticity. At the lesion site, infiltrating leukocytes release MMP-9 to degrade tight junctions of the endothelial barrier to allow extravasation and subsequent pathology (Zhang et al., 2011). MMP-9 also cleaves a number of cytokine pathways like TNFα that facilitate AMPA receptor insertion into the synaptic cleft, resulting in neural hyperexcitability and toxicity (Ethell and Ethell, 2007; Zhang et al., 2011; Beattie et al., 2002). MMP-3 plays a similar paradoxical role when it not only regulates the cleavage and transcription of MMP-9, but also functions as a novel-signaling molecule to activate microglia (Rajashekar et al., 2011; Kim et al., 2005b). There appears to be a fine line between adaptive or maladaptive functions within the metalloproteinase family (Agrawal et al., 2008). It seems fitting that we explore the complex interactions
between MMP members and their relation to competing plasticity in locomotor CPG networks after SCI.

1.7 Remote injury mechanisms may jeopardize locomotor networks

A Spinal Cord Lesion, or a Central Nervous System Condition?

Injury to the spinal cord results in a multifaceted and ill-defined lesion border. Elegant historical accounts of functional recovery after spinal cord injury have been limited by the view of a focal lesion defined by cell death and a distinct glial border (Popovich et al., 1997; Basso et al., 1995; Bunge, 2008; Gale et al., 1985). It has become increasingly clear that inhibitory boundaries to function and plasticity are not only defined by physical barriers that prevent axon conductance, sprouting, and growth, but also include phenotypic alterations in glia and bystander production of signaling molecules that influence neurotransmission, receptor expression, and long term potentiation (Yirmiya and Goshen, 2011; Ethell and Ethell, 2007; Huie et al., 2012; Vichaya et al., 2009). Indeed, recent findings of cellular and molecular changes occurring substantial distances rostral and caudal to the lesion epicenter have precipitated an evolving view of the injured spinal cord (Detloff et al., 2008; Nesic et al., 2005; Hains and Waxman, 2006; Andrews et al., 2012). Of interest is the distant response in the highly vascularized lumbar enlargement where endothelial junctions are particularly vulnerable to inflammation (Arima et al., 2012). Here, intact interneurons and motoneurons isolated from the primary injury are subject to
changes in the surrounding microenvironment. We suggest that profound fluctuations in the remote cellular milieu around CPG networks have made interpretations of locomotor performance based solely on lesion site tissue loss obsolete and require detailed examination of function and plasticity in locomotor networks.

Remote Neuroinflammation May Limit Function and Plasticity of Locomotor Networks

Distant inflammatory signaling may blunt the integration of sensory and motor signals that produce locomotor patterns after SCI. Within days of a thoracic insult, activation of resident microglia and increased production of pro-inflammatory cytokines occur at least 10 segments below the injury around sensorimotor circuits of the lumbar enlargement (Detloff et al., 2008; Hains and Waxman, 2006). The genesis and mechanism of these remote events remain phenomenological. As a result of remote neuroinflammation in the dorsal horn, dendritic spine composition and presumed receptor expression are drastically altered and influence function (Tan et al., 2008). Indeed, we and others have shown that dorsal horn microglial activation and cytokine expression create robust hypersensitivity of sensory afferents (Detloff et al., 2008; Hains and Waxman, 2006; Nesic et al., 2005). Given the profound impairments in remote sensory transduction, it is surprising that none have asked whether the same mechanisms hinder the production of locomotor patterns. Locomotion results from the precise integration of afferent sensory and descending motor signals to
produce a dynamic relay across interneuron networks to initiate oscillatory excitation and inhibition among CPG networks (Edgerton et al., 2004; Rossignol et al., 2006; Rossignol et al., 1988). It is possible that hypersensitization of primary afferents and secondary interneurons may produce spurious firing or inappropriate connectivity throughout locomotor networks after SCI. This dissertation asks a novel question: *Does a spinal cord lesion create regional locomotor impairments independent of tissue loss at the lesion site?*

Inflammatory signaling throughout the lumbar parenchyma may further prevent activity-dependent plasticity and learning in locomotor networks. While inflammation is a clear impediment to learning and LTP in the hippocampus (Yirmiya and Goshen, 2011), it is unclear whether remote inflammation after SCI will impede the activity-dependent delivery of sensory cues to the lumbar enlargement to promote plasticity and motor relearning. This is likely, given that exogenous administration of either TNFα or lippopolysacharide to intact lumbar circuitry prevents activity-dependent spinal learning (Huie et al., 2012; Vichaya et al., 2009). Our previous findings of remote TNFα production and microglial activation begin to outline signaling mechanisms that may undermine adaptive plasticity and learning after SCI. This interaction will depend on injury progression, as cytokine profiles fluctuate during acute and chronic phases of injury (Detloff et al., 2008). Recent work has shown that activity-based interventions fail to harness adaptive plasticity early after CNS injury and instead create functional impairment (Kozlowski et al., 1996; Jones and Schallert, 1994;
Griesbach et al., 2004; Smith et al., 2009). Together, it appears that a complex and evolving microenvironment that impedes synaptic efficacy within lumbar networks may be refractive to activity-based interventions. From here, we suggest that motor relearning after SCI will depend on injury severity, time after injury, and the type of activity-based intervention (Figure 1.1).

1.8 Proposed role of motor relearning and recovery after SCI

We propose that SCI results in remote neuroinflammation that produces maladaptive plasticity in locomotor networks and prevents activity-dependent motor relearning. By attenuating remote inflammatory processes, we may facilitate locomotor plasticity and promote learning and recovery after SCI. Work from this dissertation identifies a paradigm shift for clinical focus in neurorehabilitation: optimal recovery after SCI will depend on combinatorial therapies that consider both lesion-site and segmental lumbar plasticity. Our objective, as addressed in the following chapters, was to characterize lumbar-focused deficits and identify cellular and molecular mechanisms that may jeopardize plasticity in locomotor networks. By examining post injury dynamics remote to the lesion and the functional interplay of sensory and motor systems, we seek to broaden a formerly limited view of the lesioned spinal cord. Throughout this dissertation, we use systems neurobiology to call into question current directions for therapeutic intervention in basic science and clinical models. This is the first attempt to systematically examine the intricacies of motor
relearning and the ubiquitous nature of lumbar spinal plasticity in response to remote inflammation after SCI.

1.9 HYPOTHESIS

Our laboratory has shown that microglial activation and pro-inflammatory cytokine production extend at least 10-segments caudal to the lesion in the dorsal horn of the lumbar enlargement. A resulting disruption of sensory systems was identified by profound mechanical hypersensitivity. Surprisingly, the implications of remote neuroinflammation within shared interneuron networks for locomotor central pattern generation remain unexamined. Therefore, the primary hypothesis of this dissertation was:

*Spinal cord injury disrupts segmental function, plasticity, and learning in locomotor networks. Remote neuroinflammation impairs sensory-motor interactions and presents a barrier to motor relearning and recovery.*

Thus, we first characterized the recovery of segmental motor control during afferent-driven phases of locomotion. Consequently, we confirmed the importance of eccentric motor control and found a novel therapeutic direction for clinical neurorehabilitation. We then identified a time dependent interaction between axonal sparing and activity-based learning in locomotor networks after SCI. Here, we found an opportunity to harness axonal substrate to induce task-
specific plasticity and learning. To identify potential mediating factors of lumbar-centric impairments, we exposed novel mechanisms of inflammation that produce maladaptive plasticity in locomotor networks. Finally, we attenuated remote mechanisms of neuroinflammation to harness a previously refractive period of plasticity and promoted robust recovery of locomotion.
Figure 1.2 Proposed questions regarding motor relearning and recovery after SCI. Image depicts a spinal cord injury that results in axonal sparing of ascending (spinothalamic- dashed yellow) and descending (rubrospinal- dashed red, reticulospinal- dashed green, vestibulospinal- dashed purple, raphespinal- dashed blue). Notice an absence of dorsal column sparing (gracile- dashed light blue, cuneate- dashed gold, and corticospinal- dashed gray). Questions are raised as to the influence of SCI on remote central pattern generator networks. This dissertation attempts to address function, mechanism, and plasticity in locomotor CPG networks that influence motor relearning and recovery after SCI.
CHAPTER 2

Characterization of Recovered Walking Patterns and Motor Control after Contusive Spinal Cord Injury in Rats

2.1 Introduction

Spinal cord injury (SCI) results in a diverse range of behavioral outcomes that depend on the type, severity, and level of injury. To date, the extent of recovered central nervous system (CNS) control over locomotion has been best elucidated in reductionistic lesion models (Johnson et al., 2012; Edgerton et al., 2004). Surprisingly, less is understood about recovery from contusion-type lesions, which replicate human SCI. Contusive SCI results in complex pathology with distinct anatomical, behavioral, and cellular sequella along the neuroaxis (Stokes and Jakeman, 2002). It is well-accepted that greater sparing of descending midbrain/brainstem pathways improve motor function after contusion
(Basso et al., 2002). However, factors that promote supraspinal and afferent integration during locomotion have received little attention.

Differential recovery after contusive SCI may be identified by changes in gait biomechanics and muscle activation patterns. After hemisection, postural elevation, interlimb uncoupling, and aberrant coactivation patterns between adjacent muscles persist and indicate the limits of recovery (Ballermann et al., 2006). Given the compensatory nature of this injury, it is unclear whether similar factors delineate recovery after bilateral contusion. We previously identified at least one motor feature that remains impaired after SCI – the yield phase during weight acceptance (Basso et al., 1994). Here, we ask whether the kinematics or EMG metrics of yield may be associated with the extent of recovery. Eccentric motor control represents a hallmark of skilled locomotion that is impaired across CNS injury models, but remains unexamined after contusive SCI (Basso et al., 1994). In this ubiquitous action, motor units are partially recruited to keep muscle force below the external load. To attain effective eccentric muscle lengthening, descending drive is precisely controlled to match the afferent input of the movement (Enoka, 1996). A predominant eccentric period in the step cycle occurs prior to ground contact and during weight acceptance, when hamstring muscles like the semitendinosus (ST) lengthen to decelerate the HL and dissipate impact forces during yield (E2). Importantly, recruitment of ST adapts to a variety of locomotor conditions and requires descending control for optimal function (Smith et al., 1998). Our previous work in the cat shows that the
eccentric phase of locomotion remains impaired despite marked recovery from a hemisection (Basso et al., 1994). To further this observation and identify mechanisms of eccentric control after contusion, we examined ST recruitment patterns over time and at recovery plateau. Whether poor eccentric activity in ST or other HL muscles prevents optimal recovery is unknown.

The present study was designed to identify features of recovered walking patterns that differentiate functional restitution after a mild/moderate, midthoracic contusion injury. Detailed assessment of HL muscle recruitment and joint kinematics described the extent of motor control. Our findings suggest that eccentric actions of ST provide novel insight into mechanisms of locomotor recovery after SCI.

2.2 Materials and Methods

Subjects and surgeries

Experiments were conducted in 14 female Sprague-Dawley rats (250-300g, Harlan) that were randomly assigned to control laminectomy (LAM) or SCI groups following EMG implantation. Naïve data collection for all rats served as baseline. Comparisons included Naïve (n=13), LAM (n=5), and SCI (n=9). Animals were housed 2-3 per cage in a controlled environment (12h light/dark cycle) with food and water available ad libitum. Housing, surgical procedures, and assessment of behavior was done in accordance with The Ohio State
University Laboratory Animal Care and Use Committee. For all surgeries, rats were anesthetized intraperitoneal (i.p.) with ketamine (80mg/kg) and xylazine (20mg/kg). During each surgical procedure, a heating pad maintained body temperature. Prophylactic antibiotics (gentomycin sulfate 1mg/kg) and saline were given post surgery to prevent infection and dehydration.

**EMG Implantation**

Subjects were acclimated to the treadmill and trained to walk steadily prior to EMG implantation; this training required 2-3 weeks. During the first surgery, bipolar EMG electrodes were implanted into the tibialis anterior (TA), lateral gastrocnemius (LG), and the semitendinosus (ST) of the left HL. These muscles were selected based on electrode stability and locomotor biomechanics. A small rostral-caudal incision was made along the sagittal suture of the skull and four screws were anchored on each side. Teflon-coated, multi-stranded stainless steel wires fixed to a head plug were routed subcutaneously to the HL and implanted into exposed muscles with a hypodermic needle. Electrode functionality was confirmed by electrical stimulation through each lead (~0.2 – 0.8 mA, 0.2 ms cathodal pulse) to elicit a muscle twitch. A ground electrode remained subcutaneous to serve as reference. The head connector was cemented with varnish and dental acrylic to the screws, and incisions were closed with suture.
Spinal Cord Injury

In the time between EMG implant and SCI (11d), normal open field locomotion was confirmed for each rat. Over this period, rats were re-acclimated to the treadmill and learned to walk with the EMG wire connected to the headplug. This data collection was used for naïve comparison. In the second surgery, rats were anesthetized as described previously, and a midthoracic T8 laminectomy exposed the spinal cord. Animals randomized to the SCI group received a mild/moderate injury produced by rapidly impacting the spinal cord using the OSU Electromagnetic Spinal Cord Injury Device or the Infinite Horizons (IH) Device (Stokes et al., 1992). Following contusion or laminectomy (LAM) control, dorsal musculature was sutured and skin was closed using surgical clips. Sterile saline was administered subcutaneously to prevent dehydration. Antibiotics were delivered daily and bladders were manually expressed 2x/day until the bladder reflex returned. Vitamin C pellets were given to prevent urinary tract infections (Behrmann et al., 1992). Animals that exhibited wiring problems or bladder infection following surgery were not used for EMG collection (n=2).

EMG Recording

To examine muscle recruitment patterns after SCI, EMG signals were recorded and synchronized with joint kinematics for 6 animals and averaged across at least 20 steps on the TM (Columbus Instruments). For downhill recordings, the TM belt was set to a 10% (5.7 degrees) downslope grade. Flexible insulated cables were attached to a head plug, and connected via a
commutator to the amplifier, allowing free movement of the subjects on the treadmill belt. A sugar water dispenser at the front of the belt prompted forward locomotion. Pre-operative training frequency and duration was adjusted per rat until long bouts of sustained stepping occurred while drinking. Post-operatively, brief exposure to the TM occurred to maintain comfort with the task. Collection occurred at the same speed (12m/min) and while drinking to eliminate backward drift.

The EMG signals were amplified at a gain of 1K with an AM-Systems model 1700 differential amplifier. The bandpass filters were set for 20 Hz – 5 KHz, and a 60 Hz notch filter was engaged. Computerized data acquisition was accomplished with a sampling rate of 2 KHz using either a 16-bit Datapac 2K2 system (Run Technologies) or a 16-bit CED Power 1401 system with Spike2 software (CED, Cambridge, UK). EMG records were adjusted to remove DC offsets, rectified, and averaged across 20+ steps off-line using a custom script that used initial contact times as a triggering event. A burst detection program determined the beginning (onset) and end (offset) of each EMG burst and calculated relative to initial contact by determining when the EMG level crossed a threshold set to 2 standard deviations above the mean activity level during quiescence for each muscle. Visual inspection was used to adjust onset and offset times as required to eliminate spurious bursts and locate the main burst periods associated with locomotion. Burst durations were calculated based on the onset and offset times. Digital video records were synchronized with the
EMG recordings by means of an LED light that was visible to the camera, with the voltage pulse for the light recorded along with the EMG.

**Locomotor Assessments**

Locomotor recovery was assessed using the 21-point Basso, Beattie, Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). Scores range from no HL movement (0) to normal locomotor function (21). Rating criteria considered joint movement, weight support, plantar stepping, coordination, toe clearance, paw position, as well as trunk and tail control. Open field activity of each rat occurred for 4 minutes by two raters blind to group assignment. Assessments were done prior to injury, at 1 and 7 days post operatively (dpo), and weekly thereafter.

**Two-Dimensional Kinematics**

All rats had two-dimensional (2D) kinematic recordings of TM walking before and 3 weeks after SCI. Left HLs were shaved and bony prominences were marked with permanent marker preoperatively. The prominences included the iliac crest, greater trochanter, femoral condyle, lateral malleolus, and head of the fifth metatarsal. A videotape record of quadrupedal locomotion (10-20 step bouts) was collected using a Panasonic WV-CL350 camera (60Hz) with a time-code generator. The same LED light used to synchronize the EMG and digital video records was visible to the analog video camera and was used to synchronize the records. HL kinematic markers were digitized using PEAK
Motus. To account for skin movement of the knee marker, a triangulation program was used to estimate its position (Goslow, Jr. et al., 1973). Actual femur and tibia bone lengths were collected at sacrifice and used with the hip and ankle X, Y positions to derive location. Angular excursions were calculated for the hip, knee and ankle during each phase of quadruped gait: Initial Contact (E1), Yield (E2), Lift Off (E3), and Peak Flexion (F) (Basso et al., 1994). Timing of initial contact along with the LED synchronization light served as the reference times to synchronize EMG and kinematic data. Angle-angle diagrams were constructed by plotting joint excursions (hip-knee or knee-ankle) against one another to assess intralimb coordination.

**Histology**

Rats were perfused with 0.1 M phosphate buffered saline followed by 4% paraformaldehyde. Tissue was collected and cryoprotected in sucrose. The lesion site was transversely sectioned (20um) and stained for myelin using eriochrome cyanine. The section with the largest lesion and least amount of stained white matter represented the lesion epicenter. Area of stained white matter at the epicenter was divided by the total cross sectional area of an uninjured cord at the same vertebral level to serve as a measure of injury severity (Kloos et al., 2005).
**Statistics**

All outcome measures were analyzed compared to naive. Kinematic comparisons were done using a repeated measures ANOVA and Tukey’s post hoc test. Significance observed with BBB scores was determined using a Mann-Whitney U test to account for unequal sample size. Correlations between EMG burst duration, BBB score, and white matter sparing were done using Pearson’s correlation analysis. Significance was set at \( p<0.05 \) and mean ± SEM are shown.

### 2.3 Results

*Residual deficits contribute to a new walking strategy after mild SCI*

Using the BBB scale, spontaneous recovery occurred over 21 days after mild SCI but residual impairments prevented normal locomotion (Figure 2.1). Mild SCI resulted in severe paresis with slight and extensive HL movements 1 day after SCI (Mean BBB=6.83 ± 0.655). Weight supported stepping recovered within 7 days. Despite rapid improvement, recovery plateaued at levels significantly below normal at 21d (Mean BBB=15.75 ± 1.085; \( p<.05 \)). While 1 animal attained near normal locomotion (BBB=19), remaining animals had persistent trunk instability (100%), toe dragging (37.5%), and paw rotation at lift off (100%) or initial contact (37.5%).
Spontaneous recovery occurred in the open field after mild SCI. BBB scores plateaued by 21d and remained significantly lower than control (Mean SCI=15.7 ± 1.085; n=14). Residual deficits at 21d included toe dragging, paw rotation, and trunk instability. Note that controls showed a nonsignificant reduction in performance at 14d due to mild trunk instability. Data are reported as means ± standard error of the mean (Significance determined by a Mann Whitney U Test; p<.05).

Using 2D treadmill kinematics, we quantified the plateaued walking behavior across subphases of locomotion (Figure 2.2;(Basso et al., 1994). Hip movements are biphasic and include two subphases, flexion (F) and extension (E). Knee and ankle movements are more complex and are divided into four subphases (E1, E2, E3, F). The first extension subphase (E1) occurs from peak flexion in swing until initial paw contact on the ground. The E2 subphase, from initial contact through weight acceptance, represents joint flexion during yield and relies on eccentric muscle lengthening. During E3, midstance to lift off, all joints
extend. Lift off to peak flexion represents the flexion (F) subphase. Thus, stance includes E2 and E3 and swing includes F and E1 (Figure 2.2).

**Figure 2.2.** *Stick figure diagrams at the end of each phase of gait illustrate prolonged extension during TM locomotion.* Subphases of locomotion include E1, E2, E3, and F. The third extension phase (E3) occurs from midstance to lift off where knee and ankle extension is greatest. The flexion phase (F) runs from lift off to maximum knee and ankle flexion. The first extension phase (E1) occurs from peak flexion to initial contact. Weight acceptance of the limb results in flexion of the knee and ankle called yield (E2). A caudal shift is evident during all phases of locomotion after SCI (dotted line) compared to normal (solid line). On average, lift off occurs 2.39 cm $\pm$ 0.23 and initial contact occurs 1.24 cm $\pm$ 0.29 more caudal than normal ($p<.01$). Lower toe height occurred at peak flexion (1.88 cm $\pm$ 0.151 SCI, 2.10 cm $\pm$ 0.174 Naïve) and decreased limb advancement during E1 (3.35 cm $\pm$ 0.473 SCI, 5.88 cm $\pm$ 0.488 Naïve; $p<.05$) indicate hypometria during swing. An elevated crest was observed in 60% of animals (5.38 cm $\pm$ 0.29 SCI, 5.20 cm $\pm$ 0.14 Naïve).
Figure 2.3. Angular excursion profiles of hip, knee, and ankle joints. Precise kinematic analysis of joint excursion between different phases of gait reveal altered biomechanics after SCI. Extension of the knee and ankle significantly increased from late stance to midswing (E2-E3, E3-F) and decreased from midswing to midstance (F-E1, E1-E2) during TM locomotion. Hip extension increased relative to naïve and control. (Significance determined by repeated measures ANOVA and Tukey’s post hoc test; p<.05).

After recovery from SCI, the position of the paw relative to the pelvis showed significant caudal displacement during all phases of gait (Figure 2.2). The caudal shift for injured rats (dotted lines) was 2.39 ± 0.23 cm (p<.01) at lift off and 1.24 cm ± 0.29 (p<.01) at initial contact compared to naïve (solid lines; Figure 2.2). During E1, a 43% reduction in forward swing occurred after SCI (3.35cm ± 0.473 SCI; 5.88cm ± 0.488 Naïve; p<.05). This caudal shift was
reflected in significant differences in angular excursion of all HL joints (Figure 2.3). Knee and ankle extension decreased during late swing (E1) and yield (E2) (p<.05). Significantly greater extension occurred in the hip, knee, and ankle during late stance (E3), leading to more excursion during flexion (F) after SCI (p<.05). The increase in flexion was not due to hypermetria since toe height was comparable between SCI and Naive (toe height: 1.88cm ± 0.151 SCI, 2.10cm ± 0.174 Naive; Figure 2.2); rather, greater flexion represented the return from prolonged extension at lift off. At lift off, the pelvis was on average 0.78 cm higher after SCI in 60% of animals. Implantation of EMG electrodes did not affect joint angular excursion (compare Naive and LAM groups, Figure 2.3).

Recovery of intralimb coordination occurs in a proximal to distal manner

To examine coordinated movement between HL joints during locomotion, angle-angle diagrams were constructed by plotting the excursion of one joint against another. Coordination between proximal (hip-knee) or distal (knee-ankle) joints was compared to determine the extent of recovery. Angle-angle diagrams display joint excursion, position of the joints during excursion, and the coordination between joints (Basso et al., 1994). In normal locomotion, a curvilinear shape emerges when one joint moves to a greater extent (more excursion) than the other joint (Figure 2.4). Fine motor control is made evident by fractionated movement, or independent control of joints. Fractionation is most clearly demonstrated in E2, where HL joints are required to flex while another
extends. Intralimb coordination results when a reproducible and precise curvilinear pattern of movement occurs over multiple step cycles.

After recovery from SCI, coordination between distal (knee and ankle) joints is most impaired. Linear rather than curvilinear paths depict poor fractionated joint movements. The linear pattern during stance results from a lack of E2 or yield phase (Figure 2.4). The knee and ankle had equivalent changes in excursion and did not flex or extend in opposition to each other. Proximal coordination between the hip and knee was less impaired but a change in shape and position of the angle-angle plot was apparent (Figure 2.4). A second flexion occurred at the knee during E2 (arrow, Figure 2.4). A double yield was observed in 55% of animals. Prolonged extension is evident by the rightward and upward shift in position of the post op hip-knee and knee-ankle plots. At E3, the hip becomes approximately two times more extended than the knee, demonstrating greater proximal extension (Figure 2.4).
Figure 2.4. Fractionated movement in proximal and distal joints. Angle-angle plots were used to describe intralimb coordination between proximal (hip-knee) or distal (knee-ankle) joints. Naïve plots depict curvilinear patterns between the hip-knee and knee-ankle to indicate normal locomotion. After recovery from SCI, coordination between distal joints is most impaired, as knee-ankle plots depict a linear rather than curvilinear pattern. Hip-knee coordination is less impaired, but shows changes in shape and position. The most obvious change in shape was caused by an additional yield. A double yield was observed between the hip and knee in 54.5% of animals (arrows). A shift in plot position after recovery reflects greater extension, as the hip becomes approximately two times more extended than the knee at E3.

Joint kinematics and timing of muscle activity

In naive animals, TA onset occurs with ankle dorsiflexion while LG onset occurs with plantar flexion before ground contact (Figure 2.5). Both muscles are briefly coactive during terminal swing. TA offset occurs prior to plantar flexion and E1 (mean duration=210.8msec), and LG remains active during stance (mean duration=442.9msec). The dual burst pattern of ST coincides with extension and flexion in the hip and knee. Onset of ST1 occurs during hip extension (mean
duration=156.8msec) and ST2 during knee flexion through weight acceptance (mean duration=248.2msec). The double burst is separated by a brief pause during E1 while the hip flexes and the knee extends in midswing to move the paw forward.

Timing and overall pattern of muscle recruitment changed after injury alongside altered joint kinematics. At the ankle, marked changes were evident compared to naïve that were maintained throughout recovery. At 21d, plantar flexion is absent at the ankle and LG onset instead occurs during a period of prolonged dorsiflexion before ground contact (Figure 2.5). A reduction in burst duration is apparent in both muscles relative to naive- TA (-25.6% ± 7.5); LG (-44.1% ± 12.0). These reductions were independent of recovery in the open field (Figure 2.6).
**Figure 2.5.** Comparison of HL muscle activity with changes in angular kinematics before and after SCI. EMG activity is aligned with kinematics of the hip, knee, and ankle in the same animal before and 21d after mild SCI. The vertical line marks stance onset. Black bars represent an overlay of naïve burst duration and mean onset relative to ground contact. Before injury, TA onset precedes dorsiflexion while the ankle and knee are still extending. LG precedes plantar flexion prior to ground contact. The double burst of ST aligns with hip and knee extension/flexion prior to initial contact. After recovery from SCI, TA and LG show delayed activation and shorter duration relative to naïve. Plantar flexion is absent at the ankle before ground contact. Prolonged dorsiflexion is apparent as TA fires through swing and closer to ground contact. Consequently, LG onset occurs in the absence of plantar flexion and during prolonged dorsiflexion (arrow at ankle). ST2 activation occurs with a second knee flexion before ground contact (arrows at knee) and fires for longer duration after SCI. An absence of E2 in the knee and ankle after SCI may represent variability in step cycles.
Figure 2.6. Activation patterns of the semitendinosus change with recovery.
EMG activity is plotted in the same animal over time. The vertical line marks stance onset. Seven days after SCI, forelimb-hindlimb coordination was unattainable and plantar stepping was not consistent (BBB=12). A loss in a critical reset period between ST1 and ST2 is evident (dashed). ST1 duration (black bar) is noticeably shorter and ST2 is longer (gray bar) after injury. A recovery trend is apparent, but normal bursting does not occur by 21d. With established coordination and higher stepping frequency in the open field by 21d, a reset between ST1 and ST2 emerges and ST2 offset is earlier in stance.

Activity of ST changed over time but did not return to normal by 21d. Early after SCI, with only frequent stepping and no forelimb-hindlimb coordination (BBB=12) at 7d, the dual burst pattern of the ST is lost and only a single prolonged burst occurs. Dual bursts return by 21d when coordination and stepping frequency recover (BBB=15; Figure 2.6). ST1 fires later throughout recovery and occurs ~ 101.9 msec closer to initial contact, and for shorter
duration (-11.3% ± 24.5) compared to naive (Figure 2.7). After recovery, ST2 activation is delayed (35.9 msec) and fires at higher amplitude compared to 7d. There is notable variability in ST2 firing patterns, as ST2 duration was on average +33.6% ± 46.13 longer at 21d (Figure 2.6). In low (BBB=16), but not high performing animals, ST2 activation occurs with knee flexion instead of extension during yield (Figure 2.5). To determine if differences in ST2 duration were linear with recovery, burst durations were normalized (percent change post-injury) and correlated with open field BBB scores. A high correlation between ST2 burst duration and BBB scores ($r^2=0.9697; p<.05$) indicates that smaller changes in burst duration occur in high performing animals (Figure 2.8).
Figure 2.7. *Average burst duration relative to stance onset.* Burst durations were measured relative to stance onset (“0”) and averaged before (solid) and 21d (hatched) after injury. Average EMG onset and offset times are marked by the beginning or end of the shaded regions. Unshaded regions represent the standard deviation of burst onset or offset. TA, LG, and ST1 exhibit shortened burst patterns that occur closer to initial contact after SCI (-25.6% ± 7.5, -44.1% ± 12.0, and -11.3% ± 24.5 reduction respectively). Activity of ST2 shows increased burst duration +33.6% ± 46.13, with an earlier onset and later offset and marked variability between animals.
Figure 2.8. ST2 burst duration predicts recovery in the open field. Normalized burst durations were correlated with BBB scores ranging from 15-19. TA, LG, and ST1 display shortened burst durations relative to normal that do not correlate with open field performance. Variability observed in ST2 duration correlated ($r^2=.9697$) with over ground BBB scores. Longer bursting in ST2 is associated with greater residual deficits in the open field (p<.05; significance determined with Pearson's correlation analyses).

Changes in ST reflect task specificity

To determine if different forms of treadmill locomotion alter muscle recruitment after SCI, we compared flat or 10% downslope grade treadmill walking in the same animals. Similar to 7 and 21d, flat TM walking at 13d showed delayed activation of ST1 and shorter burst durations relative to normal. During flat walking, a single prolonged burst with an indiscriminate reset period occurs in ST and ST2 is negligible (Figure 2.9). Treadmill walking at a downslope grade required a different recruitment pattern that was identified by changes in
the ST. Downslope walking produced later, and less activation of TA for ankle
dorsiflexion and recruitment of LG was unchanged (data not shown). In the ST,
downslope walking re-established a dual burst pattern (Figure 2.9). Notably, ST2
fired at a greater amplitude with a more defined onset/offset period during
downslope walking than flat TM walking (Figure 2.9). While downslope walking
produced a reset period between ST1 and ST2 within the time period described
for Naives, the muscle was not silent.

Figure 2.9. Task specific changes in locomotion alter ST recruitment after mild
SCI. EMG recordings are shown for the same animal as Naive, and 13d after
injury while walking on flat or 10% downhill treadmill surface grades. Stick figure
diagrams at 60Hz show a representative step cycle from the same animal for
each condition. For EMG records, black bars illustrate burst durations during flat
or downhill TM walking. White bars represent a defined reset period between
ST1 and ST2, while hatched regions identify a nondistinct reset. The dotted line
represents peak activation of ST2. Flat TM walking resulted in very little
activation of ST2 after SCI, compared to Naive. During downhill walking, a more
defined reset period between ST1 and ST2 is evident and ST2 fires at greater
amplitude.
Figure 2.10. The extent of open field recovery correlates with white matter sparing. Endpoint BBB scores are plotted against the percentage of spared white matter. $r^2=0.8502$; $p<.01$. Significance determined using Pearson's correlation analysis.

Figure 2.11. Mild contusion injury results in a central core lesion and peripheral rim of spared white matter. Image depicts the injury epicenter of an animal with a final BBB score of 18 and 64.9% white matter sparing.
2.4 Discussion

Overview of the current study

The current work identifies fundamental components of locomotor control that are impaired after recovery from SCI. Despite rapid improvements acutely after injury, deficits persist and normal locomotion does not return by chronic periods. Plateaued walking behavior was characterized by kinematic impairments in yield depicted by a significant decline in angular excursion during the eccentric period of stance (E1-E2). Walking patterns were further characterized by changes in HL muscle recruitment. Delays in activation of knee and ankle muscles occurred during all phases of locomotion. Eccentric actions of the ST (ST2) were notably impaired during yield and significantly correlated with gross open field recovery. Moreover, we found that ST2 activation responds to downslope TM walking after SCI. Our work suggests that the temporal profile of ST serves as a sensitive indicator of gross recovery and that simple changes in locomotor specificity restore its activity.

Locomotion in the naïve rat

To date, few studies have combined EMG and kinematic measures to describe normal locomotion in the rat (Thota et al., 2005; Gruner et al., 1980; Gillis and Biewener, 2001). Even fewer have characterized stepping after SCI (Kaegi et al., 2002; Ballermann et al., 2006). Muscle recruitment patterns and gait biomechanics for quadrupedal locomotion are better defined in feline models
(Buford et al., 1990; Buford and Smith, 1990). Across models, normal gait patterns require eccentric contractions of the hamstrings to slow the HL during the transition from swing to stance (late E1 and E2) and activation of medial and lateral gastrocnemius to dissipate impact forces and facilitate weight acceptance after ground contact. Our assessment of naïve locomotion agrees with work in the rat and cat (Smith et al., 1998; Buford et al., 1990; Buford and Smith, 1990).

We show that peak activation of TA occurs during ankle dorsiflexion and LG during plantarflexion. Similar to what is shown in the cat, a dual burst pattern of ST occurs during hip and knee movements (Smith et al., 1998). The first burst (ST1) starts before liftoff and continues through peak flexion in swing and is separated from the second burst by a reset period during early E1. The second burst (ST2) decelerates the HL prior to ground contact in late E1 and remains active during the E2 yield phase (Figure 2.5).

Changes in neuromotor control after mild SCI

Contusive SCI produces distinct neuropathology with a central core lesion and a peripheral rim of spared white matter that replicates clinical SCI (Bunge et al., 1993). Even with partial sparing of ascending and descending systems, the complex cellular sequellae prevents complete locomotor recovery (Profyris et al., 2004). Previously, we showed that toe dragging, trunk instability and paw rotation was associated with white matter sparing between 25-60% (Kloos et al., 2005). Here, mild contusion with 34-65% sparing not only produced these persistent deficits during open field locomotion but also significant changes in treadmill
kinematics. The new walking pattern included a more caudal limb position during all phases of gait. As a result, joint angular excursions increased from late stance to mid swing (E3, F phases) but decreased from late swing into yield (E1, E2 phases). Thus, it appears that greater excursion is required to overcome the caudal shift in limb position during the propulsive phases of the step cycle. Unlike the cat, greater flexion was not associated with hypermetria since the paw height during swing was normal (Basso et al., 1994). Interestingly, the locomotor phases with prominent joint deceleration and lengthening contractions had below normal excursions. This reduction in kinematics during E1 and E2 may be due in part to aberrant motor control strategies. Indeed, alterations in fine control of intralimb coordination are prominent during E1 and E2 phases for both proximal and distal joints (Figure 2.4). Moreover, a prevalent, almost uniform delay in neural recruitment of distal HL muscles occurred for the TA, LG, and ST1 (+37.1%, +41.04%, +45.1% respectively; Figure 2.7). To our knowledge, we are the first to quantify recruitment latencies after experimental SCI in rats. Contrary to other SCI models, we did not observe increased recruitment of erector spinae musculature (data not shown), nor did we find aberrant co-activation between muscle pairs or across adjacent joints (Ballermann et al., 2006).

_Eccentric motor control is impaired after SCI_

Eccentric motor control is a complex skill that emerges late in development (Enoka, 1996). During an eccentric contraction, the CNS regulates motor neuron activation to produce muscle forces below an external load
resulting in active lengthening. Thus, each lengthening contraction represents the integration of afferent input regarding load and stretch with descending recruitment of motor neurons. Precise CNS modulation prevents muscle spindle-induced stretch reflexes from triggering uncontrolled spasticity after SCI. Other benefits of eccentric contractions include priming the contralateral limb for increased force production, reduced fatigue, and increased metabolic efficiency (Enoka, 1996; Grabiner and Owings, 1999; Lindstedt et al., 2001). While eccentric actions occur in various parts of the step cycle, the clearest and most predominant occurrence is during weight acceptance or yield phase (E2) when ST and other hamstring muscles lengthen to dissipate impact forces. Our finding that eccentric excursion during yield is markedly impaired across the knee and ankle after contusion confirms previous findings in cats with hemisection SCI (Basso et al., 1994). It appears that eccentric control of weight acceptance is negatively impacted after SCI and little recovery occurs regardless of injury mechanism or severity.

The ST has a distinct eccentric period of activation that helps determine CPG-directed locomotion. Activity in the ST reflects the integration of descending motor drive and afferent input from the limb (Pratt et al., 1996). Phasic sensory signals provided by the second, eccentric burst (ST2) appear to be most important given that it is completely abolished by deafferentation in decerebrate cats and is absent in fictive locomotion unless excitatory drugs are applied (Grillner and Zangger, 1984; Grillner and Wallen, 1985). The magnitude
of ST2 activation relates to the rate of knee extension, which suggests that stretch sensitive receptors in ST provide afferent signals to CPGs for locomotion (Wisleder et al., 1990).

We show that recruitment of ST changes over time with recovery. In acute stages, the dual burst pattern in ST is absent (Figure 2.6). A lack in reset between ST1 and ST2 presents a major challenge for a transition to eccentric deceleration in preparation for ground contact. This loss may explain why stepping is not consistent at 7d. The reset between bursts re-emerges alongside greater activation of ST2 by plateau, but normal patterns are not restored. Interestingly, burst onset and duration of ST2 was the most variable between animals (Figure 2.8). Moreover, ST2 activation fails to initiate knee extension before ground contact in low, but not high performing animals (Figure 2.5). Thus, it is possible that the integrative function of ST improves with recovery. To determine if changes in ST were linear with recovery, we compared burst durations of all muscles against open field performance. We found a striking correlation between ST2 duration and BBB scores (Figure 2.8). Walking patterns with refined burst duration and a re-established reset period between ST1 and ST2 occurred in animals with greater recovery in the open field. Our work suggests that the temporal profile of ST2 provides a sensitive indication of the spared motor control after SCI. Activity in ST likely reflects the successful integration of spared descending and afferent-driven signals. Facilitating sensorimotor integration in ST may optimize recovery.
Targeted changes in locomotor specificity restore eccentric control after SCI

Activity in ST reflects task specific changes in locomotion. In the cat, Buford and colleagues show that recruitment of ST changes between forward and backward walking (Buford et al., 1990; Buford and Smith, 1990). Similar to our findings early after SCI (Figure 2.6), backwards walking eliminated dual bursting and instead elicited a prolonged single burst. The author suggests that the single ST burst may reflect a generic pattern that is modulated by afferent input to produce a double burst pattern typical in normal locomotion. Clearly, our findings after SCI suggest a lack of supraspinal control across lumbar CPGs. Whether changes in locomotor specificity facilitate activation across lumbar centers after SCI remains unexplored.

Eccentric actions of the ST are accentuated by changing the grade of the TM belt. Steeper grades of downhill TM walking generate progressively greater activation in both bursts of the ST (Smith et al., 1998). After SCI, we find that downslope walking restores a previously dormant ST2 burst (Figure 2.9). In early stages of recovery, we show that flat TM walking produces a single prolonged burst in the ST. By tilting the TM belt to a downslope grade, the same animal at the same point in time produces a completely new motor pattern. Indeed, downslope walking restored a reset period and produced greater and more defined activation of ST2. Thus, the rat retained the capacity to produce controlled ST activation in a task-specific manner. This effect may not be
observed after more severe lesions, as feline models show an inability to modulate amplitude with slope changes (Brustein and Rossignol, 1998).

Conclusions, limitations and future directions

This study identifies essential features of motor control that do not recover after SCI. Impaired eccentric activity during yield is made evident by changes in kinematics and muscle recruitment. Activity in the ST plays a unique role in locomotor integration and reflects task specificity. Here, we show that impaired actions in ST occur with deficits in yield. Furthermore, we show that improvements in ST functionality indicate the extent of recovery. Whether residual impairments may be resolved after SCI by employing targeted task that accentuate eccentric control remains unexplored and warrants further investigation. Changes in locomotor specificity would provide a simple adaptation for current clinical practice. A limitation to our study is that we could not measure relative amplitude of EMG patterns. Because electrodes were implanted to a chronic time period, we expected exact measurements to be unreliable. In same day recordings (ie, Figure 2.9), interpretations of amplitude are more reliable.
CHAPTER 3

Tissue Sparing After Thoracic SCI Facilitates Spinal Learning that is Regulated by Task-specific Training

3.1 Introduction

Axonal sparing after spinal cord injury (SCI) facilitates neuroplasticity at great distances rostral and caudal to the lesion. Adaptive plasticity facilitates functional recovery and is identified in cortical re-mapping, propriospinal bridging past the lesion, and enhancement of below-level motor capacity (Aguilar et al., 2010; Fawcett, 2009; Dunlop, 2008; Basso et al., 1996). The ability to influence spinal plasticity to promote recovery has greatly improved rehabilitation strategies (Buehner et al., 2012; Harkema et al., 2011; Thompson et al., 2013). Unfortunately, not all plasticity after SCI is beneficial. Indeed, hypersensitization of nociceptive pathways, hyperreflexia, and autonomic dysfunction identify lasting mechanisms of maladaptive plasticity that prevent functional recovery (Detloff et al., 2008; Hains and Waxman, 2006; Rabchevsky, 2006). Of interest is the integrity of remote central pattern generator (CPG) networks that ubiquitously
generate learning and plasticity during locomotion. Whether axonal sparing after SCI facilitates spinal-centric learning in locomotor networks is unknown.

An opportunity to harness spontaneous plasticity to promote motor relearning and recovery is lost early after SCI. We and others have shown that activity-dependent training delivered to the lumbar enlargement early after SCI is ineffective and potentially deleterious (Smith et al., 2009; Hansen et al., 2013). It becomes clear that remote changes in the lumbar microenvironment render locomotor networks refractive to activity-based intervention. Within the first week after SCI, robust inflammatory signaling around locomotor networks likely disrupts neurophysiological mechanisms of synaptic plasticity (Detloff et al., 2008; Hains and Waxman, 2006; Hansen et al., 2013; Yirmiya and Goshen, 2011). Late after SCI, inflammatory signaling resolves and training no longer jeopardizes function but imposes little benefit (Detloff et al., 2008; Hansen et al., 2013). Sparing-induced plasticity of remote networks may display a similar phenomenon over time. After recovery to chronic phases of SCI, isolation of the lumbar cord results in greater motor capacity than normal cord systems (Basso et al., 1996). Thus, recovery in the presence of even a few spared axons greatly influences the segmental plasticity of locomotor networks. It remains unclear whether the influence of sparing on remote learning and plasticity coincides the resolution of inflammation in the lumbar cord.
Task-specific delivery of sensory cues to the lumbar enlargement differentially regulates spinal learning. Segmental lumbar systems learn (and forget) to stand, step, or avoid obstacles based on repetitive afferent signals that are specific to the training task (Hodgson et al., 1994; Edgerton et al., 2004). Eccentric motor actions provide novel sensory cues that may facilitate spinal-centric learning during locomotion. During an eccentric contraction, the CNS regulates motoneuron activation to keep force production below an environmental demand (Enoka, 1996). As a result, active muscle lengthening is determined by precise task-specific feedback. During locomotion, eccentric actions are predominant when slowing the leg before ground contact and during weight acceptance. Tasks like downhill treadmill walking require precise eccentric motor control. After SCI, eccentric actions are limited during locomotion but are restored with downhill treadmill walking (Hansen et al., 2012; Basso et al., 1994; Griffin et al., 1994). Whether task-specific training with targeted-eccentrics can regulate spinal learning and the recovery of locomotion is unknown.

This article evaluates the role of axonal sparing in remote plasticity and spinal learning in the lumbar cord after thoracic SCI. Our study had three objectives. The first was to adopt and validate an instrumental learning paradigm to measure segmental learning and plasticity in the isolated lumbar cord. The second objective was to determine if axonal sparing differentially influences
learning early or late after SCI. The last objective was to determine if task-specific eccentric training regulates learning after recovery from SCI (Figure 3.1).

**Figure 3.1.** A conceptual model of locomotor plasticity after incomplete SCI. After SCI, plasticity in locomotor networks is subjected to conflicting processes that change over time. Axonal sparing facilitates adaptive modulation of lumbar systems that enhance motor capacity (A; Basso et al., 1996). Remote neuroinflammation in the lumbar enlargement create maladaptive sensory and motor plasticity (B; Detloff et al., 2008; Hansen et al., 2013). Locomotor training delivers task-specific sensory cues that generate plasticity in the lumbar enlargement to facilitate recovery (C; Hodgson et al., 1994; Basso and Hansen, 2011).
3.2 Materials and Methods

Subjects and surgeries

Experiments were conducted in accordance with The Ohio State University Institutional Laboratory Animal Care and Use Committee. Ninety-eight adult female Sprague Dawley rats (204-290g, Harlan, Indianapolis, Indiana) were housed 2-3 per cage in a controlled environment (12h light/dark cycle) with ad libitum access to food and water. All subjects were partitioned into three experiments (Figure 3.2). Experiment 1 was conducted in paired Master (n=8) and Yoked (n=8) rats that received a T2 TX and were tested using the instrumental learning paradigm (ILP) at 24h. The Master-Yoked experiment served as a proof-of-principle replication of the spinal learning phenomenon and matched surgical and stimulation methods described by Grau and colleagues (1998). The 24h timepoint represents learning in the normal cord (Grau et al., 1998; Ferguson et al., 2008; Baumbauer et al., 2009). Experiment 2 examined the role of sparing on remote spinal learning. For rats with sparing, we created a severe contusion at T8 that results in ≤10% spared white matter (Kloos et al., 2005; Basso et al., 1996). This severity SCI ensures remote microglial activation and pro-inflammatory cytokine production that is maximal by 7d and resolves by 35d (Detloff et al., 2008). For no-sparing controls, we compared rats with a complete T8 TX. We then tested the segmental lumbar cord in the instrumental learning paradigm at acute (7d) or chronic (42d) time points. The lumbar cord
was isolated from supraspinal input 24h prior to ILP testing using a T2 TX. Thus, to identify the role of acute axonal sparing on lumbar plasticity, we compared learning at 7d in rats that recovered with sparing (AcuteSCI; n=6) to rats that recovered without sparing (AcuteTX; n=6). To identify the role of chronic axonal sparing on lumbar plasticity, we compared learning at 42d in rats that recovered with sparing (ChronicSCI; n=14) to rats that recovered without sparing (ChronicTX; n=5). To examine the influence of time and sparing on remote dendritic spine morphology, sister groups were run for Golgi cox processing without T2 retranssection or testing in the learning paradigm (24h TX, 7d TX, 7d SCI, 7d TX, 42d SCI, 42d TX; n=24). Experiment 3 determined the influence of eccentric task-specific training on spinal learning. All rats recovered with sparing following a severe T8 contusion. Chronic treadmill training was delivered 34-41d at either a 0° flat (ChronicFlat; n=6) or 5.7° downhill (ChronicDH; n=6) surface grade. Lumbar isolation occurred 24h before ILP testing at 42d. To examine the influence of eccentric training on the remote lumbar microenvironment, sister groups were run for ELISA without retranssection or testing in the learning paradigm (ChonicFlat, ChronicDH, ChronicSCI unexercised; n=15).
Experiment 1: Instrumental Learning Paradigm (n=16)
- Master Control vs. Yoked Control (n=8, n=8)
- Complete Transection (n=16)

Experiment 2: Sparing Study (n=55)
- Severe Contusion (SCI) (n=31)
  - Instrumental Learning Paradigm (n=31)
  - Sister Groups for Golgi Stain (n=24)
  - Acute SCI vs. Acute TX (n=6, n=6)
  - Chronic SCI vs. Chronic TX (n=14, n=16)
  - 24h SCI vs. Acute SCI (n=4)
  - 7d SCI vs. Acute SCI (n=4, n=4)

Experiment 3: Training Study (n=27)
- Severe Contusion (n=27)
  - Instrumental Learning Paradigm (n=12)
  - Sister Groups for ELISA (n=15)
  - Chronic Flat or Chronic DH (n=3, n=3)
  - Chronic Unexercised (n=9)
  - Pre-TM (n=2)

Figure 3.2. Experimental designs. Flow diagrams describe group allocation across 3 experiments. Experiment 1 was conducted 24h following a complete T2 TX in Master (controllable) or Yoked (uncontrollable) stimulation paradigms. Experiment 2 determined the influence of sparing on controllable spinal learning at acute (7d) or chronic (42d) time points. The role of sparing was determined in the isolated lumbar cord by comparing severe contusions to time-matched TX controls. Underlying neural substrate was described using Golgi-staining in sister groups. Experiment 3 determined whether task-specific training regulates spinal learning after recovery from severe SCI. Late treadmill training was delivered from 34-41d before lumbar isolation. Inflammatory signaling was described using ELISA in sister groups.
Laminectomy and spinal cord contusions were performed as described previously (Detloff et al., 2008; Hansen et al., 2012). Briefly, rats were anesthetized with a ketamine (80 mg/kg)-xylazine (20 mg/kg) cocktail and given prophylactic antibiotics (gentocin, 1 mg/kg, s.q.). The fur over the thoracic portion of the vertebral column, and the surface of both hindlimbs were shaved prior to surgery. Using aseptic techniques, an anterior-posterior incision was made over the second (T2) or eighth (T8) thoracic vertebrae. Removal of the spinous process and lamina of T8 exposed the dura for contusion injuries. After stabilizing the vertebral column, the Infinite Horizon (IH) device delivered a severe contusion injury. For complete spinal cord transection (TX), visible blood vessels were lightly cauterized, and the spinal cord was treated with lidocaine for 15 seconds before being cut with spring scissors. To ensure that no fibers of passage crossed the lesion site after the cut, the ventral lamina was cauterized along the bone surface. The remaining gap was filled with gelfoam for acute, but not chronic TX studies. The incision was closed in layers and 5cc of sterile saline was given subcutaneously to prevent dehydration. During recovery, rats received antibiotics (1 mg/kg gentocin, s.q.) and saline for 5 days and bladders were manually expressed twice per day until the bladder reflex returned. Vitamin C pellets were given to prevent urinary tract infections (Behrmann et al., 1992). Animals were excluded for behavioral abnormalities prior to surgery (n=1), hit biomechanics (n=2), low bodyweight (n=1), or surgical complications and death (n=1).
**Learning paradigm**

The apparatus used was modeled off of that described by Grau et al. (1998). All subjects were loosely restrained in plexiglass tubes with slots (4cm apart, 1.5cm tube edge) that allowed hindlimbs to hang freely above a plastic container that contained a saline solution. Holes were drilled in the front of the restraint tubes to allow ventilation. A stainless-steel rod served as a contact electrode to monitor leg position. The contact electrode was taped to the plantar surface of the right hindpaw. Heat-shrink tubing electrically insulated the rod from the skin. A wire extended from the contact electrode and was attached to a digital input board monitored by a Dell Latitude D830 computer. A custom script was developed (JAB) to monitor leg position and coordinate controllable or uncontrollable shock delivery.

Two electrodes were inserted into the right hindlimb using fine, teflon-coated, stainless steel wires (Lot#528066, AM Systems, WA). The first electrode was inserted through the skin over the tibia, 1.5cm from tarsus. The second electrode was inserted perpendicular through the body of the tibialis anterior (TA) muscle, 1.7cm above the first electrode. Legshock was applied by delivering a constant 60Hz current from an analog stimulus isolator (Model 220, AM Systems, WA). Stimulus intensity was adjusted for each subject to a level that produced a flexion response between 0.40-0.49N. Flexion force was determined by attaching a monofilament plastic line (25 lb test strength, South Bend) directly
caudal to the lateral malleolus. The end of the line was attached to a strain
gauge (GSO-500-C Compression, Transducer Techniques, CA). To determine
the necessary flexion force, a single 300 ms stimulus was delivered to the TA.
After calibration of flexion force, the monofilament line was removed from the
rat’s paw and the saline solution was adjusted so the contact electrode was
submerged 4mm below the surface of the salt solution (Grau et al., 1998).
During the test period, if the hindlimb extended, the contact electrode touched
the salt-water solution to complete a circuit monitored by a custom script using
Spike2 Software (CED, Cambridge, UK). Completion of the circuit resulted in the
delivery of a stimulus at the predetermined intensity (0.1-3.0 mA). A flexion
response would break the circuit and terminate stimulus. Instrumental training
took place over a 30-minute period. In rats that demonstrated learning, the task
was made more difficult by increasing the water level to 6mm for a 5-minute post-
test.

*Learning measures*

The training period was divided into thirty, one-minute bins to examine
learning behavior. The number of responses, total contact time in/out of water,
and total stimulation time were collected by the computer and separately
averaged across each bin. From these measurements, we identified three
measures of learning: response duration, response number, and stimulation
duration. Response duration calculations were made as described by Grau and
colleagues: response duration = (time out of solution) / (response number + 1).
Response number served as a measure of mistakes, however, a “0” could result from either 60 seconds out or 60 seconds in the saline solution. Stimulation delivery times described the magnitude of the mistake. Stimulus output was determined by the amount of time in the water. A greater mistake identified increased time in the water that produced longer stimulation. Area under the curve was calculated by comparing the proportion of response duration or stimulation time to the overall session duration (60 seconds x 30 minutes).

**Treadmill training paradigm**

To determine if task-specific training differentially regulates spinal learning after recovery from midthoracic SCI, we delivered flat or downhill treadmill training. Treadmill training was delivered in subgroups (n=9 Flat, n=9 Downhill) of rats from 34 - 41d post injury. Eccentric (downhill) treadmill training occurred at a 10% (5.7°) downhill grade as described previously (Hansen et al., 2012). Training consisted of eight consecutive days of manually delivered weight supported stepping during quadrupedal locomotion on a custom-built treadmill (Columbus Instruments). Trunk support and hindlimb stepping was manually assisted as needed to achieve toe clearance and plantar placement of the paw on the treadmill belt (Hutchinson et al., 2004; Hansen et al., 2013). The trunk was maintained in a typical horizontal posture to avoid confounding afferent input to locomotor CPGs (Slawinska et al., 2012). Each session included four, five-minute bouts separated by a twenty-minute rest interval to prevent delayed onset muscle soreness (McHugh, 2003).
**Protein isolation and quantification**

Fresh tissue was collected from the lumbar enlargement at L4-L5 (naïve, 42d-unexercised, 42d-Chronic downhill trained, and 42d-Chronic flat trained; n=15). ELISA was run in replicate groups alongside naïve controls (n=3-4 naives/replicate group). Rats were perfused with sterile saline 1.5 hr after the final training session. Spinal cords were quickly dissected, snap-frozen, and stored in a -80°C freezer until further processing. Segments from L4-L5 were homogenized in RIPA lysis buffer (Pierce) and a protease inhibitor cocktail (Roche). Samples underwent centrifugation at 10,000 rpm for 5 min and protein concentrations were then determined with a BSA protein assay. Quantification of proteins was measured with capture antibodies specific to tumor necrosis factor-alpha (TNFα) or interleukin-1 beta (IL-1β). Bound proteins were detected with a biotinylated detection antibody followed by streptavidin-HRP and then visualized with SuperSignal ELISA Femto Chemiluminescent substrate. The luminescent signal produced by the HRP-catalyzed oxidation of the substrate was measured using the SearchLight Imaging System (Pierce) and protein concentrations were extrapolated from a standard curve using ArrayVision (Pierce). Training data were normalized and expressed throughout as percent unexercised control.

**Dendritic Spine Morphology**

Unperfused fresh tissue was collected from the lumbar enlargement from L3-L6 (Naïve; n=4, 7d SCI, 7d TX, 42d SCI, 42d TX; n=24, Figure 3.2). Tissue
was then processed for Golgi-Cox staining using a Rapid GolgiStain Kit (FD Neurotechnologies). Samples were submerged in Golgi-Cox solution and stored for 14 days in the dark. The tissue was then rapidly frozen in pentobarbital and 200µm coronal sections were sliced on a cryostat and collected on gelatin-coated glass slides. The stain developed in NH4OH for 2-10 min. Finally, slides were dehydrated through a series of graded ethanol washes and cleared with xylene. Slides were coverslipped with Permount and dried in the dark for at least 24h. Images were acquired using brightfield microscopy (Nikon Eclipse E800).

**Locomotor Assessments**

Open Field locomotor recovery was assessed using the Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale (Basso et al., 1995). Scores range from no hindlimb movement (0) to normal locomotor function (21). Joint movement, weight support, plantar stepping, toe clearance, trunk, and tail control were considered for scoring. Two raters blind to group assignment examined the open field activity of each rat for 4 min. Assessments were done prior to injury, at 1 and 7 d post injury and weekly thereafter.

**Two-dimensional Kinematics**

Quantitative metrics of locomotion were examined using two-dimensional (2D) kinematic analysis of treadmill walking as described previously (Basso et al., 1994; Hansen et al., 2012; Hansen et al., 2013). The left hindlimb was shaved and bony prominences were labelled with permanent marker: the iliac crest,
greater trochanter, femoral condyle, lateral malleolus, and head of the fifth metatarsal. All rats had 2D recordings of treadmill walking before (34d) and after (41d) late treadmill training. A video tape record of quadrupedal locomotion (20 steps) was collected using a Panasonic WV-CL350 camera (60 Hz) with a time-code generator. Hindlimb kinematic markers were digitized using PEAK Motus. To account for movement of the knee joint under the skin, a triangulation program was used to estimate joint position (Goslow, Jr. et al., 1973). Femur and tibia bone lengths were collected at sacrifice and used with the hip and ankle X, Y positions to derive location. Angle-angle diagrams were constructed by plotting knee vs ankle joint excursions to assess intralimb coordination.

Assessments of mechanical hypersensitivity

Mechanical withdrawal thresholds were determined using von Frey hair filaments (VFH, Stoelting Co., Wood Dale, Illinois) that were applied to the dorsal surface of the hind paw at the L5 dermatome (Detloff et al., 2012). Rats were acclimated to loose restraint in a towel and positioned so that hind paws rested on a flat surface. Temperature and ground surface were monitored to ensure stability across sessions. Each VFH was applied three times (~30 s apart) in ascending order until 2 positive withdrawals occurred for a given filament (Detloff et al., 2008; Detloff et al., 2010). Testing occurred at naïve, 7, 35, and 41d.
Statistics

All behavioral learning measures were analyzed using ANOVA with Tukey’s post hoc testing. Means and standard error of the mean (SEM) are reported throughout. Protein levels are displayed as percent of unexercised SCI values and analyzed using a one-way ANOVA. A principal component analysis (PCA) was conducted on lumbar-centric variables: sensory (mechanical withdrawal thresholds, stimulus amplitude), motor capacity (pre-TX BBB, post-TX BBB), and learning (response duration and stimulation time). Significance set at p<0.05.

3.3 Results

The instrumental learning paradigm

Isolated from the brain, lumbar segments (L4-S2; Liu et al., 2005) were conditioned to avoid hindpaw stimulation by maintaining ankle flexion. A proof of principle Master-Yoke experiment distinguished learning from adaptation by comparing controllable (response-outcome) and uncontrollable (stimulus-response) conditioning 24h after complete T2 TX. The master rat was able to control whether or not it was stimulated. Each time the ankle was extended, a contact electrode completed a circuit that resulted in a nociceptive stimulus. By flexing the ankle, the stimulus stopped. Essentially, the master rat predicted stimulus delivery based on leg position. Thus, normal spinal learning is identified by maintenance of ankle flexion that is measured by increased response duration
The yoked rat was not able to control whether or not it was stimulated. Flexion of the ankle did not eliminate stimulus delivery. Instead, the yoked rat received uncontrollable stimulation based on delivery times for the master rat. An absence of learning is identified by a reduction in overall response duration (Mean = 6.51 ± 4.21 seconds; 10.76 ± 4.58 %Area under curve; p<0.001 compared to Master). Stimulation parameters are identical between groups (Mean stimulation time for Master and Yoked: 0.35 ± 0.13 seconds; 0.58 ± 0.21% Area Under Curve). Thus, response number does not statistically differ between Master and Yoked groups (Master Mean = 16.52 ± 6.09 responses/bin; Yoked Mean= 6.45 ± 2.75 responses/bin; p=0.154; Figure 3b). These results are comparable to normal spinal learning described by Grau and colleagues (Grau et al., 1998; Crown et al., 2002; Baumbauer et al., 2009; Huie et al., 2012; Ferguson et al., 2008).
Figure 3.3. *The instrumental learning paradigm.* In isolation, lumbar segments learn an instrumental (response-outcome) task. Each time the ankle is extended, a contact electrode completes a circuit that results in nociceptive stimulation. The master rat can control stimulation by flexing the ankle to stop delivery. The yoked rat cannot control stimulation, as it receives the same stimulus parameters as its master. Learning is identified by maintenance of ankle flexion that is measured by increased response duration and decreased response number over time. Only master rats demonstrate learning by increased response duration (A). Yoked rats fail to learn. Identical stimulation parameters result in similar response numbers (B). (*p<0.05 ANOVA)

**Axonal sparing differentially regulates learning early and late after SCI.**

The influence of sparing on spinal learning was determined using a complete or incomplete SCI at T8 before spinal isolation and testing in the learning paradigm. A T2 transection served to isolate lumbar segments for
functional interpretation at early or late time points. Similar to the Master-Yoked experiment, testing in the learning paradigm occurred 24h post T2 transection.

Sparing is insufficient to promote spinal learning early after SCI.

Spinal learning deficits were evident early after SCI regardless of axonal sparing (Figure 3.4A-C). At 7d, AcuteSCI rats displayed similar response durations to AcuteTX rats (AcuteSCI Mean: 28.7 ± 17.7 % area under curve; AcuteTX mean: 22.35 ± 13.6 % area under curve; p=0.78). The number of responses did not differ between groups (AcuteSCI Mean: 99.28 ± 36.8; AcuteTX Mean: 109.28 ± 38.48; p=0.85). To measure the magnitude of the mistakes, we compared the total stimulation time that was allowed during the test period. Stimulation time is determined by the amount of time the contact electrode is in the water. Thus, a greater mistake identifies increased time in the water that produces a longer stimulus. AcuteSCI and AcuteTX groups displayed similar stimulation times (AcuteSCI Mean: 37.35 ± 15.35, AcuteTX Mean: 12.99 ± 5.05 seconds). Response number and stimulation time were significantly greater than normal Master rats in both acute groups (*p<0.05).

Sparing facilitates spinal learning late after SCI.

Sparing that resulted from the same severity SCI allowed near-normal learning later in recovery. At 42d, only rats with sparing demonstrated learning (Figure 3.4D-F). ChronicSCI rats displayed greater response durations than ChronicTX rats (ChronicSCI Mean: 54.32 ± 8.69%, ChronicTX Mean: 17.78 ±
17.59 % area under curve; p<0.02). Response numbers were similar between groups (ChronicSCI Mean: 19.74 ± 8.15%; ChronicTX: 37.24 ± 27.24; p= 0.32). However, the magnitude of the mistake was less in ChronicSCI rats as identified by significantly less stimulation time (ChronicSCI Mean: 9.33 ± 6.08, ChronicTX Mean: 70.39 ± 18.33 % area under curve; *p<0.0001). ChronicSCI rats did not differ from normal Master performance. Response number and stimulation times were significantly greater than normal Master rats for ChronicTX rats (p<0.05).
Figure 3.4. Axonal sparing differentially regulates learning early and late after SCI. Early after SCI, learning impairments are evident in both AcuteSCI rats with sparing and AcuteTX rats without sparing. Measures of response duration do not differ between groups and fall significantly below normal learning (yellow line) (A). Similarly, response number does not differ between groups and is significantly higher than normal learning trends (B). The magnitude of the mistake is identified by increased stimulus delivery during the training period that does not differ between groups (C). The presence of sparing over a longer recovery period promoted learning in the ChronicSCI group compared to the ChronicTX group with no sparing. Sparing-induced improvements in spinal learning are identified by significantly higher response durations (D). Response numbers do not differ between chronic groups (E), however the magnitude of the mistake is significantly less in ChronicSCI rats with sparing (F). In all cases, yellow line denotes normal learning as identified by mean value from Master rats (Figure 3.3). (*p<0.05 ANOVA)
Task-specific treadmill training improves locomotion and regulates spinal learning.

Severe SCI resulted in immediate paralysis in open field locomotion with only slight hindlimb movements at 24h (mean BBB= 0.63 ± 0.16). By 14d, weight support was evident in both groups (mean BBB = 9.33 ± 0.62) and recovery of stepping plateaued between 21-34d (Figure 3.5A). Overall BBB scores did not change after late flat or downhill training delivered 34-41d (Mean change Flat: -0.5 ± 0.3; DH: 0.2 ± 0.2). Training-induced changes in the open field were identified by stepping sub-categories: stepping frequency, toe clearance, and coordination. Downhill training improved stepping categories in 66.6% of trained rats, with minimal deterioration in 1 rat. Flat training both improved and worsened performance in sub-categories in 50% of rats (Figure 3.5B). Two-dimensional treadmill kinematics identified a similar trend. Measures of trunk instability and paw contact time were significantly increased following flat, but not downhill training (Mean Trunk %Change DH: -3.21 ± 5.67; Flat: 32.26 ± 14.28 *p<0.05; Mean %Change Contact time DH: -3.05 ± 4.07; Flat: 14.58 ± 4.85 *p<0.05; Figure 3.5C,D). The proportion of swing that was spent toe dragging was also influenced by training method. Toe dragging was reduced following downhill training but was not altered by flat training (Mean %Change Toe dragging DH: -11.35 ± 4.85; Flat: 3.86 ± 7.73).
Figure 3.5. *Task specific treadmill training improves open field and treadmill locomotion.* Rats recovered over 5 weeks after SCI before lumbar isolation and testing in the learning paradigm (A). After 8 days of downhill training at a chronic timepoint, 66.6% rats had improved stepping parameters in the open field (stepping frequency, toe clearance or coordination). After flat training, stepping parameters deteriorated in 50% of rats (B). Only 1 rat had worsened open field stepping after downhill training. On the treadmill, flat training induced significantly greater trunk instability and total contact time (C,D). Reductions of toe dragging are evident only after downhill training (E). (*p<0.05)

To describe distal motor control between hindlimb joints, kinematic angle-angle diagrams were constructed as described previously (Hansen et al., 2012). Qualitative analysis of angle-angle diagrams includes excursion (pattern location), coordination (curvilinear shape), and reproducibility of the pattern. In normal locomotion, a curvilinear shape identifies reciprocal extension and flexion
between joints to create fractionated movement (Figure 3.6B). A reproducible curvilinear pattern over multiple step cycles demonstrates fine motor control. After recovery from SCI, there are marked reductions in knee extension and an absence of fractionated movement during yield (Figure 3.6C,D). Step cycles also lacked a reproducible pattern between steps. After downhill training, intralimb knee-ankle coordination was improved in 66.6% of rats (Figure 3.6A). No rats had worsened knee-ankle coordination after downhill training (p<0.05 determined using chi square). A reproducible curvilinear pattern identified clear fractionation that was restored during yield (arrow, Figure 3.6C). After flat training, 83.3% of rats displayed worse knee-ankle coordination (p<0.05 determined using chi square). A repeatable coordination strategy was not attainable and fractionation during yield was absent (Figure 3.6C).
Figure 3.6. Task specific training improves fractionated movement in distal joints. Representative angle-angle plots describe intralimb coordination between knee and ankle joints during flat treadmill walking. Qualitative assessments considered joint excursion (pattern location), joint coordination (curvilinear shape), and reproducibility of the pattern (A). Normal locomotion results in a reproducible, curvilinear angle-angle plot between the knee and ankle (B). Precise fractionation is made evident by a curvilinear shape that displays cooperative flexion and extension of the knee and ankle during eccentric yielding (arrow, B). Knee-ankle coordination improved in 66.6% of downhill trained rats, but deteriorated in 83.3% of flat trained rats (A). In downhill trained rats, a reproducible pattern is evident between the knee and ankle (C). Most notably, restoration of a curvilinear pattern identifies better knee-ankle coordination in the eccentric, yield phase of locomotion (C, arrow). In flat trained rats, the new walking strategy is not reproducible and displays less knee extension at lift off (D). (*p<0.05 determined using chi-square)
Task-specific treadmill training produced differential spinal learning trends in the isolated lumbar cord. At 42d, sustained learning was evident in downhill, but not flat trained rats (Figure 3.7). Rats that received eccentric downhill treadmill training showed similar overall response durations to flat trained controls (ChronicDH Mean: 60.67 ± 10.81\%, ChronicFlat Mean: 42.34 ± 16.98 \% area under curve; p=0.38). Response number was also comparable (Chronic DH Mean: 6.07 ± 1.52; Chronic Flat Mean: 6.31 ± 3.54; p=0.95). However, the magnitude of the mistakes significantly differed between groups. Rats that had received downhill treadmill training had less stimulus delivery compared to flat trained rats and did not differ from Master rats (ChronicDH Mean: 0.21 ± 0.05, ChronicFlat Mean: 33.02 ± 12.01 seconds; *p<0.003).
Figure 3.7. Task specific treadmill training differentially regulates spinal learning after SCI. In rats that received downhill training, response duration is sustained above Master-rats throughout the 30-minute test period. Flat trained rats display inconsistent response durations over time (A). Response number is similar in flat and downhill trained groups (B). The magnitude of mistakes significantly increased over time for the flat trained group but were negligible in the downhill group(c). Yellow line denotes normal learning as identified by mean value from Master rats (Figure 3.3). (*p<0.05 ANOVA)
Inflammatory signaling at the site of training-induced neural activity is regulated by task specificity

Remote production of pro-inflammatory cytokines in regions of central pattern generation (L4/5) responds to task-specific treadmill training (Figure 3.8). Downhill training significantly reduced expression of TNFα compared to rats with flat treadmill training (ChronicDH Mean TNFα= 82.1 ± 3.4 %; ChronicFlat Mean TNFα=115.0 ± 10.1 %; data is shown as percent of ChronicSCI Unexercised values). A similar difference was observed in the reduced remote production of IL-1β in rats with downhill training (ChronicDH Mean IL-1β: 77.6 ± 4.34%; ChronicFlat Mean IL-1β: 118.5 ± 10.06 %; p<0.05).

Figure 3.8. Task specific training regulates inflammatory signaling at the site of remote neural activity. Protein assessments of the lumbar enlargement (L4/5) show that chronic downhill (DH) treadmill training attenuates the production of pro-inflammatory TNFα (A) and IL-1β (B). Flat training increases cytokine production. Training data is displayed relative to unexercised SCI controls. (*p<0.05 ANOVA)
**Treadmill kinematics predict segmental spinal learning**

Improved treadmill locomotion identified central learning improvement in rats that received eccentric training (Figure 3.9). We compared kinematic measures of distal motor control with spinal learning measures in ChronicFlat and ChronicDH groups. Downhill training resulted in reductions in toe dragging that significantly correlated with increased response duration in the learning paradigm (p<0.05, Pearsons). Rats with flat treadmill training did not reduce measures of toe dragging that did not correlate with learning performance.

![Figure 3.9](image)

**Figure 3.9. Improvements in toe dragging correspond with better learning in downhill trained rats.** In downhill trained rats, reductions in toe drag kinematics were significantly correlated with greater learning measured with response duration (*p*<0.05, Pearsons). This relationship did not exist for flat trained rats whereby most rats did not learn and had little change in toe drags after treadmill training.

**Multivariate analysis of lumbar-centric functions after SCI**

Pattern-detection of lumbar-centric variables (sensory, motor, learning) was performed using principal components analysis. This method transforms a set of multivariate observations into linear sets of uncorrelated variables,
identified as principal components. The first principal component identifies the maximal variance and the second principal component identifies maximal variance that is not correlated with the first. Our PCA identified two significant (*p<0.001) principal components with eigenvalue>1. PC1 identifies a relationship between sensory (stimulus amplitude post-TX, mechanical withdrawal threshold pre-TX), sparing related motor behavior (BBB scores pre-TX), and learning (stimulus delivery post-TX, response duration) that accounts for 43.97% of the variance in the sample. PC2 identifies a relationship between sensory (mechanical withdrawal threshold pre-TX), segmental motor capacity (BBB scores post-TX), and learning (response duration) that accounts for 22.19% of variance in the sample.
Figure 3.10. Multivariate analysis of lumbar-centric variables. A principal components analysis was used to reduce data correlations into multivariable components (PC1, PC2) determined by eigenvalue. Each component reflects variance that is shared by outcome measures: sensory (mechanical withdrawal thresholds pre-TX, stimulus amplitude post-TX), motor (BBB scores pre-TX, BBB scores post-TX), and learning (response duration, stimulation time). PC1 and PC2 accounted for 66.25% of the variance in the sample (43.97% and 22.19%, respectively; p<0.001). Each arrow value represents a Pearson correlation between individual outcomes and the principal component. The magnitude of the correlation is represented by arrow width (high/low) and color (green is positive correlation and red is negative correlation).
Recovery in the absence of sparing increases dendritic spine density in lumbar networks

Qualitative assessments of dendritic spine morphology identify aberrant spine formation in the lumbar cord (L3-L6) of rats that recover from SCI without sparing. Intermediate interneuron dendrites display thin branches with regularly spaced, thin spines in naïve rats (Figure 3.11). Ventral horn motoneurons show larger branches and thin spines. Alternatively, ChronicTX rats show thicker dendritic branches and increased spine density in both interneuron and motoneurons of the lumbar cord.
Figure 3.11. Dendritic spine densities increase on lumbar neurons in the absence of sparing. Golgi-cox staining labels neurons and glia at L3-6 in Naïve and ChronicTX rats. Increased spine densities are evident after recovery without sparing in intermediate laminae interneurons compared to naïve controls (A). Dendritic branches are wider and display more dense spine expression than naives (B; arrows).
3.4 Discussion

This is the first study to systematically examine whether sparing after incomplete SCI facilitates spinal learning in the lumbar enlargement. Our work shows that early after SCI, sparing is insufficient to promote learning. However, exposure to spared axons for six weeks produced near-normal learning in the lumbar cord. Importantly, we found that eccentric task-specific treadmill training regulates spinal learning after recovery from SCI. Together, our experiments identify a time dependent interaction between lesion-site sparing and task-specific plasticity in locomotor networks that influences the efficacy of rehabilitation.

Axonal sparing after SCI facilitates motor capacity in remote locomotor networks. This was first evidenced when isolation of the lumbar enlargement late after recovery from an incomplete SCI generated greater behavioral output than TX alone (Basso et al., 1996). Similar work in the opossum showed that regrowth of a small complement of descending axons after a neonatal lesion modifies segmental lumbar networks to generate near-normal locomotion after retransection (Wang et al., 1998). Likewise, we found that sparing facilitates segmental learning in the lumbar cord but the degree of improvement depends on time post injury. Early after SCI, sparing-induced improvements in learning are negligible. However, recovery in the presence of even a few spared axons
produces plasticity in locomotor networks that allows significantly greater learning to occur late after SCI. When compared to time-matched TX controls without sparing, no learning occurred. These experiments identify a critical interaction between remote plasticity and injury progression. We suggest that resolution of below-level injury processes late after SCI allow spared axons to elicit adaptive plasticity in the lumbar enlargement (Figure 3.1).

Several remote injury processes may undermine sparing-induced plasticity and learning in locomotor networks. Increasing evidence shows that remote inflammation after thoracic SCI jeopardizes sensory and motor systems (Detloff et al., 2008; Hains and Waxman, 2006). Early after SCI, neurovascular and glial reactivity around putative CPG networks prevent motor relearning and recovery (Hansen et al., 2013; Smith et al., 2009). Of interest is remote signaling in pro-inflammatory cytokine pathways that predict below-level mechanical hypersensitivity. Expression of both TNFα and IL-1β are maximal in the lumbar enlargement at 7d after thoracic SCI and return to homeostatic levels by 35d (Detloff et al., 2008). Indeed, both TNFα and IL-1β impede spinal learning in isolated preparations (Huie et al., 2012; Young et al., 2007). Mechanistic evaluations of TNFα and downstream targets identify maladaptive synaptic plasticity that results from AMPA receptor overexpression and aberrant excitability (Beattie et al., 2002; Huie et al., 2012). Thus, the resolution of remote neuroinflammation after SCI appears to coincide with sparing-induced facilitation of learning. We suggest that the remote microenvironment early after SCI
creates maladaptive synaptic plasticity that prevents learning. Our finding of superior learning at 42d in rats with sparing demonstrates a natural period of efficacy for activity-based intervention. However, combinatorial strategies that reduce early cytokine signaling in the lumbar enlargement will likely harness a period of reactive plasticity that is unattainable with a late intervention (Hansen et al., 2013).

Our findings of impaired learning early but not late after SCI may describe an interaction between sparing and the resolution of spinal shock. Originally termed diaschisis in 1914, this response is characterized by an ephemeral suppression of below-level reflexes that gradually return and often display an exaggerated response or hyperreflexia (von Monakow, 1969; Finger et al., 2004). Only recently has the timecourse and some of the underlying mechanisms been identified (DiTunno et al., 2004; Silver, 2005). In our model, we show that a uniform depression of below level reflexes does not adequately describe the condition. Instead, we suggest that spinal shock may describe a transient impairment of more complex circuitry. Across experiments, we found that suppression of the flexor withdrawal reflex did not occur at any time point following complete or incomplete SCI. We did, however, see differences in the learned modulation of this reflex pathway. In the absence of sparing, the flexor withdrawal reflex is maintained early and late after SCI but fails to produce learning. When sparing is maintained, considerable reflex-based learning occurs late after SCI. Thus, it is likely that the influence of spinal shock extends to the
ability of locomotor networks to integrate sensory and motor signals to generate learning. We show that over time, even partial sparing of ascending and descending systems allow substantial learned modulation of reflex pathways.

An important finding from our study was that task-specific treadmill training differentially regulates spinal learning and recovery after SCI. Task specific sensory cues preferentially activate CPG networks to initiate locomotion (Hodgson et al., 1994). Even modest task alterations elicit new motor control strategies. Downhill treadmill walking requires eccentric motor control that precisely integrates sensory and motor signals during locomotion (Smith et al., 1998). After recovery from SCI, eccentric motor control is severely limited but is restored during downhill treadmill walking (Hansen et al., 2012). Here, only eight days of downhill treadmill training improved walking that had previously plateaued. In fact, improvements in locomotion predicted central learning trends. Maintenance of learning was only evident in the lumbar cord of rats with downhill training compared to flat trained controls. Thus, it appears that late after SCI, potential exists to harness ascending and descending substrates to promote task-specific learning in locomotor networks. It is further possible that improved central integration after downhill training will reflect learned modulation of reflex gains in spasticity (Thompson et al., 2013). Eccentric treadmill training provides a simple adaptation for clinical rehabilitation that may greatly enhance function.
Interestingly, we found that training-induced activity in locomotor networks altered local inflammatory signaling. However, this was not a uniform finding. In our downhill paradigm, our work suggests that sensory cues provided by predominantly eccentric actions attenuate TNFα and IL-1β production and improve learning in the lumbar cord. Flat treadmill training increased cytokine production. While the systemic influence of exercise on inflammation has been well established (Woods et al., 2009), task-specific regulation of cytokine pathways within the CNS has received little attention. Recent work suggests that sensory neurons are novel regulators of immunity (Tracey, 2011; Olofsson et al., 2012). It may be possible that differential activation patterns of dorsal horn neurons in the spinal cord regulate cytokine signaling. We found a similar relationship in the task-specific regulation of trophic factors (Hutchinson et al., 2004). The cellular mechanisms involved in task-specific modulation of cytokine production are largely speculative. It becomes clear, however, that combinatorial therapies must consider the type of neural activity that is delivered and how it interacts with the local microenvironment.

Our multivariate principal component analysis examined three sets of lumbar-centric variables: sensory (stimulation amplitude, mechanical withdrawal threshold), motor capacity (BBB scores pre-TX, BBB scores post-TX), and learning (response duration, stimulation delivery). The pattern detection showed that the largest variance in the sample was accounted for by sensory and learning trends that correlated with motor behavior before lumbar isolation (PC1).
This suggests a positive relationship between axonal sparing and tactile sensitivity on spinal learning. After lumbar isolation, BBB scores corresponded with higher sensory thresholds that negatively relate to learning performance (PC2). It appears that the second component suggests a segmental relationship: greater motor output and dorsal horn sensitivity conjunctively prevents learning. Thus, sparing induced plasticity of lumbar networks may generate a new sensory–motor threshold for segmental learning. It is possible that maladaptive plasticity in afferent pathways may elicit aberrant motor responses similar to hyperreflexia that are not conducive to learning. Taken together, our model suggests that the presence of even a few spared axons differentially regulates the role of sensory and motor systems in learning.

It may be possible to examine neural substrates that prevent spinal learning by examining dendrite morphology. In the intact spinal cord, motoneurons and interneurons receive continuous excitation or inhibition from descending supraspinal axons (DiTunno et al., 2004). After SCI, a loss of descending modulation creates a state of disorder in remote CPG interneurons. As a result, competitive sprouting occurs in locomotor networks that can produce adaptive or maladaptive functional plasticity (Murray and Goldberger, 1974). Sprouting and new synapse formation may identify a process that leads to maladaptive hyperreflexia. Indeed, increased synthesis of excitatory glutamate receptors occurs in caudal motoneurons after SCI (Grossman et al., 2001; Grossman et al., 2000). Increases in receptor expression are typically
accompanied by increases in dendritic spine density (Matsuzaki et al., 2001). Preliminary data shows that late after complete transection, increases in both ventral and intermediate-laminae neuron spine density are evident compared to naïve controls (Figure 3.11). Thus, we may identify a critical substrate of plasticity that initiates hyperexcitability and hyperreflexia but undermines learning.

Conclusions, Limitations, and Future Directions

A limitation to our study is that we cannot discount the influence of muscle fatigue on learning. Similar to all behavioral evaluations after SCI, it is possible that peripheral changes in muscle phenotype can secondarily influence learning. Previous work in our lab showed a significant decrease in wet weight that occurs in the tibialis anterior at 7d (Hutchinson et al., 2001). After recovery to chronic time points, muscle wet weights return to baseline levels. In treadmill training groups, muscle wet weights do not differ from unexercised controls (data not shown). Nevertheless, our stimulus paradigm used a square-wave pulse that was delivered at thresholds known to selectively recruit nociceptive (a-δ and C) fibers and produce a well-characterized flexor-withdrawal response (Mouraux et al., 2010; Grau et al., 1998). Stimulus delivery did not directly recruit efferent motor pathways that may have undergone phenotypic alterations in contractile properties as a result of SCI.
Our work identifies a time-dependent interaction between spared sensory and motor systems that influence spinal centric learning. It becomes clear that competing forms of adaptive and maladaptive processes in the lumbar enlargement influence plasticity. Early after SCI, this interaction renders locomotor networks refractive to learning. Therapeutic strategies that attenuate early inflammatory signaling in locomotor networks may harness reactive plasticity to promote learning and recovery. Late after SCI, recovery in the presence of even a few spared axons induces plasticity that is permissive for learning. Importantly, task-specific training can be used to regulate local inflammatory signaling and improve spinal centric learning and recovery. Future studies will determine if eccentric training results in central improvements that facilitate learned modulation of reflex gains during locomotion.
4.1 Introduction

A loss in mobility is one of the most noticeable and debilitating consequences of spinal cord injury (SCI). Activity-dependent tasks like treadmill training can harness endogenous spinal plasticity to promote motor relearning and recovery after injury (Hodgson et al., 1994; Basso and Hansen, 2011). However, despite modest improvements with treadmill training in the clinic, deficits persist and complete recovery is rare (Buehner et al., 2012; Harkema et al., 2012). The reasons for limited improvements are poorly understood. We theorize that the efficacy of training is related to interactions between the timing of training and the local microenvironment at the site of training-induced neural activity. Previous studies have defined a robust period of plasticity early after injury, comprised of structural and synaptic changes throughout the neuroaxis.
Delivering locomotor training when plasticity is primed has the potential to produce greater functional improvement. Surprisingly, some forms of early exercise instead prove detrimental to recovery, possibly via mechanisms that disrupt neurovascular integrity (Smith et al., 2009; Kozłowski et al., 1996; Maldonado et al., 2008; Griesbach et al., 2007).

Neuroinflammation is a known impediment to spinal learning and plasticity (Yirmiya and Goshen, 2011; Vichaya et al., 2009; Huie et al., 2012). Glial reactivity and production of inflammatory signaling molecules prevent synaptic plasticity and molecular mechanisms of learning in the hippocampus (Yirmiya and Goshen, 2011). After rat SCI, we showed that activated microglia and cytokine expression extends caudal to the lesion at least 10 segments to the lumbar enlargement and contributes to sensory dysfunction but the effects on spinal centric learning are unknown (Detloff et al., 2008). Changes in extracellular matrix composition in the lumbar enlargement after SCI also identify an inhibitory microenvironment for plasticity in locomotor interneuron networks (Andrews et al., 2012). Matrix metalloproteinases (MMPs) regulate diverse functions, including tissue remodeling, inflammation and learning (Ethell and Ethell, 2007; Zhang et al., 2011). In particular, the gelatinase MMP-9 amplifies proinflammatory cytokine production, increases blood spinal cord barrier (BSCB) permeability, and regulates synaptic long term potentiation (LTP) (Noble et al., 2002; Kawasaki et al., 2008; Nagy et al., 2006). MMP-9 is produced by various cell types including glial cells, vascular endothelia, and leukocytes at the lesion
site in rodent and human SCI (Zhang et al., 2011; Buss et al., 2007). If MMP-9 is produced in remote lumbar regions after SCI, it may contribute to an inhibitory microenvironment and interfere with plasticity and recovery of function even when treadmill training is delivered.

Here we hypothesize a remote production of MMP-9 after T9 SCI impairs the effectiveness of motor relearning and recovery of function. We present the first evidence of MMP-9 upregulation in the lumbar enlargement, which results in remote inflammation during the first week after midthoracic SCI in C57BL/6 mice. Lumbar-focused treadmill training administered during this early period impaired locomotor recovery and resulted in greater deficits in wild-type (WT) mice, while robust training-induced recovery occurred in MMP-9 null (KO) mice. Such findings support a time-sensitive adverse interaction between MMP-9 and treadmill training that influences recovery.

4.2 Materials and Methods

Subjects and surgeries

Experiments were conducted in accordance with The Ohio State University Institutional Laboratory Animal Care and Use Committee. Adult (3-4 months of age) female MMP-9 null (B6.FVB(Cg)-Mmp9<sup>tm1Tvu</sup>/J) and C57BL/6J wild-type (WT) mice were obtained from Jackson Laboratories. The MMP-9 null mouse shows a mild delay in bone formation (Vu et al., 1998), which was
accounted for in kinematic assessments by collecting actual femur and tibia bone lengths at the time of sacrifice. Laminectomy and spinal cord contusions were performed as described previously (Jakeman et al., 2000; Kigerl et al., 2006). Briefly, mice were anesthetized with a ketamine (138 mg/kg)-xylazine (20 mg/kg) cocktail and given prophylactic antibiotics (gentocin, 1mg/kg). Using aseptic techniques, removal of the spinous process and lamina of T9 exposed the dura. After stabilizing the vertebral column, the Infinite Horizon (IH) device delivered 75 kilodynes of force to induce a severe contusion injury. Biomechanics of the injury were screened on day 0, and outlier forces or displacements were excluded (n=8; Figure 4.1). Force and displacement trends were similar to those established by Ghasemlou and colleagues (2005) (mean force: 77 ± .2; mean displacement: 718 ± 19.9). The incision was closed in layers and 2cc of sterile saline was given subcutaneously to prevent dehydration. During recovery, mice received antibiotics (1mg/kg gentocin, s.q.) and saline for 5 days and bladders were manually expressed twice per day until tissue harvest (Hoschouer et al., 2010). Based on previous experience, BMS scores were screened on day 1 to be less than 0.5 for training studies, and 2.5 or less for ELISA or histology studies. Any outliers were removed from the study (n=4). Disposition of all mice entered into the study is shown in Figure 4.1. Randomized group assignment and blinded behavioral assessments occurred.
Figure 4.1. Flow diagram of experimental enrollment, screening, and group allocation. To reduce risk of spontaneous recovery, we applied stringent exclusion criteria to training and behavioral studies. For early assessments of recovery (7d), pre-trained LTT groups were combined with unexercised controls for independent statistical comparisons.

Protein isolation and quantification

Fresh tissue was harvested from the lumbar enlargement (Naïve, 1, 2, 3, 7, and 9 days post-injury; n=48) of unexercised KO and WT mice. ELISA was run in replicate groups alongside naïve controls (n=3-5/SCI groups, n=2-3 naives/replicate group). Mice were perfused with sterile saline, and spinal cords were quickly dissected, snap frozen, and stored at -80°C. Segments from L4-5
were homogenized in RIPA lysis buffer (Pierce) and protease inhibitor cocktail (Roche). After centrifugation at 10,000 rpm for 5 min, protein concentrations were determined by a BSA protein assay. Quantification of matrix and cytokine proteins was determined using a custom SearchLight Multiplex ELISA Array and performed by Aushon Biosystems (Billerica, MA, USA). Custom arrays were spotted with capture antibodies specific to tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), MMP-2, MMP-3, and MMP-9. The bound proteins were then detected with a biotinylated detection antibody, followed by streptavidin-horseradish peroxidase (HRP) then were visualized with SuperSignal ELISA Femto Chemiluminescent substrate. The luminescent signal produced from the HRP-catalyzed oxidation of the substrate was measured using the SearchLight Imaging System (Pierce) and protein concentrations extrapolated from a standard curve using ArrayVision (Pierce). All protein levels were analyzed relative to uninjured WT controls and are expressed throughout as percent naïve.

**In situ gelatinase zymography**

*In situ* zymography was used to detect and localize gelatinase activity in fresh tissue sections (Oh et al., 1999; Noble et al., 2002; Goussev et al., 2003). Segments from L1-3 from the same mice used for ELISA (n=48 detailed above) were then blocked and cut in 20um transverse cryostat sections for *in situ* gelatinase zymography. Sections were incubated in 0.05 M Tris-HCL, 0.15 M NaCl, 5 mM CaCl2, and 0.2 mM NaN3, pH 7.6, containing 40 ug of FITC-labeled gelatin (Molecular Probes), at 37°C for 1 hour. The gelatin is tagged to a peptide
that fluoresces when cleaved by gelatinolytic activity. This reaction was then visualized with fluorescent microscopy (Nikon Eclipse E800 B-2E/C FITC filter). This assay localizes gelatinase activity but does not distinguish between members. Thus, we used ELISA in the same mice to distinguish in situ activity MMP-2 and MMP-9. Measurements of gelatinase positive vascular-like structures were made after identifying the largest stained structure in each section and quantitatively measuring width and length using Stereoinvestigator (MBF Biosciences).

Gelatin zymography.

Gelatin zymography was used to detect MMP-2 and MMP-9 in the remote lumbar cord after T9 SCI. Trained and unexercised WT mice were sacrificed at 9dpi (n=3/group) and segments from L4-5 were collected and quick-frozen at -80°C. Epicenter tissue at 24h served as a positive control (n=3). Each sample was homogenized in lysis buffer containing 50mM Tris-HCl, pH 8.0, 150mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS. After centrifugation, supernatants were collected, and concentrated by a Microcon filter (50K Membrane, Millipore). Equal amounts of protein were loaded on a 10% zymogram gel. After electrophoresis, the gel was incubated with renaturing buffer (Bio-Rad Laboratories) at room temperature for 30 min to restore the gelatinolytic activity of the proteins, then incubated with developing buffer (Bio-Rad Laboratories) at 37°C for 48 h. The gel was stained with Coomassie Blue and destained until clear bands became evident.
**Histology**

WT (n= 11) and MMP-9 null mice (n=3) were transcardially perfused with 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (pH 7.2), i.e., naive (n=3 WT) at 1 (n=4 WT), 7 days post injury (dpi) (n=4 WT, n=3 KO Figure 4.1). Spinal cord segments at the T9 lesion site and from spinal levels L1-L5 were post-fixed for 1h in 4% paraformaldehyde, rinsed overnight in 0.2M phosphate buffer (PB, pH 7.4) then cryoprotected in 30% sucrose before being embedded in M-1 Embedding Matrix (Thermoscientific) and frozen on dry ice (Detloff et al., 2008; Ma et al., 2004; Ma et al., 2001; Basso et al., 2006). Epicenter and lumbar blocks were sectioned in their entirety at 20 um on a Microm HM505E cryostat and collected in series of equally spaced sections on ColorFrost Plus slides (Fisher Scientific).

Fluorescent immunohistochemistry was performed to identify microglia and macrophages in L1-L2 transverse spinal cord sections of lumbar segments. A 1:200 dilution was used for Rabbit anti Iba-1 (Wako, 019-19741). The antibody was prepared in blocking solution containing 1%BSA, 0.1%FG, 3%NGS, 0.2%Tx-100 in PBS. Incubation of the primary antibody occurred overnight at 4°C. A goat anti-rabbit secondary antibody was used at a 1:400 dilution (AlexaFluor 488; Molecular Probes, A21103). Final detection of signal was visualized under fluorescence. For all staining, control sections were processed by eliminating the primary antibody and replacing with blocking solution to ensure
positive labeling. Sets from L1-L2 were stained for all groups. Sections were imaged using a Zeiss 510 Laser Confocal Microscope (The Ohio State University Confocal Microscopy Imaging Facility).

To quantify phenotypic changes in microglia and macrophages, digital image analysis of Iba-1 staining was performed on representative sections in the L1-L2 region of the remote spinal cord (Donnelly et al., 2009). Thresholds for positive staining were performed by a blinded individual. Images were then processed by densitometric scanning of threshold targets using ImageJ software (Wayne Rasband, ImageJ, NIH). Proportional area is reported as the average percent area in the positive threshold for all pictures. To quantify morphological changes in microglia cell body sizes, ImageJ was used to measure cell body width in the same sections used for proportional area measurement. Width measurements were made in each section by randomly choosing 10 microglia throughout the gray matter (4 dorsal horn, 2 intermediate laminae, 4 ventral horn).

The lesion site was transversely sectioned (20um) and every 10th section was stained for myelin using eriochrome cyanine and differentiated with 5% iron alum and borax ferricyanide solutions (Rabchevsky et al., 2001). The lesion epicenter was identified as the section with the largest central core lesion and least amount of stained myelin. Spared white matter or lesioned tissue areas were measured with point counting methods using Stereoinvestigator software.
(Microbrightfield, Burlington, VT). A grid was randomly placed on each spinal cord section and located intersections on spared white matter, spared gray matter, and lesioned tissue. Each intersection counted as a point (p). Point counts for each section were converted to estimated area using the equation: estimated area=($\sum P$) * (a/p). $\sum P$ is the sum of the points for an individual mouse and a/p is the area of each point. Area of stained white matter at the epicenter was divided by total cross sectional area to serve as a measure of injury severity.

Training paradigm

Treadmill training was delivered in subgroups of mice at early (ETT ; 2 - 9dpi; n= 9 KO, n=6 WT) or late (LTT ; 35 - 42dpi; n=6 KO, n=5 WT) timepoints alongside unexercised controls (n=4 KO, and n=5 WT). LTT mice served as unexercised controls at the 7d timepoint (total n=10 KO Unex, n=10 WT Unex). An independent group of Naïve controls (n=10) were used for kinematic comparisons. Training consisted of eight consecutive days of manually delivered weight supported stepping during quadrupedal locomotion on a custom-built treadmill (Columbus Instruments). Hindlimb (HL) stepping was manually assisted as needed using small rounded pestles to achieve toe clearance and plantar placement of the paw on the treadmill belt (Hutchinson et al., 2004). Adjustable harnesses provided partial body weight support while maintaining the trunk in a typical horizontal murine posture (Figure 4.6). Each session included two 10-minute bouts separated by a 20-minute rest interval to prevent delayed onset muscle soreness (McHugh, 2003).
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**Table 4.1. Relevance of behavioral strategies.**
Locomotor Assessments

Open Field Locomotor recovery was assessed using the Basso Mouse Scale for Locomotion (BMS) by two raters blind to group assignment (Basso et al., 2006). Rating criteria considered hindlimb (HL) joint movement, weight support, plantar stepping, coordination, paw position, and trunk and tail control. Scores range from no HL movement (0) to normal locomotor function (9). The rationale for selection of each behavioral outcome measures is discussed in Table 1.

Gridwalk

A 2.54cm square metal grid apparatus (41x28x36mm) was used to measure precise paw placement during forward locomotion. Mice were acclimated to the grid pre-operatively, and then tested at 35 and 42d. Stepping performance was analyzed for 5 volitional passes. Passes were recorded with a digital camcorder (Sony Hancam HDR SR11) and then analyzed in slow motion. Success was determined by a weight-supported step from rung to rung. The number of HL stepping failures are expressed relative to the number of forelimb steps and shown as percent success.

Two-Dimensional Kinematics

All mice had two-dimensional kinematic analysis of walking using a novel application for mice based on established criteria for rat and feline models.
Kinematic assessments were done for naïve animals and at 7, 35, and 42dpi. Retention of training effects was determined in early treadmill (ETT) trained groups by evaluating locomotor function 4 weeks after training ended (35dpi). To demarcate the HL joints pre injury, mice were briefly anesthetized with 2% isofluorane, the left HLs were shaved and bony prominences were identified by palpation. Appropriate marker placement was later confirmed during locomotion. We used depilatory cream to remove hair and maintain marker locations for the study duration. Marked prominences included the iliac crest, greater trochanter, femoral condyle, lateral malleolus, and head of the fifth metatarsal (Figure 4.6). Treadmill locomotion was captured using a Panasonic WV-CL350 camera (60Hz) with a time-code generator. Kinematic markers were digitized using PEAK Motus. To account for movement of the knee joint under the skin during locomotion, a triangulation program was used to estimate its position (Goslow, et al., 1973) from actual femur and tibia bone lengths were collected at sacrifice. Trunk instability was determined by calculating maximal change in vertical displacement of the iliac crest during each step. Toe dragging was measured by the difference in time between initial forward movement of the toe and visual confirmation of lift off from the treadmill belt. Total time in swing and stance was calculated from visually confirmed lift off and initial contact from video. The relevance and application of kinematic measures are described in Table 1. These assessments were averaged over a total of 20 steps. Animals that failed to produce a minimum of 18 plantar steps on the treadmill were statistically penalized for nonperformance.
Statistics

All behavioral outcome measures were analyzed using MANOVA (ETT) with Dunnett’s post-hoc testing or repeated measures (LTT) ANOVA with Tukey’s post hoc testing. Means and standard error of the mean (SEM) are reported throughout. For kinematic data collection, not all mice were able to perform the task (ie dragged or dorsal stepped). Nonperformance was corrected by assigning the maximal kinematic value plus one standard deviation from the mean or “0”. Group comparisons for open field plantar stepping and weight support were made using a Chi square analysis. Protein levels are displayed as percent naïve and were analyzed using a one-way ANOVA. When appropriate, differences between protein measures were evaluated with a Students t-test. Correlations between white matter sparing and proportional area of iba1-labeling were made using Pearson’s correlation analysis. Significance set at p<.05.

4.3 Results

Acute injury mechanisms extend to the lumbar enlargement after thoracic SCI.

Thoracic spinal cord contusion results in increased TNFα and metalloproteinase expression in the lumbar enlargement (L4/5) in wild type mice during acute stages following injury (Figure 4.2). Within 24 hr, pro-gelatinase MMP-3 protein was significantly elevated (423.9% ±105.9 vs Naive; p<.001) but returned to basal levels by 2 dpi. In contrast, MMP-9 expression reached a more than a 5-fold increase at 7dpi and persisted at 9dpi (526.4% ±73.3 and 547.2%
±144.1 respectively vs Naive; p<.001), a time when TNFα expression is also increased. Gelatin zymograms confirm lumbar expression of pro-MMP-9 at 9d (Figure 4.7c). Protein expression of the pro-inflammatory cytokine, TNFα, reached a nearly 2-fold increase by 9dpi (162.1% ± 86.3 vs. Naïve; p<.05), while levels of IL-1β remained at baseline in the lumbar enlargement during the first week after SCI (Figure 4.2B).
Figure 4.2. Injury mechanisms extend to the lumbar enlargement during acute stages of SCI. Protein analyses reveal increased metalloproteinase and cytokine expression in the lumbar enlargement (L4-5) of wild type mice over the first week after SCI (n=3-5/group). A) Within 24 hours, MMP-3 was significantly upregulated (p<.001 compared to Naive) but returned to baseline by 2 dpi. MMP-9 reached peak values 7-9dpi (547.2%; p<.001 compared to Naive). MMP-2 protein was unchanged. B) TNFα expression was increased at 9dpi (p<.002 compared to Naive), while IL-1β was unchanged. In situ gelatinase zymography localized gelatinase activity around microvascular endothelia in lumbar segments. Sparse labeling of cellular structures is evident in the naïve cord (C). After SCI, there is pronounced gelatinase activity around vascular-like phenotypes within 24h (D,H) and remains apparent at 3, 7 and 9dpi(E,F,G). Scale bar is 130 um in C,D, E, F,G and 32 um in H.

In situ gelatinase zymography localized gelatinolytic activity in tissue sections from L1-3 blocks of the same lumbar spinal cords used for ELISA. In the uninjured spinal cord, low gelatinase activity was observed in the meninges
and sparse labeling of vascular-like structures corresponding to locations reported previously (Figure 4.2C; Noble et al., 2002; Goussev et al., 2003). Within 24h after SCI, a pronounced increase in gelatinase activity was evident in similar structures in the lumbar cord (Figure 4.2D), which remained elevated at 3, 7 and 9dpi (Figure 4.2 E-G). The number of branches and width of vessels labeled with gelatinase activity was greatest in the lumbar cord at 3dpi (mean branches: 5.33 ± 1.86; mean width: 14.5 ± 2.86 um; p<.05 compared to Naïve mean branches: 1.0 ± .58; mean width: 6.2 ± 1.1um).

WT mice showed robust and rapid microglial activation in the lumbar enlargement after thoracic SCI. Within 24 hr, iba1-labeled microglia displayed significantly larger cell bodies (mean width: 19.2um ±1.7 at 24h WT compared to 7.73um ± 0.53 in Naive; p<.001) and a bushy phenotype (Figure 4.3) similar to those described previously (Soltys et al., 2001). By 7dpi, pronounced microglial activation remained with a hypertrophic phenotype (mean width: 14.1um ± 1.1) compared to finely ramified microglia in naïve controls (mean width: 7.73 ± .53; p<.05). Increased microglial activation occurred throughout sectional gray and white matter from L1-2 (mean area: 1.8% ± 0.5 area in naïve and 8.0% ±0.8 area in 7dpi WT p<.05). The greatest microglial density occurred in the dorsal horn relative to the ventral horn after SCI (Figure 4.3). Changes in microglia phenotype occurred alongside a rise in pro-inflammatory cytokine production.
Figure 4.3. **Thoracic SCI results in rapid activation of microglia in the lumbar enlargement.** Within 24 hr, Iba1 labeling shows that resident microglia display an activated bushy phenotype made evident by increased cell body width (p<.001 compared to naïve; n=3-4/group). By 7dpi in WT mice, microglia still exhibit hypertrophy compared with naives and total staining covers a greater proportional area in the dorsal horn (p<.01 compared to naïve, 24 hr WT, and 7d KO) and ventral horn (p<.05 compared to Naïve, 24 hr WT, and 7d KO). MMP-9 deficiency resulted in a significant attenuation of microglia phenotype and proportional area. MMP-9 null mice showed reduced cell body width (10.2um; p<.01 compared to 24 hr) and reduced labeling in dorsal horn (p<.001 compared to 7d WT) and ventral horn (p<.01 compared to 7d WT). Scale bar in panel A is 150 um and 100 um in panel B and panel C.
Deletion of MMP-9 improves white matter sparing and reduces inflammation in the lumbar enlargement

Similar to findings of Noble and colleagues (2002), in a more severe injury model we show that MMP-9 deficiency results in a modest increase in white matter sparing at the lesion site (Figure 4.4). After severe SCI, lesion size was smaller (78.80% ± 1.49 Area in KO, and 87.06% ± 2.32 in WT; p<.05) and the amount of white matter sparing was significantly greater in KO mice (21.10% ± 1.49 WMS in KO, and 12.93% ± 2.33 in WT; p<.05)(Figure 4.4B). White matter sparing at the lesion site predicted the extent of microglial activation in the lumbar enlargement. At 7d, mice with greater sparing at T9 had significantly less cross-sectional iba-1 labeling throughout L1/L2 (r²=.67; p<.01)(Figure 4.4C).
**Figure 4.4. MMP-9 deficiency results in white matter sparing that predicts remote activation of microglia.** Representative epicenters are shown from KO and WT mice stained for myelin after severe SCI (A) at 7d. MMP-9 KO mice had greater myelin sparing (B; p<.05) and smaller lesion size (B; p<.05; n=3-4/group). The extent of white matter sparing at the lesion epicenter was highly predictive of remote microglial activation. Greater white matter sparing at T9 corresponded to less Iba1-labeling at L1-2 throughout the entire cross section at 7d (C; WT black, KO red; p<.05).

Deletion of MMP-9 attenuated inflammation in the lumbar enlargement.

MMP-9 deficiency did not alter MMP-3 or MMP-2 expression compared to WT.

Pro-gelatinase MMP-3 expression was elevated in MMP-9 null mice and reached an almost 4-fold increased within 24 hr (386.5% Naïve ± 57.9; p<.05) (Figure...
4.5A) but returned to baseline by 3dpi. MMP-2 levels remained at baseline in WT mice, and did not show a compensatory increase in MMP-9 null mice that confirms work by Hsu et al. 2008 (Figure 4.5B). Microglial activation was markedly reduced in MMP-9 KO mice compared to WT at 7dpi (Figure 4.3). In both the dorsal and ventral horn, microglia maintained a hypertrophic phenotype but over much less area (328.4% reduction in dorsal horn, 193.4% reduction in ventral horn; p<.05; Figure 4.3). MMP-9 deficiency reduced pro-inflammatory TNFα production in the lumbar enlargement over the first week after SCI (Figure 4.2B, 4.5C). In MMP-9 null mice, TNFα expression was significantly reduced in L4/5 at 24 hr (52% reduction relative to naïve; Figure 4.5C; p<.05). At 7dpi, deletion of MMP-9 restored TNFα levels to homeostatic levels (79.7% Naïve ±15.1; p<.05 compared to naïve) (Figure 4.5C).
Figure 4.5. MMP-9 deficiency attenuates pro-inflammatory cytokine but not metalloproteinase expression. A) ELISA confirms depletion of MMP-9 in null mice at 24 hr, 3d, and 7d. MMP-9 null mice maintain an acute upregulation of pro-gelatinase MMP-3 at 24 hr (p<.05). Compensatory increases in MMP-2 protein are not observed at 42d (B). Increased expression of TNFα protein is evident at 7d in L4-5 of WT mice (p<.01 compared to Naïve; 24 hr WT used are same as Figure 4.2). In MMP-9 null mice, TNFα expression is greatly reduced at 24 hr (p<.01 compared to WT Naïve) but returns to homeostatic levels at 7dpi.

*Early but not late treadmill training promotes robust recovery only in MMP-9 null mice*

Severe SCI resulted in paresis during overground locomotion with only occasional, slight HL movements at 24 hr in all groups (Mean BMS= 0.3 ± 0.1 KO, 0.3 +/-0.1 WT). Early treadmill training (ETT) delivered 2-9dpi resulted in
substantial locomotor improvements by 7dpi in MMP-9 KO but not unexercised KO or trained or unexercised WT groups (Figure 4.7A). At 7dpi, recovery was negligible, with plantar stepping occurring in only 9.1% of ETT WT mice, 20% of unexercised WT mice and 23.1% of unexercised KO mice (Figure 4.7A). Conversely, plantar stepping occurred in 66.7% of ETT KO mice in the open field at 7d (Figure 4.7A). This was similarly observed during treadmill locomotion, as ETT MMP-9 KO mice were the only group capable of generating kinematic metrics (Figure 4.7B). Unexercised KO and trained and unexercised WT groups required an assistive harness for bodyweight support and consistently dragged HLs on the treadmill. Neither training alone nor MMP-9 depletion alone promoted recovery. MMP-9 expression persisted in the lumbar cord of trained and untrained WT mice at 9d (Figure 4.7C).
Figure 4.6. Mouse kinematics and treadmill training paradigm. Recovery was examined in two treatment paradigms. Quadrupedal treadmill training was delivered early (ETT) from 2-9d post SCI, or late (LTT) from 35-42d post SCI. For ETT, recovery was assessed at 7dpi and retention was examined at 35dpi. For LTT, recovery was assessed before (35dpi) and after (42dpi) the intervention. Bodyweight support was provided using a harness and manual trunk assistance. A pestle was used to ensure plantar placement of steps. Recovery was quantified using two dimensional mouse kinematics. Markers were placed on the iliac crest, greater trochanter, femoral condyle, lateral malleolus, and the head of the 5th metatarsal.

Functional improvements in ETT KO were retained to 4 weeks after the training period ended. Groups did not differ in open field recovery at 35dpi (Mean BMS= 5.6 ± 0.2 ETT KO, 5.6 ± 0.3 Unex KO, 4.6 ± 0.5 ETT WT, and 5.1 ± 0.2 Unex WT). Kinematic metrics identified significant differences in treadmill performance. At 35dpi, early trained MMP-9 null mice stepped with near normal measures of toe dragging, swing time, stance time, and ankle velocity that did not statistically differ from Naïve (Figure 4.8B,C,D,E). In contrast, early training of WT mice resulted in significant locomotor deficits at 35d. During treadmill walking, ETT WT mice displayed measures of trunk instability, toe dragging, swing time, stance time, and ankle velocity that showed the greatest mean
deviation from Naïve (Figure 4.8 A,B,C,D,E; p<.05). On the gridwalk, early training resulted in significantly worse stepping precision. Weight supported, rung-to-rung stepping was only successful in 5.49% ± 2.54 of attempts in ETT WT, while unexercised WT stepped with a 13.58% ± 1.81-success rate (Figure 4.8F).

**Figure 4.7. Robust locomotor improvements occur with early treadmill training in MMP-9 null mice.** By 7dpi, ETT MMP-9 null mice plantar stepped in the open field while all other groups failed to step (A; p<.01). Weight support and plantar stepping performance in the open field was significantly higher in the treadmill trained MMP-9 null group (100% Weight support and 66.7% plantar stepping; p<.05). On the treadmill, early trained KO mice independently plantar stepped and no longer required a supportive harness (B). Only ETT KO mice were capable of producing quantifiable kinematic metrics. All other groups required bodyweight support and dragged the hindlimbs. Gelatin zymograms (C) show that MMP-9 expression persists in the lumbar cord (L4/L5) of both trained and unexercised WT mice.
Figure 4.8. Retention of training effects. Four weeks after training ended (35dpi), motor control was assessed using gridwalk and treadmill kinematics. Early training in MMP-9 null mice (ETT KO) resulted in near-normal measures of trunk displacement, toe dragging, time spent in swing/stance, and peak velocity of the ankle during swing. Trained WT, and unexercised controls displayed significant motor deficits compared to naive in almost all kinematic measures (p<.05). Early training resulted in significantly worse rung-rung paw placement on gridwalk in WT mice (p<.05). n=6-10/group;* = significantly different from naïve.

The same duration of treadmill intervention delivered in late stages of recovery (35-42dpi) failed to result in any significant locomotor improvements in either MMP-9 KO or WT mice (Table 2). Because late treadmill training (LTT) was delivered following spontaneous recovery when stepping was evident for 100% of mice in the open field, the training intervention differed in that they did not require body weight support or manual assistance of the hindlimbs. The intensity of training, as measured by the number of hindlimb steps performed in the 20 minute session, was significantly greater than the early intervention (ETT:
1295.7 ± 27.1; LTT: 3622.2 ± 254.6; p<.001). Despite more intensive training, open field scores showed no benefit in recovery, as predominant weight supported plantar stepping was typical in both groups before and after the delayed intervention. In addition, kinematic metrics of trunk instability, toe dragging, swing time, and ankle velocity showed no significant improvements after training (Table 2). Unlike an early intervention, LTT did not result in motor deficits in KO or WT groups.
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<tr>
<td>BMS</td>
<td>9</td>
<td>5.7 ± 0.4</td>
<td>5.6 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>Trunk displacement (cm)</td>
<td>0.54 ± 0.04</td>
<td>1.01 ± 0.31</td>
<td>0.64 ± 0.11</td>
<td>1.24 ± 0.40</td>
<td>0.87 ± 0.26</td>
</tr>
<tr>
<td>Toe dragging (s)</td>
<td>0.031 ± 0.001</td>
<td>0.067 ± 0.010</td>
<td>0.051 ± 0.004</td>
<td>0.077 ± 0.013</td>
<td>0.078 ± 0.007</td>
</tr>
<tr>
<td>Swing time (s)</td>
<td>0.113 ± 0.005</td>
<td>0.060 ± 0.012</td>
<td>0.078 ± 0.005</td>
<td>0.055 ± 0.023</td>
<td>0.068 ± 0.019</td>
</tr>
<tr>
<td>Stance time (s)</td>
<td>0.30 ± 0.01</td>
<td>0.69 ± 0.09</td>
<td>0.52 ± 0.04</td>
<td>0.82 ± 0.14</td>
<td>0.91 ± 0.09</td>
</tr>
<tr>
<td>Peak ankle velocity (cm/s)</td>
<td>22.9 ± 1.5</td>
<td>15.5 ± 3.3</td>
<td>19.3 ± 1.6</td>
<td>11.4 ± 4.7</td>
<td>12.3 ± 3.7</td>
</tr>
<tr>
<td>Peak toe velocity (cm/s)</td>
<td>30.0 ± 1.8</td>
<td>21.8 ± 4.7</td>
<td>27.7 ± 2.0</td>
<td>16.4 ± 6.8</td>
<td>19.6 ± 5.5</td>
</tr>
<tr>
<td>Gridwalk (% success)</td>
<td>84.3 ± 1.8</td>
<td>12.3 ± 3.2</td>
<td>11.3 ± 3.1</td>
<td>13.1 ± 1.2</td>
<td>14.6 ± 3.0</td>
</tr>
</tbody>
</table>

Recovery was examined before (35 d) and after (42 dpi) LTI in KO and WT mice using a battery of locomotor tests. Open field (BMS), gridwalk, and kinematic measures revealed no improvements or deficits that resulted from training in KO and WT groups. Data are shown as means ± SEM.

**Table 4.2.** *Late training is ineffective in KO and WT mice.* Recovery was examined before (35d) and after (42dpi) late treadmill training in KO and WT mice using a battery of locomotor tests. Open field (BMS), gridwalk, and kinematic measures revealed no improvements or deficits that resulted from training in KO and WT groups.
4.4 Discussion

Spinal cord injury creates dual and conflicting cellular processes along the neuroaxis. In addition to well-documented events at the lesion site, mechanisms of both plasticity and inflammation occur remote to the lesion in the lumbar enlargement that influences function (Detloff et al., 2008; Andrews et al., 2012; Hains and Waxman, 2006; Hougland et al., 2012). Given recent evidence that remote neural activity provides an essential substrate for locomotor recovery (Harkema et al., 2011), we propose that remote inflammatory processes alter the cellular microenvironment and dramatically influence retraining and function. We show for the first time that elevated MMP-9 contributes to the failure of motor relearning early after SCI alongside an inflammatory microenvironment in remote lumbar segments. Deletion of MMP-9 reduces the inflammatory signature in the lumbar cord and allows robust locomotor plasticity and improved recovery that is otherwise refractory to early intervention. For the first time, we identify the blunting effect of remote inflammation on activity-dependent plasticity within locomotor interneuron networks. Our work demonstrates a negative, time-dependent interaction between lumbar MMP-9 production and motor relearning.

MMP members display a distinct temporal expression in the lumbar enlargement during the first week after SCI. Numerous cell types including resident glia, trafficking leukocytes, and vascular endothelia produce MMPs in
response to CNS trauma (Zhang et al., 2011). Pro-gelatinase MMP-3 shows rapid upregulation within 24 hr that precedes MMP-9 at 3, 7, and 9dpi. A similar trend is observed in the brain when an entorhinal cortex lesion results in distant expression of MMP-3 in the dentate gyrus (Kim et al., 2005a; Falo et al., 2006). Notably, MMP-3 is considered to be the primary activator of MMP-9 and likely plays a critical role in the transcription and activation of MMP-9 at later time points in the lumbar cord (Vempati et al., 2007). In situ and gelatin zymograms confirm increased MMP-9 expression and localize activity primarily to structures with a vascular phenotype in lumbar segments. Evidence of gelatinase activity around BSCB constituents suggests abnormal permeability or neurovascular reactivity far distant to the site of direct trauma. MMP-9 activity may contribute to demonstrated alterations in gray matter permeability and vascular uptake mechanisms extending to at least L1-L2 in a rat midthoracic contusion model (Popovich et al., 1996).

Midthoracic contusion produced a rapid, pro-inflammatory shift in resident microglial morphology and increased cytokine expression around putative locomotor networks. At 24 hr during peak MMP-3 expression, microglia displayed an exaggerated activation profile, or bushy phenotype, that may indicate a pre-migratory activation state (Kumar et al., 2013). In culture, MMP-3 functions as a novel signaling protein to activate microglia and may be one of the earliest microglial regulators remote to the injury site (Kim et al., 2005b). Hypertrophic microglia were evident throughout dorsal and ventral regions of the
lumbar cord during the first week after SCI. The prolonged hypertrophic phenotype may be part of a paracrine/autocrine loop in which MMP-9 from endothelial cells regulates pro-inflammatory cytokine availability and increased MMP-9 production in microglia via NFKB and p38 signaling as shown in other cell types (Rajashekhar et al., 2011). Additionally, the possibility exists that MMP-9 has an indirect influence on lumbar microglia after injury by regulating axonal dieback and demyelination at the injury site (Busch et al., 2009; Liu and Shubayev, 2011). Regardless of the downstream actuators, we demonstrate that MMP-9 activity is prominent in the lumbar spinal cord and that microglial regulation is MMP-9 dependent given that deletion of MMP-9 maintained microglia in a resting phenotype and reduced TNFα expression.

In our training paradigm, repetitive sensorimotor cues delivered to the lumbar enlargement facilitate oscillatory motor output from CPGs. Synaptic strengthening and reorganization within interneuronal networks promote locomotor recovery, a process collectively termed motor relearning (Rossignol et al., 2006). Spinal centric learning is ubiquitous and task-dependent (Hodgson et al., 1994). Indeed, the lumbar spinal cord can learn to modulate motor responses which improves locomotor recovery (Chen et al., 2006). Recently, the same learned-modulation was effective in human SCI (Thompson et al., 2013; Chen et al., 2010; Wolpaw et al., 2010). Neuroinflammation limits synaptic formation and long-term potentiation (LTP), two necessary features of motor relearning (Yirmiya and Goshen, 2011). We show for the first time that MMP-9
and downstream factors interact negatively with treadmill training to impede locomotor recovery after SCI. By eliminating MMP-9 in combination with early training, we restored plantar stepping by 7 days – an extent of recovery unattained by training alone or MMP-9 deficiency alone. Moreover, these training effects extended 4 weeks after the intervention ended. We propose that after SCI, MMP-9 regulated neuroinflammation is inhibitory to synaptic plasticity and spinal centric learning. The inhibitory role of inflammatory signaling on spinal learning has been well characterized in instrumental conditioning paradigms (Vichaya et al., 2009; Huie et al., 2012).

Surprisingly, white matter sparing at the epicenter was insufficient to produce functional gains in our severe SCI model. Unexercised MMP-9 null mice did not differ from WT in the open field which contrasts with the improved locomotor recovery reported by Noble and colleagues after reducing or eliminating MMP-9 in a less severe injury (Noble et al., 2002). We propose that modest improvement in white matter sparing with this lesion severity is likely too small to support the robust recovery attained with training in MMP-9 null mice. However, the changes at the epicenter may have influenced lumbar neuroinflammation since we found a relationship between sparing and lumbar microglial activation. Additionally, during the late timepoint when inflammatory processes have subsided in the lumbar cord, sparing of descending systems did not facilitate recovery even when combined with training and/or MMP-9 elimination. In rat SCI, we observed that early treadmill training in the presence
of moderate or substantial white matter sparing does not produce behavioral benefit (unpublished). Thus, attenuating inflammation throughout the neuroaxis may be a necessary first step to induce activity-dependent plasticity of segmental and spared descending systems.

Treadmill training is not universally effective but rather must be optimally timed to capture injury-induced plasticity. Despite lower levels of inflammation in MMP-9 null mice, the same training that produced robust and lasting improvement early after SCI failed to induce recovery when delivered late after SCI. Distinct alterations in extracellular matrix proteins occur in the lumbar enlargement that may prevent the efficacy of late training (Andrews et al., 2012). Extracellular matrix components such as chondroitin sulfate proteoglycans stabilize and regulate synaptic plasticity. Full-length neurocan and NG2 are known MMP substrates that increase after the first week in the lumbar enlargement and persist to chronic times post injury (Rauch, 2004; Larsen et al., 2003; Andrews et al., 2012). Lasting increases in chondroitin sulfate proteoglycan production around locomotor networks may increase synaptic stability at the cost of reducing plasticity. The inefficacy of late treadmill training identifies a reduction of lumbar plasticity in both MMP-9 null and WT mice. Thus, a critical period of spontaneous plasticity acutely after SCI offers a novel opportunity to influence locomotor recovery. In our mouse model, this period is defined by a period of days. However, marked differences in the onset and duration of inflammation in human SCI suggest that an optimal window to create
a permissive environment for rehabilitation may be weeks or months in the clinic (Donnelly and Popovich, 2008; Fleming et al., 2006).

An important finding was that intervening with treadmill training early after SCI during a period of marked neuroinflammation exacerbates deficits more than had no exercise been applied. In WT mice, we found that early treadmill training produced substantial impairments. These findings are consistent with work in SCI and other CNS injury models (Kozlowski et al., 1996; Smith et al., 2009). Early after SCI, increasing vascular demand when BSCB integrity is compromised may exacerbate pathology. In fact, a single session of swim training delivered in acute stages of SCI increases lesion-site permeability (Smith et al., 2009). Whether MMP-9 facilitates exercise-mediated disruption of BSCB components is unknown, although it is likely given that acute phase BSCB breakdown is reduced in MMP-9 null mice (Noble et al., 2002). Because early expression of MMP-9 persists in lumbar segments of both trained and unexercised WT mice, mitigation of remote neurovascular reactivity in MMP-9 null mice likely facilitated plasticity and recovery. In contrast, late after SCI when reconstitution of the BSCB is evident, MMP-9 inhibition may be insufficient to facilitate lumbar plasticity (Whetstone et al., 2003).

Our results suggest that disrupted neurovascular interactions around locomotor circuitry far distant to the SCI can result in profound functional consequences. We show that locomotor training can be quite effective when
administered early after SCI and in combination with reduced inflammation.

However, the efficacy of treadmill training is lost when delivered chronically and carries the risk of inducing neurotoxicity in an inflammatory microenvironment. Combinatorial therapies that consider the interaction between spinal centric learning and remote lumbar inflammation will likely permit the most robust synaptic and structural remodeling in locomotor networks for recovery.
CHAPTER 5

General Discussion

A spinal cord lesion results in a CNS syndrome identified by sensory, motor, autonomic, and psychological deficits. Impaired locomotion may be the most obvious consequence. Of the many questions left unanswered, perhaps the most striking quandary is our conceptual lack of understanding of lesion boundary and the functional consequences thereof. Increasing evidence suggests that impaired function and plasticity in the CNS are not solely attributed to physical barriers. Indeed, competitive cellular and molecular processes along the neuroaxis influence synaptic actions (Hains and Waxman 2006; Detloff et al., 2008; Nesic et al., 2005; McAdoo et al., 1999; Aguilar et al., 2010). A better understanding of remote injury processes will advance therapeutic aims to promote recovery from the SCI syndrome. The primary interest of this dissertation is the remote function and plasticity of putative locomotor networks after SCI (Figure 1.2). Hence, we examined behavioral, physiological, cellular, and molecular evidence in the lumbar enlargement following a standardized
midthoracic SCI. Chapter 2 identifies profound deficits in eccentric motor control that require segmental integration of sensory and motor signals during locomotion. These lumbar deficits not only serve as an indicator of recovery, but also provide a therapeutic venue for task-specific rehabilitation. Chapter 3 describes the role of axonal sparing on remote spinal learning early and late after SCI. We found that late after SCI an opportunity exists to harness lumbar plasticity to promote task-specific learning in locomotor networks. Finally, Chapter 4 identifies novel mechanisms of inflammation in the lumbar enlargement that jeopardize locomotor plasticity early after SCI. We showed that by attenuating remote inflammation, lumbar-focused treadmill training produces robust plasticity and recovery. Our data collectively reveal injury processes along the neuroaxis that jeopardize the segmental function and plasticity of locomotion after SCI. Thus, a spinal cord lesion creates regional locomotor impairments that only compound the influence of functional axon loss at the injury site. Future therapeutic aims must consider a lesion without boundaries to address function and cure the SCI syndrome.

5.1 Redefining the Locomotor Consequence of a Spinal Cord Lesion

Injury to the spinal cord results in a central core lesion and a peripheral rim of spared motor and sensory axons that are not sole determinants of locomotor recovery. Over the last century, a one-dimensional assumption has been made regarding function after SCI: locomotor recovery is determined by the boundaries of axonal sparing (Schucht et al., 2002; Fehlings and Tator, 1995;
Blight and Descrescito, 1986). However, it becomes clear that SCI is a multifaceted condition and the concept of a lesion border is, functionally, obsolete. “Intact” neural networks far removed from the site of mechanical damage are directly influenced by the surrounding microenvironment. At least 10 segments below the SCI, compelling evidence demonstrates that inflammatory signaling hypersensitizes nociceptive pathways (Detloff et al., 2008; Hains and Waxman, 2006; Nesic et al., 2005). Similarly, our present work identifies impairments within shared interneuron networks for locomotion. We first explored this possibility in Chapter 2, when we found profound deficits in the segmental integration of sensory and motor signals during locomotion. Importantly, we showed that these eccentric deficits in the semitendinosus improved over time. From our findings, we postulate that eccentric bursting in the semitendinosus may not only serve as an indicator of recovery, but may also reflect the extent of below level pathology. We continued to explore this notion in Chapter 3, where we examined the isolated capacity of lumbar segments and found that the same severity SCI allowed segmental learning late, but not early. This finding identified a transient impairment of locomotor networks that was independent of lesion-site sparing. Finally, we identified potent regulators of the lumbar microenvironment in Chapter 4. Activated microglia, pro-inflammatory cytokine production, and neurovascular reactivity were evident throughout lumbar gray matter early after SCI. This inflammatory signature impedes function by disrupting neurophysiological actions at the synapse (Yirmiya and Goshen, 2011; Beattie et al., 2002; Cunningham et al., 1996). Alongside this nonpermissive
milieu, we again found that early locomotor networks are refractive to activity-based learning. Only when we attenuated remote neuroinflammation were we able to produce early recovery with lumbar-focused training. Our findings warrant further examinations of neural structure, extracellular and intracellular protein expression, and synaptic physiology. Nevertheless, it seems that the locomotor consequences of remote injury mechanisms after SCI have been vastly underestimated.

Clearly, sparing of motor and sensory axons provide essential substrate for recovery. Descending raphe-, reticulo-, rubro-, and vestibulo-spinal tracts fundamentally mediate the restoration of locomotion (Basso et al., 2002). Even sparing of ascending spinothalamic tracts provide opportunities for cortical remapping and the recovery of sensation, but also present risks for the development of neuropathic allodynia (Cruz-Almeida et al., 2012; Wall et al., 2002; Detloff et al., 2008). These findings have made examinations of white matter sparing critical to the explanation of locomotor deficits (Basso et al., 2006; Basso et al., 1995; Kloos et al., 2005). However, this dissertation finds an inconsistent benefit of axon sparing on locomotor function. After recovery plateau, Chapter 2 shows that superior locomotor performance corresponds to better eccentric bursting in the semitendinosus. This supports the likelihood that increased sparing facilitates segmental integration of sensory and motor signals. However, this relationship depends on time because eccentric bursting was indiscriminant early after SCI. Similarly, our work in Chapter 3 shows that the
same amount of axonal sparing produced remote plasticity that facilitated learning late but not early after SCI. It becomes clear that the ability of axons to elicit targeted plasticity in the lumbar enlargement will depend on factors in the surrounding microenvironment (Chapter 4). Our work begins to uncover underlying events that may describe the elusive mechanisms of spinal shock and spontaneous recovery after SCI. Given the myriad of complex biological changes that coincide with preserved axonal substrate, identifying recovery after SCI will depend on a syndromic variable approach to therapeutic testing (Ferguson et al., 2013).

5.2 The Genesis of Remote Neuroinflammation after SCI

An evolving view of the spinal cord lesion identifies neuroinflammation at great distances rostral and caudal to the site of injury. As such, the term “neuroinflammation” should be approached with caution. Basal signaling of pro-inflammatory cytokine pathways are involved in the majority of neuronal and glial communication, and their elimination prevents function (Yirmiya and Goshen, 2011; Kaneko et al., 2008). However, potential also exists for maladaptive interactions around the synapse. Pathological neuroinflammation describes both hematogenous trafficking of immune cells into the CNS, as well as reactivity of resident glia (termed gliopathy; Hulsebosch, 2008). Previous work has identified gliopathy in the remote lumbar dorsal horn that induces intracellular signaling and hypersensitizes nociceptive pathways (Detloff et al., 2008; Tan et al., 2008). Our present work identifies the inflammatory signature throughout dorsal and ventral
gray matter, and suggests a peripheral origin. Chapter 4 shows remote upregulation of the vascular permeabilizing factor, MMP-9 in the lumbar cord. Indeed, leukocytes release MMP-9 to degrade tight junctions of the blood spinal cord barrier (BSCB) and allow extravasation (Zhang et al., 2011; Zhang et al., 2010). Our findings of gelatinase activity around lumbar vasculature support the likelihood of immune cell infiltration. Yet, the notion that active diapedesis occurs 10 segments away from the lesion has received little, if any, attention (Popovich et al., 1996). It seems that our preliminary experiments have identified a novel manifestation. Using vascular tracers and bone-marrow chimeric mice, we have not only identified parenchymal trafficking but found evidence of leaky vasculature at least 13 segments below the lesion in the sacral cord (data not shown). Above the lesion, we found peripheral infiltrates as far rostral as the hippocampus. The influence of inflammatory signaling on trophic support in regions of dynamic plasticity requires further examination. Both the hippocampus and lumbar enlargement are subject to lasting declines in BDNF and synapsin I after thoracic SCI (Gomez-Pinilla et al., 2012; Hutchinson et al., 2004). These findings continue to identify our limited knowledge of post-injury dynamics and the functional implications thereof.

To date, the initiating factors of remote neuroinflammation have been phenomenological. Chapter 4 begins to uncover a rapid mechanism. Within 24 hours of SCI, we found that resident microglia display an activated phenotype alongside increased expression of pro-gelatinase MMP-3. At this same time, our
preliminary studies have identified myeloid phenotypes throughout the lumbar and sacral parenchyma. The rapidity of this response is curious. Future studies will question the role of astrocytes in remote dysfunction. Astrocytic endfeet border the endothelial barrier to maintain neurovascular stability (Abbott et al., 2006; Bechman et al., 2006). While astroglial reactivity in the lumbar cord appears to be negligible after thoracic SCI (Andrews et al., 2012), long-distance astrocyte communication may still be possible. Astrocytes initiate rapid, long-distance propagation of pulsating calcium waves via Gap junctions (Goldberg et al., 2010). Following a select cortex lesion, astrocytes rapidly produce MMP-3 in the remote dentate gyrus to facilitate synaptogenesis (Kim et al., 2005a; Falo et al., 2006). It is unclear whether such a response occurs in the lumbar enlargement after thoracic SCI. However, our findings of microglial hypertrophy alongside MMP-3 production at 24h may not be coincidental. MMP-3 is a novel signaling protein involved in the activation of microglia and may identify early regulatory signaling within the neurovascular unit (Kim et al., 2005b). Real-time in vivo imaging of lumbar microglia and astrocytes after SCI would provide invaluable information regarding glial behavior. It will further be critical to identify neurovascular signaling that occurs in lumbar vasculature to recruit myeloid cell types. A cooperative interaction between MMP-9 degradation and CXCL12 production facilitates hematogenous trafficking at the epicenter, and may also occur in the lumbar cord (Zhang et al., 2011b). Preliminary experiments found increases in endothelial ICAM-1 expression that identify vascular adhesion in the lumbar cord (data not shown). Ultimately, the identification of peripherally
derived inflammation provides greater opportunities for therapeutic intervention, as systemic treatments are more effective than attempts to target a resident glial response (Lee et al., 2011; Popovich et al., 1999).

5.3 Plasticity of Locomotor Networks after SCI

Spinal cord injury results in spontaneous structural, functional, and biochemical indices of neuroplasticity in locomotor networks. In the intact nervous system, descending supraspinal axons maintain excitation or inhibition within CPG networks. After SCI, a loss of descending control results in competitive sprouting and new synapse formation throughout the lumbar cord (Murray and Goldberger, 1974). Spontaneous circuit formation is also evident along propriospinal relays that increase projections to lumbar motoneurons (Bareyre et al., 2004). Work in this dissertation examines activity-dependent plasticity within these already plastic networks. It seems, then, that we are measuring plasticity of plasticity. When the history of a synapse determines future plasticity, it describes a state of metaplasitcicity (Abraham and Bear, 1996; Abraham, 2008). The degree and direction of future plasticity (LTP, LTD) is subsequently conditioned by cues in the microenvironment or stimulus. After SCI, differential periods of lumbar plasticity predispose activity-based interventions to adaptive or maladaptive function. Chapter 3 uses an activity-dependent learning task to measure the segmental capacity of a small part of the rat CPG (L4-S2; Liu et al., 2005). We found that only late after SCI does sparing produce plasticity that is permissive for activity-dependent learning. Early after
SCI, negligible learning trends identify a nonpermissive, or maladaptive period of plasticity. At this early time point, a potent upregulation of TNFα occurs in the lumbar enlargement that is known to produce metaplastic inhibition of spinal learning (Detloff et al., 2008; Ferguson et al., 2012; Huie et al., 2012). TNFα and downstream signaling through TNFR1 initiate hyperexcitability by increasing calcium-permeable AMPA receptor expression (Beattie et al., 2002). By late time points after SCI, TNFα signaling returns to homeostatic levels (Detloff et al., 2008). We identify a similar phenomenon in Chapter 4. Early after SCI in mice, we engaged the entire CPG network using treadmill training and found not only inefficacy, but worsened behavior. This time period coincided with remote upregulation of MMP-9 dependent TNFα production. It seems that inhibitory metaplasticity in the lumbar enlargement produced maladaptive function. Together, our work shows that the early microenvironment of the lumbar cord renders locomotor networks refractive to activity-dependent plasticity and learning. Current work is underway to examine dendritic spine morphology of intermediate and ventral laminae neurons to identify functional substrates of inhibitory neuroplasticity.

5.4 Clinical Neurorehabilitation: An Opportunity for Combinatorial and Task Specific Intervention

Despite decades of clinical and basic science attempts to cure paralysis and repair the lesion, there are currently no therapeutic standards for intervention
(Hurlbert et al., 2013). Work from this dissertation identifies two novel directions for clinical neurorehabilitation in patients with SCI. First, we suggest that an optimal intervention will consider the consequence of SCI at both lesion site and in the lumbar enlargement. In both cases, the influence of at- and below-level pathology will depend on time. Combinatorial drug interventions may best promote recovery by select inhibition of cellular processes that prevent adaptive plasticity. At the epicenter, sparing of axonal systems will be critical. Efforts to improve neuroprotection should also consider the influence of the surrounding microenvironment on spared axonal relays and saltatory conduction (Maier et al., 2009; Bittner and Meuth, 2013; Kobayashi et al., 2008). In the lumbar cord, our work clearly demonstrates the inhibitory role of inflammation on locomotor plasticity (Chapter 3, Chapter 4). When we attenuated MMP-9 regulated inflammation in lumbar segments (and epicenter, data not shown), we gained access to a period of plasticity that promoted recovery not attainable with a late intervention. Thus, inhibition of at- and below-level MMP expression may be a promising therapeutic direction to both influence neuroprotection and attenuate the toxic remote microenvironment (Zhang et al., 2011). With careful timing, localized delivery of MMP-9 siRNA inhibitors to the lumbar cord has demonstrated promising outcomes in pain models (Kawasaki et al., 2008).

Second, we found that task-specific treadmill training promotes learning and recovery. By adapting environmental demands of the training task, novel sensory cues can better activate lumbar CPG networks. In Chapter 2, we
showed that downhill treadmill walking facilitates eccentric motor control. Eccentric bursting in the semitendinosus displayed greater activation during downhill treadmill walking. During hip extension, stretch receptors in the semitendinosus provide distinct afferent signals to initiate central pattern generation (Wisleder et al., 1990; Grillner and Zangger, 1984). Thus, it is likely that our downhill training paradigm in Chapter 3 delivered greater afferent drive to CPG networks that promoted locomotor improvements. This resulted in task-specific plasticity within lumbar networks that facilitated spinal learning. In the clinic, our findings may further translate to improvements in learned modulation of reflex gains during locomotion (Thompson et al., 2013). Task specific treadmill training would provide a simple adaptation for current interventions. Together, the clinical implications of this dissertation suggest that delivering a combinatorial task-specific intervention at the correct time in a permissive microenvironment will promote robust recovery. Indeed, preliminary studies show that early downhill training in MMP-9 null mice produced even greater functional improvement that restored open field coordination by 42d post injury (data not shown).

Potential further exists to examine biomarkers that predict the efficacy of intervention. It is estimated that 30% of patients that receive locomotor training show no improvement in functional ambulation (Buehner et al., 2012). It may be possible to evaluate circulating factors in the blood or CSF that identify underlying neuropathology that prevents motor relearning and recovery in these
subjects. A relatively noninvasive lumbar puncture could provide information regarding circulating immune cells and cytokine profiles that bathe the CNS. In fact, lumbar punctures have been used in dogs to identify an early expression and activation profile of MMP-9 in CSF (Levine et al., 2006; Levine et al., 2011). It may also be possible to examine genetic predispositions that prevent training efficacy. It was recently found that human BDNFVal66Met polymorphisms result in hypothalamic-pituitary-adrenal axis hyperreactivity and impaired supraspinal learning (Yu et al., 2012). Ultimately, screening of biomarkers may provide critical information that decides patient enrollment, intervention timing, and the appropriate drug to deliver in combination with training.

5.5 Significance and Conclusions

A loss in mobility is a devastating consequence of SCI. In order to fully treat the SCI syndrome, we must first understand the functional implications of neuropathology that influences locomotor networks. This dissertation provides cellular, molecular, behavioral, and physiological evidence showing that injury to the spinal cord results in an expansive affliction along neuroaxis that jeopardizes lumbar-centric function and plasticity. Functionally, we found profound deficits in eccentric motor control that identified impaired integration of sensory and motor signals in the lumbar cord (Chapter 2). We also found that locomotor networks were incapable of sparing-induced facilitation of learning early after SCI (Chapter 3). Underlying these lumbar-centric impairments, we uncovered mechanisms that propagated early remote neuroinflammation around locomotor networks.
When we attenuated remote inflammatory processes, we were able to harness a formerly refractive period of plasticity that allowed robust recovery (Chapter 4). Taken together, our work collectively identifies a paradigm shift for clinical practice and basic science models of spinal cord injury that refocuses the treatment of a lesion to that of a CNS syndrome.
Figure 5.1 Identified barriers to motor relearning and recovery after SCI. Image depicts a spinal cord injury that results in axonal sparing of ascending (spinothalamic- dashed yellow) and descending (rubrospinal- dashed red, reticulospinal- dashed green, vestibulospinal- dashed purple, raphespinal- dashed blue). Notice an absence of dorsal column sparing (gracile- dashed light blue, cuneate- dashed gold, and corticospinal- dashed gray). This dissertation provides novel evidence that shows remote neuroinflammation (image depicts activated microglia, blood spinal cord barrier (astrocytic border, endothelia, tight junctions, perivascular macrophage), MMPs-red, and cytokines-green), learning and maladaptive plasticity (image depicts impaired synaptic transmission and LTP), and eccentric motor control (image depicts matched motor output for sensory input) collectively impede motor relearning and recovery after SCI.
BIBLIOGRAPHY


