INVESTIGATION OF PLASMA MEMBRANE COMPROMISE AND CITICOLINE-MEDIATED REPAIR AFTER SPINAL CORD INJURY

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INVESTIGATION OF PLASMA MEMBRANE COMPROMISE AND CITICOLINE-MEDIATED REPAIR AFTER SPINAL CORD INJURY

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Success is a journey, not a destination.
The doing is often more important than the outcome.
-Arthur Ashe

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

3dex	3kDa FITC-conjugated dextran
10dex	10kDa FITC-conjugated dextran
BBB	Basso Beattie Bresnahan open field locomoter test
Ca^{2+}	calcium
CNS	central nervous system
cPLA2	cytoplasmic PLA2
CSF	cerebral spinal fluid
IP	intraperitoneal
NF-160	neurofilament-160
PC	phosphatidylcholine
PLA2	phospolipase A_2
SCI	spinal cord injury

SUMMARY

Although spinal cord injury (SCI) is a debilitating condition that presents a large socioeconomic problem in the United States, there is currently no treatment that reliably reduces morbidity and mortality. Current research is aimed at identifying mechanisms involved in the pathophysiology of SCI and using this knowledge to develop rational treatments. We have observed plasma membrane compromise in the acute (within 10 minutes), sub-acute (3 days), and chronic phases (5 weeks) in a rat model of contusion SCI and postulate that it negatively affects neurological outcome. Holes/tears in the plasma membrane were assessed with a dye exclusion assay, in which a fluorescent cell-impermeant dye was injected into the cerebrospinal fluid prior to sacrifice; therefore, cellular uptake of the dye is indicative of plasma membrane compromise. As early as 10 minutes after SCI, widespread uptake of permeability markers was evident in neuronal cell bodies as well as axonal projections. The number of permeable cells and the size of the membrane breaches (measured by using permeability markers of various sizes) varied with distance from the injury site, with larger disruptions located closer to the epicenter. Greater cellular uptake was observed when the impact force was increased (200 > 150 > 100 kdyn > sham). At longer time points (3 days and 5 weeks), substantial permeability marker uptake was observed in axons but not in cell bodies. Cells with increased permeability displayed a variety of pathomorphological alterations, including swelling, blebbing, retraction bulb formation, neurofilament loss, and fragmentation, suggesting that increased plasma membrane permeability is detrimental to cell survival and function. We therefore investigated a clinically-relevant treatment strategy designed to restore plasma membrane integrity. Animals were treated with citicoline, a molecule utilized in

the endogenous synthesis of phosphatidylcholine (the major membrane component in mammalian cells). Citicoline has been shown to be beneficial in numerous studies of neurological disease, improving overall outcome by increasing phospholipid synthesis and attenuating phospholipid destruction (by reducing phospholipase A2 activity). However, these mechanisms have not been explored in a model of SCI. When compared to injured animals receiving vehicle (saline) injections, citicoline treatment after SCI did not have a statistically significant effect on cytoplasmic PLA2 activity (at 24h post-injury), the density of permeable axons (at 3 days post-injury), or the lesion volume (at 3 days post-injury). Since citicoline may improve neurological outcome after SCI through mechanisms we did not directly assess, we then conducted a longer-term study to evaluate the overall efficacy of citicoline treatment in terms of longer-term functional and histological consequences. Citicoline did not have a biologically significant effect on behavioral recovery (evaluated during open field locomotion, grid walk and hyperalgesia testing weekly for up to 5 weeks post-injury) or lesion volume (at 5 weeks post-injury). The lack of citicoline-mediated effect may be attributed to experimental parameters (e.g., dosing or sensitivity of outcome measures) or biological inefficacy. Although we were not able to demonstrate that citicoline improves outcome after SCI, the finding that plasma membrane damage occurs in a persistent fashion and is associated with pathophysiological cellular alterations may provide fundamental knowledge necessary for developing treatments targeted at membrane repair. Future work examining the complex mechanisms causing prolonged membrane damage after SCI and evaluating strategies for manipulating these pathways (potentially using citicoline in combination with other pharmacological agents) may lead to a clinically effective therapy.

CHAPTER 1

INTRODUCTION

Overview of Spinal Cord Injury

There are an estimated 12,000 new cases of spinal cord injury (SCI) each year in the United States (Albin and White 1987; Sekhon and Fehlings 2001). Depending on the location and severity of the injury, persons with an SCI may experience neurological dysfunction such as paralysis, neuropathic pain, bladder and bowel dysfunction, and impotency. This is particularly devastating to the patient, since most are under the age of 30 and are otherwise healthy (Nobunaga et al. 1999). Although physical and emotional states are dramatically affected in these individuals, the currently available treatments offer modest benefit if any at all (Baptiste and Fehlings 2007; Hall and Springer 2004; Sayer et al. 2006). Thus, SCI remains a major socioeconomic problem in the United States, with lifetime costs ranging from $500,000 to more than $2 million per patient in addition to a loss of income (Sekhon and Fehlings 2001).

The pathophysiology of SCI begins with a mechanical insult that surpasses structural thresholds to inflict damage on tissue and cellular levels. Trauma is most commonly inflicted by vehicle accidents, but also occurs as a result of falls, violence, or sports-related injuries (Nobunaga et al. 1999). Typically the injury is incomplete, meaning that the spinal cord is damaged but not completely transected. Hemorrhage, necrosis, and cell rupture occur immediately at the injury site in an irreparable fashion. These early events (collectively termed primary injury) set into motion a complex series

of events that result in prolonged cell death and axonal degeneration (secondary injury). Factors contributing to secondary injury include ischemia, blood-spinal cord barrier breakdown, formation of the glial scar, and neuroinflammation among many other pathophysiological phenomena. As a result of these secondary injury mechanisms, the lesion expands over the following days, weeks, and even months to create a fluid-filled cystic cavity within the spinal cord parenchyma (for review, see (Dumont et al. 2001; Hagg and Oudega 2006; Kwon et al. 2004)).

Since a large portion of the cell death inflicted by SCI accumulates over time due to secondary injury mechanisms, it is feasible that a treatment given after the injury can reduce the total amount of tissue damage. Methylprednisolone, the current standard of care for persons with SCI, is a neuroprotective agent shown to attenuate tissue damage and promote functional recovery in animal models by attenuating lipid peroxidation (Braughler and Hall 1982; Constantini and Young 1994; Hall 1992). However, use of methylprednisolone in humans remains controversial because of the risk of harmful side effects (e.g, septic shock, pneumonia, gastrointestinal bleeding, and wound infection) in the face of only minor functional improvements (Fehlings 2001; Hall and Springer 2004; Hurlbert 2000; Sayer et al. 2006). Therefore, the development of a safe and effective treatment for SCI remains a priority for researchers and clinicians. Accordingly, the goal of this thesis project was to identify and characterize a pathophysiological mechanism of SCI, and then test a clinically relevant treatment strategy that targets this source of damage.

Plasma membrane damage as a therapeutic target for SCI treatment

Plasma membrane damage after CNS injury

The plasma membrane serves as a barrier that maintains the structure and organization of cell, regulates molecular transport, and sustains concentration gradients essential for cell survival and function. Plasma membrane disruptions have been observed in many models of traumatic CNS injury (Choo et al. 2007; Geddes-Klein et al. 2006; Geddes et al. 2003; LaPlaca et al. 1997; Pettus et al. 1994; Prado et al. 2005; Shi 2004; Shi et al. 2000; Whalen et al. 2007). Non-specific pores in the plasma membrane occur in the initial phase of traumatic CNS injury (within minutes) as a direct result of mechanical forces that have surpassed the structural threshold of the cell. This is not entirely surprising, considering the plasma membrane of other cell types are known to rupture as a result of a mechanical perturbation (Clarke et al. 1995; Guzman et al. 2001; McNeil 1989; Schlicher et al. 2006; Yu and McNeil 1992). However, this phenomenon has largely been unexplored in models of CNS trauma and many questions remain unanswered in regard to the fate of permeabilized cells.

Plasma membrane disruptions likely play a large role in the pathophysiology of SCI, especially given the importance of the lipid bilayer in maintaining homeostasis and the finding that permeability changes have been shown to persist for days to weeks after traumatic CNS injury (Farkas et al. 2006; Shi 2004; Whalen et al. 2007). It is currently unknown whether long-term membrane damage is caused by: 1) an inability to reseal mechanically-induced holes in the plasma membrane, 2) the presence of a persistent stimulus in the post-injury environment that generates new membrane breaches (e.g., free radicals or phospholipase activity), or 3) a combination of the two. Previous work

suggests that a combination of these mechanisms is responsible (Farkas et al. 2006; Whalen et al. 2007); therefore, the working hypothesis is that a subset of plasma membrane breaches are formed through mechanical destruction in the acute phase whereas others are created through secondary injury mechanisms (as depicted in Figure 1.1).

The mechanisms involved in plasma membrane resealing after traumatic CNS injury have been explored in previous studies. Membrane repair requires calcium (Ca^{2+}) entry into the cytoplasm (presumably via diffusion through the hole created in the membrane). Ca^{2+} influx triggers vesicles to fuse with each other and also with the adjacent plasma membrane, thus transporting membrane components to the outermost cell membrane (Eddleman et al. 1997; McNeil and Terasaki 2001). In addition, Ca^{2+} influx activates the cysteine protease calpain (Howard et al. 1999; Shi et al. 2000; Xie and Barrett 1991; Yawo and Kuno 1985; Yoo et al. 2003). When combining this evidence with studies conducted in other cell types and injury models, it is postulated that calpain-mediated degradation of the actin cytoskeleton is necessary for removing the barrier between vesicles and the plasma membrane, thus clearing the path for vesicle-mediated resealing (Eitzen 2003; McNeil and Kirchhausen 2005; Miyake et al. 2001).

Although these studies have provided valuable information about plasma membrane resealing after traumatic injury, it should be noted that they were conducted *in vitro* and thus are somewhat limited in their interpretation. The *in vivo* scenario is more complex and presents different environmental conditions that may affect plasma membrane damage and the subsequent repair response. Notably, the resealing time course is much shorter *in vitro* (Farkas et al. 2006; Geddes et al. 2003; Prado et al. 2005;

Shi 2004; Shi et al. 2000; Whalen et al. 2007), suggesting that factors found *in vivo* differentially affect membrane resealing in comparison to those represented *in vitro*. For example, one important variable not modeled *in vitro* is that extracellular Ca^{2+} concentrations are dynamic and greatly depressed after SCI (Stokes et al. 1983; Young et al. 1982), which would presumably slow down or prevent the Ca^{2+}-mediated membrane repair processes. In addition, *in vitro* CNS trauma models do not typically represent ischemia and the inflammatory response, both of which may cause plasma membrane damage and affect the cell's ability to reseal. Thus, the mechanisms contributing to plasma membrane compromise and repair *in vivo* are complex and are not completely understood.

Alterations in phospholipid metabolism following SCI may introduce additional factors that can exacerbate membrane permeability or delay/prevent membrane repair *in vivo*. In general, SCI causes a shift that enhances membrane breakdown and decreases membrane synthesis. Previous work has shown that SCI causes a reduction in phospholipid levels and an accumulation of free fatty acids (Demediuk et al. 1989a; Lemke et al. 1990; Murphy et al. 1994). Phospholipids are destroyed mainly through two mechanisms: hydrolysis and peroxidation. Phospholipid hydrolysis occurs through activation of the phospholipase A_2 (PLA2) family of enzymes (Farooqui et al. 1997; Liu et al. 2006; Olivas and Noble-Haeusslein 2006), whereas lipid peroxidation is due to oxidative stress caused by an accumulation of free radicals (Anderson and Hall 1993; Carlson et al. 1998; Hall and Braughler 1986).

The interplay between these mechanisms is complex and lead to a vicious positive feedback cycle that destroys membrane integrity (see Figure 1.1). PLA2 activation

occurs as a result of Ca^{2+} influx, which can be generated by ion diffusion through membrane disruptions (Bauldry et al. 1996). Activated PLA2 then hydrolyzes phospholipids to release fatty acids. This removal of phospholipids can compromise membrane integrity and lead to further Ca^{2+} increase, thereby activating more PLA2 (O'Regan et al. 1996). Moreover, the PLA2-mediated generation of fatty acids (arachidonic acid in particular (Demediuk et al. 1985)) leads to eicosonoid synthesis and generation of free radicals (Farooqui et al. 1997; Liu et al. 2006; Olivas and Noble-Haeusslein 2006). Free radical accumulation, generated through arachidonic acid metabolism as well as the inflammatory response, can cause lipid peroxidation. To further intensify the problem, the chemical reaction between a lipid and a free radical creates a lipid peroxide radical, which can subsequently react with other phospholipids in the membrane to produce more lipid peroxide radicals (for review see (Anderson and Hall 1993; Hall and Braughler 1986; McCall et al. 1987). Thus, under pathophysiological conditions, the interplay between these mechanisms causes a chain reaction that compromises the integrity of the plasma membrane (Kumar et al. 2003).

In summary, plasma membrane compromise occurs after SCI and is a dynamic, complex pathophysiological process. Plasma membrane damage is a result of both the primary insult (which causes mechanical disruption) and subsequent activation of secondary injury mechanisms (e.g., inflammation and enzymatic activation), as shown in Figure 1.1. Traumatic CNS injury causes membrane defects that are quite large (allowing entry of molecules as large as 44 kDa (Pettus et al. 1994; Pettus and Povlishock 1996; Shi et al. 2000; Shi and Whitebone 2006)), which may lead to cell death, degeneration, and/or dysfunction.

Targeting membrane repair for treatment of SCI

Although persistent plasma membrane damage is likely to negatively influence post-SCI outcome, it also presents a therapeutic opportunity to restore function and prevent cell death/dysfunction. Plasma membrane compromise is postulated to initiate numerous detrimental cellular responses by preventing the cell from maintaining homeostasis. Therefore, targeting membrane repair may be an effective approach because it can alleviate numerous deleterious pathways at once. Compromise of the plasma membrane can directly lead to cell death if the defect is large enough, but some cells are able to reseal or remain permeable without dying (Farkas et al. 2006; Geddes-Klein et al. 2006; Geddes et al. 2003; Prado et al. 2005; Whalen et al. 2007). However, it is likely that permeabilized cells that are able to reseal or survive without resealing can experience longer-term cell death and/or dysfunction through the activation of aberrant signaling pathways. For example, compromise of the plasma membrane results in Ca^{2+} flux down its concentration gradient into the cell. Ca^{2+} influx after CNS injury has been correlated with many damaging downstream events, including endonuclease activation, cytoskeletal breakdown, mitochondrial damage, and apoptotic cell death (for review, see (Dumont et al. 2001)). In fact, increases in plasma membrane permeability have been associated with pathophysiological events such as protease activation (Farkas et al. 2006), conduction block (Shi and Borgens 2000), and delayed cell death (Whalen et al. 2007).

Previous research has shown that membrane resealing agents are able to provide benefit following CNS trauma. It has been demonstrated the polyethylene glycol (PEG) mediates resealing and promote functional recovery following SCI (Borgens 2001;

Borgens and Shi 2000; Laverty et al. 2004; Luo et al. 2002; Luo and Shi 2007; Shi and Borgens 2000). PEG is a hydrophilic polymer that promotes fusion of opposing membranes, thereby enhancing vesicle fusion and joining adjacent cellular membranes (e.g., transected axons) (Krause and Bittner 1990; Lentz and Lee 1999; Lore et al. 1999). Although PEG has been effective in animal models, clinical translation will require caution. PEG has also been used to fuse healthy cells together (Davidson and Gerald 1976), which may lead to unintentional harmful effects. Furthermore, prolonged PEG application has been shown to negatively affect both healthy and injured spinal cords (Cole and Shi 2005). Therefore, patient safety will require great care in ensuring that the appropriate treatment regimens are followed.

Poloxamer 188 has also been shown to promote spinal cord repair following traumatic injury (Borgens et al. 2004; Follis et al. 1996; Kilinc et al. 2007; Laverty et al. 2004; Serbest et al. 2005; Serbest et al. 2006). The amphiphilic structure (a tri-block copolymer consisting of a central hydrophobic chain surrounded by 2 hydropholic chains), allows it to insert directly into the cell membrane, thereby promoting membrane repair and cell survival (Baekmark et al. 1997; Marks et al. 2001; Maskarinec et al. 2002; Serbest et al. 2006; Sharma et al. 1996). These findings provide supporting evidence that plasma membrane damage is detrimental to post-injury outcome and that targeting membrane repair is an effective approach for SCI treatment. However, a treatment that augments endogenous membrane repair may be safer and more clinically effective for the treatment of SCI.

Citicoline as a membrane resealing agent and therapy for SCI

Citicoline (a pharmaceutical agent identical to the endogenous molecule CDP-choline or cytidine diphosphate choline) is a nucleotide that serves as the endogenous choline donor in the synthesis of acetylcholine and phospholipids (mainly phosphatidylcholine (PC), the major membrane component in mammalian cells) (Kennedy and Weiss 1956). Citicoline cannot be made by the body and is therefore ingested through the diet (Best and Huntsman 1932) or found in cell culture medium (Vance and Vance 2004). When ingested, citicoline is absorbed in the digestive tract then broken down into its components, cytidine and choline, in the gut wall and liver. Cytidine and choline then circulate throughout the blood stream and are utilized in various parts of the body for phospholipid synthesis (for review see ((Conant and Schauss 2004; Weiss 1995)). Choline enters cells through transporter proteins (Yamamura and Snyder 1972) and is then utilized for PC synthesis through several enzymatic reactions, as summarized in Figure 1.2 (for review see (Vance and Vance 2004)). First, choline is converted to phosphocholine by the enzyme choline kinase. Phosphocholine is then converted to CDP-choline by CTP:phosphocholine cytidylyltransferase. Finally, cholinephosphotransferase transfers phosphocholine to diacylglycerol from citicoline to create PC. The importance of this mechanism in maintaining cellular function is underscored by experimental evidence demonstrating that blocking this pathway is sufficient to induce cell death *in vitro* (Cui and Houweling 2002; Cui et al. 1996).

Citicoline has shown promising results for treatment of various neurological diseases and disorders in humans and animals, where citicoline plays a role in both phospholipid production and membrane preservation (for review see (Adibhatla and

Hatcher 2002; Conant and Schauss 2004; Zweifler 2002)). Exogenous citicoline enhanced PC levels as well as sphingomyelin and cardiolipin (a major lipid component of the inner mitochondrial membrane) in an animal model of stroke (Rao et al. 2000). In addition to promoting plasma membrane synthesis after an ischemic insult, citicoline also prevents plasma membrane damage by reducing oxidative damage, decreasing arachidonic acid production (which reduces free radicals and lipid peroxidation), and attenuating PLA2 activity (Adibhatla and Hatcher 2003; Arrigoni et al. 1987; Fresta et al. 1994; Rao et al. 1999). Citicoline is currently approved for treatment of stroke in over 70 countries worldwide and is undergoing clinical trials in the United States. Initial US trials showed a beneficial effect (Clark et al. 1997), but subsequent trials were only moderately successful and depended on the criteria used (Clark et al. 2001; Davalos et al. 2002). As a treatment for SCI, citicoline may offer advantages over methylprednisolone, as almost no side effects have been observed (for review see (Adibhatla and Hatcher 2002; Conant and Schauss 2004; Zweifler 2002)).

Recent work has assessed the effects of citicoline as a treatment for SCI. Cakir et al (2005) demonstrated that citicoline treatment improved hindlimb locomotion at 24 and 48 hours post-SCI and reduced lipid peroxidation at 48 hours. Yucel et al (2006) compared the effects of citicoline to methylprednisolone as well as a combination of citicoline and methylprednisolone. At 24 hours post-SCI, citicoline attenuated lipid peroxidation, increased levels of the antioxidant glutathione, and decreased nitric oxide levels (indicative of a reduction in oxidative stress). In addition, citicoline improved functional outcome (locomotor function and ability to balance on an inclined plane) and reduced the area of the lesion at 6 weeks post-injury. These effects were equivalent to

methylprednisolone treatment but did not offer additional benefit when used in combination with methylprednisolone. Thus, citicoline offers great promise as a treatment for SCI, but more extensive pre-clinical work can provide more information about the mechanisms involved and ensure that its benefits can be reproduced in various laboratories. Reproducibility and detailed information about the mechanisms of action can provide more confidence when translating a treatment from the benchtop to the bedside and are therefore necessary aspects of pre-clinical research.

Current research

This research project addresses the need to improve the current understanding of the pathophysiology of SCI and develop effective, clinically relevant, and mechanistically-driven treatments. Our specific objectives were to better understand the characteristics of SCI-induced plasma membrane compromise and to test the therapeutic efficacy and membrane stabilizing properties of citicoline. Since little is currently known about the extent of primary damage after contusion SCI, we first characterized acute plasma membrane damage as a function of injury severity and pore size (Chapter 2). Results showed that the vast majority of cells and axons at the injury site were damaged by the mechanical insult and that plasma membrane defects were associated with pathological changes in morphology. Our findings show that membrane permeability is widespread and suggest that it can have a significant role in cell death/dysfunction following SCI.

Membrane damage persisted for at least 5 weeks post-SCI (Chapter 4), which provided motivation for administering the membrane stabilization agent citicoline as a treatment. We assessed the effects of citicoline on membrane integrity and PLA2 activity

(Chapter 3), since these are the primary mechanisms postulated to lead to citicoline-mediated improvements after stroke and they have not been investigated in a model of SCI (Adibhatla and Hatcher 2003; Arrigoni et al. 1987). However, citicoline treatment did not reduce PLA2 activity at 24 hours, nor did it attenuate plasma membrane compromise or lesion volume at 3 days post-SCI.

To address the possibility that citicoline requires more than 3 days to have an effect, we conducted a longer-term study assessing behavioral and histological outcomes (Chapter 4). Treatment with citicoline did not have a biologically significant effect on hindlimb function or lesion volume. Although we were unable to reproduce the findings from other studies (Cakir et al. 2005; Yucel et al. 2006), it should be noted that we delivered citicoline at higher doses and more frequently (which was more consistent with the stroke literature but different from previous SCI research). Future work assessing citicoline dosing and the mechanisms of chronic plasma membrane damage may lead to an effective membrane repair strategy for SCI treatment.

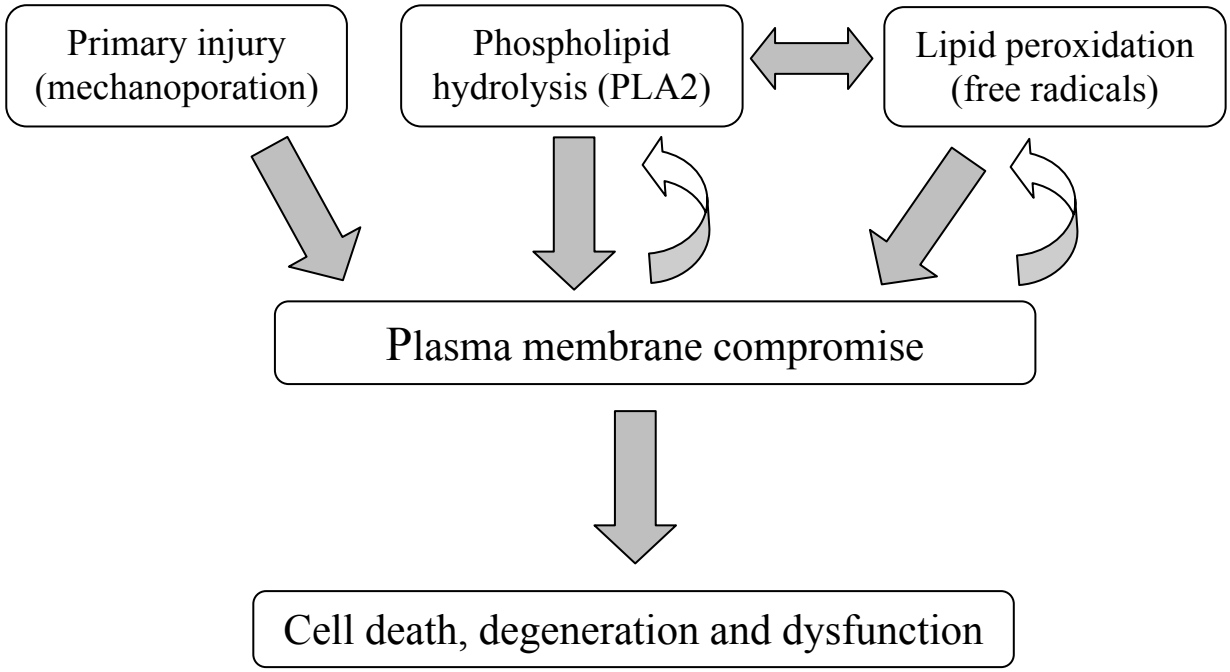


Figure 1.1: Mechanisms involved in SCI-induced plasma membrane damage. Plasma membrane compromise is caused by both primary and secondary injury pathways, ultimately affecting cell survival and function. The mechanical insult can disrupt the membrane in the acute phase by surpassing structural thresholds. Sustained membrane damage can occur through phospholipid hydrolysis or lipid peroxidation. Phospholipids are hydrolyzed by PLA2, which results in the production of arachidonic acid, a mediator of free radical formation. Free radicals destroy the membrane through lipid peroxidation, which generates additional free radicals in a positive-feedback cycle.

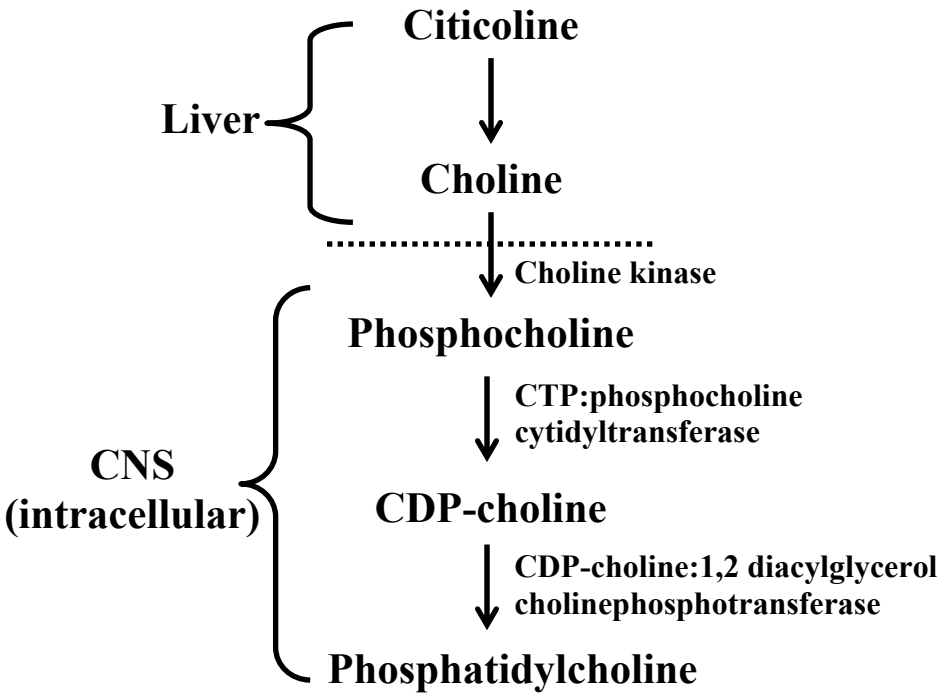


Figure 1.2: Schematic of endogenous phosphatidylcholine synthesis. Citicoline plays a fundamental role in the maintenance and preservation of the plasma membrane. Citicoline enters the digestive tract and is broken down into its components cytidine and choline in the gut and liver (not shown in this diagram). Choline enters the tissue and is transported intracellularly, where it is utilized for the synthesis of phosphatidylcholine according to the pathway illustrated here.

CHAPTER 2

ACUTE PLASMA MEMBRANE COMPROMISE CAUSED BY SPINAL CORD CONTUSION INJURY

Introduction

Spinal cord injury (SCI) is a debilitating condition that dramatically affects quality of life for the affected individual and their families. Clinically available treatments provide modest benefit, if any at all; therefore ongoing research is aimed at developing more effective therapies for spinal cord repair and regeneration (for review see (Baptiste and Fehlings 2007; Hall and Springer 2004; Kwon et al. 2004)). A greater understanding of the time course of destructive and degenerative mechanisms involved in SCI is expected to improve the ability to develop targeted therapeutic strategies.

In the acute phase of SCI, primary damage occurs as a direct result of trauma when structural thresholds are surpassed, leading to immediate physical and biochemical cellular alterations. As a result of these immediate changes, a complex secondary injury cascade is initiated and ensues in the following hours, weeks, and months. Secondary injury mechanisms such as apoptotic cell death, inflammation, and formation of the glial scar are considered to be largely deleterious to regeneration and are commonly targeted for treatment of SCI (Dumont et al. 2001; Hagg and Oudega 2006). Although it is well-documented that increasing the severity of loading parameters exacerbates tissue damage and neurological dysfunction (Basso et al. 1996a; Beattie et al. 1997; Kloos et al. 2005; Scheff et al. 2003; Wrathall et al. 1985), relatively little is known about how the

mechanical impact (primary injury) initiates secondary injury. Improving the current understanding of the characteristics of primary damage after contusion SCI can facilitate a better understanding of acute injury mechanisms and their relationship to the overall pathophysiology.

One direct result of the mechanical impact in traumatic CNS injury is the formation of non-specific breaches in the neuronal plasma membrane. Plasma membrane compromise has been observed in many models of neuronal injury and is postulated to be detrimental to post-injury outcomes (Farkas and Povlishock 2007; LaPlaca et al. 1997; LaPlaca et al. 2007; Pettus et al. 1994; Pettus and Povlishock 1996; Povlishock and Pettus 1996; Shi 2004; Shi et al. 2000; Singleton and Povlishock 2004; Stone et al. 2004; Whalen et al. 2007). Under physiologic conditions, the plasma membrane serves as a barrier that maintains the structure and organization of the cell, regulates molecular transport, and preserves concentration gradients essential for cell survival and function. However, these homeostatic mechanisms are disrupted when mechanical impact to CNS tissue physically ruptures the plasma membrane. Although many permeabilized cells die immediately through necrosis, a subset are able to reseal without undergoing acute cell death or degeneration (Farkas et al. 2006; Shi 2004; Shi et al. 2000; Whalen et al. 2007). Surviving cells, however, may experience downstream consequences of plasma membrane compromise (for review see (Barbee 2005; Farkas and Povlishock 2007)). By temporarily or permanently destroying the barrier between the cytosol and the extracellular fluid, ions and other molecules could freely enter or exit the cell. This unregulated molecular flux would be expected to negatively affect cell function and survival. For example, Ca^{2+} influx down its concentration gradient would result in

mitochondrial damage, aberrant enzyme activation, and apoptotic cell death (for review see (Dumont et al. 2001)). In fact, increases in plasma membrane permeability after CNS injury have been associated with pathophysioglogical events such as protease activation (Farkas et al. 2006), conduction block (Shi and Borgens 2000), and delayed cell death (Whalen et al. 2007). Although membrane compromise may play a critical role in the pathophysiology of SCI, little is known about the anatomical distribution of permeable cells within the injured spinal cord or the size of the membrane defects in those cells.

The objective of this study was to better characterize acute plasma membrane damage induced by *in vivo* contusion SCI, since membrane compromise may be an initiating factor for numerous deleterious events. The spatial profile of cellular damage was assessed as a function of injury severity and the size of the plasma membrane defect in a rat contusion model. Results obtained in this study aid in defining the extent of SCI-induced primary damage and can facilitate the discovery of pathophysiological mechanisms involved in transducing a mechanical input into a cellular response.

Methods

Surgical procedures

All procedures involving animals were approved by the Georgia Tech Institutional Animal Care and Use Committee (protocol A05003). Male Sprague-Dawley rats (275-400g; total n = 35) were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg). Isoflurane (1-3%) was used to maintain anesthesia when necessary. Animals were placed on a stereotaxic apparatus and the atlantoocipital membrane was surgically exposed. An incision was made in the dura, and a catheter composed of PE10 tubing was attached to the needle of a Hamilton syringe and inserted into the intrathecal space. A

fluorescent dye normally unable to penetrate the cell membrane was injected slowly over 10 minutes (4% lucifer yellow in deionized water, 2.5% FITC-conjugated 10kDa dextran (10dex) in saline, or 2.5% FITC-conjugated 3kDa dextran (3dex) in saline; 15-20 µl total volume). All permeability markers were obtained from Invitrogen (Carlsbad, CA). A summary of the molecular weight and corresponding radius can be found in Table 2.1. Concentrations and solvents were based on previous studies assessing plasma membrane damage in neural tissue (Choo et al. 2007; Farkas et al. 2006; Howard et al. 1999). Ten minutes after the dye injection, the catheter was removed and the muscle and skin at the surgical site was sutured closed.

Permeability dyes were allowed to diffuse throughout the CSF for 3h prior to injury. SCI was induced according to methods described in detail elsewhere in this thesis (Appendix A). Briefly, a laminectomy was performed at T10 vertebral level to expose the underlying spinal cord tissue. Three injury severities were used to examine the distribution of membrane damage: 100, 150, or 200 kdyn impact force (Infinite Horizons device; Precision Systems and Instrumentation, Lexington, KY). These forces correspond to mild, moderate, and severe injury levels, respectively according to histological and behavioral outcome measures (Scheff et al. 2003). Sham animals were subjected to all surgical procedures including the dye injection and laminectomy, but the spinal cord was not impacted. Animals were transcardially perfused 10 minutes following SCI with phosphate buffered saline (PBS) followed by fixative (4% paraformaldehyde / 0.1% gluteraldehyde in PBS). The vertebral column was subsequently removed from the rat and immersed in fixative at 4ºC. After 24h, spinal cord tissue was dissected from the vertebral column and placed in sucrose at 4ºC. Tissue

was cut into 2cm segments, frozen in OCT, and then cryosectioned in the transverse plane.

Verification of cellular uptake of permeability markers

Immunohistochemistry was performed using thin sections (10 μm) to verify that permeability marker uptake was neuronal. Primary antibodies specific for neurofilament-160 (NF-160, 1:100; Sigma NF264, St. Louis, MO), NeuN (1:100; Millipore MAB377, Billerica, MA), and glial fibrillary acidic protein (GFAP, 1:500; Millipore MAB360) were used to identify axonal projections, neuronal cell bodies, and astrocytes in the tissue, respectively. Briefly, slides were rinsed in PBS then immersed in blocking/permeabilization solution (8% goat serum and 0.4% Triton X100 in PBS) for 1 hour at room temperature. Sections were then incubated in primary antibody solution (primary antibody diluted in 8% goat serum and 0.4% Triton X100 in PBS) overnight at 4°C in a humidified chamber. Slides were rinsed in PBS then incubated in AlexaFluor 546 or 568 (1:500, Invitrogen) secondary antibody solution (2% goat serum in PBS) for 2h at room temperature in a humidified chamber. Following a final rinse in PBS, slides were coverslipped using an aqueous mounting medium. Images were obtained at 40x magnification using a confocal microscope (LSM 510, Carl Zeiss MicroImaging Inc., Thornwood, NY).

Quantification of permeability marker uptake in cell bodies

Three different fluorescent dyes (lucifer yellow, 3dex, or10dex) were used to assess membrane damage in neuronal cell bodies. Lucifer yellow uptake was assessed after sham surgery or mild, moderate, and severe injury (100, 150, or 200 kdyn impact

force) in order to investigate whether plasma membrane damage is dependent upon impact force. Additional experiments compared uptake of 3dex and 10dex in order to better characterize the size of SCI-induced membrane defects.

Uptake of permeability markers was quantified at 1mm increments up to 3mm from the injury epicenter in both the rostral and caudal directions (n=3-5 rats per group). The number of permeable cells was quantified in thick sections (35 μm) to allow for visualization of large motor neurons. Cells were counted manually with the assistance of StereoInvestigator software (MBF Bioscience, Williston, VT). Although the fluorescent dyes accumulated around blood vessels, only cells with neuronal morphology (as shown in Figure 2.1) were included in the quantification. Sections at the injury epicenter were excluded from the analysis because the extensive tissue damage did not permit reliable cell counts. In order to reduce variability, two adjacent sections were counted at each 1mm increment distance from the epicenter, and these values were averaged together to yield one data point per animal at each distance. Data were analyzed using two-way repeated measures ANOVA followed by Tukey's pairwise comparisons, with distance from the epicenter and impact force or dextran size as the independent variables and the number of positive cells as the dependent variable.

Quantification of axonal uptake of dextran molecules

The density of dextran-positive axons was quantified within thin sections (10 μm) using ImageJ software (National Institutes of Health, Bethesda, Maryland). Lucifer yellow was not observed within axonal projections, possibly due to dye leakage or rapid diffusion of the small molecule along the axon. Montage images of each section were acquired at 10x magnification using NeuroLucida software (MBF Bioscience). The

density of permeable axons was determined within an area of approximately 85 μm^2 in ventral, dorsal, and lateral regions. Within these areas, thresholds for fluorescence intensity (determined by a blinded observer) and size (0.25-25 μm^2) were used to eliminate autofluorescent debris from the analysis. Each section was quantified using two independent measurements in each quadrant of the section (ventral, dorsal, lateral left, and lateral right) and an average was taken. Data were analyzed using two-way repeated measures ANOVA followed by Tukey's pairwise comparisons, with distance from the epicenter and dextran size as the independent variables and the number of positive cells as the dependent variable.

Assessment of cellular morphology

Cellular morphology was evaluated in paraffin sections taken from two adult male Sprague-Dawley rats. 3dex was injected intrathecally 3h prior to 150kdyn spinal cord impact, and animals were perfused 10 min after SCI with PBS followed by 4% paraformaldehyde and 0.1% gluteraldehyde in PBS (as described above). Longitudinal sections (5μm thick) were stained with cresyl echt violet prior to imaging. Fluorescent and bright field images were obtained at 63x magnification using a confocal microscope (Zeiss LSM 510, Carl Zeiss Corporation, Oberkochen, Germany).

Results

Intra-axonal and intra-cellular uptake of fluorescent dyes following SCI

Immunohistochemistry confirmed that regions with intense fluorescence represented cellular uptake of the permeability markers. NeuN staining demonstrated that dye uptake within the gray matter was neuronal (Figure 2.1, A-C). All three

permeability markers (lucifer yellow, 10dex, and 3dex) were easily visualized within cell bodies, allowing for quantification of cells that had become permeable to the fluorescent dye. Dye uptake was distributed homogenously throughout the gray matter, with no preferential uptake in the dorsal or ventral regions.

Axonal uptake of the permeability markers was verified using immunohistochemistry specific for NF-160. Regions with dye accumulation co-localized with NF-160 staining, confirming intra-axonal uptake of the permeability marker (Figure 2.1, D-F). Accumulation of 10dex and 3dex was observed within axolemma. Although influx of the smallest dye (lucifer yellow) was expected within axons, the presence of this molecule was not observed at levels above background. The absence of intra-axonal lucifer yellow may be attributed to a low signal-to-noise ratio due to diffusion of the dye along the axon or leakage of the molecule out of large tears in the plasma membrane. Axonal dextran uptake was evenly dispersed at the injury epicenter, with the vast majority of axons taking up the dye.

Additionally, permeability marker uptake was observed within astrocytes (mainly located adjacent to blood vessels) within the spinal cord white and gray matter, as confirmed with immunohistochemistry specific for GFAP (data not shown). This was seen in both injured and sham animals; therefore, we did not interpret this to be an SCI-induced response. Permeability marker uptake within cells with this morphology was not included in the quantification.

Lucifer yellow uptake within cell bodies following sham surgery or injury at mild, moderate, or severe impact forces

Lucifer yellow uptake within cell bodies was quantified at 1mm increments up to 3mm from the epicenter in the rostral and caudal directions. Increasing the impact force caused a corresponding increase in the rostral-caudal extent of permeability marker uptake (Figure 2.2). Interestingly, lucifer yellow uptake was asymmetrical in nature, with more positive cells found in the caudal direction. Severe injury (200 kdyn) resulted in the greatest membrane permeability alterations, with significant differences compared to sham out to 2mm rostrally and 3mm caudally. Significant lucifer yellow uptake was observed after mild (100 kdyn) and moderate (150 kdyn) injury in the caudal direction up to 1mm and 2mm, respectively. Additionally, permeability marker uptake after impact of 150 and 200 kdyn was significantly higher compared to mild injury at 2mm in the caudal direction, and 200 kdyn impact induced significantly more uptake compared to 100 kdyn impact at 3mm caudally. These data demonstrate that the impact force dictates the anatomical distribution of plasma membrane damage.

Plasma membrane defect size within cell bodies following SCI contusion

We compared the cellular uptake of two different permeability markers (3dex and 10dex) in order to characterize the size of SCI-induced defects in the plasma membrane. More dye uptake was observed in shams receiving lucifer yellow compared to 10dex or 3dex, which did not allow for direct comparison of injured animals among these groups. Sham uptake was not significantly different for animals receiving injections of 3dex and 10dex; therefore, these groups were combined for analysis. Significant differences between sham and 3dex uptake were observed up to 2mm in the rostral and caudal

directions, whereas 10dex uptake was limited to 1mm in either direction (Figure 2.3). In addition, 3dex uptake was greater than 10dex uptake at 1 and 2 mm caudal of the injury epicenter; therefore we can infer that some cells incorporating 3dex were able to exclude 10dex in these anatomical regions. These results show that non-specific membrane defects induced by contusion SCI range in size (a subset larger than 5.4 nm in diameter, a subset between 5.4 and 3.2 nm in diameter, and a subset excluding both molecules). The size of membrane defects varied as a function of distance from the injury epicenter, with larger membrane defects located closer to the site of impact.

Plasma membrane defect size in axons following SCI contusion

Axonal uptake of 10dex and 3dex was assessed at discrete distances from the injury epicenter in order to determine the size of membrane defects. Permeability marker uptake in shams in the 10dex and 3dex group was statistically the same at all distances; therefore, these animals were combined in the analysis. Uptake of 3dex within axons extended 2 mm in both the rostral and caudal directions, and 10dex uptake was significant compared to sham up to 2 mm rostrally and 1 mm caudally. No differences between 3dex and 10dex uptake were observed at any location (Figure 2.4). From these results, we concluded that axonal permeability occurs following SCI and that pores are at least as large as 5.4 nm in diameter.

Morphological alterations associated with plasma membrane compromise

High magnification confocal imaging of cells within spinal cord tissue revealed abnormal cellular morphology in many cells taking up the permeability marker 3dex. In many permeable cells, we observed pericellular blebs that were not filled with the 3dex

permeability marker. Blebs were visibly less dense than the cytoplasm within the cell and varied in size from very large blister-like morphologies that surrounded almost the entire cell (typically accompanied by cellular shrinkage, Figure 2.5, D-F) to small circular extensions on a small portion of the cell surface (Figure 2.5, A-C and G-I). Blebbing was observed in a very small subset of cells that did not take up 3dex (<1%) but was far more common in 3dex-positive cells. We therefore concluded that neuronal blebbing is associated with plasma membrane permeability. Although others have observed blebbing in response to chemical or mechanical cellular perturbation in other cell types (Barros et al. 2003; Keller et al. 2002; Marin-Castano et al. 2005; Mills et al. 1998; Shi et al. 2005), we are the first to our knowledge to report neuronal blebbing *in vivo* after traumatic CNS injury.

Discussion

Investigation of primary membrane permeability changes in SCI may facilitate an improved understanding of the overall pathophysiology and thus may lead to the development of treatment strategies targeted at spinal cord repair. To this end, we characterized acute plasma membrane damage in a clinically relevant contusion model by quantifying the plasma membrane defect size in both cell bodies and axons as a function of insult severity and anatomical location. Our investigation of the severity-dependence of membrane permeability revealed that the rostral-caudal extent of plasma membrane damage is related to impact force. In this study, increasing the impact force caused a corresponding increase in permeability marker uptake in distal regions. Previous studies have examined the relationship between injury biomechanics and plasma membrane permeability (Choo et al. 2007; Geddes-Klein et al. 2006; Geddes et al. 2003; Shi and

Whitebone 2006), but the relationship between impact force and cell membrane damage has not previously been investigated in an *in vivo* model of CNS trauma. *In vitro* work has demonstrated that membrane permeability increases with increasing strain rate and magnitude in axons as well as cell bodies (Geddes et al. 2003; LaPlaca et al. 1997; Shi and Whitebone 2006). In addition to a dependence upon injury parameters, the extent of primary damage has been shown to be a function of the mode of injury: a recent *in vivo* study demonstrated that distraction and dislocation injury results in more extensive plasma membrane damage in comparison to contusion SCI (Choo et al. 2007). Our data provide further supporting evidence that the parameters of the mechanical impact dictate acute biophysical plasma membrane failure.

We also evaluated membrane pore size by quantifying uptake of 10dex and 3dex molecules after moderate contusion SCI. Examination of both permeability markers confirmed that spinal cord impact causes plasma membrane damage in axons and cell bodies. Direct comparison of 3dex and 10dex uptake revealed differences in permeability marker uptake within the cell bodies located in the gray matter but not within axons. Cells with larger membrane disruptions were located closer to the injury epicenter. Our group has previously shown that more severe mechanical damage results in larger membrane defects in an *in vitro* model of neuronal injury (Geddes et al. 2003). Results obtained here further support this concept, as more severe mechanical damage is expected to be located near the injury epicenter (and thus corresponded to greater permeability marker uptake).

Uptake of 3dex and lucifer yellow exhibited an asymmetrical pattern with a larger number of permeable cells in the caudal direction compared to rostral of the injury site,

suggesting that the caudal direction in more susceptible to primary damage. These results are intriguing in light of previous studies that have shown that spinal cord tissue caudal to the injury experiences more gross tissue damage (Nossin-Manor et al. 2007), increased DNA fragmentation (Sribnick et al. 2007), and enhanced resistance to anti-apoptotic drugs (Ling and Liu 2007) in rats at 24 h after injury. Taken together, the data suggests that events occurring within minutes of SCI (such as plasma membrane damage) may initiate cell death and other deleterious mechanisms manifested later in time. One possible explanation for this asymmetrical cell and tissue damage is that all of these studies utilized a mid-thoracic contusion injury, in which the mechanical impact was located just rostral to the lumbar enlargement. This may have an effect on the loading distribution along the spinal cord since there is proportionally more gray matter in the caudal direction compared to rostral, and gray matter tissue has been shown to have a lower threshold for mechanical failure (Ichihara et al. 2001). In addition, this skewed gray-to-white matter ratio may have influenced these results because one would expect a larger number of cell bodies in the gray matter in the caudal direction. Although we did not normalize to the total number of cells, there was no asymmetrical distribution of 10dex or sham uptake (with any of the permeability markers we used), which would be expected if this effect were purely based on the total number of cells within the histological section.

Quantification of axonal permeability marker uptake showed no significant difference between SCI-induced uptake of 10dex and 3dex; therefore, we concluded that axolemmal plasma membrane defects are at least 5.4 nm in diameter. Previous studies have shown axonal uptake of molecules as large as 44 kDa (horseradish peroxidase)

(Pettus et al. 1994; Pettus and Povlishock 1996; Shi et al. 2000; Shi and Whitebone 2006). These data indicate that contusion injury causes larger membrane defects in axons compared to cell bodies. Axons may be more vulnerable to mechanical failure because of their elongated morphology or anatomical location on the outer edges of the spinal cord. In addition, *in vitro* evidence suggests that axons require much longer for resealing (on the order of 1 h) (Eddleman et al. 1997; Shi et al. 2000; Yoo et al. 2003), whereas neuronal cell bodies are able to reseal within minutes (Geddes et al. 2003; Prado et al. 2005). Although it is difficult to directly compare these studies due to variations in cell type/species, mechanical insult parameters, and the size of permeability markers, the results collectively suggest that cell bodies and axons differ in their structural thresholds and subsequent repair response. It should also be noted that the time required for resealing appears to be much longer *in vivo* (Farkas et al. 2006; Howard et al. 1999; Shi 2004). Future work assessing the time course and mechanisms involved in plasma membrane damage in both cell bodies and axons may elucidate the reasons for this discrepancy and provide the foundations for developing treatment strategies aimed at membrane repair.

In this study we also examined cellular morphology in permeable and non-permeable cells at 10 min post-injury. Blebbing was observed in cells within the injured spinal cord, and was almost exclusively found in the cells that took up the (3dex) permeability marker. No blebs were seen in cells located distally from the injury epicenter, suggesting that the blebbing phenomenon is specific to cells affected by the mechanical impact. Blebbing has been observed in other cell types in a process by which a decrease in membrane tension initiates a bulk flow of lipids and separation of the

plasma membrane from the underlying actin cytoskeleton (Charras et al. 2007; Charras et al. 2006; Keller and Eggli 1998; Keller et al. 2002). Although mechanoporation induced by the mechanical insult may be one initiating factor for membrane blebbing (by directly decreasing membrane tension through the formation of holes/tears), subsequent events such as oxidative stress, increased intracellular pressure, cytoskeletal alterations, and sodium influx may also cause post-SCI blebbing as observed in other cell types (Cunningham 1995; Marin-Castano et al. 2005; Rentsch and Keller 2000; Shi et al. 2005). Plasma membrane blebbing has been associated with apoptosis and necrosis (Barros et al. 2003; Laster and Mackenzie 1996; Mills et al. 1998; Tomiyoshi et al. 2004), but bleb formation does not necessarily lead to cell death (Hiruma et al. 2007; Marin-Castano et al. 2005; Shi et al. 2005). Future work should be aimed at a better understanding of the causes and effects of membrane blebbing and its relationship to plasma membrane disruptions.

Although plasma membrane damage has been observed in many models of traumatic CNS injury (Farkas et al. 2006; Geddes-Klein et al. 2006; Geddes et al. 2003; LaPlaca et al. 2007; Povlishock and Pettus 1996; Prado et al. 2005; Shi et al. 2000; Shi and Whitebone 2006; Whalen et al. 2007), the fate of cells experiencing membrane rupture remains unclear. Although plasma membrane failure may be indicative of necrotic cell death, permeable cells do not necessarily die at acute time points (Geddes-Klein et al. 2006; Guzman et al. 2001; Prado et al. 2005; Stroetz et al. 2001). Some cell types have been shown to survive much larger tears in the plasma membrane, most notably up to 1 μm^2 in sea urchin eggs with no evidence of cellular dysfunction (McNeil et al. 2000). Although the fate of cells experiencing membrane damage after CNS trauma

remains rather elusive, previous studies have correlated axonal membrane permeability marker uptake after CNS injury with irregular morphology (Kilinc et al. 2007; Stone et al. 2004) and abnormal electrophysiology (Prado et al. 2005; Shi and Whitebone 2006). In neuronal somata, acute plasma membrane permeability after traumatic brain injury has been correlated with cell death after cortical contusion (Whalen et al. 2007), but other reports utilizing a model of diffuse brain injury have found no strong correlations between dye uptake and morphological changes, fluorojade staining, or calpain activity (Farkas et al. 2006; Singleton and Povlishock 2004). It is therefore likely that plasma membrane defects can lead to a variety of cellular consequences depending on the magnitude and duration of damage.

Conclusions

This study investigated the anatomical distribution of plasma membrane permeability changes occurring acutely after SCI. Our results demonstrate that non-specific plasma membrane tears /defects form occur as a result of mechanical impact, with increased impact force causing more extensive damage. Furthermore, we observed that membrane damage of cell bodies within spinal cord grey matter are larger near the injury epicenter then decrease in size distally. Axonal membrane defects, however, were not variable in size for the range of permeability markers tested. Cellular uptake of permeability marker was associated with morphological changes indicative of blebbing and shrinkage, suggesting that breaches in the plasma membrane lead to cell death and/or dysfunction. Collectively, these results provide an enhanced foundation for understanding acute SCI pathophysiology and may offer insights for developing more mechanistically-driven treatment strategies.

Table 2.1. Permeability markers used to assess plasma membrane damage. Cellular uptake of three different fluorescent aldehyde-fixable molecules was quantified at 10 minutes following acute spinal cord injury. Dextran radii were approximated according to the equation $r = 0.488MW^{0.437}$ (to account for its chain-like configuration), whereas the radius of Lucifer yellow was calculated using the equation $r = 0.421MW^{0.427}$ (which assumes a globular conformation). These equations are based on a previous study in which the relationship between molecular weight and size was derived from experimental data examining particle movement across biological membranes (Oliver et al. 1992). Note that dextran size is reported as an average but actually contains a range of molecular masses.

Permeability marker	Molecular size (Da)	Molecular radius (nm)
Lucifer yellow	457	0.6
FITC-conjugated dextran	3,000	1.6
FITC-conjugated dextran	10,000	2.7

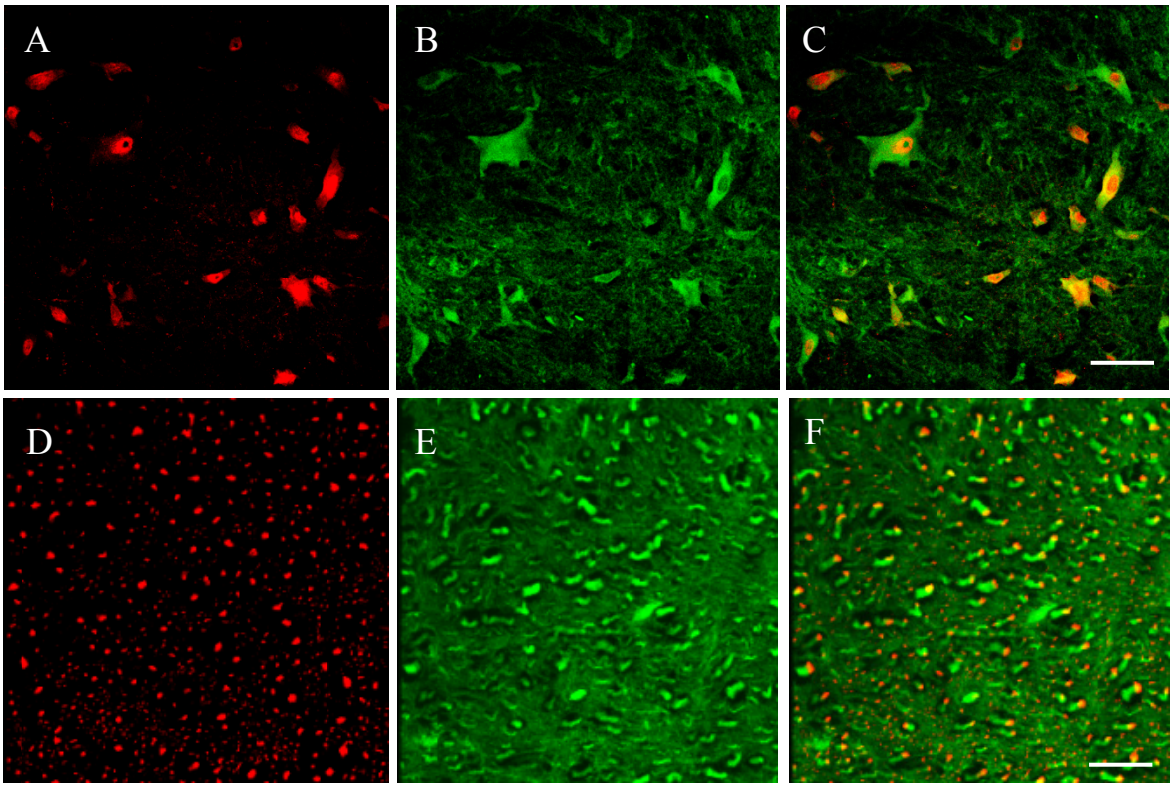


Figure 2.1. Permeability marker uptake following SCI. Histological assessment revealed permeability marker uptake in neuronal cell bodies and axons 10 minutes following SCI. Images shown here represent uptake of FITC-conjugated dextrans within 1mm caudal of the injury epicenter (3kDa, B-C; 10kDa, E-F). Immunohistochemistry specific for NeuN (A, C) and NF-160 (D, F) verified that dye uptake was within neuronal cell bodies and axons, respectively. Uptake was similar for both dextrans used in the study. However, Lucifer yellow was only visualized within cell bodies and not axonal projections, presumably due to dye leakage or rapid diffusion of the small molecule along the axon. Scale bar = 50μm (A-C) and 100 μm (D-F).

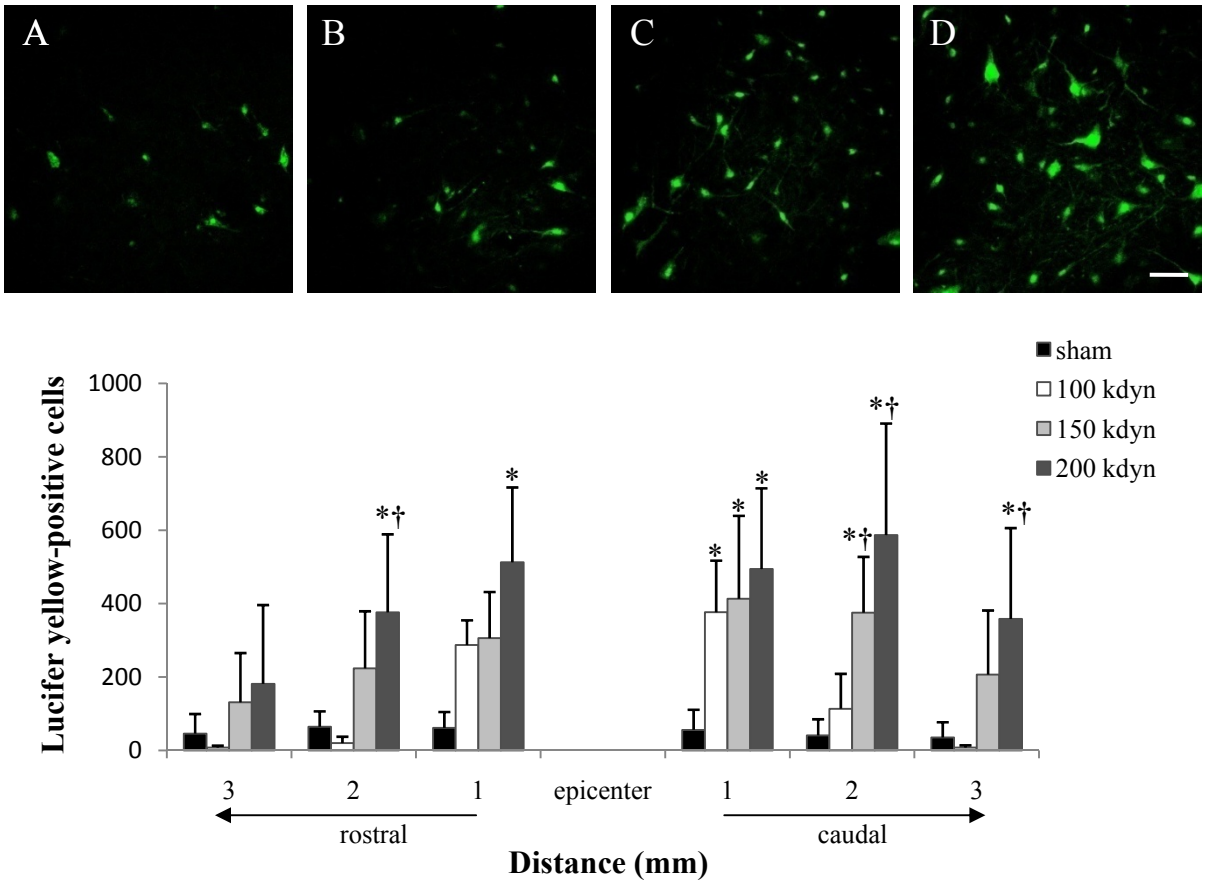


Figure 2.2. Lucifer yellow uptake as a function of injury severity. A dye exclusion assay was used to characterize plasma membrane damage 10 min following sham surgery (A) or contusion SCI with an impact force of either 100 kdyn (B), 150 kdyn (C), or 200 kdyn (D). Images shown here were taken from representative sections 2mm caudal of the epicenter. Quantification of cellular uptake in the gray matter indicated that the rostral-caudal extent of plasma membrane damage increased with the severity of the injury (mean ± standard deviation; *$p<0.05$ compared to sham, †$p<0.05$ compared to 100 kdyn; n=4-5 per group). Scale bar = 50 µm.

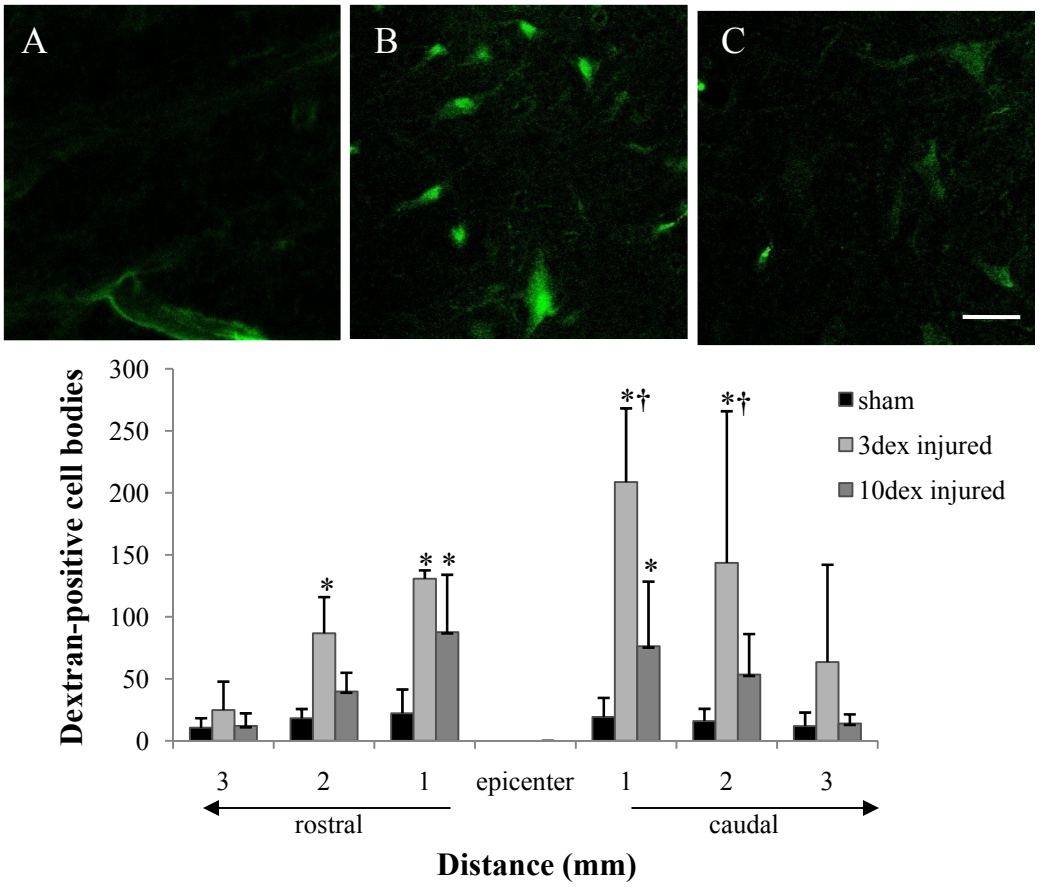


Figure 2.3. FITC-dextran uptake in cell bodies within gray matter. The number of cells permeable to 3kDa FITC-conjugated dextran (3dex) and 10kDa FITC-conjugated dextran (10dex) was quantified within tissue sections taken at discrete distances from the injury epicenter (n=3-4 per group; mean ± standard deviation). Results demonstrate acute SCI-induced permeability marker uptake of 3dex and 10dex after moderate injury (B and C, respectively) compared to sham controls (A) as a function of distance from the injury epicenter. More extensive uptake of the 3dex molecule was observed compared to 10dex, indicating that membrane defects of larger size are located proximal to the injury epicenter while smaller membrane tears extend distally. Images shown are confocal reconstructions at 1mm caudal of the epicenter. Scale bar = 40 μm.

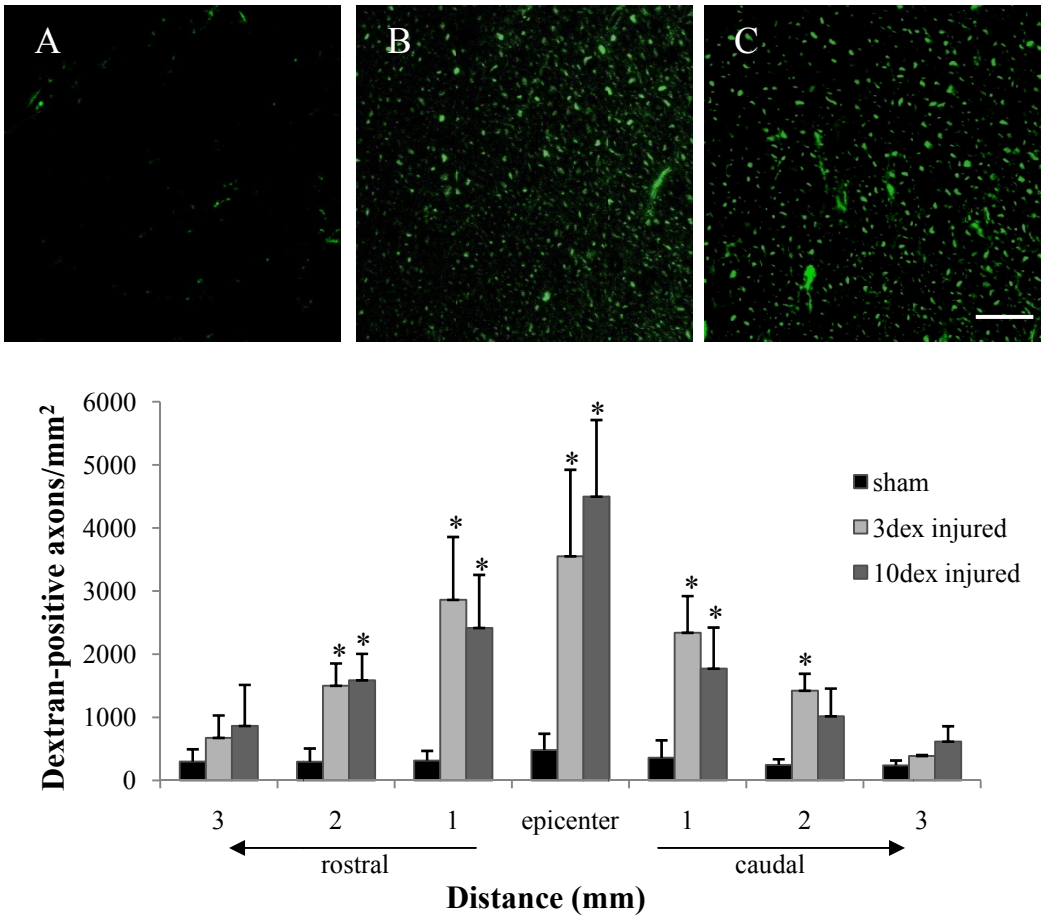


Figure 2.4. FITC-dextran uptake in axons of the white matter. Quantification of the density of dextran-positive axons revealed significantly greater permeability marker uptake extended up to 2mm in the rostral-caudal direction in injured animals compared to sham control. No significant differences between 3dex and 10dex uptake were found, demonstrating that axonal membrane defects are at least 2.7nm in radius (n=3-4 per group; mean ± standard deviation). Images shown here represent confocal reconstructions of sham (A), 3dex (B), and 10dex (C) permeability marker uptake 1mm caudal from the injury epicenter. Scale bar = 40μm.

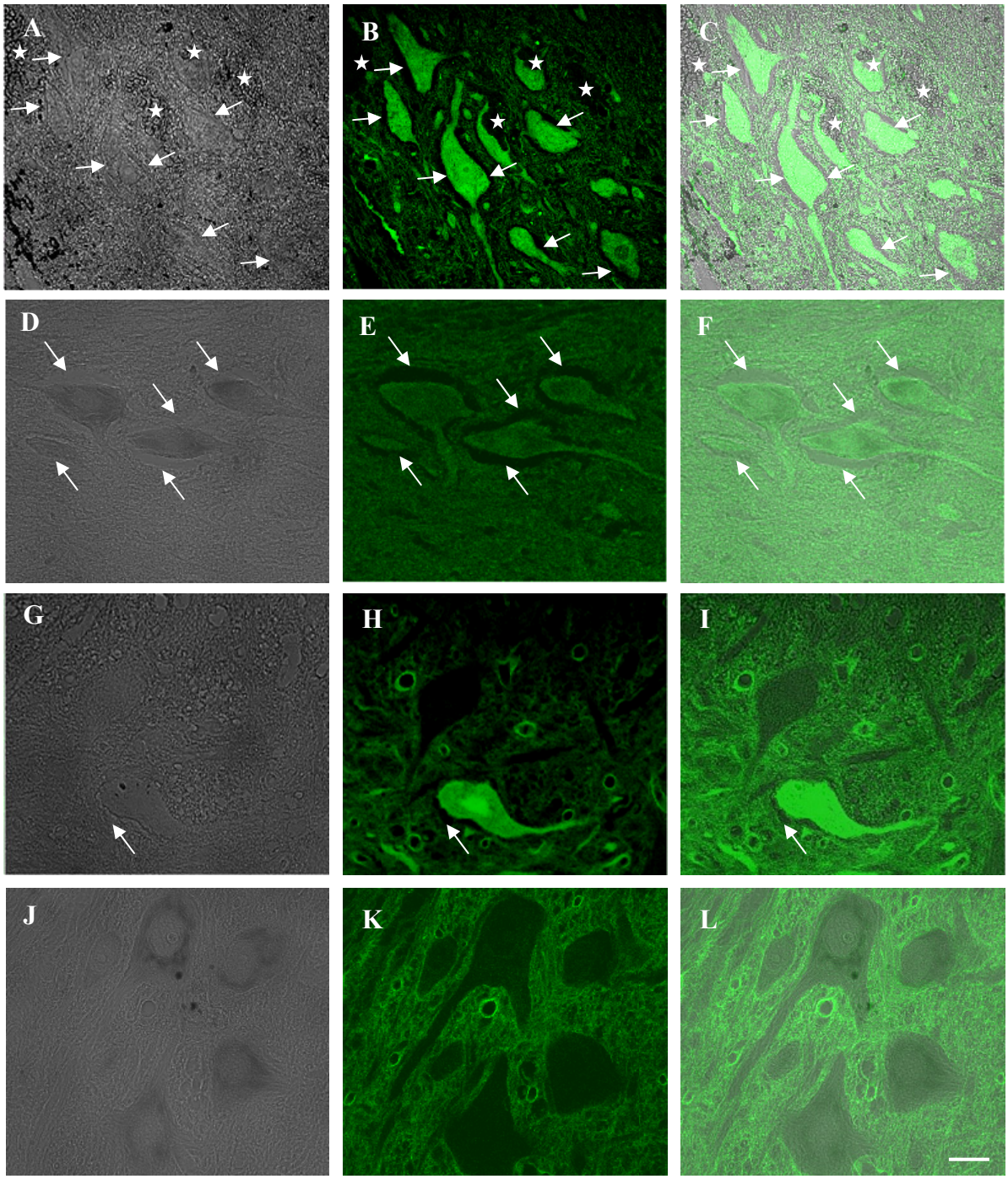


Figure 2.5: Membrane blebbing is associated with plasma membrane permeability. High magnification confocal imaging (63x) of 3dex-filled cells within histological sections fixed 10 min after SCI revealed that membrane permeability is associated with SCI-induced cellular blebbing. Images shown here are representative phase (left panel), fluorescence (middle panel), and phase overlayed with fluorescence (right panel). A subset of cells near the epicenter (A-C) displayed a necrotic appearance (stars) but did not

take up the fluorescent dye, whereas other neighboring cells became 3dex-positive. Most permeable cells were comprised of a dense, dextran-filled cytoplasm surrounded by a less dense layer of fluid that did not fill with the fluorescent dextran molecule (arrows, A-I). The majority of dextran-impermeable cells did not exhibit blebbing (G-L). These results indicate that plasma membrane damage is associated with pathophysiological alterations in cellular morphology. Scale bar = 25 μm.

CHAPTER 3

CITICOLINE AS A TREATMENT FOR SPINAL CORD INJURY: SUB-ACUTE ASSESSMENT

Introduction

SCI is a debilitating condition that affects and estimated 12,000 Americans each year, but there is currently no reliable treatment (for review see (Baptiste and Fehlings 2007)). Use of high dose methylprednisolone, the current standard of care, has raised controversy because it has shown only modest benefit and may even cause harm to patients (Hall and Springer 2004; Hurlbert 2000; Sayer et al. 2006; Young and Bracken 1992). It is therefore necessary to investigate new therapeutic approaches for reducing morbidity and mortality caused by SCI.

In the previous chapter, we demonstrated that SCI contusion causes the plasma membrane to rupture, allowing molecules at least as large as 2.7nm in radius to enter the cell. We and others have postulated that plasma membrane damage is a major contributor to cell death and dysfunction as well as neurological impairments. Under normal physiological conditions, the plasma membrane serves as a barrier between the cytosol and the extracellular fluid. Disruption of this barrier would be expected to destroy cellular homeostatic mechanisms and lead to death, electrophysiological dysfunction, and/or activation of aberrant signaling pathways. Plasma membrane compromise can occur immediately after injury due to mechanical disruption (as shown in Chapter 2 as well as other reports (Choo et al. 2007; Shi et al. 2000; Shi and Pryor

2002; Shi and Whitebone 2006)), but can also persist up to several weeks (Shi 2004). Mechanisms causing persistent membrane damage are largely unknown but may be related to the state of the post-injury environment, which could either prevent the cell from resealing or increase the amount of damage.

A decrease in phospholipid levels has been observed after SCI (Demediuk et al. 1989a; Faden et al. 1987), supporting the hypothesis that persistent plasma membrane compromise may be due to abnormal phospholipid metabolism. Loss of phospholipids can occur through lipid hydrolysis (mediated by phospholipase A2 [PLA2] activity) or lipid peroxidation (mediated by free radicals). Free radical-induced damage has long been hypothesized to be a major mechanism involved in the pathophysiology of SCI (for review see (Braughler and Hall 1989; Hall and Braughler 1986; McCall et al. 1987), and more recent studies have shown that PLA2 levels also increase after injury (Liu et al. 2007; Liu et al. 2006). Loss of phosphatidylcholine (PC), the most abundant molecule in the plasma membrane of mammalian cells, is undoubtedly important in maintaining homeostasis. In fact, loss of PC through inhibition of the citicoline pathway is sufficient to cause cell death under normal culture conditions *in vitro* (Cui and Houweling 2002; Cui et al. 1996). Given the importance of maintaining PC and the fact that SCI causes the destruction of this molecule, we investigated a treatment strategy intended to restore PC levels and ultimately enhance post-SCI outcome.

More specifically, we explored use of citicoline as an agent for facilitating membrane repair after SCI. Citicoline is the precursor to PC and has been shown to increase phospholipid levels in models of CNS trauma and disease (Adibhatla et al. 2004; Adibhatla et al. 2006; Rao et al. 2000; Weiss 1995). In addition to promoting PC

synthesis, citicoline also prevents its loss (Adibhatla and Hatcher 2003; Arrigoni et al. 1987; Dorman et al. 1983; Trovarelli et al. 1981; Trovarelli et al. 1982a). Sparing of PC has been attributed to a citicoline-mediated reduction in PLA2 activity (Adibhatla and Hatcher 2003; Adibhatla et al. 2006; Arrigoni et al. 1987). Previous work has shown that PLA2 activation occurs when cells are permeabilized (Bauldry et al. 1996; Yawo and Kuno 1985) and that its activation can enhance membrane permeability (Maher et al. 2007a; Maher et al. 2007b; O'Regan et al. 1996; Pratt et al. 2005). Attenuation of PLA2 can improve membrane integrity by reducing phospholipid hydrolysis and lipid peroxidation, since arachidonic acid created by PLA2 hydrolysis leads to oxidative damage. PLA2 activity is elevated after SCI and has been postulated to be a major player in the resulting pathophysiology (Liu et al. 2006).

In addition to stabilizing the membrane, citicoline's ability to attenuate PLA2 activity may have considerable benefits for post-SCI outcomes. PLA2 serves as a major point of convergence for multiple signaling pathways involved in cellular responses including apoptosis, differentiation, membrane trafficking, and proliferation (for review see (Farooqui et al. 2006; Olivas and Noble-Haeusslein 2006)). Rampant activation of PLA2 after SCI can lead to unchecked activation of these cell signaling pathways, causing cell death and/or dysfunction. Notably, it has been demonstrated that experimental activation of PLA2 in the intact healthy rat spinal cord is sufficient for inducing paralysis and histological lesions that are comparable to the effects of SCI (Liu et al. 2006).

By promoting membrane repair through a variety of mechanisms (increasing PC synthesis as well as decreasing PLA2 activity and free radical production), citicoline is

expected to facilitate plasma membrane resealing and enhance post-SCI outcomes. Numerous animal and clinical studies support the rationale of using citicoline as a treatment for SCI. It has been shown to be safe for human use and effective as a treatment in animal models of CNS diseases and disorders (Barrachina et al. 2003; Baskaya et al. 2000; Cakir et al. 2005; Clark et al. 1997; Davalos et al. 2002; Yucel et al. 2006). No notable side effects have been observed, making it an appealing alternative to methylprednisolone therapy or a complement to lower dosing of methylprednisolone. Citicoline is currently approved for human stroke treatment in over 70 countries around the world and is now undergoing clinical trials in the United States.

When administered after SCI in animals, citicoline has been shown to reduce lipid peroxidation, promote tissue sparing, and enhance functional recovery (Cakir et al. 2005; Yucel et al. 2006). However, citicoline-mediated membrane resealing has not been directly assessed after SCI or in any other model of neurological dysfunction. Therefore, although citicoline has been shown to reduce some potential causes of membrane compromise, it is currently unknown whether the drug is actually able to restore plasma membrane integrity. In addition, citicoline-mediated alterations in PLA2 activity have not been explored in a model of SCI, even though this has been proposed as the primary mechanism of action after stroke and traumatic brain injury (Adibhatla et al. 2002; Arrigoni et al. 1987). Due to this gap in the knowledge, we investigated the membrane stabilizing properties of citicoline (using a dye exclusion assay as a direct measure) and its effects on PLA2 activity in a rat contusion model of SCI. We hypothesized that citicoline would promote membrane resealing, attenuate PLA2 activity, and reduce the size of the lesion.

Methods

Surgical procedures

All experiments were conducted according to methods approved by the Georgia Tech Institutional Animal Care and Use Committee (protocol A05003). Moderate SCI was induced in adult male Sprague-Dawley rats (350-450g, total n=27) as described in detail elsewhere in this dissertation (Appendix A). Briefly, animals were anesthetized with 50 mg/kg Nembutal (IP), and isoflurane was used as a supplement when necessary. Surgical procedures were conducted using aseptic technique. Laminectomy was performed at T10 vertebral level, and then a force of 150 kdyn was imparted to the exposed spinal cord. Muscle was sutured together and skin was stapled in order to close the wound. Post-operational monitoring included bladder expression and maintenance of hydration until the time of sacrifice.

Citicoline (500 mg/kg in saline, IP) was administered immediately and 3h after SCI and then once per day thereafter. This dose was chosen according to a previous study demonstrating that prolonged high dose administration of citicoline was more effective than lower doses or less frequent delivery (Schabitz et al. 1996). This dose is commonly used in studies conducted in animal models of ischemia (Adibhatla and Hatcher 2003; Krupinski et al. 2002). Furthermore, clinical studies utilizing citicoline have administered high doses once daily (although much lower than those given in animals) (Clark et al. 1997; Clark et al. 2001). All other animals received injections of the vehicle (saline) in order to control for the effect of fluid administration as well as any effects of the injection procedure.

Assessment of cPLA2 activity

cPLA2 activity was measured at 24h post-injury, a time point that has previously been shown to have elevated levels of PLA2 (Liu et al. 2007; Liu et al. 2006). In addition, 24h allows enough time for citicoline to reach the CNS tissue (Lopez-Coviella et al. 1995) and provided greater attenuation of PLA2 activity compared to more acute time points after an ischemic insult (Adibhatla et al. 2006). Experimental groups (n=4 per group) included sham, injured animals receiving citicoline treatment, and injured animals receiving vehicle treatment. Rats were anesthetized with Nembutal and then transcardially perfused with cold heparinized saline (0.16 mg/ml). A 1cm piece of spinal cord tissue was extracted as quickly as possible (within 3-4 minutes) and placed in cold buffer (50 mM HEPES containing 1mM EDTA; 5ml per gram of tissue). Tissue was mechanically homogenized on ice then centrifuged at 10,000g for 15 min at 4ºC. Supernatant was collected and stored at -80ºC until the assay was performed.

PLA2 activity was assessed with a commercially available kit (Cayman Chemical Catalog # 765021; Ann Arbor, MI). Techniques were performed according to the manufacturer's instructions. Quantitative assessment of PLA2 activity is based on the reaction of the enzyme with the synthetic substrate arachidonyl-thio PC. PLA2 hydrolyzes the arachidonyl thioester bond to release a free thiol group, which can then be detected through a reaction with DTNB (5,5' dithio-*bis*(2-nitrobenzoic acid), also known as Ellmans' reagent).

Two main subtypes of PLA2 are present in biological tissues (secretory [sPLA2; 14kDa] and cytosolic [cPLA2; 85-110 kDa]) and can be measured by this assay; however, samples were centrifuged in filter device with a 30kDa cutoff in order to obtain

a sample containing only cPLA2. We chose to assess cPLA2 activity because it shows much greater specificity for phospholipids containing arachidonic acid in the *sn*-2 position. This is significant for the pathophysiology of SCI because arachidonic acid metabolism leads to the formation of free radicals and is known to be a major player in secondary injury cascades (Clark et al. 1995; Farooqui et al. 2006). Furthermore, cPLA2 is prevalent in CNS tissue (Ong et al. 1999) and is up-regulated after SCI (Liu et al. 2006).

All samples were run in multiples of 2-3, depending on the volume available after centrifugation. Samples were incubated for 1h in arachidonyl-thio PC substrate at room temperature, and then 10 µl of DTNB/EGTA solution was added to each well and incubated for 5 minutes. Absorbance was measured at 405 nm wavelength using a plate reader. Measurements were normalized to the total protein concentration within the sample used in the assay using a bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). Comparisons were made using one-way ANOVA followed by Tukey's pairwise comparisons.

Histological assessments: plasma membrane damage and lesion volume

Rats were randomly divided into 3 experimental groups: sham, injured receiving citicoline treatment, and injured receiving saline treatment. Lesion volume and plasma membrane damage was assessed in animals euthanized 3 days post-SCI. This time point was chosen after a pilot study was conducted in which a dye exclusion assay was performed at 1d, 3d, and 7d post-SCI (n=1-2 per time point, data not shown). The 1d post-SCI time point was ruled out because it did not allow for reliable quantification of permeable cells due to a large amount of tissue debris caused by the injury. No

noticeable differences were observed at 3d and 7d, so the most acute time point of the two was chosen for this study in order to be most time and cost efficient. The study began with n=5 per group. However, 2 animals were excluded from the citicoline group (one animal was a statistical outlier when lesion volume was measures [Grubbs' test], and one animal was given citicoline that was not properly stored overnight).

To perform the dye exclusion assay, rats were anesthetized with a low dose of Nembutal (40 mg/kg) on day 3 post-SCI. A low dose was required to reduce the risk of animal death during the surgical procedure, as this had been observed in other experiments when surgery was performed on animals that had undergone a prior surgical procedure (Chapter 4). Isoflurane was used as a supplemental anesthetic when needed. Permeability marker (FITC-conjugated 3kDa dextran [3dex]) was injected into the cerebrospinal fluid as previously described in this manuscript (Chapter 2). 3dex was selected because it was the most sensitive dye in the previous study (Chapter 2), detecting both cell body and axonal plasma membrane damage with minimal sham uptake. Briefly, the animal was mounted on a stereotaxic apparatus and the atlanto-occipital membrane was punctured. A catheter was inserted intrathecally and 3dex was injected (25 mg/ml in saline, 15 µl over 10 minutes). Three hours later, rats were heavily anesthetized with Nembutal and then transcardially perfused with PBS followed by 4% paraformaldehyde and 0.1% gluteraldehyde. Tissue was cryoprotected in sucrose, frozen in OCT, and sectioned longitudinally.

Plasma membrane damage assessment

Axonal plasma membrane permeability was assessed in citicoline and vehicle groups by a blinded observer. Slides were selected in random order and the level of

permeability was ranked on a scale from 0 to 5 (higher score corresponding to more permeable cells). Two adjacent sections at 200 μm increments were assessed for each animal. Values obtained from all slides were then averaged to generate one score per animal, and groups were compared using a t-test.

In order to visualize the morphology of permeabilized axons, sections were immunostained with an antibody specific for neurofilament-160 (NF-160) and imaged at 40x using confocal microscopy (Zeiss LSM 510). Immunohistochemistry was performed according to the protocol described in Chapter 2. Briefly, sections were rinsed in PBS and then blocking/permeabilization was performed for 1h at room temperature (8% goat serum and 0.8% Triton X100 in PBS). Sections were incubated in primary antibody (1:100; Sigma NF264) overnight at 4ºC, rinsed thoroughly in PBS, and then incubated in Alexa 568 goat anti-mouse secondary antibody (1:250; Invitrogen) for 2h at room temperature. A negative control subjected to all solutions except primary antibody was used to determine whether staining was specific for NF-160. Sections were rinsed then coverslipped using aqueous mounting medium.

Determination of lesion size

The volume of the lesion was compared in citicoline-treated rats and vehicle controls at 3 days post-SCI. Two adjacent sections were stained with hematoxylin and eosin at 200 μm increments. The Nissl-free area within each section (outlined in Figure 3.4) was calculated using StereoInvestigator software, and the average of two adjacent sections was taken. If more than one Nissl-free area was found within the same section, those areas were added together to obtain a measure of the total area at that location. The area between experimentally measured points was estimated using a linear regression, and

then these values were used to approximate the lesion volume. Groups were comparing using a t-test.

Results

cPLA2 Assay

cPLA2 activity was assessed in spinal cord homogenates taken 24h post-injury from sham as well as injured animals treated with citicoline or vehicle (Figure 3.1). SCI caused an approximately 2-fold increase in cPLA2 activity. Citicoline treatment decreased the average cPLA2 activity levels to be statistically the same as sham, but it was also not significantly different from untreated injured animals. Sample size calculation using this data set revealed that 22 animals per group would be required to determine if there was a statistically significant difference between injured animals receiving vehicle and those receiving citicoline (power = 0.8, $\alpha = 0.05$).

Plasma membrane permeability

Axonal permeability was enhanced in injured animals compared to sham controls (Figure 3.2). At this time point (3d post-SCI), 3dex uptake was minimal within cell bodies and was not noticeably different from sham; therefore our outcomes were based solely on axonal permeability. The morphology of permeable cells was heterogeneous and depended largely on the location relative to the lesion (Figure 3.2) Some axons located relatively far from the injury site (Figure 3.2, M-R) had a normal appearance, whereas others close to the lesion were fragmented or swollen on the ends (Figure 3.2, D-L). Immunostaining specific for neurofilament-160 (NF-160) showed colocalization with some but not all permeable axons.

A 5-point scale was used to assess the density of permeable axons in vehicle and citicoline-treated injured animals. A high degree of variability was observed between animals (Figure 3.2, M-R). Comparison of axonal permeability marker uptake in citicoline-treated rats compared to vehicle control showed no difference between the groups (Figure 3.3). The number of animals needed to obtain statistical power of 80% was calculated to be 22 per group ($\alpha = 0.05$).

Lesion volume

Lesion volume was examined in histological sections obtained from citicoline and vehicle-treated injured animals to determine whether citicoline had a tissue-sparing effect. Comparison showed no difference between citicoline-treated animals and the vehicle control (Figure 3.4). Sample size calculation using this data set revealed that 22 animals per group would be required to determine whether there is a statistically significant difference between the two groups (power = 0.8, $\alpha = 0.05$).

Discussion

This study assessed the ability of citicoline to reduce activation of the membrane-destabilizing enzyme cPLA2, facilitate plasma membrane resealing, and attenuate gross tissue damage at sub-acute time points. Citicoline is described as a membrane stabilizing agent and has been shown to promote plasma membrane repair and improve outcome following brain ischemia, but these properties have not been thoroughly explored in models of SCI (for review see (Adibhatla et al. 2002; Conant and Schauss 2004; Zweifler 2002). Only two studies investigating citicoline treatment for SCI have been published, and these have collectively shown that citicoline reduces lipid peroxidation and oxidative

stress at 24 hours and attenuates tissue damage and functional deficits at 6 weeks (Cakir et al. 2005; Yucel et al. 2006). If citicoline will eventually be used in the clinic, however, it will be important to have a firm understanding of the treatment mechanisms involved. This can help with selection of patients (e.g., time post-injury and insult severity) for improved efficacy. The main mechanisms of action in the stroke literature have been postulated to be restoration of membrane integrity as well as reduction in PLA2 activity (Adibhatla and Hatcher 2003; Adibhatla et al. 2006; Arrigoni et al. 1987), but this has not been evaluated in a model of SCI.

In this study we assessed cPLA2 activity at 24h post-injury. Citicoline has been shown to decrease overall PLA2 activity in models of ischemic stroke (Adibhatla and Hatcher 2003; Adibhatla et al. 2006; Arrigoni et al. 1987). Here we specifically examined cPLA2 activity instead of assessing all PLA2 isozymes. This was done for several reasons. In comparison to sPLA2, cPLA2 shows much greater specificity for phospholipids containing arachidonic acid, and this reaction is a key initiating event in inflammation and oxidative damage (for review see (Clark et al. 1995; Farooqui et al. 2006)). Furthermore, the spinal cord contains high levels of cPLA2 under normal conditions (Ong et al. 1999) and is up-regulated after SCI (Liu et al. 2006). cPLA2 is therefore expected to be a large contributor to the cell death and damage occurring after SCI. By examining this form of the enzyme (as opposed to determining the activity of all PLA2 enzymes), we expected to obtain more specific information regarding the mechanisms involved in SCI and citicoline treatment.

Our results demonstrated that SCI significantly increased cPLA2 activity compared to sham controls. This is a new finding that adds to the current knowledge of

secondary injury mechanisms, since only two previous studies have assessed PLA2 activity after SCI and the assay was not specific for cPLA2 (Liu et al. 2007; Liu et al. 2006). These previous experiments detected a similar 2-fold increase in overall PLA2 activity at 24h post-SCI, indicating that cPLA2 contributes at least a proportional amount of the overall increase in PLA2 activity levels.

Treatment with citicoline decreased cPLA2 activity to levels statistically equivalent to both sham and vehicle-treated injured animals. These data were interpreted to be encouraging even though there was not a statistically significant effect when animals receiving citicoline were compared to those not receiving the drug. Achieving a significant reduction in cPLA2 activity with citicoline treatment would be very difficult given the relatively small difference in the means between vehicle and sham and the amount of biological variability in the samples. In order to achieve a statistically significant reduction in cPLA2 compared to vehicle controls, citicoline would have to almost completely prevent cPLA2 activation after SCI (which would be difficult at best).

Although we chose to measure citicoline's effects on cPLA2 activity based on sound logic, it is possible that evaluation of sPLA2 or overall PLA2 activity could show a larger treatment-mediated effect. Citicoline has been shown to decrease overall PLA2 activity in an animal model of stroke (Adibhatla et al. 2003). It is unclear whether this effect was mediated through a reduction in sPLA2 or cPLA2 levels since the assay detected activity of both forms of the enzyme and both have been shown to increase in activity after an ischemic insult (Adibhatla and Hatcher 2003; Rordorf et al. 1991). Nevertheless, we interpreted the results of our cPLA2 assay to be somewhat promising, as citicoline treatment resulted in a modest decrease in activity levels.

In a separate group of animals, we explored plasma membrane damage induced by SCI and the effects of citicoline. A fluorescent dye (3dex) was injected into the cerebrospinal fluid 3h prior to sacrifice. 3dex is normally cell-impermeant; therefore cellular uptake of the molecule indicates that the plasma membrane has been breached. Axonal uptake of the permeability marker 3dex was higher in injured animals (treated with citicoline or vehicle) compared to sham controls (Figure 3.2). Permeable axons exhibited a variety of morphologies, ranging from a normal appearance to a pathological one (characterized by swelling and retraction bulbs). Similar results have been observed in a previous study in which dextran uptake after SCI was associated with pathophysiological axonal alterations (Lu et al. 2001). Bulb formation and axonal swelling have been observed after SCI and are postulated to precede degeneration (Anthes et al. 1995; Seif et al. 2007).

In addition to showing morphological alterations, permeable axons were inconsistently immunolabeled with NF-160. Although this result may be attributed to technical difficulties associated with immunohistochemistry, it should be noted that more acute time points showed consistent colocalization of the permability marker with NF-160 staining (Chapter 2). Neurofilament loss has been observed after SCI, presumably due to activation of cytoskeleton-degrading enzymes (Banik et al. 1982; Banik et al. 1997; Schumacher et al. 1999; Zhang et al. 2000). In models of traumatic brain injury, alterations in plasma membrane permeability have been associated with neurofilament compaction and subsequent degradation (Okonkwo et al. 1998; Pettus et al. 1994). Cytoskeletal degradation may be indicative of the axon's attempt to reseal, since disassembly has been shown to promote resealing by removing the barrier between

vesicles and the plasma membrane (Howard et al. 1999; Miyake et al. 2001; Shi et al. 2000; Xie and Barrett 1991). Our results show that plasma membrane damage 3 days post-SCI corresponds with neurofilament loss in some but not all cases. Thus, the relationship between cytoskeletal membrane damage and plasma membrane compromise is complex but may be related to the extent and duration of axonal membrane compromise.

Our imaging results led us to speculate that axonal permeability is an initiating factor in neurofilament loss, swelling, fragmentation, and degeneration. A subset of permeable axons appeared normal and others were damaged, which may represent populations of axons that are undergoing changes over time. In addition, many permeable cells were found along the injury cavity and therefore are likely to undergo degeneration. Thus, plasma membrane compromise may precede axonal degeneration. Future work tracking permeabilized axons over time would be necessary to investigate this hypothesis and determine if a cause-and-effect relationship exists.

Semi-quantitative comparison of permeability marker uptake in injured animals receiving citicoline or vehicle control showed no difference between the two groups. We postulated that citicoline would reduce axonal permeability after SCI, but the data did not support this hypothesis. A variety of experimental limitations and biological mechanisms may explain this result and are described in more detail below.

Although a loss of phospholipids would certainly explain why permeability markers are able to enter axonal projections after SCI, we do not necessarily know that this is the mechanism responsible for increased plasma membrane permeability. Therefore, even if citicoline was able to increase phospholipid levels (which we did not

directly assess), it may not have necessarily changed the results obtained in the dye exclusion assay. Alternative explanations for entry of the permeability marker into the cell membrane (other than a loss of phospholipids) include a failure in the vesicle docking mechanisms needed for endogenous repair (McNeil and Terasaki 2001; Schlicher et al. 2006; Terasaki et al. 1997) or an injury-induced alteration in protein activity involved in membrane turnover or resealing (e.g., calpain and annexin which are involved in membrane resealing) (Howard et al. 1999; Liu and Schnellmann 2003; McNeil et al. 2006; Shi et al. 2000; Yoo et al. 2003). If these alternative explanations are responsible for persistent membrane damage, citicoline would not have been expected to have an effect on axonal dye uptake (even if it increased PC levels, which we did not assess). Future work examining these mechanisms in a simplified model would provide valuable information regarding plasma membrane repair and resealing. For example, experimentally manipulating the amount of PC molecules *in vitro* would provide evidence to determine whether membrane permeability could be related to a decrease in PC after SCI.

An additional possibility is that citicoline may have altered plasma membrane permeability at a time point we did not evaluate in this study. The 3d time point was chosen because of the relative ease with which plasma membrane damage could be evaluated (although it remained a difficult task due to difficulty in distinguishing intracellular uptake from tissue debris and extracellular accumulation around blood vessels and myelin). It is possible that citicoline may have changed the time course of membrane permeability (i.e., decreased the time required for resealing), but we only assessed one time point and therefore are limited in our ability to draw conclusions.

In addition to measuring plasma membrane damage and cPLA2 activity, we also assessed the size of the lesion in citicoline-treated animals and vehicle controls at 3d post-SCI. Results showed no treatment effect. In a previous study, Yucel et al (2006) demonstrated that citicoline reduced the size of the injury cavity 6 weeks post-SCI. Although we did not achieve the same result in our study, differences may be attributed to variations in the dose administered or the time point selected. It is possible that the time point we evaluated did not allow enough time for citicoline treatment to have a tissue-sparing effect. Therefore, we evaluated this outcome at a more chronic time point in a subsequent study (Chapter 4).

In summary, we did not observe notable benefit of citicoline treatment at sub-acute time points. Although the data did not support our hypotheses, it is possible that we did not select appropriate time points or outcome measures. In addition, it is plausible that citicoline improves outcome following SCI through mechanisms we did not test here. Therefore, in a subsequent study, we evaluated the effects of citicoline on functional recovery and longer-term tissue sparing in order to determine if the overall affects of citicoline treatment are beneficial.

Conclusions

In this study we assessed the effects of citicoline as a membrane-stabilizing neuroprotective agent administered after SCI. The rationale for investigating this treatment was based on a thorough review of the literature, which has shown beneficial effects of citicoline in numerous pre-clinical studies of neurological dysfunction. Based on these previous reports and our knowledge of SCI pathophysiological mechanisms, we

hypothesized that citicoline would attenuate cPLA2 activity (24h post-SCI), plasma membrane compromise (3 days post-SCI), and lesion size (3 days post-SCI).

Our data show that SCI causes a 2-fold increase in cPLA2 activity at 24h. This finding is significant because it may prove to be an important target for pharmaceutical intervention, since cPLA2 is a major point of convergence for multiple signaling pathways. In addition, we detected plasma membrane compromise at 3 days post-SCI utilizing a dye exclusion assay. Permeability marker uptake was associated with morphological alterations (swelling, retraction bulbs, and fragmentation) as well as neurofilament loss.

Citicoline treatment caused a moderate decrease in cPLA2 activity (statistically the same as sham as well as vehicle control). No effect on lesion volume or axonal permeability was observed. The lack of citicoline-mediated effect could be a result of the experimental methods chosen (e.g., doses, time points, outcome measures) or treatment inefficacy. Future work assessing a variety of time points, doses, and/or combinational therapies may support use of citicoline for treatment of SCI.

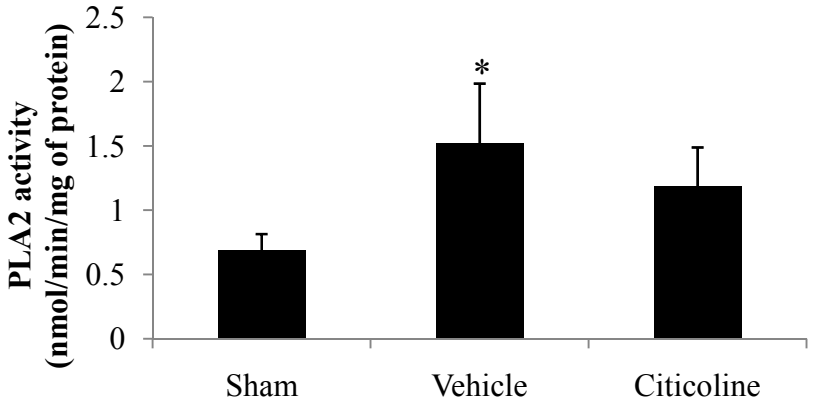
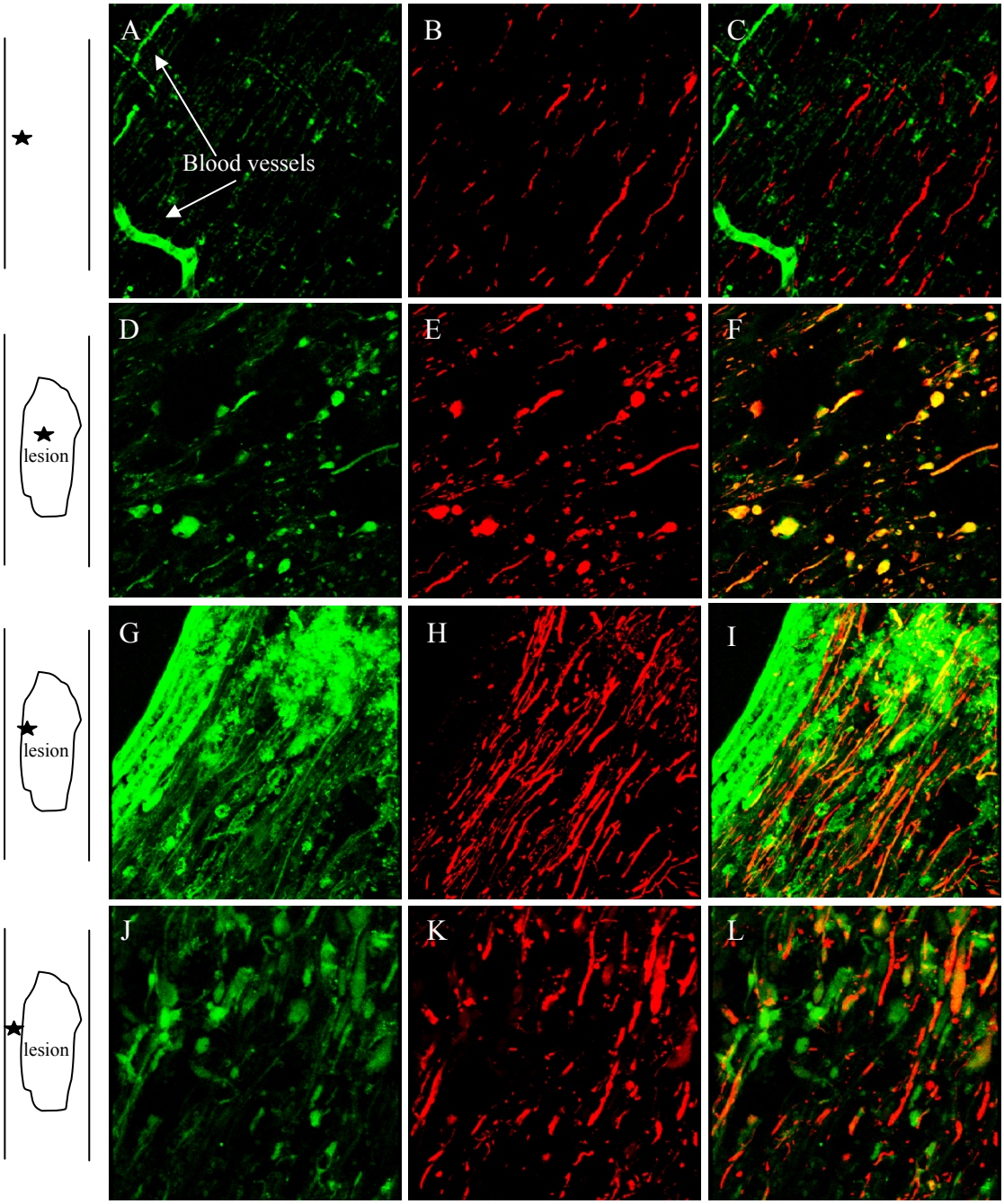


Figure 3.1: Effect of citicoline on cPLA2 activity at 24h post-SCI. cPLA2 activity levels were assessed within spinal cord tissue homogenates taken 24 hours after SCI (n=4 per group). Injured animals receiving vehicle injections had significantly greater cPLA2 activity than sham controls ($p<0.05$). Citicoline-treated injured animals did not have statistically different PLA2 activity compared to sham ($p=0.14$) or vehicle controls ($p=0.38$). Data is represented here as mean ± standard deviation.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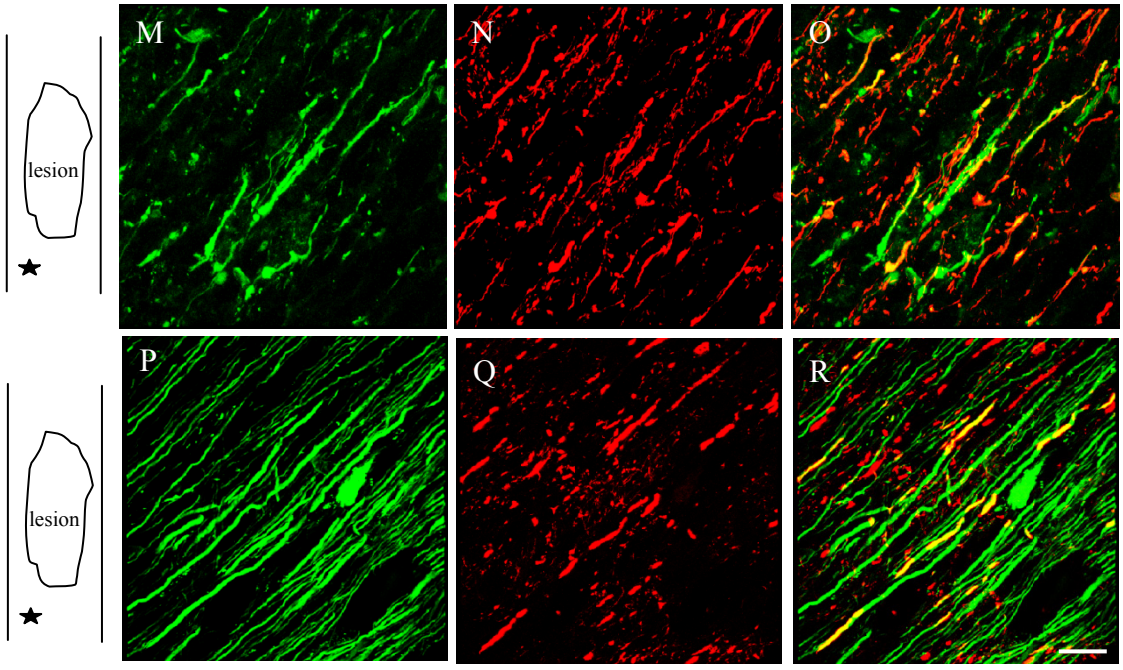


Figure 3.2: Axonal permeability marker uptake 3 days post-SCI. Plasma membrane damage was identified in axons taking up the 3dex permeability marker (left panel). Immunostaining with NF160 (middle panel) and subsequent confocal imaging showed colocalization with 3dex (right panel). Low uptake was observed in sham animals (A-C), whereas SCI caused uptake of the permeability marker (D-R). The morphology of axons taking up the permeability marker was heterogeneous and depended largely upon anatomical location relative to the epicenter. Cellular fragments taking up 3dex could be seen in the lesion (D-F). In addition, many axons lining the injury cavity were 3dex-positive, and a large portion of those axons terminated into large bulbs (G-L). Axons farther from the epicenter also took up the permeability marker in some cases (M-R). The density of permeable axons in this region was highly variable, with low uptake in some animals (M-O) and larger amounts of uptake in others (P-R). Scale bar = 50 µm.

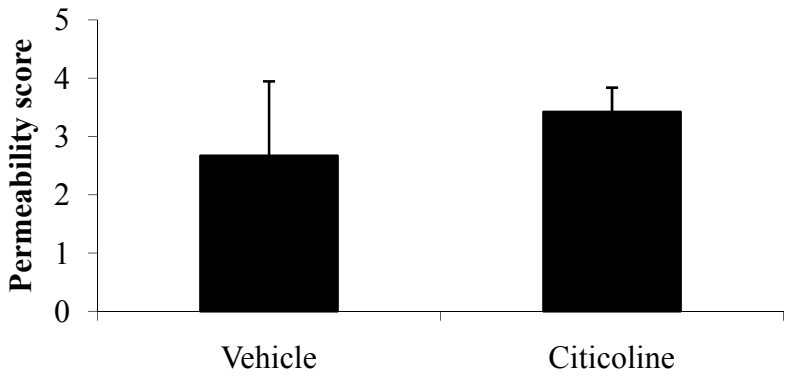


Figure 3.3: Effect of citicoline treatment on axonal uptake at 3 days post-SCI. 3dex axonal uptake was evaluated in two adjacent sections at 200 µm increments throughout the thickness of the spinal cord. Scores were assigned on a scale ranging from 0 to 5, with higher scores indicating a greater density of axonal permeability marker uptake. The average score for each animal was determined, and data were analyzed using a t-test. Results indicate that citicoline had no effect on axonal plasma membrane permeability ($p=0.38$). Mean ± standard deviation, n=5 (vehicle) and n=3 (citicoline).

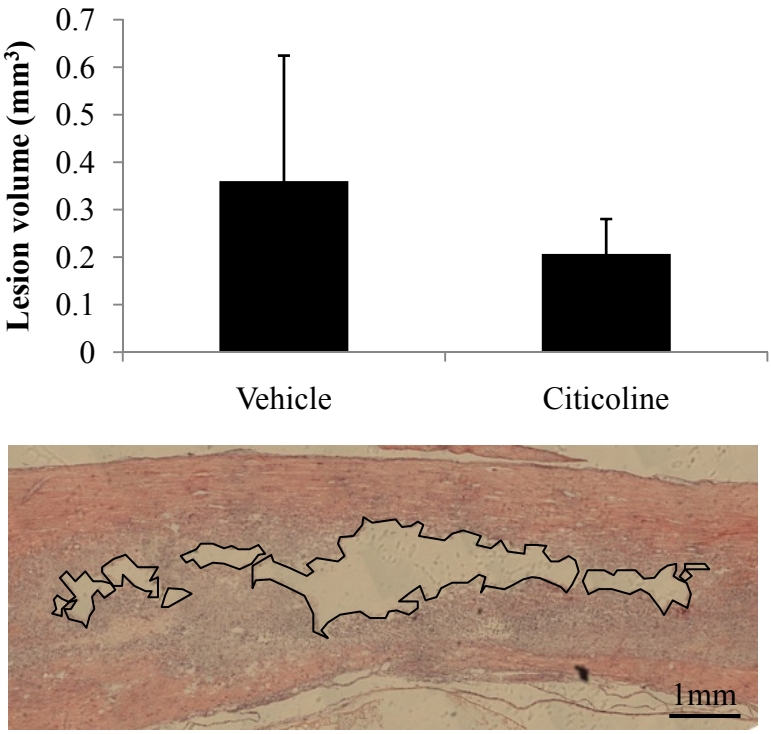


Figure 3.4: Effect of citicoline treatment on lesion volume at 3 days post-SCI. The size of the injury cavity was measured in longitudinal sections stained with hemotoxylin and eosin. A representative section is shown above with the area of the lesion outlined in black. Citicoline treatment did not have a statistically significant effect on the size of the lesion at this time point (p=0.38). Mean ± standard deviation, n=5 (vehicle) and n=3 (citicoline).

CHAPTER 4

CITICOLINE AS A TREATMENT FOR SPINAL CORD INJURY: BEHAVIORAL AND HISTOLOGICAL ASSESSMENT

Introduction

Depending on the anatomical location and severity of the impact, SCI can result in functional impairments including sensory and motor paralysis, impotence, spasticity, bladder and bowel dysfunction, and neuropathic pain. These consequences are particularly devastating because most individuals with SCI are otherwise healthy and under the age of 30 (Nobunaga et al. 1999). Although SCI dramatically affects individuals, families, and society, there is currently no reliable treatment for reducing morbidity and mortality (for review see (Baptiste and Fehlings 2007; Kwon et al. 2004)). High dose methylprednisolone is the only treatment found to be clinically effective in controlled multi-center clinical trials and therefore has been considered the current standard of care when given within 8h of SCI (Bracken and Holford 1993; Bracken et al. 1992; Bracken et al. 1997; Bracken et al. 1998). However, the benefits provided by methylprednisolone are modest at best. In fact, several clinical studies have shown no improvement when methylprednisolone was administered after SCI (George et al. 1995; Levy et al. 1996; Pollard and Apple 2003; Prendergast et al. 1994; Wang et al. 2004). As a result of the questionable efficacy and serious side effects (e.g., severe sepsis, pneumonia, gastrointestinal disorders), use of methylprednisolone has become controversial (Coleman et al. 2000; Fehlings 2001; Hurlbert 2000; Lammertse 2004;

Sayer et al. 2006). On one hand, these modest benefits may make a big difference in quality of life in these patients. However, on the other hand, these individuals may suffer deleterious side effects but no improvements. Therefore, there is a need to explore treatments that are safe, effective, and well-tolerated.

Neuroprotective treatments are one strategy for reducing the damage caused by SCI. Much of the cell death and degeneration caused by SCI does not occur immediately after impact, but instead accumulates over time in the following days, weeks, and months due to a variety of secondary injury mechanisms (for review see (Blight 2002; Dumont et al. 2001)). Therefore, the prolonged time course of cell death and dysfunction provides an opportunity to deliver a therapeutic agent that prevents damage or augments repair. We have observed plasma membrane damage occurring in both the acute (Chapter 2) and sub-acute phases of SCI (Chapter 3) and subsequently investigated a treatment intended to augment endogenous membrane repair mechanisms.

The membrane stabilizer citicoline offers an attractive strategy for reducing cell and tissue damage occurring after SCI. Citicoline has been shown to improve functional outcomes and attenuate cell death in many models of traumatic CNS injury and disease and is currently undergoing clinical trials for stroke treatment in the United States (Adibhatla and Hatcher 2003; Adibhatla et al. 2002; Adibhatla et al. 2006; Arrigoni et al. 1987; Cacabelos et al. 1993; Fioravanti and Yanagi 2004; Krupinski et al. 2002; Mir et al. 2003). Citicoline is postulated to reduce damage in the diseased brain by repairing the damaged plasma membrane components (increasing synthesis of phosphatidylcholine [PC] and preserving other membrane components) and also preventing membrane damage (decreasing PLA2 activity and free radical-induced damage) (Adibhatla and

Hatcher 2003; Adibhatla et al. 2003; Arrigoni et al. 1987). Citicoline is safe for human use, and no notable side effects have been observed (Conant and Schauss 2004; Secades and Lorenzo 2006; Zweifler 2002).

Citicoline has been extensively studied in models of stroke but has only recently been investigated as a treatment for SCI (Cakir et al. 2005; Yucel et al. 2006). Cakir et al. (2005) assessed the effects of citicoline treatment within 48h after SCI, demonstrating improved locomoter function and reduced levels of lipid peroxidation. In addition, Yucel et al. (2006) compared the efficacy of citicoline to that of methylprednisolone and a combination of the two drugs. Citicoline reduced lipid peroxidation, nitric oxide levels, lesion size, and functional deficits. Treatment with citicoline was comparable to methylprednisolone treatment, but did not offer additional benefit when given in conjunction with methylprednisolone. Although these studies show promise for citicoline treatment, more extensive pre-clinical research is needed to support clinical use and demonstrate reproducibility in various laboratories.

Our previous studies (Chapter 3) demonstrated no citicoline-mediated effect on cytoplasmic PLA2 activity (24h post-SCI), plasma membrane permeability (3d post-SCI), or lesion size (3d post-SCI). However, the lack of effect may have been due to the outcome measures or time points selected. Therefore, in this study, we proceeded to evaluate the longer-term histological and functional effects of prolonged high dose citicoline treatment. Results obtained from these studies were intended to provide evidence to either support or oppose use of citicoline as a treatment for SCI, regardless of the mechanisms involved.

Materials and Methods

Surgical procedures and treatment administration

All procedures involving animals were approved by the Georgia Tech Institute of Animal Care and Use Committee (protocol A05003). SCI was performed according to methods described elsewhere (Appendix A). Briefly, adult male Sprague-Dawley rats (365-430g) were anesthetized with Nembutal (50mg/kg, IP), and isoflurane (1-2%) was used as an anesthesia supplement when necessary. A laminectomy was performed at vertebral level T10 to expose the underlying spinal cord. Moderate SCI (150 kdyn impact force) was induced using the Infinite Horizons device. Animals were randomly assigned to one of the following groups: sham (n=6), injured then treated with vehicle (saline; n=6), or injured then treated with citicoline (500 mg/kg of 200 mg/ml solution; n=6). All animals received IP injections with either citicoline or saline immediately after injury, 3h after injury, and daily thereafter until sacrifice. Shams and vehicle control animals received saline injections of an equal volume:weight ratio as the treated animals.

Behavioral testing was conducted up to 36 days post-injury and animals were euthanized on day 37. A permeability marker (10 kDa FITC-conjugated dextran [10dex]; Invitrogen) was injected into the cerebrospinal fluid three hours prior to sacrifice as previously described in Chapter 2. Briefly, animals were anesthetized with 50 mg/kg Nembutal, and isoflurane (1-2%) was used when necessary. An incision was made in the atlantooccipital membrane, and PE10 tubing was inserted into the intrathecal space. Fifteen µl of 10dex (2.5% in saline) was injected over 10 minutes. A lethal dose of Nembutal was administered three hours following dye injection and animals were perfused with phosphate buffered saline (PBS) then cold 4% paraformaldehyde/0.1%

gluteraldehyde in PBS. The vertebral column was excised and post-fixed for 24h. Spinal cord tissue was dissected, immersed in sucrose, and then frozen in OCT. Transverse sections at a thickness of 35 μm and 10 μm were generated using a cryostat for histological analysis.

Behavioral outcome measures

Prior to injury, animals were placed on behavioral testing devices for mock trials until signs of fear (such as crouching, freezing, and frequent urination/defecation) were eliminated. Pre-surgery scores were obtained for each animal on the BBB and plantar heat tests to ensure that each could perform the task and that none were statistical outliers that needed to be withdrawn from the study. Animals were not placed on the grid prior to injury in order to prevent any training effects that may occur during repeated performance of a skilled task.

Basso Beattie Bresnahan (BBB) open field locomoter

Animals were placed in an open field and encouraged to locomote for 4 minutes while blinded observers assigned a score ranging from 0 to 21. As previously described, scores reflected a wide range of hindlimb function including joint movement, weight supported stepping, forelimb-hindlimb coordination, paw rotation, and toe drags (Basso et al. 1995). A low score is considered a poor performance (limited hindlimb function) whereas a higher score is a reflection of greater function. Each hindlimb was assigned an independent score and those scores were then averaged together to yield a single number at each time point. Testing was conducted once a week for 5 weeks following injury.

BBB open field scores were also converted to a subscore that has been shown to improve the sensitivity of the test (Lankhorst et al. 1999). In this post-hoc analysis, the following are assigned to point values as follows: forelimb-hindlimb coordination (one point), trunk stability (one point), tail lift (one point), paw rotation at placement (0.5 points per hindlimb), paw rotation at liftoff (0.5 points per himdlimb), and toe drags (one point per hindlimb). Points were added together to create a single score with a maximum of 7 points. BBB subscore was not generated at 1d post-SCI because injured animals were not consistently stepping at this time point.

Grid walk

Animals were placed on an elevated wire mesh grid (4" x 2" spacing between rungs, 8"x 48"x 12") for 3 minutes while a blinded observer counted the number of steps and the number of times the foot slipped below the grid. Scores were calculated as the percentage of times the hindlimb stepped correctly (did not fall below the grid) relative to the total number of steps. Testing was conducted at 29 and 37 days post-SCI.

Plantar heat testing

Hyperalgesia was assessed using the Thermal Plantar Analgesia Instrument (Stoelting Co., Wood Dale, IL). Animals were placed on a clear glass platform and a heat stimulus was applied to the plantar surface of the hindlimb using a near-infrared controller. The latency for hindlimb withdrawal was quantified. Great care was taken to control the environment in which testing was performed. Only one animal was allowed in the room at a time to prevent high frequency communication between rats, and the platform was thoroughly cleaned between trials. Each hindlimb was tested three times in

a random testing order and scores were averaged together. At least 30 seconds was allowed between trials to prevent a sensory wind-up effect. A decrease in the response latency was interpreted as a hyperalgesic effect, which has been observed in previous animal studies after SCI (Horiuchi et al. 2003; Mills et al. 2001).

Statistical analysis

Each behavioral test was analyzed as a two-way repeated measures ANOVA followed by Tukey's pairwise comparison (α=0.05). Independent variables included the time point (day post-injury) and treatment (citicoline, sham, and vehicle). The dependent variable was the behavioral score. Scores more than two standard deviations from the mean were treated as outliers and were removed from the analysis.

Lesion volume

Lesion volume was quantified using the Cavalieri method. Thick sections (35 μm) spaced 250 μm apart were stained with Hoechst and examined using Stereoinvestigator software (MBF Bioscience). The area of the lesion was estimated by marking the points in a randomly placed grid (150 μm spacing) located within the lesion. These areas were then extrapolated to approximate the volume of the lesion according to the assumptions of stereology and the Cavalieri method (Mouton 2002).

The size of the cystic cavity was compared between vehicle and citicoline-treated animals (n=6 vehicle; n=4 citicoline). Not all animals tested for behavior were used in this analysis because of a high accidental death rate during permeability marker injection. Death was likely a result of difficulties in controlling anesthesia during a second surgery under Nembutal anesthesia (Laura O'Farrell, PhD/DVM, personal communication).

Results

Behavioral assessment of citicoline treatment following SCI

BBB open field locomoter and BBB subscore

BBB testing demonstrated an injury deficit at all time points assessed (Figure 4.1). Both citicoline and vehicle-treated animals performed significantly worse than sham controls, but no citicoline-mediated effect was observed. Injured animals recovered to a score of approximately 15, which traditionally indicates that animals achieved weight supported stepping, forelimb-hindlimb coordination, and parallel paw placement at initial contact. This score reflects deficits in paw rotation at lift-off, trunk stability, toe clearance, and tail position (Basso et al. 1995).

Although it is not reflected in the average BBB score, animals in this study typically fell into one of two categories: a score of 13 (frequent but not consistent forelimb-hindlimb coordination) or a score of 18 (consistent coordination but paw rotation during lift-off). Scores between 13 and 18 represent consistent coordination but deficits in toe clearance; therefore, the BBB score average of 15 did not represent the population of animals tested in this experiment. To address this mis-representation of data, we utilized a BBB subscore system, which has been previously published (Lankhorst et al. 1999). Transformation of data into this subscore also revealed significant differences between sham and injured groups at all time points tested (Figure 4.1). Citicoline-treated rats performed significantly worse than vehicle controls on day 29 post-injury. This effect was not sustained on day 36.

Sample size needed to achieve statistical power of 80% with $\alpha = 0.05$ on day 36 was calculated using Sigma Stat 3.0 software. At this time point, the difference in means

between vehicle and citicoline-treated animals was 1.1, and the average standard deviation was 1.7. Results showed that 39 rats in each group would be needed to determine whether there was a statistically significant effect at this time point.

Grid walk

Animals were placed on a wire mesh grid for three minutes and the number of footfalls relative to the total number of steps was quantified. Results demonstrated that injured animals (citicoline and vehicle-treated) performed worse than sham controls at all time points assessed. Citicoline-treated animals exhibited improved scores at 29 days post-injury, but this treatment-mediated effect was not observed on day 36 (Figure 4.2).

Sample size needed to achieve statistical power of 80% with $\alpha = 0.05$ on day 36 was approximated using Sigma Stat 3.0 software. At this time point, the difference in means between vehicle and citicoline-treated animals was 4.79, and the average standard deviation was 12.8. Results showed that 114 rats would be needed in each group to determine whether there was a statistically significant effect at this time point.

Plantar heat

Plantar heat testing was conducted weekly up to 36 days post-SCI in order to quantify the withdrawal latency to a heat stimulus applied to the hindlimb. Although we expected a shorter response time in injured animals compared to sham controls, there were no statistically significant differences between any of the groups (Figure 4.3). No signs of pain were observed during testing (vocalizations, attempts to escape, etc.); therefore, we concluded that contusion SCI did not induce hyperalgesia in these animals.

Histological assessment

The size of the lesion was estimated using the Cavalieri method in tissue sections obtained 37 days post-SCI. SCI resulted in a large fluid-filled cyst at the center of the tissue, which is consistent with numerous studies utilizing a rat contusion model (Basso et al. 1996a; Scheff et al. 2003; Stokes et al. 1992). Results demonstrated that citicoline treatment had no effect on the size of the lesion (Figure 4.4).

The power of the statistical test was 0.097, a number far below the desired power of 0.8. Sample size was calculated to determine the number of animals needed per group to achieve statistical significance (difference between means = 2.24, average standard deviation = 2.85, desired power = 0.8, $\alpha = 0.05$). Approximately 27 rats would be needed to determine if there is a statistically significant effect.

Assessment of permeability marker uptake

Plasma membrane damage was assessed at 37d post-SCI by injecting 10dex into the cerebrospinal fluid 3h prior to sacrifice, processing tissue for histology, and then visualizing axonal membrane damage with fluorescence microscopy. As shown in Figure 4.5, punctuate uptake of the FITC-conjugated dextran molecule was evident at this time point. Tissue was stained with NF-160 in order to verify that permeability marker uptake was intra-axonal. Some but not all dextran-positive regions colocalized with NF-160 staining, providing evidence that the axonal membrane was compromised enough to allow influx of the dye. Although we could not confirm definitively, the size and shape of regions of intense dextran not colocalizing with NF-160 suggested that axons with

neurofilament loss (a previously observed post-SCI response (Banik et al. 1982; Banik et al. 1997; Schumacher et al. 1999; Zhang et al. 2000)) also sequestered the dye.

Discussion

In this study, we examined the functional and histological effects of treatment with citicoline, a clinically appealing therapeutic agent. Citicoline has been shown to be provide benefit in numerous studies of stroke (Adibhatla and Hatcher 2003; Adibhatla et al. 2002; Adibhatla et al. 2005; Clark et al. 2001; Davalos et al. 2002; Kakihana et al. 1988; Krupinski et al. 2005; Trovarelli et al. 1982a) as well as CNS trauma (Baskaya et al. 2000; Cakir et al. 2005; Yucel et al. 2006). Clinically, citicoline is an attractive drug because it targets a well-studied endogenous biochemical pathway (allowing better prediction of the effects) and has been shown to be safe.

Here we investigated the effects of daily administration of high dose citicoline. Functional outcome measures used in this study included two sensorimotor tasks (BBB and grid walk) as well as a measure of pain sensitivity (plantar heat). SCI-induced deficits were observed in both the BBB and grid walk testing but not in the plantar heat test. Interestingly, treatment with citicoline caused improved grid walk accuracy at the same time point that it decreased the BBB subscore. Neither of these effects was observed at any other time point in this study. Although these data are *statistically significant*, they cannot be considered *biologically significant* because the effects were fairly modest and not maintained over time.

Although we did not observe notable citicoline-mediated behavioral effects, the discrepancy between treatment effects observed in grid walk and BBB testing are worthy

of discussion. Differences between neurological mechanisms involved with each task may partially explain the apparent inconsistency in results. BBB open field assesses normal locomoter patterns (which mostly involve local circuits at the spinal cord level in rats) while grid walk consists of a more skilled task (which would be expected to involve a larger contribution from higher order processing centers in the brain). Therefore, these results suggest that citicoline may differentially affect these regions of the CNS. Additionally, these differences in outcome may be explained by variation in the motivating factors required for the test. In the BBB, animals walk about the open field and are not punished or rewarded according to their performance. In the grid walk, however, animals are inadvertently punished for missteps as they fall through the wire mesh. Due to the added motivation to perform well on the grid, animals would be more likely to compensate or change patterns of movement. Although we did not directly test cognitive function in this study, there is evidence that citicoline improves mental performance in animal models of Alzheimer's disease and in humans suffering from dementia (Fioravanti and Yanagi 2004; Fioravanti and Yanagi 2005; Franco-Maside et al. 1994). Therefore, it is possible that citicoline-treated rats performed better on the grid walk due to improvements in higher level neurological processing. In addition, citicoline treatment may have promoted sparing of corticospinal tracts and other circuits involved in skilled motor control and sensorimotor integration, thus improving performance on the grid.

Plantar heat testing was utilized for assessment of pain hypersensitivity following SCI. Nociceptive thresholds are commonly reduced following SCI in both humans and animals, causing unbearable pain that can sometimes lead to human depression or suicide

(Hao and Xu 2003; Horiuchi et al. 2003; Mills et al. 2001; Rintala et al. 1998; Stormer et al. 1997; Ullrich 2007). Therefore, chronic pain is a clinically relevant outcome measure of high importance to humans with SCI and should be tested in pre-clinical studies. In this study we did not observe an SCI-induced hyperalgesic response to the heat stimulus. Importantly, however, citicoline did not augment this response (in other words, citicoline did not cause pain in the animals). Although it is possible that our detection methods were not sensitive enough to detect an SCI-induced deficit, we did not observe any signs of pain during the testing (i.e., vocalizations, attempts to escape, crouching, etc.). This is consistent with the result we obtained when von Frey testing was performed (Appendix A); therefore the results are unlikely to be due to experimental artifact. We therefore conclude that this contusion SCI model does not induce a hyperalgesic effect in our hands.

In this study, treatment with citicoline after rat contusion SCI was beneficial in some behavioral outcome measures (grid walk) and detrimental in others (BBB subscore). In addition, citicoline did not reduce the size of the lesion. One interpretation of these results is that citicoline is not an effective treatment of SCI. However, studies conducted in other laboratories have shown enhanced functional recovery and increased tissue sparing with citicoline treatment after SCI in the rat (Cakir et al. 2005; Yucel et al. 2006). Yucel et al. (2006) demonstrated that citicoline was just as effective as methylprednisolone but did not offer additional benefit when used in combination. We were unable to show a citicoline-mediated benefit in our studies. This lack of treatment effect may be explained by experimental parameters (e.g., dosing, time points, outcome measures) or real biological inefficacy.

One notable difference in our study compared to those previously published by Yucel et al (2006) and Cakir et al (2005) is the selected dose. In the current study, we chose to administer multiple high doses of citicoline (500 mg/kg given immediately after injury, 3h after injury, then daily thereafter until sacrifice). In contrast, the published studies demonstrating functional improvement with citicoline treatment after SCI gave a slightly lower dose without prolonged treatment (300-400 mg/kg immediately after injury). We chose to deliver such high doses because this amount has been shown to be more beneficial than lower doses or fewer doses after an ischemic insult to the rat brain (Schabitz et al. 1996). In addition, previous work indicates that daily doses of 500 mg/kg for 42 days were needed to increase PC levels in normal animals (although the number of doses needed in the injured spinal cord is currently unknown). Furthermore, human stroke clinical trials have utilized prolonged dosages of 500mg, 1000mg, and 2000mg given daily for six weeks (Clark et al. 1997). We chose to deliver long-term high doses in order to be consistent with previous work conducted in other models of neurological dysfunction and based on the rationale that repeated dosing is necessary to increase PC levels in the CNS.

It is not surprising that increasing the drug dose can reduce or even eliminate treatment effects. Most pharmacological agents exhibit a dose-response curve with low and high extremes that are ineffective. In this case, prolonged high-dose treatment may have exceeded the effective range of citicoline treatment and thus we did not observe improvements in functional recovery or lesion size. The efficacy range of citicoline appears to be narrow and unpredictable, as clinical trials showed beneficial effects at 500mg and 2000mg but not at an intermediate dose of 1000mg (Clark et al. 1997).

However, future work will be necessary to determine if the lack of efficacy we observed is due to a dose-response effect. In other words, lower dose and/or shorter-term citicoline treatment would need to be directly compared to prolonged high-dose treatment. Such experiments would also determine whether previously published reports are reproducible in other laboratories.

An additional concern in this study is the sensitivity of the behavioral tests used. Hindlimb deficits occurring as a result of moderate contusion injury are subtle in nature, which may render small improvements difficult to assess. Moreover, behavioral impairments observed in this study were less severe than previous experiments (Appendix A and others not shown) as well as other previously published studies (Basso et al. 1996a; Ferguson et al. 2004; Lankhorst et al. 1999; Scheff et al. 2003; Scheff et al. 2002), which would make deficits more difficult to assess and decrease the likelihood of detecting a treatment effect. In response to the lack of sensitivity in the testing methods, we have validated the use of the CatWalk^{TM} in our laboratory (Appendix B). The CatWalk^{TM} is expected to improve the sensitivity of behavioral testing in future studies by providing a quantitative, objective, and reliable measure of gait parameters, thus improving the ability to detect treatment effects and distinguish sub domains of gait that may be differentially affected (Hamers et al. 2006; Hamers et al. 2001; Koopmans et al. 2005).

Another experimental limitation is the possibility that our study did not assess behavioral outcomes at time points that were long enough from the time of injury. One previous study did not observe citicoline-mediated effects until six weeks post-injury (Yucel et al. 2006), a time point we did not evaluate. However, this study indicated a

steady increase in functional outcome in the citicoline-treated group prior to achieving statistical significance at six weeks, an effect we did not observe. Furthermore, another study reported functional improvements with citicoline treatment as early as 24 hours after SCI (Cakir et al. 2005). Although outstanding, it is rare that a treatment mitigates its effects so rapidly post-SCI; therefore it is unexpected that others would observe the same effect.

Additional differences between this study and the previous may explain variations in the findings, including: injury severity (moderate in our study versus severe in the previous work), injury location (T10 in our study versus T7-T10 in previous work), surgical anesthesia (Nembutal/isoflurane in our study versus ketamine:xylazine cocktail in the previous work), citicoline vendor (Bio-Mol [Plymouth Meeting, PA] in our study versus Sigma [Steinheim, Germany] in the previous work), as well as rat strain and weight (Sasco Sprague Dawley 365-430 g in our study versus Wistar 210-280g in the previous work). Future work investigating whether these parameters affect the efficacy of citicoline can lead to improved methods for selecting patients for treatment if citicoline is translated into clinical use (i.e., based on injury severity, injury location, and age/weight).

Although these logistical reasons and experimental caveats may explain the lack of citicoline-mediated improvement, it may also be explained by a lack of a real biological effect. When citicoline is administered, numerous biochemical steps must be completed to produce PC (summarized in Figure 1.2). If SCI decreases the activity of any enzymes involved in PC synthesis, precursors to PC will accumulate and the desired end product will not be synthesized. Previous work has shown that exogenous citicoline

administration increases the activity of CTP: phosphocholine cytidylyltransferase in an animal model of stroke (Adibhatla et al. 2004; Adibhatla et al. 2006), but this has not been tested in SCI. Since we did not directly measure PC or choline levels, we cannot draw conclusions on this possibility.

Due to the potential of encountering rate-limiting enzymatic steps, others have sought to minimize the number of steps in the synthesis pathway. More specifically, delivery of citicoline in liposomes has been shown to be more effective than delivery of the free drug (Adibhatla et al. 2005; Fresta et al. 1994). When free citicoline is given IP as we did here, it is broken down into its components by the liver so that it can cross the blood-brain-barrier and then become resynthesized intracellularly (Trovarelli et al. 1982b). Therefore, delivering citicoline within a liposome would be expected to bypassing these steps in the PC pathway and thus increase efficacy. Future work combining citicoline treatment with a drug that augments the necessary enzymatic activity may also improve the efficacy of citicoline treatment.

One somewhat surprising finding from this work was that plasma membrane damage was evident at such a chronic time point (37 d post-SCI). Since the plasma membrane plays an essential role in cellular survival and function, compromise of this structure is likely to be a major detriment to neurological outcome. Future work examining strategies intended to facilitate membrane resealing may lead to an effective treatment for SCI.

Conclusions

This study assessed the behavioral and histological effects of prolonged high-dose citicoline treatment. Our results demonstrate minimal to no benefit of citicoline

treatment, as behavioral outcome measures were not able to detect sustained improvements and assessment of the lesion volume showed no treatment effect. Long-term high dose administration of citicoline, however, was not detrimental to post-SCI outcomes. Future work designed to optimize citicoline dosing regimens or combine it with other therapies may show more promise for clinical application of citicoline following SCI.

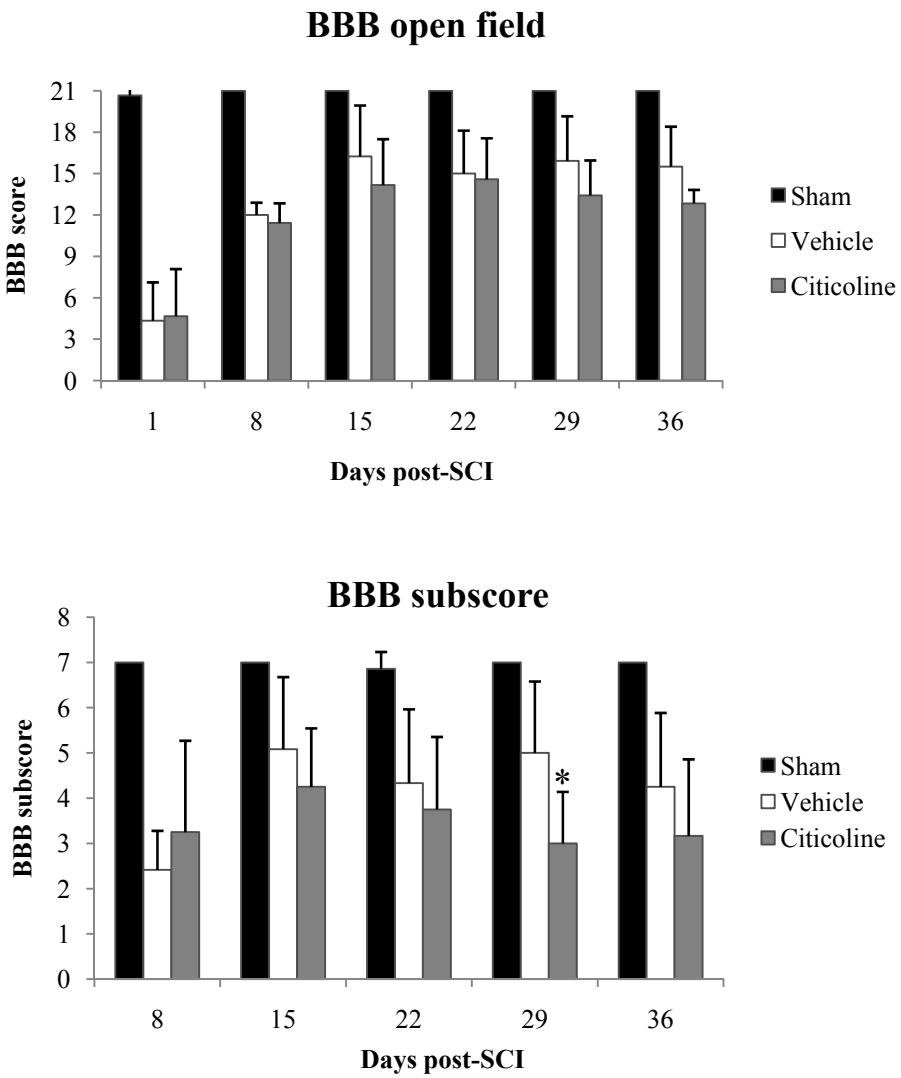


Figure 4.1. Assessment of citicoline-mediated effects on normal locomotion patterns. Traditional BBB open field testing revealed no significant difference in hindlimb locomotion in animals treated with citicoline compared to vehicle controls. However, conversion of the BBB score to a subscore revealed that citicoline-treated animals performed worse than controls on day 29 post-injury. This effect was not observed at any other time point.

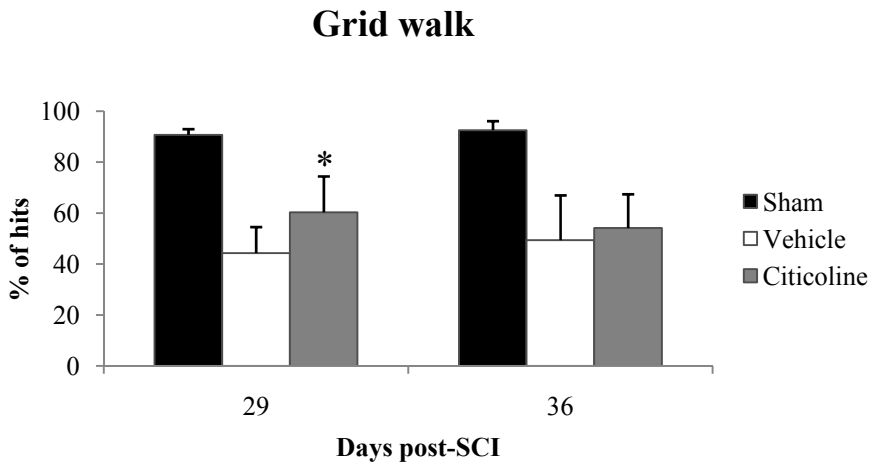


Figure 4.2. Effect of citicoline on skilled hindlimb movements. Animals were placed on a wire mesh grid and the number of footfalls relative to the total number of steps was quantified. Treatment with citicoline increased placement accuracy on the grid at day 29 but this effect was not observed on day 36.

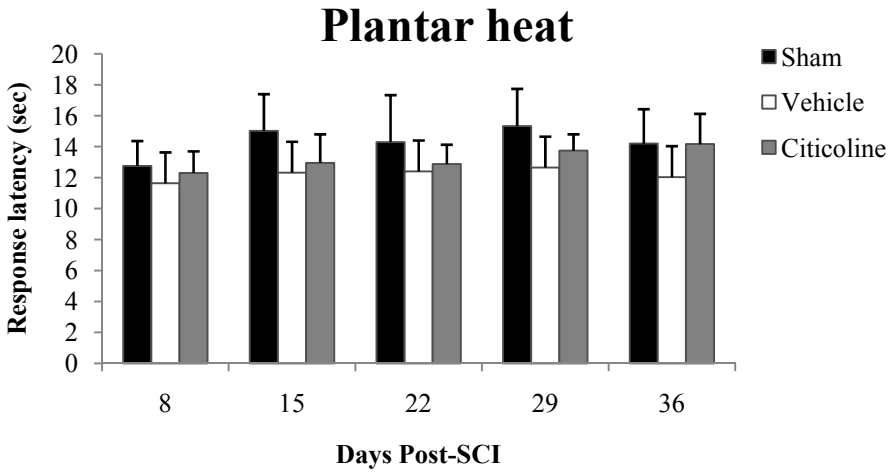


Figure 4.3. Evaluation of sensitivity to a stimulus. A heat source was applied to hindlimbs and the withdrawal latency (time required for hindlimb removal) was quantified. Although scores did not demonstrate a deficit in injured animals compared to sham, citicoline did not increase pain sensitivity.

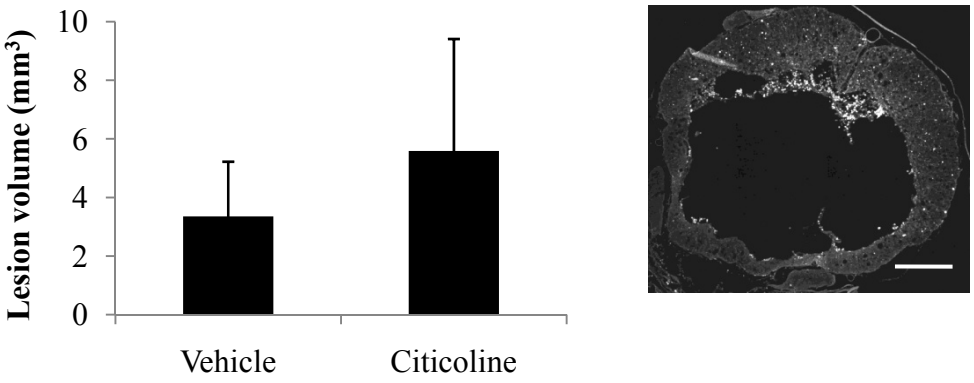


Figure 4.4: Effect of citicoline treatment on lesion volume at 37 days post-SCI. The volume of the injury cavity was estimated in transverse histological sections using the Cavalieri method (n=4 citicoline treated rats, n=6 vehicle controls). Treatment with citicoline after SCI had no effect on the size of the lesion ($p>0.05$). A representative lesion epicenter is shown on the right (scale bar = 500 μm).

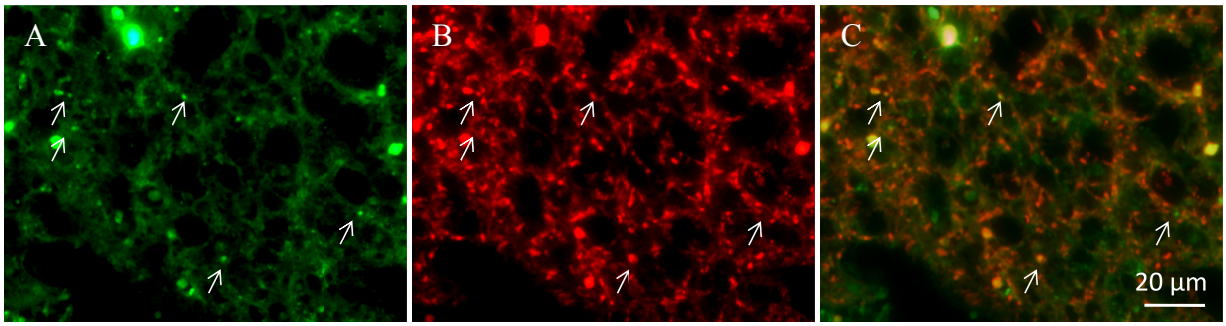


Figure 4.5: Axonal permeability marker uptake 37 days following SCI. Uptake of 10dex was apparent at 37 days post-SCI. The permeability marker was injected 3h prior to sacrifice, and histological sections in the transverse plane were visualized with fluorescence microscopy. 10dex uptake (A) colocalized with NF160 immunostaining in some but not all instances (B; overlay in C). Lack of colocalization in some dextran-positive regions may be explained by dextran uptake of axons with neurofilament loss (a common hallmark of SCI). These data indicate that chronic membrane damage occurs out to at least 37d in this model of SCI.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

The incidence and long-term debilitating consequences of SCI make it a major socioeconomic concern. Because there are no reliable clinically available treatments, there is a need to uncover and explore alternative mechanisms of cellular damage and dysfunction then target those mechanisms with rational treatment strategies. Accordingly, this research project was focused on achieving a better understanding of plasma membrane compromise occurring after SCI and investigating a treatment approach intended to augment membrane repair.

Through a series of experiments utilizing a dye exclusion assay in an *in vivo* rat contusion model, we observed plasma membrane compromise at acute (within 10 min), subacute (3d post-SCI), and chronic (5w post-SCI) time points. Our assessment of permeability marker uptake revealed that SCI causes immediate as well as persistent breaches in the plasma membrane that are large enough to allow the influx of normally cell-impermeant molecules. Alterations in membrane permeability within neuronal cell bodies in the gray matter occurred in the acute phase but did not persist in the subsequent time points we assessed. Cell bodies taking up the permeability marker at the 10 min time point often had a pathological appearance, including pericellular blebbing and cell shrinkage. Axonal permeability changes, however, were observed at acute, sub-acute, and chronic time points. Morphology of permeable axons was most extensively examined 3d post-SCI. A subset of permeable axons adjacent to the lesion were fragmented and/or swollen, whereas others farther from the injury site had a normal

appearance. These findings indicate that plasma membrane damage is linked with a variety of morphological alterations, which are potentially related to the size of the membrane breach and/or the amount of time that it is present.

These results provide the foundation for future work examining the causes and effects of plasma membrane permeability after SCI. For example, our results demonstrate that membrane damage persists for several weeks post-injury. Although it is understood that the mechanical impact can cause immediate damage by physically rupturing the membrane, the factors causing longer-term damage are highly complex and not fully understood. Future experiments performed in a more simplified *in vitro* axonal injury model would allow for well-controlled manipulation of certain variables in order to examine the major players that contribute to the loss of plasma membrane integrity. Potential mediators of membrane damage include a decrease in phospholipid synthesis (e.g., through decreased CTP: phosphocholine cytidyltransferase activity), increased phospholipid damage (e.g., through increased PLA2 activity or free radical production), and/or a failure to reseal (e.g., through blocking of vesicle fusion with the cell membrane). These parameters could be experimentally altered by genetically modifying the cells or using pharmacological agents to either augment or block each variable individually. Measuring the duration and extent of permeability increases due to these modifications can help elucidate their role and lead to a better understanding of the mechanisms involved.

Our experiments also suggested that persistent plasma membrane compromise is associated with morphology consistent with axonal degeneration. However, we did not explicitly show that there is a cause and effect relationship. Future work aimed at

isolating plasma membrane permeability from other post-injury variable would be useful for examining this more closely. For example, holes/tears of known sizes and duration time could be created in axons *in vitro* using a laser or a similar tightly controlled stimulus, and these cells could be tracked over time to determine the effects. Varying the pore size, number of pores, and pulse duration could help determine the parameters that lead to pathological changes, thus allowing us to better define the critical cellular thresholds.

This research also investigated citicoline as a treatment intended to facilitate membrane repair and improve post-SCI outcomes. Our data showed little to no benefit when citicoline was administered to rats at high doses and at frequent repeated delivery times (500 mg/kg given immediately and 3h after injury then daily thereafter). We assessed a plethora of outcome measures (cPLA2 activity, plasma membrane permeability to 3kDa dextran, lesion volume, and a variety of behavioral tests). However, none of these experiments demonstrated a biologically significant treatment effect.

Future experiments comparing the levels of citicoline, phosphatidylcholine (PC), and the intermediates in the synthesis process (see Figure 1.2) in sham, injured, and citicoline-treated injured rat spinal cords could be performed in order to allow a more complete interpretation of our results. At present it is unclear whether citicoline was able to increase PC levels after SCI. If citicoline was able to increase PC to levels equivalent to sham, we could infer that PC loss has minimal (if any) effect on plasma membrane permeability (at 3d post-injury), lesion volume (at 3d and 5w post-injury), or behavioral outcomes (up to 5w post-injury). If, however, citicoline did not enhance PC levels, it

would be useful to know which step in the synthesis process was rate-limiting. For example, an accumulation of choline would suggest that choline kinase activity limited the synthesis of PC. This assessment could provide the opportunity to deliver combinational therapies to increase PC synthesis (e.g., delivering citicoline in combination with a choline kinase activator).

Our results using citicoline as a treatment contradict previous reports showing that citicoline is beneficial as a treatment for SCI (Cakir et al. 2005; Yucel et al. 2006). However, these studies differed from ours in the dosing regimen administered (we administered immediate and daily doses, whereas previous reports delivered only a single bolus immediately after SCI). Thus, one interpretation is that citicoline loses its efficacy when administered more frequently. A study directly comparing these dosing regimens would be necessary to determine if this is the case. Taken together, these findings indicate that clinical translation of citicoline treatment will require additional animal studies in various laboratories to verify efficacy, determine a dosing strategy (e.g., number of doses and time administered post-SCI), and define the conditions under which it provides a favorable outcome (e.g., varying injury severity). If citicoline is determined to have a reliable treatment effect, such studies will facilitate essential planning for clinical trials. The need for extensive pre-clinical studies should not be overlooked, since ill-planned clinical trials have plagued the field of neurotrauma for years and may have led to inconclusive results in some instances (Lammertse et al. 2007; Machado et al. 1999; Sayer et al. 2006; Tolias and Bullock 2004).

Although citicoline was not efficacious in our studies, there is great potential for targeting plasma membrane compromise to treat SCI. The plasma membrane plays a

fundamental role in maintaining cellular homeostasis. Its integrity is crucial for all aspects of cell function; therefore the finding that SCI causes persistent membrane compromise may be significant for developing new treatment strategies. Future work identifying the mechanisms compromising the plasma membrane may lead to the design and efficacy of novel therapies. Although we have yet to find the "magic bullet" for curing SCI, this research may bring us one step closer to finding a clinically effective treatment.

APPENDIX A

VALIDATION OF SPINAL CORD CONTUSION MODEL

Introduction

Spinal cord injury (SCI) is most commonly initiated by fracture or dislocation of the spinal column, which results in tissue damage in the spinal cord parenchyma. The mechanical impact causes displacement of bone fragments, intervertebral discs, or ligaments, resulting in transient compression or contusion of spinal cord tissue. When mechanical forces surpass structural thresholds, cell death (necrosis) occurs and blood vessels become ruptured. In addition, this mechanical perturbation sets into motion a series of events which includes many complex pathophysiological alterations such as blood-spinal cord-barrier breakdown, edema, inflammation, excitotoxicity, and apoptotic cell death (for review see (Dumont et al. 2001; Hagg and Oudega 2006; Hausmann 2003)). Collectively, these events result in the formation of a fluid-filled cystic cavity that gradually expands over the days, weeks, and months after injury.

The contusion SCI model mimics the transient compression of spinal cord tissue, as a brief mechanical impact is applied bilaterally to the dorsal surface of the exposed spinal cord. When animals are injured at a mid-thoracic vertebral level (T10 in this case), they exhibit symptoms of SCI including hindlimb, trunk, and tail sensorimotor dysfunction. In some cases, rodents also display other characteristics of SCI including neuropathic pain and a loss of bladder function. The lesion created by the mechanical impact is similar to human SCI, characterized by a fluid-filled cystic cavity that expands over time then eventually stabilizes (for review of animal models see (Kwon et al. 2002)).

T10 was chosen as the injury location because it induces functional deficits that can be assessed with behavioral testing without causing unnecessary forelimb paralysis or respiratory dysfunction (which would be induced with a cervical injury).

Although a variety of animal species and modes of injury have been used to study SCI, contusion injury in the rat is a commonly used animal model. The first contusion model consisted of a weight dropped onto the spinal cord from a known height (Allen 1911). The severity of injury could be altered by dropping the weight from various heights, resulting in graded histological and functional effects (Black et al. 1988; Noble and Wrathall 1987; Wrathall et al. 1985). Further technological advances in the field resulted in the creation of more sophisticated devices that induce a single rapid impact. The user can prescribe the severity of injury by altering the piston displacement (Stokes et al. 1992) or force (Scheff et al. 2003). These devices are more consistent and reproducible compared to weight drop because they eliminate any bouncing that may occur. In addition, feedback control allows the investigator to determine the actual force and/or displacement applied, which can then assist in ruling out instances when bone or other tissue was impacted inadvertently.

We chose to use the commercially available Infinite Horizons device (Precision Systems and Instrumentation, Lexington, KY) in these studies because of its widespread use in the field of neurotrauma research (thus allowing for direct comparisons between research groups) as well as logistical reasons (portability, ease of use, cost, etc.). This device utilizes a piston driven by a servo-controlled motor to impact the spinal cord at a user-defined force. This model has been previously characterized and has demonstrated behavioral and histological characteristics of human SCI (Scheff et al. 2003).

The purpose of this study was to validate the SCI rat contusion model in our laboratory and establish behavioral outcome measures that can be used to assess potential treatment strategies. Accordingly, we performed moderate contusion SCI (150 kdyn impact force) on rats and compared functional outcomes to sham and naïve controls. Our results demonstrate that SCI resulted in measurable functional deficits and validate that the injury model used in our studies are similar to those previously published.

Materials and Methods

Surgical procedures

All procedures involving animals were approved by Georgia Tech's Institutional Animal Care and Use Committee. Male Sasco Sprague-Dawley rats (300-400g, n=18 in total) were anesthetized with sodium pentobarbital (50 mg/kg IP). Prior to surgery, rats were shaved, eye ointment was applied, and the local anesthetic Marcaine was administered (0.2cc, subcutaneous). Skin was scrubbed with three applications of alcohol then chlorohexiderm. An incision was made along midline, and the muscle overlying T10 vertebrae was removed. In injured animals (n=8), the spinal cord was impacted with a force of 150 kdyn (Precision Systems and Instumentation, Lexington, KY). Following injury, muscle was sutured and skin was stapled in order to close the wound. Saline was administered to prevent post-operational dehydration (5cc, subcutaneous). Shams (n=6) were subjected to all surgical procedures except for mechanical impact (to control for all surgical procedures including laminectomy). Naïve animals (n=4) received only anesthesia, pre-surgery preparation, and an incision along midline, which was necessary for keeping observers blinded to the identity of animals undergoing testing.

A strict post-operational care regimen was implemented to ensure ethical treatment of animals and reduce unnecessary pain and health complications. Rats were weighed three times in the first week after surgery and once per week thereafter. If rats lost 10% of their body weight, they were given a nutritional supplement until body mass was restored. Bladders were expressed twice a day until voiding function was consistent for three consecutive days. Hydration status was monitored daily in the first week with a gentle dorsal skin pinch. Fluids were administered subcutaneously (saline, 5cc) if skin was slow to retract.

Following behavioral testing, animals were euthanized via transcardial perfusion with phosphate buffered saline followed by 4% paraformaldehyde. Spinal cord tissue was removed, post-fixed overnight, and then immersed in sucrose. Tissue was frozen in OCT medium and stored at -80ºC.

Behavioral assessment

For approximately two weeks prior to surgery, animals were handled by researchers and placed in testing devices until signs of fear were no longer present (crouching/freezing, frequent urination and defecation, etc.). All behavioral testing was conducted once a week up to 57 days after surgery, and observers were blinded. The time of day for testing was kept as consistent as possible to reduce variability. As recommended by others, a range of behavioral activities were tested, including spontaneous locomotion, skilled motor performance, and sensory function (Basso 2004; Muir and Webb 2000). Testing was conducted once prior to surgery to ensure that each rat was able to perform within the normal range of hindlimb function. Scores more than two standard deviations from the mean were treated as outliers and removed from the analysis. Groups were compared using two-way repeated measures ANOVA followed by Tukey's pairwise comparisons ($\alpha = 0.05$).

Basso Beattie Bresnahan (BBB) open field locomoter

BBB testing was performed according to previously described methods (Basso et al. 1995). Briefly, each animal was placed in the open field for four minutes, and hindlimb function was scored by two observers based on parameters such as joint movement, weight-supported stepping, coordination, paw rotation, and trunk stability. Possible scores ranged from 0 to 21, with higher scores corresponding to greater hindlimb function. Scores obtained for the left and right hindlimb were averaged together since the injury was bilateral. Although this scoring system is ordinal, the ANOVA was deemed an appropriate statistical test due to its robust nature (Scheff et al. 2002). In addition, others have noted that the scale is ordinal in the scoring range we observed (Ferguson et al. 2004).

Grid walk

Animals were placed on an elevated wire grid (4" x 2" spacing between rungs, 8"x 48"x 12") for three minutes and encouraged to locomote with gentle prodding. During the testing session, the total of number of steps taken, the number of times the hindlimbs missed a rung, and the number of accurate steps were quantified and recorded. Data were expressed as the percentage of accurate steps relative to the total number of steps.

Von Frey Testing

The von Frey sensory test was used to evaluate the recovery of sensory function. Typically, animals with more severe spinal cord injuries will be more sensitive to a normally innocuous stimulus (Hutchinson et al. 2004; Lindsey et al. 2000; Mills et al. 2001; Yoon et al. 2004). Animals were placed in a clear plastic box above a wire mesh grid (¼" x ¼") and given a food reward (fruit cereal) to distract the animal during testing. In addition, marks were applied to the hindpaw using a permanent marker in order to maintain a consistent location of stimulus application. A known force was applied to the plantar surface of the hindpaw with a set of Semmes-Weinstein monofilaments (Stoelting Co., Wood Dale, Illinois). Testing was conducted only when the animal was eating in an attempt to normalize the stress level and to prevent responses due to visualization of the monofilament. A positive reaction to the stimulus was scored if the animal responded by lifting the hindlimb, and the next smallest monofilament was used. If no response was observed, the next largest monofilament was used. This process continued until 20 consecutive stimuli were conducted per hindlimb. At least five seconds was allowed between trials to prevent a sensory wind-up response. The score was reported as the lowest force that caused a response >50% of the time, and the threshold values for the right and left hindlimb were averaged together. Testing was restricted to animals exhibiting weight support in the open field because otherwise the monofilament lifted the hindlimb before a response was elicited. Thus, injured animals were not tested on day two and the number of injured rats undergoing testing was reduced on days nine (n=5), sixteen (n=7), and twenty-three (n=7).

Results

BBB Open Field Locomotor

BBB scores revealed that spinal cord impact produced sustained deficits in locomotion (Figure A.1). Specifically, animals receiving spinal cord impact performed significantly worse at all time points compared to sham and incision only/"naïve" rats. Thus, we can conclude that the mechanical impact (and not the surgical procedure of laminectomy) was responsible for alterations in locomotor function. These results are comparable to those previously reported when moderate contusion injury was applied to the rat spinal cord (Basso et al. 1995; Lankhorst et al. 1999; Scheff et al. 2003). Injured rats averaged a score of approximately thirteen on the BBB scale, which signifies that recovery was limited to consistent weight supported stepping with frequent but not consistent forelimb-hindlimb coordination. Observed deficits also included toe drags, rotation of the hindlimbs during locomotion, trunk instability, and inability to maintain the tail in an elevated position.

Grid Walk

Grid walk testing resulted in significantly greater footfalls in injured animals compared to sham and naïve controls (Figure A.2). Sham surgery did not alter grid walk performance compared to naïve animals, demonstrating that the surgical procedure was not detrimental to hindlimb function in this behavioral task. Although it is difficult to directly compare our data to other published reports (due to slight variations with injury models and grid walk specifications, etc.), these results are similar to other reports in the literature using rat models of moderate contusion SCI (Dijkstra et al. 2006; Lankhorst et al. 1999; Metz et al. 2000).

Von Frey Testing

We explored hindlimb sensitivity to a tactile stimulus by performing von Frey testing. This was considered an important outcome measures since neuropathic pain is a clinically relevant problem that dramatically affects human quality of life after SCI (and thus would be a useful outcome measure when assessing potential therapeutic agents). However, we did not observe an SCI-induced deficit in touch sensitivity at any of the time points assessed (Figure A.3).

Although it is quite possible that our injury model does not induce hypersensitivity, unforeseen errors in the testing methods may also explain our results. Pain testing must be carefully performed so that factors such as stress and environmental cues are carefully controlled, and it is possible that we may have overlooked an external factor that masked any injury-induced effects. Some researchers perform testing with only one animal in the room to prevent communication between animals. This high frequency communication can heighten the next animal's sensitivity and thus alter the threshold for response. It has also been noted that environmental factors such as temperature, relative humidity, and the surface the rat stands on can alter the properties of von Frey hairs and thus change the force applied (Andrews 1993; Pitcher et al. 1999). Such precautions were not taken in this study; animals were all placed in the same room where testing occurred and environmental conditions were not tightly controlled. Lack of control of these factors may have caused the high degree of variability we observed.

Although these technical concerns may have led to erroneous results, some observations led us to believe that the injury simply did not induce hypersensitivity in the animals. Naïve and sham animals were within the normal range (60-80g), indicating that methods were performed in a manner consistent with previous reports (Hutchinson et al.

2004; Lindsey et al. 2000). Injured rats did not have an exacerbated response to the stimulus (vocalizations, spasticity, jerky reflexive movements, etc.), which would suggest that they did not perceive pain when the stimulus was applied. Results obtained from a subsequent study (Chapter 4) further support this explanation, as we were unable to detect injury-induced hypersensitivity to a heat stimulus in this model.

Discussion

Results obtained from this study demonstrated deficits in locomotor function and grid walk performance for at least eight weeks post-injury. Importantly, sham surgery did not decrease performance on any task, demonstrating that surgical procedures (specifically laminectomy) did not inadvertently injure the spinal cord. Von Frey testing revealed no SCI-induced hypersensitivity, which may be explained by absence of this phenomenon in our hands or limitations in the testing methods employed. Overall, these results validate the SCI model used in the studies throughout this thesis, as the findings are similar to those previously reported.

Contusion injury in the rat creates histological and functional deficits that closely mimic the human condition. Rodent contusion injury results in a tissue response that starts with progressive hemorrhage and edema followed by a period of partial repair and regeneration and eventually results in a chronic lesion comprised of a fluid-filled cystic cavity surrounded by intact tissue (Dumont et al. 2001; Kwon et al. 2004; Rosenzweig and McDonald 2004). These SCI-induced histological alterations are consistent with those observed in humans (Hayes and Kakulas 1997; Stokes and Jakeman 2002) and therefore provide a clinically-relevant experimental model to explore mechanisms and treatments.

In addition to the changes occurring within the tissue, we and others have shown that contusion SCI results in functional deficits that can be measured over time in an animal model. This aspect of animal research provides a method for assessing recovery of neurological function, a clinically relevant outcome for persons with SCI. The results obtained in this study demonstrate that we are able to perform contusion SCI in rats and assess behavioral outcomes. Optimization of these methods was necessary before any new scientific research could be conducted. Therefore, the findings presented here provide the foundation for performing the remainder of the experiments described in this dissertation.

Although the contusion model is accepted by the scientific community as a clinically relevant model of SCI, there are some limitations that must be addressed. SCI often occurs as a result of a traumatic injury (such as a motor vehicle accident) in which many other wounds may be inflicted. In fact, approximately 50% of the SCI patients also sustain a brain injury (Macciocchi et al. 2004; Tolonen et al. 2007). These concurrent injuries may alter the systemic response to SCI but are not modeled here. In addition, exposure of the spinal cord by laminectomy allows for more reproducible injury; however, most injuries occur when the bone is fractured or dislocated and inflicts tissue damage to the spinal cord. In addition to altering the biomechanics of injury, performing a laminectomy may decrease the intrathecal pressure caused by SCI and thus alter the post-injury response.

Another limitation to this model is that animals must be anesthetized in order to perform surgery. Anesthetic agents cause a loss of consciousness by altering neuronal firing patterns. Although anesthetics are necessary for the surgical procedure, their use

may change the effects of injury. In fact, studies have shown that experimental outcomes are dependent upon the type of anesthetic used in the surgical procedure (O'Connor et al. 2003; Salzman et al. 1990; Statler et al. 2006). Nembutal was used as the anesthetic of choice in our studies due to technical considerations (e.g., long period of anesthesia and decreased bleeding during surgery). Nembutal suppresses the central nervous system by potentiating the effects of the inhibitory neurotransmitter GABA and also blocking excitatory AMPA receptors. These receptors are known to play a role in traumatic CNS injury (Demediuk et al. 1989b; Diaz-Ruiz et al. 2007; Goda et al. 2002; Goforth et al. 1999; Gwak et al. 2007). Therefore, altering receptor activity with an anesthetic may have an effect on the injury response. In addition to these direct effects on the nervous system, Nembutal causes systemic effects such as a reduction in blood pressure and suppression of the respiratory system, which may alter the systemic response to trauma. Although the effects of Nembutal are pointed out here because of its relevance to these studies, all anesthetics alter the central nervous system and the overall physiology in some way that is likely to affect the results of SCI. However, anesthetics must be used in animal research due to ethical and technical considerations and are therefore a necessary limitation in the model.

Although the contusion model is not a perfect replica of human SCI, it is nonetheless a powerful tool in SCI research. Animal models provide a platform for obtaining valuable information about injury mechanisms and potential treatment strategies. The ability to induce SCI at a defined anatomical location and with consistent mechanical parameters provides a well-controlled system for studying SCI. Pre-clinical testing in animal models such as the one described here can provide evidence that

supports new therapies, ultimately aiding in the development and implementation of clinically relevant treatments.

Conclusions

Rat spinal cord contusion provides a clinically-relevant model for studying SCI. Here we conducted an experiment to validate the use of this model in our laboratory, and results demonstrated that we were able to perform surgeries and behavioral testing in a manner that produced results similar to previous work. Contusion SCI caused sustained functional impairments in open-field locomotion as well as stepping accuracy on the grid walk, although hypersensitivity to a touch stimulus was not observed. Overall, this study provides a foundation for experimental SCI research in our laboratory by optimizing methods for performing surgical procedures and behavioral testing so that SCI-induced functional deficits can be reliably measured.

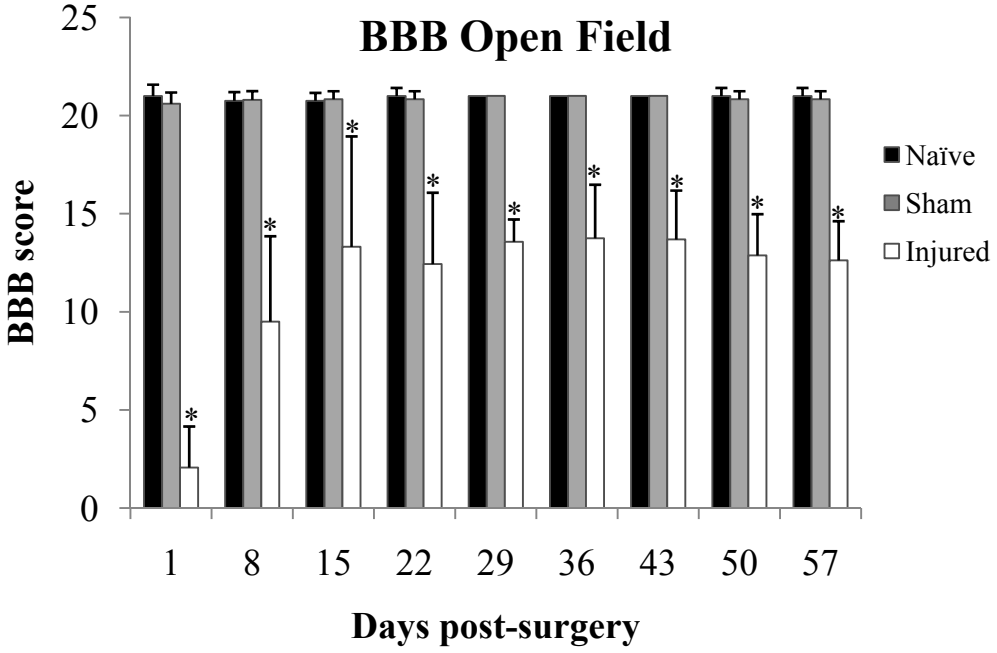


Figure A.1. Comparison of BBB scores in naïve, sham, and injured animals. Locomotor patterns were observed and scored according to the Basso Beattie Bresnahan (BBB) criteria in naïve (n=4), sham (n=6), and injured (n=8) animals up to 57 days post-surgery. A sustained behavioral deficit was observed in injured rats compared to sham and naïve controls. There was no difference between sham and naïve animals, indicating that surgical procedures were not detrimental to locomotor function (*$p<0.001$, mean ± standard deviation).

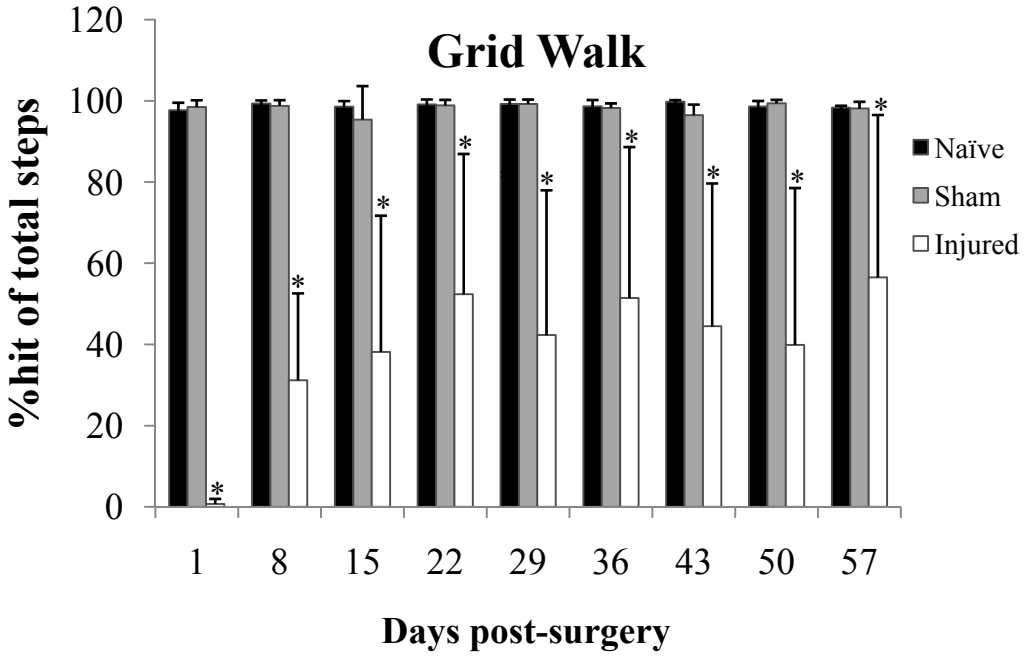


Figure A.2. Comparison of grid walk accuracy in naïve, sham, and injured animals. Grid walk testing revealed that injured rats (n=8) had significantly more footfalls compared to shams (n=6) and naives (n=4) at least up to 57 days post-surgery. Shams and naives were statistically the same, demonstrating that the surgical procedure did not impair performance on the grid walk task (*$p<0.005$, mean ± standard deviation).

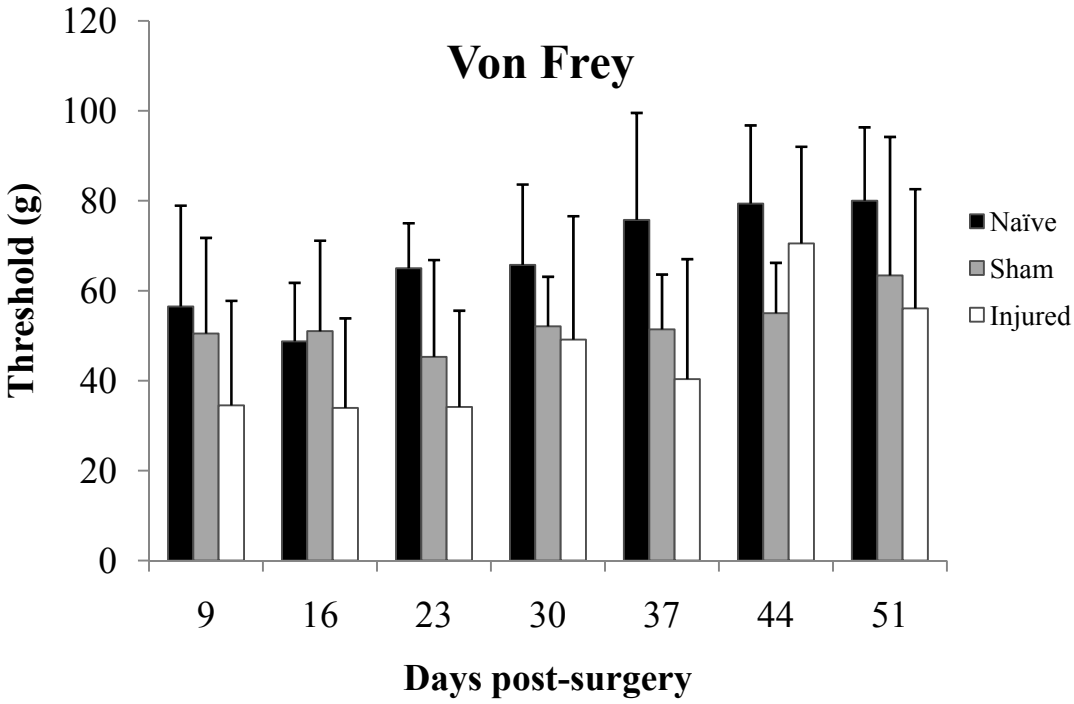


Figure A.3. Comparison of von Frey sensitivity in naïve, sham, and injured animals. Von Frey testing was conducted in order to determine the threshold for hindlimb withdrawal when a known force was applied to the plantar surface of the paw. No significant differences were found between naïve, sham, and injured rats ($p>0.05$).

APPENDIX B

AUTOMATED GAIT ANALYSIS SYSTEM FOR QUANTITATIVE ASSESSMENT OF LOCOMOTION FOLLOWING SPINAL CORD INJURY

Introduction

Assessment of functional outcome is an invaluable aspect of spinal cord injury (SCI) animal research. The animal model provides a unique system in which treatments and experimental conditions can be assessed in terms of neurological function. Therefore, animal studies with a behavioral component are a powerful and clinically-relevant experimental test bed for evaluating potential SCI treatments. Although the importance of behavioral assessment is well-understood, there is room to improve upon the reliability and sensitivity of the testing methods.

Assessment of spontaneous locomotion in the open field is far and away the most common form of behavioral testing in SCI research (for review see ((Basso 2004; Goldberger et al. 1990; Metz et al. 2000; Muir and Webb 2000)). Typically, rat hindlimb function is assessed in the open field using the Basso Beattie Bresnahan (BBB) scale (Basso et al. 1995). In this test, several qualitative outcomes are combined to form a single score that is related to hindlimb locomotion patterns. Although quick and simple to perform, there are several limitations involved with this testing method. The categories in the BBB are defined rather subjectively and require great attention to detail during the assessment. As a result, comparisons between laboratories are not easily made

(although it is less problematic if individuals are properly trained (Basso et al. 1996b)). In addition, the BBB lacks sensitivity at certain points along the scale (scores between 2 and 4 and scores above 14), which decreases the statistical power and thus the ability to detect a treatment effect (Ferguson et al. 2004; Lankhorst et al. 1999; Popovich et al. 1999). Furthermore, scores are assigned based on broad categories (i.e., greater or less than 50%) and therefore are not sensitive to smaller changes.

The CatWalk™ system (Noldus, Leesburg, VA) is a commercially available testing apparatus that may address some of the limitations of BBB testing. The goal is to provide a quantitative and objective method for gait analysis for enhancing the sensitivity and accuracy of behavioral testing in neuroscience research (Hamers et al. 2006; Hamers et al. 2001). The CatWalk is comprised of a lighted glass walkway, a camera located beneath the walkway, and a software analysis system. When the animal crosses the walkway, light reflects down onto the camera and the intensity is recorded. Using the data acquired on video, the software then converts light intensity into gait parameters such as stepping pattern, regularity of stepping, stride length, stance duration, timing of steps, and base of support (parameters that are not quantitatively measured in the BBB). The user can then assess gait characteristics according to what is relevant for that particular experimental model.

In the study presented here, we utilized the CatWalk system for assessment of hindlimb function following contusion SCI at T10 vertebral level. This has been performed in other studies of SCI (Deumens et al. 2006; Joosten et al. 2004; Kloos et al. 2005; Koopmans et al. 2006; Van Meeteren et al. 2003), but has not been established in our laboratory. This study established protocols and baseline parameters for CatWalk

testing in our model and demonstrated that SCI-induced deficits can be measured using this system in our laboratory. Future work may utilize this testing method for examining the effects of various interventions after SCI.

Materials and Methods

Surgical procedures

All procedures involving animals were approved by the Georgia Tech Institute of Animal Care and Use Committee (protocol A05003). Six adult male Sprague-Dawley rats (250-276g upon arrival) were subjected to moderate SCI at T10 vertebral level, as described elsewhere in this dissertation (the most detailed description can be found in Appendix A). Briefly, rats were anesthetized with Nembutal (50 mg/kg) and supplemented with isoflurane as necessary. Asceptic techniques were used. Laminectomy was performed at T10 vertebral level, and then the Infinite Horizons spinal cord impactor was used to deliver a force of 150 kdyn to the exposed spinal cord. The surgical site was closed with suture in the muscle and staples in the skin. Post-operational care ensured that animals remained hydrated and were able to eliminate bodily waste.

Upon completion of the testing period, animals were euthanized via heavy anesthesia with Nembutal followed by transcardial perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Three animals were perfused at five weeks post-SCI, and the three remaining animals were perfused at eight weeks, in order to provide tissue for future histological pilot studies at a variety of time points.

CatWalk testing

Animals were trained to cross the CatWalk prior to baseline testing. During the training period (approximately 6 weeks), a protocol for motivating the animals to cross the runway with a consistent velocity was established. Testing was conducted at night during the animal's wake cycle. The home cage was placed at the end of the runway with a box over the top to darken the cage compared to the lit runway, and animals were allowed to enter the home cage at the end of each of the three trials. Cages were changed no less than four days prior to testing in order to intensify the odor. Placing a gate (provided with the CatWalk system) at the beginning of the runway then removing it just prior to the trial also improved motivation. Testing was conducted in the dark to enhance the contrast when a paw was placed on the walkway. All animals underwent baseline testing the day before SCI surgery was performed and once per week thereafter. Three sequential trials were captured and analyzed for each animal at each time point.

After trials were acquired on video, researchers utilized CatWalk software (version 7.1) to label limb placement on the runway as right forelimb, right hindlimb, left forelimb, or left hindlimb. An example of the software application can be seen in Figure B.1. The CatWalk system then calculated quantitative values for various gait parameters. Data were analyzed using one-way repeated measures ANOVA followed by Dunnett's pairwise test to compare each post-SCI value to baseline ($\alpha = 0.05$).

Results and Discussion

Although the CatWalk system is capable of generating a very large amount of data, only a subset is presented here. These data were chosen for display because they were selected as those that are potentially the most useful for future studies (as

determined by a large and sustained injury deficit accompanied by a relatively small standard deviation). A description of the selected parameters can be seen in Table B.1.

Many of the key parameters demonstrating a behavioral deficit after moderate contusion SCI are related to forelimb-hindlimb coordination (specifically shown in Figure B.2 as regularity index and phase dispersion mismatch). As defined by Basso et al., a coordinated pass is one in which "for every forelimb step a hindlimb step is taken and the hindlimbs alternate". Objective evaluation of coordination is a valuable tool offered by the CatWalk system, since it is difficult to assess even when the observers are experienced. In addition, achieving coordination is an important and biologically significant milestone in post-SCI recovery, since we and others have noted that BBB scores after moderate SCI contusion plateau at 13 (Basso et al. 1995; Ferguson et al. 2004; Scheff et al. 2003). A score above 13 indicates consistent forelimb-hindlimb coordination with deficits in toe clearance, paw rotation, trunk stability, and/or elevation of the tail. A rat must obtain consistent coordination in order to obtain a score above 13; therefore, the BBB will not detect an effect if a treatment alters aspects of fine motor control but has no effect on coordination. Several post-hoc transformations that diminish have been developed in order to minimize this limitation in the BBB (Ferguson et al. 2004; Lankhorst et al. 1999; Popovich et al. 1999). Thus, objective analysis of forelimb-hindlimb coordination can improve the sensitivity and reliability of open field testing (Koopmans et al. 2005).

Here we observed an SCI-induced decrease in the regularity index, a quantitative and objective measure of forelimb-hindlimb coordination. The regularity index is defined as the percent of normal step patterns out of the total number of step sequences.

In this study, we observed statistically significant SCI-induced deficits in the regularity index at the first week post-injury, but these deficits were not sustained thereafter (Figure B.2). Others have observed longer-term deficits in the regularity index after moderate contusion SCI (Hamers et al. 2001; Koopmans et al. 2005; Van Meeteren et al. 2003). Diagonal phase dispersion mismatch was also statistically different in injured animals one week post-SCI but not at later time points (Figure B.2). Phase dispersion mismatch would be sufficient to decrease the regularity index, and these parameters changed on the same time scale; therefore, they are likely to be related.

In addition to impairments in forelimb-hindlimb coordination, the step sequence changed over time after SCI (Figure B.3). Step sequence patterns and their alterations following SCI were first noted by Cheng et al. (1997). It is important to note that variations in the step sequence would not be detected in the regularity index outcome, since these are considered normal stepping patterns. Throughout this study, sequence Ab was most commonly used by rats to cross the walkway. The baseline percentage of Ab sequence was a mean value of 88%. This value dropped to 67% at week one, then increased to approximately pre-injury levels and above in subsequent weeks (85%, 98%, and 90% at weeks two, three, and four respectively). In addition, SCI caused animals to increase the number of rotate stepping patterns (Ra and Rb) in the first and second week post-injury (Figure B.3). These results show that moderate T10 contusion SCI alters the step sequence used by rats. Others have noted similar alterations in step sequence patterns after contusion SCI (Hamers et al. 2001), although a different study showed dissimilar baseline and post-SCI step sequence patterns (Koopmans et al. 2005). Reasons

for the discrepancy may include the strain of rat or the pace with which animals crossed the platform.

The most robust and sustained deficit we observed in this study was in the hindlimb base of support. Hindlimb base of support was statistically greater than baseline values at every time point we assessed in this study. This measure is not assessed in the BBB and would therefore provide an additional outcome measure for assessment of locomotion when used in combination with the BBB. Others have shown a similar sustained increase in the base of support after contusion SCI, using either the CatWalk or footprint analysis as the detection method (Hamers et al. 2001; McEwen and Springer 2006; Stokes and Reier 1992).

In general, the CatWalk is a powerful tool for assessment of functional recovery following SCI under various experimental conditions. However, some important technical limitations should be noted. Although CatWalk is partially automated, it should not be misconstrued to be a "high-throughput" testing tool. Training and motivating animals to consistently cross the runway can be extremely time-consuming, and inadequate pre-injury training can lead to erroneous results and interpretation. Furthermore, labeling of hindlimbs in the CatWalk software is also a large time commitment. All in all, the CatWalk easily requires twice as much time as the BBB or grid walk test. However, this time commitment is balanced by the enormous potential offered by quantitative, objective, and accurate data acquisition.

In the study presented here, we detected SCI-induced deficits with the CatWalk system. Impairments observed were less severe compared to previous publications using moderate contusion SCI, although relatively few studies have been conducted using this

system to date (Hamers et al. 2001; Koopmans et al. 2005). There are many potential explanations for this outcome, but it is most likely that the SCI device administered an impact of less than 150 kdyn (the intended force). Observations from previous behavioral studies (Appendix A, Chapter 5, and other studies not shown) demonstrate a less severe injury deficit with each study compared to the previous. As an example, one can compare BBB scores in Appendix A (which was performed first) with those in Chapter 4. The subsequent study showed higher BBB scores, suggesting that the device delivered a less severe injury. The study presented here was performed after the one described in Chapter 4 and thus the animals may have been injured with an even lower impact force. Calibration of the device is recommended before any future studies are performed.

Another potential limitation to the study is the possibility that hindlimb locomotion may have changed over time in a way that was not directly related to the injury. During baseline testing, animals did not freely cross the CatWalk and much of the recorded video had to be discarded due to pauses in locomotion and hesitance to begin the pass at the beginning of the trial. However, videos from later time points demonstrated that the animals quickly and easily crossed the glass walkway with no pause at the beginning. The speed of crossing the walkway can have a large effect on gait parameters, which is apparent during BBB testing (personal observation). Therefore, the speed with which the animals cross the walkway is likely to be a large determinant of parameters computed by the CatWalk. A more complete study should include a sham group to control for any training effects. However, we did not include that group in this

study out of ethical considerations since this study was intended to be a pilot study for optimizing methods and establishing pre- and post-injury baseline values.

Given the advantages and limitations provided by the CatWalk system, we recommend using this test in conjunction with other behavioral outcome measures. It is generally good practice to evaluate different types of functional recovery, typically comprised of testing normal locomotion (e.g., BBB and CatWalk) in addition to a skilled sensorimotor task (e.g., grid walk and beam walk). This is necessary in the event that a treatment has a differential effect on these mechanisms of hindlimb function (as we observed in Chapter 4) and is consistent with previous recommendations (Basso 2004; Muir and Webb 2000). Utilizing the CatWalk in conjunction with a variety of functional measures can lead to a powerful, sensitive study able to detect differences between groups when they exist.

Conclusions

The CatWalk system provides a quantitative method for analyzing gait in animal models of SCI and other neurological diseases and disorders. Results obtained from this study demonstrate that we are able to utilize this system and obtain deficits in hindlimb locomotion following SCI. Although many parameters can be evaluated with the CatWalk, we recommend using this system in combination with other behavioral outcome measures such as grid walk. Assessment of multiple functional outcomes can give a better understanding of various aspects of hindlimb function, including gait during normal locomotion (as tested in BBB open field and CatWalk) and fine motor control (tested with grid and beam walk). Utilization of various tests may yield different

outcomes and paint a more complete picture of treatment effects, as some may be more sensitive than others depending on the mechanisms involved.

Table B.1. Description of gait parameters used to assess hindlimb function post-SCI with the CatWalk system.

Parameter	Description
Regularity Index	Number of normal step sequence patterns relative to the total number of steps. This value corresponds to "coordination" in the BBB scale.
Hindlimb Base of Support	Average distance (width) between the hindlimbs.
Diagonal Phase Dispersion Mismatch	The number of peculiarities that occurs between the right forelimb (anchor) relative left hindlimb (target) or left forelimb (anchor) relative to right hindlimb (target). A peculiarity is defined as an error condition comprised of either placement of the anchor but no target placement or placement of the anchor with multiple target placements. In this case, mismatches from the two anchor-target combinations were averaged for each animal to form a single score.

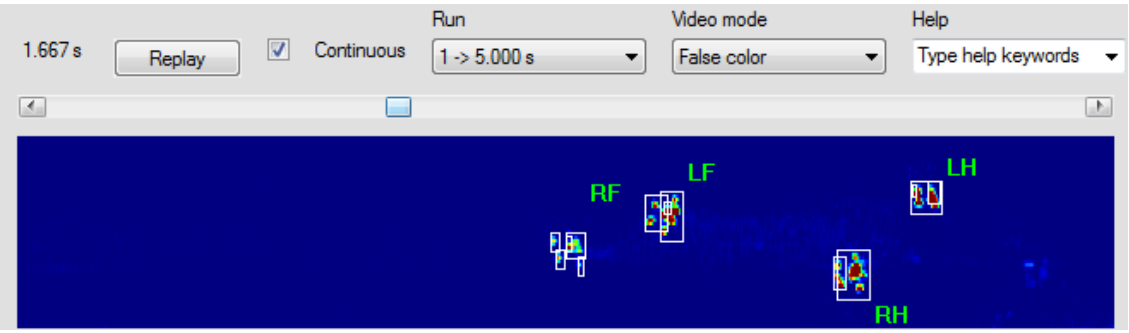


Figure B.1. Example of CatWalk analysis software screen. The video of a rat crossing the walkway is captured by the CatWalk system and can be displayed using the software. Limbs are labeled manually within the software, and then the intensity measurements are converted into quantitative data sets related to gait.

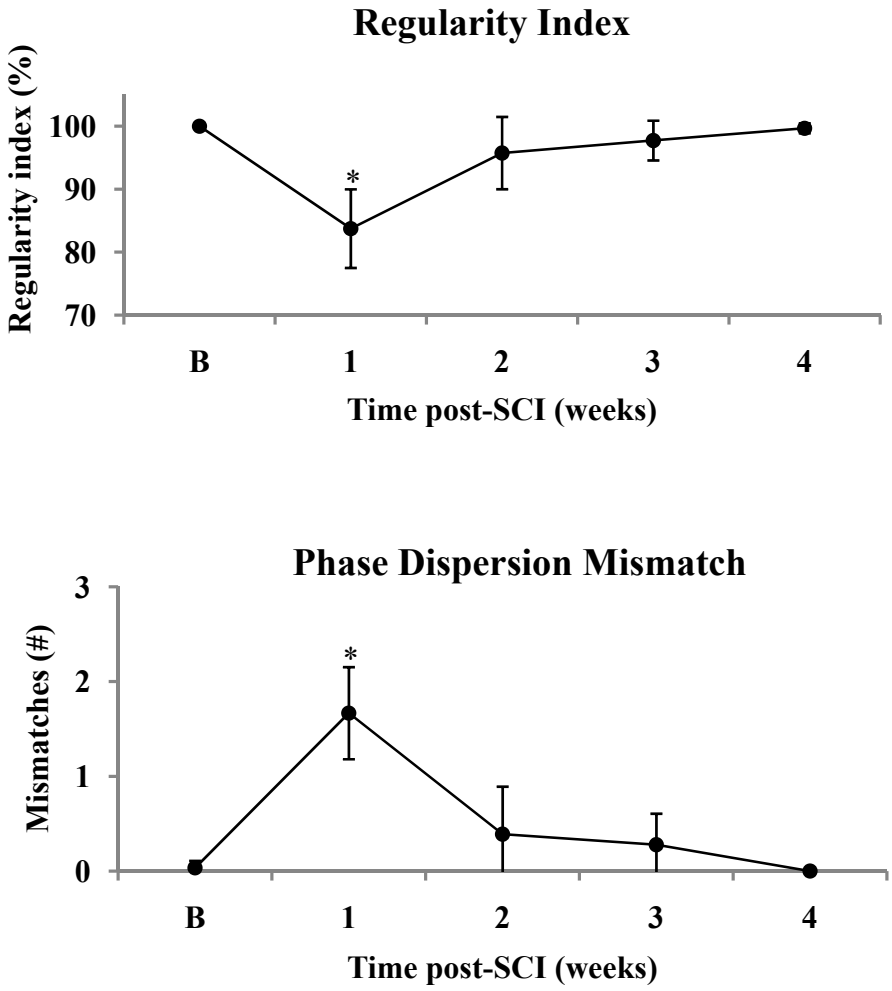
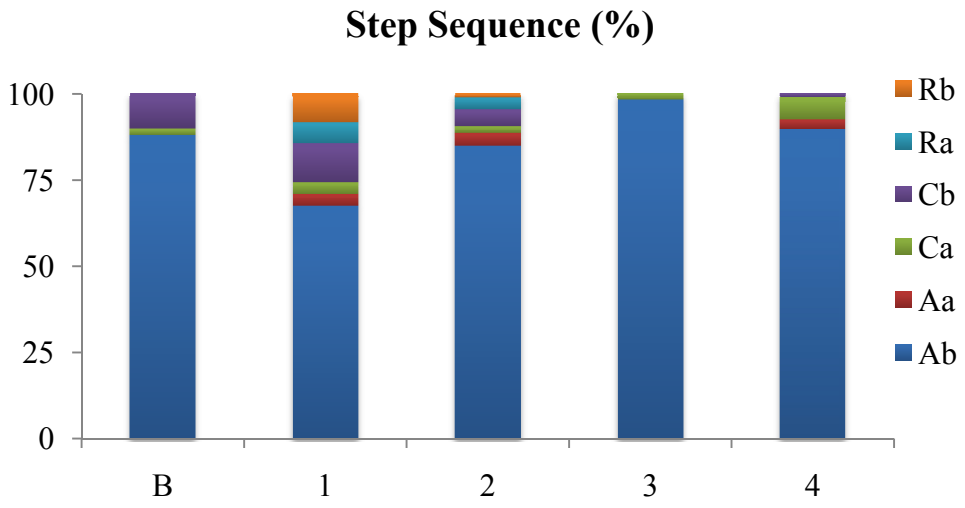


Figure B.2. SCI-induced deficits in regularity index and diagonal phase dispersions. Moderate SCI caused a decrease in the percentage of normal stepping patterns (regularity index) as well as an increase in the number of erroneous steps relative to the diagonal limb (phase dispersion mismatch). These results show that, in our laboratory, CatWalk is able to detect impairments in forelimb-hindlimb coordination after rat contusion SCI. Impairments were observed one week post-SCI but animals recovered by the second week (mean ± standard deviation, n=6, *$p<0.05$ compared to baseline, B denotes baseline score).



Step Sequence		
Category	**Abbreviation**	**Sequence**
Alternate	Aa	RF-RH-LF-LH
	Ab	LF-LH-RF-RH
Cruciate	Ca	RF-LF-RH-LH
	Cb	LF-RF-LH-RH
Rotate	Ra	RF-LF-LH-RH
	Rb	LF-RF-RH-LH

Figure B.3. SCI changes step sequence patterns. Contusion SCI decreased the frequency of alternate stepping patterns and increased the frequency of rotate patterns. A description of the possible stepping patterns of rat locomotion is shown in the table. Abbreviations: B (baseline), LF (left forelimb), LH (left hindlimb), RF (right forelimb), and RH (right hindlimb).

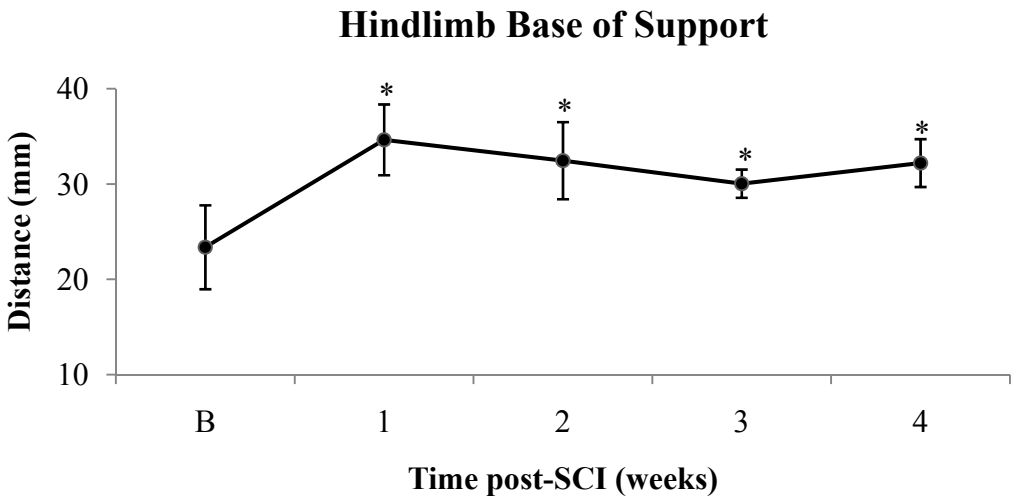


Figure B.4. Hindlimb base of support increases after SCI. Moderate contusion SCI increased the width of the hindlimbs. Deficits were sustained throughout the 4 week testing period (mean ± standard deviation, n=6, *$p<0.05$ compared to baseline, B denotes baseline score).

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