CHARACTERIZATION OF CATHEPSIN B mRNA AND PROTEIN EXPRESSION, ENZYMATIC ACTIVITY AND CELLULAR LOCALIZATION FOLLOWING CONTUSION SPINAL CORD INJURY IN RATS

By

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

UNIVERSITY OF FLORIDA

2004

This document is dedicated to my family, friends and colleagues. Their support, encouragement and guidance have made all the difference in the world.

ACKNOWLEDGMENTS

Numerous people deserve credit for making this experience as positive and fulfilling as it has been and I have been very fortunate to work with so many knowledgeable and talented individuals. There are a few people, however, that deserve to be specially recognized for their contribution.

The members of my committee, including Ronald Hayes, Richard Moyer, Harry Nick, Lucia Notterpek and Kevin Wang, are very deserving of my gratitude, as they have helped mold me and guide me in many different ways. Each individual has provided me something different, which I have incorporated into my own development. I have enjoyed our time together, and without them, my training would simply not have been as rich.

Sue Semple-Rowland has been a most wonderful influence on my scientific progression. She is an extraordinarily passionate and motivated scientist. Her enthusiasm and dedication to both her own work and the development of aspiring scientists are remarkable. I have grown tremendously from our interactions and I hope that I can be as giving to those around me as she is. I cannot express how thankful I am for all that Sue has done for me.

Michelle Deford has been by my side through these last few years. Michelle is an incredibly insightful and gifted scientist and I am constantly amazed by her depth and versatility. She has always found the time to lend me a helping hand and her assistance in this last year has been invaluable. She is truly an asset to any organization and I am blessed to have her as my friend.

Wilbur O'Steen has also played an integral part of my progression. He has been an endless source of information regarding so many different aspects of the research process including my initial surgical training, the nuances of animal care and with understanding a mountain of procedural guidelines. Wilbur is an irreplaceable resource and he has always been willing to lend a helpful hand. I appreciate his kindness through these years. He has made the lab a fun place to be and I am happy to have him as my friend.

Lastly, there is Dr. Douglas K. Anderson, my dissertation mentor. Dr. A has been wonderful throughout this process. He has been thoughtful and caring (and delightfully witty) as we worked toward the completion of these experiments. Through his incredible work ethic and dedication to his numerous responsibilities, Doug has been a very positive role model not only in my scientific endeavors but also in every aspect of my life. I have enjoyed being his student and look forward to being his colleague. I am honored to have called him "mentor."

On a personal note, I would like to thank my family. I could not have asked for a more loving and supportive family than the one I have. Above all else, my mother Barbara has been an inspiration for me throughout my life. She is unwavering in her support and I love and I respect her more than I can ever express. My mother is my rock and I am so happy to share this moment with her and with my entire family. Lastly, I want to thank Shane McFadyen for all his support, particularly during this last year. He is a constant source of love and encouragement and I am extremely thankful to have him in my life.

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December 2004

Chair: Douglas K. Anderson
Major Department: Neuroscience

Mechanical spinal cord injury (SCI) initiates a cascade of pathochemical and pathophysiological events, collectively known as the secondary injury. These processes disrupt normal homeostasis and include a prolonged inflammatory response, a decrease in energy metabolites, ionic gradient imbalances and the loss of vascular regulation. Significant tissue loss via apoptotic and necrotic cell death ensues, resulting in cavitation around the initial injury site. The subsequent loss of voluntary motor function and sensation below the injury site is accompanied by a poor prognosis for recovery.

There has been significant interest in understanding the activation and involvement of proteases in the secondary injury. Several proteases including calpain, the caspases and matrix metalloproteinases are activated by injury-induced perturbations to the spinal cord. These proteases have been linked to cell death in models of central nervous system (CNS) injury. Cathepsin B is no exception, having been associated with a brain tumor

metastasis and progression, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis and stroke.

Collectively, our studies represent the first in-depth characterization of cathepsin B mRNA and protein expression, enzymatic activity and cellular localization in the contusion-injured spinal cord. Sham-injury was a sufficient perturbation to the spinal cord to induce a transient increase in cathepsin B mRNA expression but did not affect protein expression or enzymatic activity. Contusion-injury, however, elicited significant increases in both cathepsin B expression and enzymatic activity levels and altered its cellular localization. Furthermore, the immunohistochemical analyses revealed that these increases were due to the invasion of inflammatory cells into the injured spinal cord.

In light of these findings, the powerful and indiscriminate hydrolytic properties of Cath B make it an attractive candidate for involvement in the secondary injury cascade. Thus, the reduction of SCI-induced increases in cathepsin B expression and enzymatic activity may ameliorate the secondary tissue damage, thereby providing some degree of neuroprotection to injured spinal cord tissue. However, the inhibitor used in these experiments did not suppress cathepsin B enzymatic activity *in vivo* following contusion-injury. Nevertheless, the effectiveness of cathepsin B inhibitors in other CNS insults suggests that they should continue to be tested in models of SCI.

CHAPTER 1
INTRODUCTION

Spinal Cord Injury Demographics

Currently, there are approximately 243,000 people living in the United States (1,000,000 worldwide) with spinal cord injury (SCI). Approximately 11,000 new injuries are sustained each year, the bulk (53%) of which is sustained by younger individuals (average age of 32.6 years). It is an injury that predominantly affects males (81.6%), a statistic that likely reflects life style choices. Currently, motor vehicle accidents (40.9%), falls (22.4%) and acts of violence (21.6%) are the leading causes of SCI. However, these trends change with increasing age. For example, injuries due to violent acts decrease with advancing age, and after age 45, falls overtake motor vehicle accidents as the leading cause of SCI. Furthermore, the percentage of persons 60^{+} years old at the time of injury has risen from 4.7% in the 1970s to 11.4% today (Spinal Cord Injury 2003). This age related increase most likely reflects the fact that the median age of the general population has increased from 27.9 years to 35.3 years during this same time period.

There are several terms (*e.g.*, tetraplegia/paraplegia, complete/incomplete) used by clinicians and researchers to designate the severity of an injury. Paraplegia results from a primary injury to the thoracic, lumbar and/or sacral regions of the spinal cord. It generally does not affect the sensory or motor function of the upper body. Tetraplegia occurs when at least one of the eight cervical segments is injured, resulting in paralysis of all four limbs. An incomplete injury indicates that some motor and sensory function below the injury site is still present while a complete injury is one in which the patient is devoid of

discernible voluntary movement and sensation below the injury site. Although the most severe functional deficits usually occur when the cord is transected, the vast majority of SCIs are not complete transections (Bunge *et al.,* 1993; Bracken *et al.,* 1990). The most common type of SCI is incomplete tetraplegia (30.8%), followed by complete paraplegia (26.6%), incomplete paraplegia (19.7%) and complete tetraplegia (18.6%). However, clinicians and researchers have noted a decreasing trend in injury severity, "Trends over time indicate an increasing proportion of persons with incomplete paraplegia and decreasing proportion of persons with complete tetraplegia" (Spinal Cord Injury 2000). Among the many factors contributing to this trend are 1) improved roadside care, 2) aggressive blood pressure and respiratory maintenance (Vale *et al.*, 1997; Levi *et al.*, 1993), 3) the administration of methylprednisolone following injury (Bracken *et al*., 1997, 1992, 1990; Bracken & Holford 1993) and 4) well-defined rehabilitation programs.

In addition to the loss of function and sensation below the injury site, SCI patients can experience a host of systemic complications. Injured persons are at greater risk of experiencing bladder, bowel and sexual dysfunction, neuropathic pain and muscle spasticity. Furthermore, SCI patients have special concerns regarding the development of cardiovascular disease (Garstang 2001), deep vein thrombosis (McKinney & Garstang 2001) and osteoporosis (Weiss 2004). Prior to World War II, most spinal cord injured persons died within weeks of injury due to urinary dysfunction, respiratory infection or bedsores. Renal failure was also a leading cause of death. However, since the inception of the National Spinal Cord Injury Database in 1973, the greatest contributors to the reduced life span of SCI patients are pneumonia, pulmonary embolism and septicemia. In addition to diminishing the quality of life of SCI patients, these systemic concerns contribute to a

significantly shortened life span. For example, if a 20 year old sustains a paraplegia inducing SCI, his/her life expectancy is approximately 65.3 years as compared to the 77.8 years of an uninjured individual. The discrepancy is higher for more severely injured people. The life expectancy of a person with a high C1-C4 injury decreases to 56 years.

Social and economic hardships also result from SCI. As the majority of SCIs are sustained by people aged 16-30 (50% of which are single), the probabilities of both getting married and sustaining that marriage are reduced. For those who are married at the time of injury, the likelihood of the marriage remaining intact is slightly lower than in the uninjured population. The financial burden associated with SCI is also overwhelming, costing the taxpayers alone over 12 billion dollars per year. A person sustaining a high C1-C4 injury, resulting in either complete or incomplete tetraplegia, requires approximately $626,588 of medical care during the first year post-injury. The cost of care in each subsequent year is estimated at $112,237, which excludes indirect costs such as the loss of wages, fringe benefits and productivity. Typically, indirect costs average ~$52,915 per year although this number varies tremendously with injury severity and pre-injury employment status. Similarly, an individual with paraplegia has a first year financial burden of $228,955, followed by $23,297 in subsequent years. Although these figures are directly related to injury severity, a 25-year-old individual with a high C1-C4 injury may likely incur estimated lifetime medical bills exceeding $2,393,507 (Spinal cord injury 2003).

It is clear that SCI, although not the most prevalent CNS injury/disease in today's society, is certainly one of the most physiologically complex and financially and socially burdensome. There is no cure. As previously indicated, advances in roadside and clinical

care coupled with rapid pharmacological treatment and improved rehabilitative strategies have helped to foster a trend towards less severe injuries, thereby improving quality of life and reducing the financial and social obstacles faced by injured persons. Further clinical improvements are dependent upon identifying and understanding the pathophysiological processes that comprise the secondary injury cascade. Improved communication between clinicians and researchers and a continuous influx of funding are indispensable necessities in the battle to develop a viable and promising treatment strategy.

Spinal Cord Injury Pathophysiology

Spinal cord injury is an extremely complex injury comprised of a mechanical primary injury and a delayed, prolonged secondary injury cascade. The primary injury can be contusive, shearing, stretching or concussive in nature. Most humans suffer anterior or circumferential cord compression from fracture or dislocation of a closed vertebral system (Amar & Levy 1999). The primary injury not only damages vascular and skeletal components, but also harms neuronal cell bodies, axons and oligodendrocytes. The secondary injury cascade is made up of a series of uncontrolled endogenous biochemical reactions that, together, prolong the injury process and can extend the tissue damage to segments rostral and caudal to the original site of injury. Ultimately, significant cell death occurs, leading to loss of motor and sensory function below the injury site. At the present time, the prognosis for substantial functional recovery in the vast majority of SCI individuals is extremely poor.

Research in animal models of SCI demonstrates mechanical trauma causes vasospasm of the superficial cord vessels (Ducker & Assenmacher 1969). Within 30 minutes post-injury, petechiae form at the injury epicenter and eventually expand and

converge to replace most of the spinal gray matter (Means & Anderson 1987; Ducker *et al.*, 1971). Disruption of vascular elements and structures leads to the breakdown of the blood-spinal cord barrier (BSCB) following SCI (Jaeger & Blight 1997; Popovich *et al*., 1996; Goodman *et al.*, 1976; Griffiths 1976; Griffiths & Miller 1974), release of vasoactive molecules such as cytokines, kinins, prostaglandins, leukotrienes, thromoboxane, catecholamines, platelet activating factor (Pan *et al*., 2002; Streit *et al.*, 1998; Anderson & Hall 1993, Xu *et al.*, 1991) and loss of vascular system autoregulation (Mautes *et al*., 2000; Amar & Levy 1999; Anderson & Hall 1993). Vascular disruption and blood-spinal cord barrier breach (reviewed by Mautes *et al.*, 2000) allow the rapid invasion of neutrophils into the injury epicenter, followed by the recruitment of activated microglia and T cells (Streit *et al.*, 1998; Popovich *et al*., 1997). These events are closely linked with edema, ischemia and decreased tissue O_2 levels, all of which contribute to spinal cord ischemia (Hall & Wolf 1986; Hickey *et al*., 1986). By the end of the first week post-injury, the tissue in the injury epicenter has been transformed into a large mass of eroded tissue filled with inflammatory cells.

There are many processes – some immediate, some delayed and others cyclic-that contribute to destruction of spinal cord tissue following injury. Within minutes of SCI, activation of membrane phospholipases and lipases leads to the release of fatty acids followed by the subsequent production of eicasonoids, *e.g.*, prostaglandins and leukotrienes (Anderson & Hall 1993; Demediuk *et al.*, 1987, 1985; Horrocks *et al*., 1985). Eicosanoids are known mediators of inflammation and vascular permeability. Nonenzymatic (or free radical induced) lipid peroxidation also occurs very shortly after SCI (Anderson & Hall 1993; Hall & Braughler 1986; Demopoulos *et al*., 1982), as

evidenced by the rapid decrease in tissue vitamin E levels and membrane cholesterol (Saunders *et al*., 1987; Anderson *et al*., 1985; Demopoulos *et al*., 1982). The metabolic composition of the spinal cord places it at greater risk for free radical mediated damage than other tissues because 1) it is poor in antioxidant enzymes, 2) it produces a greater number of free radicals relative to its size than other tissues and 3) it is rich in polyunsaturated fatty acids, which are particularly vulnerable to oxidation. Thus, SCI-induced over production of free radicals overwhelms the endogenous scavenging systems to damage proteins, lipids and DNA and, in the process, alters the structure and functions of cellular (neuronal, glial, vascular) and mitochondrial membranes. Membrane disruption, in turn, precipitates the loss and/or malfunction of Ca^{2+} and Na^{+}/K^{+}-ATPase ionic pumps, transporters and receptors. The resulting loss of osmotic regulation, coupled with electrolyte imbalance, interrupts cellular signaling and neurotransmitter clearance (Young & Constantini 1993; Kwo *et al.*, 1989; Goldman *et al.*, 1983). A number of phosphatases, phospholipases, kinases and endonucleases (Goldman *et al*., 1983; Stokes *et al*., 1983; Yashon *et al.*, 1975) and other proteases (Taoka *et al.*, 1998; Iwasaki *et al*., 1987; Banik *et al.*, 1986; Iizuka *et al*., 1986) are activated following SCI. The increase in both Ca^{2+}-activated and non-Ca^{2+}-activated protease activity, which will be discussed in more depth later, results in the degradation of cytoskeletal, myelin and extracellular components essential for normal cellular function and survival.

It is clear that SCI is a dynamic and complex injury. The damage sustained from the primary injury and the spreading damage inflicted by the aberrant post-injury biochemical processes ultimately determine the cytoarchitecture of the surviving spinal cord tissue as well as the extent of preserved motor and sensory function. Usually, the

injury will involve spinal cord segments both rostral and caudal to the primary injury. Although it may take more than a month (depending on the species) to establish the final three-dimensional configuration of the lesion, eventually the injury site consists of either a single or multilocular fluid filled cyst. This cyst can be sculpted for months post-injury (Quencer & Bunge 1996).

Cell Death after Spinal Cord Injury

As indicated above, most cases of SCI result in cavitation of the gray matter, a process that is reached through extensive cell death. Previously, the cell death observed following SCI was believed to be solely necrotic (Balentine 1978a, 1978b). Necrotic cell death, which is characterized by a loss of ionic homeostasis, organelle and cellular swelling, membrane disruption, cell lysis, internal content spillage and inflammation, is a consequence of the primary injury and is difficult to prevent. In many cases, 50% of the spinal cord at the injury site is necrotic by 4 h post-injury (reviewed by Velardo *et al.*, 1999). Within 2 h post-injury, inflammatory cell invasion into the injury epicenter begins, exposing the damaged tissue to additional insult via the release of reactive oxygen species and active proteases (Bethea 2003; Babior 2000; Bartholdi & Schwab 1997; Means & Anderson 1987). While necrosis plays a substantial role following the primary insult, studies now show that apoptosis also significantly contributes to SCI-related cell death (Hayashi *et al*., 1998; Lou *et al*., 1998; Mackey *et al*., 1997). Apoptosis is a very orderly process that consists of well-characterized biochemical cascades that take cells from an intact, metabolically active state to a state of cellular breakdown, *e.g.*, the contents of the cell being neatly packaged into membrane bound apoptotic bodies that are digested. Over the past decade, several studies indicate that apoptotic cell death occurs within the injured spinal cord. In models of both contusion- (Yong *et al.*, 1998; Liu *et al.*,

1997) and compression-SCI (Li *et al*., 1999) in the rat, apoptotic cell death has been observed in neurons, astrocytes, oligodendroglia and microglia. Although the peak period of apoptosis differed for the various cell types (Liu *et al.*, 1997), certain populations continued to undergo apoptosis for 2-3 weeks post-injury (Yong *et al*., 1998; Crowe *et al.*, 1997; Liu *et al*., 1997). It is now clear that both necrotic and apoptotic cell death occurs following SCI and contributes to the loss of viable tissue and the resulting decrease in motor and sensory function.

It appears that while necrosis may be a result of the primary injury, the secondary injury cascade, or certain components of it, is somehow responsible for the apoptosis observed in the injured populations of cells. The observation that apoptotic cell death occurs following SCI is important because it not only improves our understanding of SCI pathophysiology but more important, it also provides a possible avenue for therapeutic intervention. The prolonged time period during which cells undergo apoptosis increases the potential therapeutic window to treat at risk cellular populations following SCI.

Proteases and Spinal Cord Injury

Among the many pathophysiological processes that occur following SCI, one process is the activation and release of lytic enzymes such as proteases, phosphatases, lipases and endonucleases. While some proteases like the matrix metalloproteinases (MMPs) and calpains are calcium activated, others, like the caspases and cathepsins, are not. Because all of these proteases are widely expressed and extremely powerful, elaborate cellular mechanisms have evolved to regulate their expression and activity. However, following SCI, it is likely that these regulatory controls are disturbed and/or overwhelmed, thereby leading to the over-expression, over-activation and possibly aberrant release of a number of proteases. Furthermore, invading inflammatory cells may

also release these enzymes exacerbating vascular, neuronal and glial damage. The relatively ubiquitous distribution of these proteases and their documented role in the proteolysis of vital substrates make them strong candidates for involvement in the secondary injury cascade.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are zinc and calcium dependent endopeptidases capable of degrading all extracellular matrix (ECM) components. As such, they play a critical role in matrix remodeling, development and wound healing and repair (Yong *et al.*, 1998). Nearly two dozen members of the MMP family have been identified and all share a common propeptide and N terminus catalytic domain. Over the past decade, the role of MMPs in the pathogenesis of several diseases has become clearer. MMPs are believed to participate in tumor invasion and metastasis (Noel *et al.*, 2004; Khasigov *et al.*, 2003), rheumatoid arthritis, periodontal disease (Smith *et al.*, 2004; Ejeil *et al.*, 2003), atherosclerosis (Katsuda & Kaji 2003; Jones *et al.*, 2003) and in CNS pathologies including multiple sclerosis (Maeda & Sobel 1996, Opdenakker & Van Damme 1994), amyotrophic lateral sclerosis (Lim *et al*., 1996), Alzheimer's disease (Deb & Gottschall 1996), malignant glioma invasion (Stetler-Stevenson & Yu 2001) and breakdown of the blood-brain-barrier (Aoki *et al*., 2002; Rosenberg *et al.*, 1998; Chandler *et al.*, 1997).

MMPs have now been implicated in SCI pathophysiology. Following moderate contusion-SCI in mice, levels of MMP-9 enzymatic activity rapidly increased, peaked at 24 h post-injury and subsided by 72 h post-injury (Noble *et al*., 2002). MMP-9 enzymatic activity in the injured cords of these mice was localized to blood vessels, neutrophils and cells expressing astrocyte and macrophage markers (Noble *et al*., 2002). Evidence that MMP-9 contributed to the injury response was obtained in the parallel studies of MMP-9

knockout mice. Examination of these MMP-9 deficient following SCI revealed reduced breakdown of the BSCB, decreased neutrophil infiltration and improved locomotor recovery (Noble *et al*., 2002). Similarly, Wells *et al*. (2003) examined the transcript expression of 22 MMPs following compression-injury in mice and found mRNA expression of several MMPs was upregulated. The most noticeable induction occurred for MMP-12 (189 fold over basal levels) at 24 h post-injury, an increase that was still evident at 14 d post-injury. As was observed in MMP-9 knockout mice, MMP-12 knockout mice exhibited significantly improved functional recovery, decreased BSCB permeability and reduced microglial and macrophage density following compression-injury (Wells *et al.*, 2003). Although these studies implicate at least two MMPs in the destructive processes following SCI, additional studies are required to provide a more systematic examination of MMP mRNA and protein expression and enzymatic activity following SCI, as well as to identify the endogenous substrates of these enzymes in the post-SCI milieu.

Calpain

Calpain in a cytosolic cysteine protease that is expressed in every cell and tissue studied thus far (Banik *et al*., 1997). Its two forms, μ-calpain and m-calpain, are activated by micromolar and millimolar concentrations of ionic calcium, respectively. Calpain has numerous substrates including cytoskeletal (Croall & DeMartino 1991) and membrane (Schlaepfer & Zimmerman 1985) proteins. The very specific endogenous inhibitor, calpastatin, normally tightly regulates calpain activity. However, the influx of Ca^{2+} into the cell following SCI overactivates calpain thereby disturbing the normal balance between calpain activation and inhibition. In fact, increased calpain mRNA and protein expression and activity levels following SCI have been well documented (Wingrave *et*

al., 2003; Shields *et al.*, 2000; Banik *et al*., 1997; Springer *et al.*, 1997; Ray *et al.*, 1999; Li *et al.*, 1996, 1995). Increased calpain immunoreactivity, due to elevated levels of the protein, was detected in multiple cell types including macrophages, reactive astrocytes, microglia and neurons in the injury epicenter following SCI (Shields *et al.*, 2000; Li *et al.*, 1996). These increases were detected within minutes of injury and persisted, in some cases, for up to 72 hours following the initial trauma (Banik *et al.*, 1997). Furthermore, the increases in calpain protein levels and enzymatic activity have been shown to correlate with the degradation of several important proteins such as α-fodrin (Ray *et al.*, 1999), proteolipid protein (Banik *et al.*, 1985, 1980), microtubule associated protein-2 (MAP2, Springer *et al.*, 1997) and neurofilament (NF, Ray *et al.*, 2000a; Schumacher *et al*., 1999 Banik *et al*., 1997). The calpain-specific 145 kDa αII spectrin breakdown product (BDP) has also been observed to accumulate in the injury epicenter following SCI (Wingrave *et al.*, 2003; Springer *et al.*, 1997).

The most convincing evidence for calpain involvement in protein degradation and/or cell death following SCI was obtained in studies employing calpain inhibitors. Intraparenchymal administration of the water-soluble calpain inhibitor, CEP4143, just prior to compression-SCI in rats inhibited calpain activation, reduced degradation of dephosphorylated NF200 and improved performance on both the inclined plane test and the Beattie-Bresnahan-Basso (B.B.B) test for functional hindlimb recovery (Schumacher *et al*., 2000). In another series of experiments, Ray *et al.* (2003, 2001a,b, 2000a,b) showed that administration of the membrane permeable cysteine protease inhibitor E64-d over a 24 h period following contusion-SCI in rats, prevented internucelosomal DNA fragmentation and apoptosis and inhibited NFP degradation in the injury site segment.

E64-d treatment also prevented SCI-induced increases in the levels of calpain mRNA and protein and caspase-3 mRNA expression. Furthermore, inhibitor treatment restored transcription of proteolipid protein and myelin basic protein. Finally, intraspinal microinjection of the cell permeable calpain inhibitor MDL28170 prior to injury inhibited total calpain enzymatic activity and reduced spectrin and MAP2 proteolysis (Zhang *et al*., 2003). Together, these studies show that calpain contributes to the breakdown and/or modification of important cellular proteins following SCI and suggest that this protease is also involved in the secondary injury cascade.

Caspases

Caspases are intracellular cysteine proteases that play an essential role in the initiation and execution of apoptotic cell death. At least 14 members of the caspase family have been identified. These cysteine proteases, which cleave their substrates after an aspartate residue, are normally present as inactive zymogens in cells. With the discovery of apoptotic neuronal and glial cells in spinal cord tissue following SCI (Li *et al.*, 1999; Emery *et al*., 1998; Yong *et al*., 1998; Crowe *et al*., 1997; Liu *et al*., 1997; Katoh *et al*., 1996) and the link between caspase and apoptosis (Citron *et al*., 2000; Springer *et al*., 1999; Yuan *et al*., 1993), there has been heightened interest in understanding caspase-mediated apoptosis in SCI. Several recent reports have documented increases in caspase protein and mRNA expression and activity in both neuronal and non-neuronal cells following SCI. Springer *et al.* (1999) reported increased caspase-9 activation and cytochrome c levels within 30 minutes of contusion-SCI in rats. These upstream events were sustained for over 24 hours. Furthermore, Keane *et al*. (2001) noted that activation of caspases-2, -8 and –9 leads to apoptosis following SCI while Takagi *et al*. (2003) noted that the enzymatic activity of caspases-3 and -8 were

upregulated after contusion-SCI in mice. The effects of SCI on the expression of "executioner" caspase-3 have also been examined. Caspase-3 enzymatic activity levels in injured spinal cord were 600% greater than control levels within 1 hour post-injury and persisted for 24 hours (Springer *et al.*, 1999). Increases in caspase-3 mRNA and protein levels are highly correlated with apoptosis in the injury site and preceded internucleosomal DNA fragmentation and the accumulation of the 120 kDa caspase-specific αII spectrin breakdown product (Wingrave *et al*., 2003; Ray *et al*., 2001; Li *et al*., 2000) and the 160 kDa caspase-specific fodrin breakdown product (Citron *et al*., 2000). Thus, it appears that increased caspase protein and mRNA expression and enzymatic activity following SCI contribute to apoptotic cell death following SCI.

As was the case with calpain, experimental use of caspase inhibitors have helped to delineate the role that caspases have in the secondary injury cascade. Caspase inhibitors have been shown to prevent staurosporine-, kainate- and thapsigargin-induced apoptotic death of mature oligodendrocytes in culture (Benjamins *et al*., 2003). Z-VAD-fmk, a pan caspase/cathepsin B inhibitor, was found to increase the survival of bone marrow cells (BMCs) that had been grafted into an ischemic area caused by middle cerebral artery occlusion (MCAo) in adult rats (Chen *et al.*, 2002). These animals also had significantly reduced levels of apoptosis within the transplanted BMCs and demonstrated significant improvement in a test of somatosensory function (adhesive removal), but did not have a significant improvement in a test of motor function (rotarod) (Chen *et al*., 2002). Following MCAo in mice, intracerebroventricular (ICV) injections of Z-VAD-fmk, reduced ischemia-induced tissue damage, brain swelling and behavioral deficits (Hara *et al.*, 1997). In a rat model of traumatic brain injury, ICV injections of the cell permeable

caspase-3 inhibitor, Z-DEVD-fmk reduced TBI-induced DNA fragmentation and improved motor function (Yakovlev *et al*., 1997). In aggregate, these studies suggest that caspase inhibitors may provide varying degrees of neuroprotection through attenuation of apoptotic cell death following an ischemic or traumatic brain insult.

The utilization of caspase inhibitors following SCI is less conclusive. ICV injection of either a non-specific caspase inhibitor or a specific caspase-3 inhibitor immediately following contusion-SCI in mice has been shown to reduce caspase-3 enzyme activity (Takagi *et al*., 2003). Intrathecal (IT) Z-DEVD-fmk injection prior to contusion-SCI in rats was found to block cytochrome c dependent DNA fragmentation through the cleavage of DFF45/ICAD, a process likely to occur downstream of caspase-9 activation but involving the activation of caspase-3 (Springer *et al*., 1999). Mice treated with Z-VAD-fmk soaked gelfoam placed over the injury site had significantly smaller lesions and demonstrated significant functional improvements as compared to control animals (Li *et al.*, 2000). Furthermore, in a rabbit model of ischemic SCI, Lapchak *et al*. (2003) showed that the IT delivery of the non-selective cell permeable caspase inhibitor, BDFMK, decreased both the levels of caspase-3 activity and a caspase-3 cleavage product. However, this treatment but did not improve functional recovery. Similarly, administration of Z-VAD-fmk using a variety of delivery routes (*e.g*., local, subarachnoid, intravenous and intraparenchymal) following mild contusion-SCI in rats did not improve B.B.B. scores or reduce levels of apoptosis as compared to control animals (Ozawa *et al*., 2002).

Although it is clear that caspase-mediated apoptosis contributes to the cell death observed following SCI and therefore would appear to be a reasonable point for

therapeutic intervention, the variety of caspase inhibitors (specific/non-specific, permeable/impermeable), injury types (mild/moderate/severe, compression/contusion/ischemic), animal models (rat/mouse/rabbit), administration strategies (IP/ICV/IT/IV) and outcome measures (motor/sensory) used in these studies complicates the assessment of the neuroprotective capacity of caspase inhibitors following SCI. Thus, better-coordinated and controlled studies are needed to determine the effectiveness of caspases inhibitors to protect the injured spinal cord.

Cathepsins

Several human cathepsins have now been identified and sequenced. Like other proteases, cathepsins are synthesized as inactive zymogens and are activated by proteolytic removal of the N-terminus propeptide. The residue (serine, cysteine, metallo or aspartate) in the active site distinguishes the cathepsins from one another. Cathepsins play an important role in several physiological processes including homeostatic protein degradation (Bond & Butler 1987; Turk *et al*., 2000), antigen presentation (Chapman *et al.*, 1997), bone remodeling (Kakegawa *et al*., 1993) and hormone processing (Dunn *et al.*, 1991). Their expression varies across species, tissues and developmental stages of an organism (Levicar *et al*., 2002; Pungercar *et al*., 2000; San Segundo *et al*., 1996; Shi *et al*., 1994; Petanceska & Devi 1992; Qian *et al*., 1991), and over the past two decades, cathepsins have been identified as contributors to several disease and injury processes. In many of these conditions, including multiple types of cancers, asthma, arthritis, osteoporosis, muscle wasting and pycnodysostosis, the mRNA and protein expression, activity and localization of multiple cathepsins are altered. Furthermore, several cathepsins have been implicated in the pathologies of Alzheimer's disease, dementia, multiple sclerosis, Guillian-Barre syndrome, Creutzfeld-Jakob, Down syndrome and

ischemic cell death (Gan *et al.*, 2004; Nagai *et al*., 2000; Mackay *et al*., 1997; Haas & Sparks 1996; Bever & Garver 1995; Bernstein & Wiederanders 1994). Their relatively ubiquitous distribution, indiscriminate hydrolytic preferences and enzymatic robustness (*e.g*., activity in acidic and neutral pH) make it worthwhile to characterize cathepsin mRNA and protein expression, enzymatic activity and cellular localization in the context of SCI.

To date, the role of cathepsins in SCI pathophyisology has received little attention. Thus, minimal information is available concerning cathepsin expression and activity in the injured spinal cord. Microarray analyses have shown increases in the transcript expression of many cathepsins following two CNS insults and one peripheral nervous system injury. Three days post-spinal root avulsion, Hu *et al*. (2002) reported an increase in the expression of cathepsins B, C, D, H, I, L and S. The expression of cathepsins B (Cath B) and D were also both elevated after either sciatic nerve crush (Fan *et al.*, 2001) but only Cath B was upregulated following hemisection of the spinal cord (Fan *et al.*, 2001). These microarray studies suggest that insults to the spinal cord cause an increase in the mRNA expression of several of the cathepsins, particularly Cath B, which was upregulated in all three reports.

Cathepsin B

General Background

The single human Cath B gene is comprised of 12 exons (Gong *et al*., 1993), spans more than 27 kilobases (Berquin *et al*., 1995) and is localized to chromosome 8p22 (Fong *et al*., 1992; Ferrarra *et al*., 1990). While its regulation is not fully characterized, it has been suggested that more than one promoter can drive the expression of human Cath B (Berquin & Sloane 1996; Rhaissi *et al.*, 1993). Furthermore, multiple transcripts resulting

from alternative splicing in the 5' and 3' untranslated regions (UTRs), and possibly from the use of alternative promoter regions, have been detected (Berquin & Sloane 1996). While putative Cath B promoters have characteristics similar to those of housekeeping promoters, including the absence of TATA or CAAT boxes (Gong *et al*., 1993), high guanidine/cytosine (GC) content (Qian *et al.*, 1991b) and several potential binding sites for the transcription factor Sp-1 (Yan *et al*., 2000), Cath B mRNA expression still appears to vary depending on the cell type and state of differentiation.

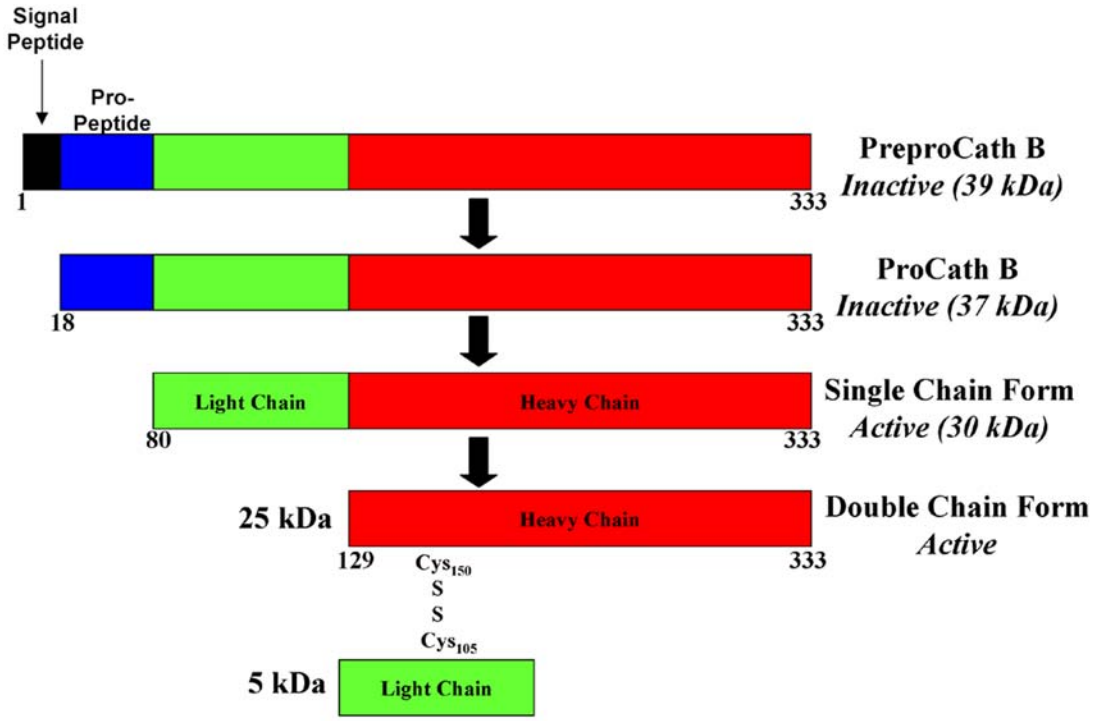

Figure 1-1: Schematic of the Cath B protein. Preprocathepsin B (333 aa) is synthesized on the rough ER where the signal peptide is cleaved co-translationally. Following transport to the Golgi apparatus, the enzyme is glycosylated and the mannose-6-phosphate signal is assembled. This receptor allows for transport to the trans-Golgi network and then onto an acidic compartment where the pro-peptide is removed and enzyme is activated. Within the lysosome, the active single chain form of Cath B (30 kDa) is further modified (loss of a dipeptide) to produce the active double chain form (25 kDa and 5 kDa) held together via disulfide bond.

As is the case with other cathepsins, Cath B is synthesized as an inactive preproenzyme (39 kDa) in the trans-golgi, which is processed to the still inactive proenzyme (37 kDa) in the lysosomal compartment (Mort & Buttle 1997). Removal of the 63-residue propeptide from its N-terminus converts the inactive proenzyme into the single chain active form (30 kDa). This single chain Cath B undergoes hydrolysis within the lysosome, converting it to a double chain form consisting of heavy (25 kDa) and light (5 kDa) components held together by a disulfide bond (Mort & Buttle 1997).

Cath B is involved in homeostatic protein turnover and digestion of cellular debris (Turk *et al*., 2000; Mort & Buttle 1997). Because Cath B is capable of hydrolyzing complex carbohydrates, nucleic acids, lipids and ECM components under a broad pH optimum (Lindebaugh *et al.*, 1999; Bajkowski & Frankfater 1983a, b), it is sequestered in the lysosomes and away from potential substrates.

Role in Peripheral Pathologies

Cath B expression and enzymatic activity are strictly regulated at several levels including transcription, post-transcriptional processing, translation and glycosylation, maturation and trafficking and protein inhibition (Yan *et al.*, 1998). Increased levels of Cath B mRNA and protein expression and enzymatic activity, as well as the intracellular redistribution of the protein, have been reported in multiple pathologies including rheumatoid (Codorean & Gabrielescu 1985; Lenarcic *et al*., 1988) and osteoarthritis (Bayliss & Ali 1978; Martel-Pelletier *et al.*, 1990). Furthermore, Cath B is markedly upregulated and aberrantly localized (and/or secreted) in several human cancers including ovarian (Scorilas *et al.*, 2002; Warwas *et al*., 1997), thyroid (Kusunoki *et al*., 1995), gastric (Watanabe *et al*., 1989), lung (Sukoh *et al*., 1994), prostate (Sinha *et al*., 1995), breast (Yano *et al.*, 2001; Sameni *et al*., 1995), colon (Campo *et al*., 1994), melanomas

(Sloane *et al*., 1981) and osteoclastomas (Page *et al*., 1992). In fact, staining for Cath B is very conspicuous at the leading edge of invading tumor cells, where it is secreted and is associated with degradation of cellular and ECM components of healthy tissue during the infiltration of tumor cells.

Role in Central Nervous System Pathologies

The potential role that Cath B plays in cell death and peripheral tissue pathologies makes it a protease that needs to be investigated in CNS diseases and injuries. However, to date, no systematic characterization of Cath B following any CNS injury has been published. While the mechanism of action remains unclear, elevated levels of Cath B mRNA and protein, increased enzymatic activity and altered protein subcellular localization have been documented in multiple sclerosis (Bever *et al*., 1995), Alzheimer's disease (Mackay *et al.,* 1997), amyotrophic lateral sclerosis (Kikuchi *et al.*, 2003) and myoclonus epilepsy (Houseweart *et al*., 2003; Rinne *et al*., 2003). In these diseases, Cath B's roles in necrotic and apoptotic cell death and tissue loss processes are under active investigation.

Cath B has been more thoroughly investigated for its role in tumor progression in the brain. Rempel *et al*. (1994) demonstrated over-expression of Cath B mRNA and protein in glioma cell lines and in biopsy samples as compared to control samples, a finding confirmed by Sivaparvathi *et al*. (1995). Increased Cath B immunostaining has been observed in both tumor and endothelial cells of high grade brain tumors (Strojnik *et al*., 1999). Similar to non-CNS tumors, invading tumor cells adjacent to malignant gliomas also exhibited increased Cath B mRNA and protein expression (Demchik *et al.*, 1999; Rempel *et al.*, 1994). Furthermore, immunohistochemial analysis of human gliomas revealed a positive correlation between increasing intensities of Cath B staining

and the grade of malignancy and an inverse correlation with shorter survival times (Mikklesen *et al*., 1995; Strojnik *et al*., 2001). Based on these observations, Cath B has become useful as a prognostic marker for human gliomas.

Because of Cath B's purported role in tumor expansion, inhibitors of this protease have been utilized in attempts to diminish the tumorigenic quality of invading cells. The invasion of malignant glioblastoma cells into collagen IV, fibronectin and laminin matrices has been shown to be inhibited by the Cath B inhibitors E64, CA-074 (specific for Cath B) and CA-074 Me (cell permeable derivative of CA-074) (Levicar *et al*., 2002). The Cath B inhibitor, K11017, was also found to significantly inhibit gliomal cell invasion into matrigel and to reduce infiltration of glioma spheroids into normal brain aggregates (Demchik *et al*. 1999). The administration of other inhibitors such as CA-074 and E64-c significantly blocked glioblastoma cell migration (Lah *et al*., 1998). Thus, Cath B appears to play a critical role in the processes that allow for the growth of tumors into CNS tissue.

For transient cerebral ischemia, a CNS insult more closely related to traumatic injury, the functional role for Cath B is becoming clearer. Indeed, ischemia is a major pathological event following SCI and contributes heavily to the tissue loss seen following this primary injury. Thus, studies characterizing Cath B following ischemic events in the brain could provide insight as to the post-injury effects of this protease on spinal cord tissue. Following a 20-minute cerebral ischemic event in both monkeys (Yamashima *et al*., 1996) and gerbils (Kohda *et al*., 1996), the CA1 region of hippocampus undergoes complete neuronal death by post-injury day 5 and Nitatori *et al*. (1995) established that the CA1 neuronal cell death in these models was apoptotic. Both calpain and Cath B were

immediately activated in the CA1 region following cerebral ischemia and that Cath B appeared to have redistributed to the cytosol (Yamashima *et al.,* 2003) In their 'calpain-cathepsin hypothesis', Yamashima *et al*. (1998) speculate that the disruption of the lysosomal membrane, partially mediated by calpain activity, results in the release of powerful hydrolytic enzymes (such as Cath B) into the cytosol where they degrade substrates vital to cell survival. Lastly, the premise of the 'calpain-cathepsin hypothesis' has recently been confirmed in *C. elegans* by Syntichaki *et al*. (2002), where it was demonstrated that two specific calpains (TRA-3 and CLP-1) function upstream of the cathepsins and are required for degradation by various necrosis initiators.

To date, the neuroprotective potential of Cath B inhibitor treatment following traumatic or ischemic CNS injury has received little attention. However, the few studies that have been published seem to indicate that inhibition of Cath B reduces cell death in models of cerebral ischemia. For example, the administration of CA-074 and E-64c immediately following transient ischemia in monkeys reduced levels of Cath B enzymatic activity and immunoreactivity and spared approximately 67 and 84%, respectively, of the CA1 neurons from cell death (Yoshida *et al*., 2002; Tsuchiya *et al.*, 1999; Yamashima *et al.*, 1998). Additional studies have shown that this inhibitor-mediated neuroprotection extended to other neuronal populations as well (*e.g*., cortical, caudate-putaminal, Purkinje) (Yoshida *et al.*, 2002). In a rat MCAo model, Cath B immunoreactivity and enzymatic activity were both increased (Seyfried *et al.*, 1997). The IV injection of CP-1, a member of the peptidyl diazomethane family specific for Cath B and L but not calpain or caspase, reduced infarct volume and improved functional scores implicating Cath B in the development of the infarct in this model (Seyfried *et al.*, 2001).

Cath B and Apoptosis

While cathepsin participation in apoptosis has long been neglected (as lysosomes appear to remain intact during the programmed cell death process), it is widely held that cathepsins do participate in cellular autolysis and the damage of nearby cells during necrosis (Nixon & Cataldo 1993). However, the emergence of new *in vitro* and *in vivo* data has forced reconsideration of the role of cathepsins in apoptosis (reviewed by Leist & Jaattela 2001). While the mechanisms of action differed, a role for Cath B in both TNF- and bile salt-induced hepatocyte apoptosis was confirmed by Guicciardi *et al*. (2001, 2000) and Roberts *et al*. (1997), respectively. Following TNF exposure (a cytokine that is also induced following SCI), Cath B in conjunction with an unidentified cytosolic substrate increased the release of cytochrome c, which subsequently led to activation of caspase-9 and –3 (Guicciardi *et al.*, 2002). Conversely, in the model of bile-salt induced hepatocyte apoptosis, Cath B's involvement was caspase-8 dependent (Roberts *et al.*, 1997). Furthermore, Cath B was essential in TNF-induced apoptosis of WEH1 fibrosarcoma cells (Foghsgaard *et al*., 2001) and the apoptotic cell death of HT22 hippocampal cells and cerebellar granular cells following microglial stimulation (Kingham & Pocock 2001).

As was the case with previously discussed proteases (*i.e.*, MMPs, calpain and the caspases), the prevention of increases in Cath B mRNA and protein expression and enzymatic activity also provides protection from apoptosis. Inhibition of Cath B activity partially attenuated TNF-induced liver damage in wild type mice (Guicciardi *et al*., 2001). In a parallel set of experiments, TNF-induced release of cytochrome c, caspase activation and apoptosis of isolated hepatocytes were markedly inhibited in Cath B knockout mice (Guicciardi *et al.*, 2001). In another study, the administration of CA-074

Me prevented all apoptotic related events in WEH1 fibrosarcoma cells (Foghsgaard *et al*., 2001). Additionally, Cath B inhibitors blocked apoptosis induced by p53 and other cytotoxic agents (Lotem & Sachs 1996) and the treatment with antisense Cath B cDNAs also proved to be anti-apoptotic (Lakka *et al.*, 2004; Monaham *et al.*, 2001). While the role of Cath B in apoptotic cell death varies from model to model (*e.g*., upstream vs. downstream, initiator vs. executioner), it appears that Cath B contributes to apoptotic cell death and may offer a target for potential therapeutic intervention.

Cath B in SCI Pathophysiology

The role of Cath B in the cell death and tissue loss in multiple CNS pathologies supports the hypothesis that Cath B may contribute to the secondary injury cascade following SCI. With a broad range of substrates at both an acidic and neutral pH, the biochemical changes following SCI may precipitate an unregulated response by Cath B, including increased mRNA and protein expression and enzymatic activity and a redistribution of the active protease. As Cath B is clearly capable of hydrolyzing important components of the cytoskeleton (*e.g.*, neurofilament, spectrin, actin), myelin sheath (MBP) and ECM (*e.g.*, laminin, fibronectin, proteoglycans), it is plausible that Cath B is, at the very least, partially responsible for the damage caused by the secondary injury cascade. Considering the number of systems that are compromised in the post-SCI milieu, the release of pre-existing and newly synthesized active Cath B from neuronal and non-neuronal sources (including invading inflammatory cells) may collectively overwhelm any endogenous inhibitors in the CNS, thus causing excessive Cath B-related damage and tissue loss through both apoptotic and necrotic cell death.

CHAPTER 2
MATERIALS AND METHODS

All experimental procedures were conducted in accordance with the guidelines set forth by the University of Florida's Institute Animal Care and Use Committee (IACUC).

Surgery

Female Long-Evans rats weighing approximately 230-300 grams (Harlan, Indianapolis, IN) were used in this study. All surgical procedures were conducted under sterile conditions with supplemental heat. Intraperitoneal administration of Nembutal (sodium pentobarbital – 50-60 mg/kg) was used to induce anesthesia. Following a T12 laminectomy (intact dura mater), injury to the spinal cord was produced with the NYU impactor device (*i.e.*, 10 g dropped 25 mm onto the exposed dura). The sham-injury animals received a laminectomy and were placed in the injury apparatus, but were not injured. The incision was closed in layers (*i.e.,* muscle then skin). Animals recovered in a heated incubator with food and water available *ad libitum*. Bladders were manually expressed and fluids were administered as frequently as required.

Tissue Harvesting

mRNA, Protein and Enzymatic Activity

Spinal cord tissue was collected after extending the laminectomy to allow three segments of tissue to be removed (*i.e.*, tissue from the injury site and tissue from the regions immediately rostral and caudal to the injury site). Each tissue segment was approximately 6 mm in length. The fresh tissue was rinsed with cold PBS and either immediately homogenized with 500 μL guanidinum thiocyanate salt solution (mRNA

experiments) or flash frozen with liquid nitrogen (protein and enzymatic activity experiments).

Immunohistochemistry

Animals prepared for immunohistochemistry were perfused intracardially initially with 0.9% saline and then 4.0% paraformaldehyde (pH 7.4). The perfused animal was stored overnight at 4° C, after which the cord was removed. After the dura was carefully dissected away, the spinal cord was cryoprotected in 30% sucrose. The segment containing the injury site was sectioned (14 μm) and mounted onto slides (Fisher Scientific, Pittsburgh, PA) using the Frigocut 2800 (Reichert-Jung).

Analysis of Cath B mRNA Expression

Cath B mRNA expression was measured at 2, 6, 24, 48 and 168 h post-injury in rats that received a either a sham-injury or contusion-injury (n = 3-6 rats/time point/group). Spinal cord tissue from 4-5 normal (*i.e.*, not receiving any surgical treatments and/or other manipulations) animals was also examined for Cath B mRNA expression.

RNA Isolation and cDNA Synthesis

Total RNA isolation from the spinal cord tissue was achieved using a modified protocol, described by Earnhardt *et al.,* 2002, based on the guanidinium thiocyanate-phenol-chloroform extraction developed by Chomczynski and Sacchi (1987). Final RNA concentrations were determined via spectrophotometry and samples were stored at –20 ^{o}C in diethyl pyrocarbonate (DEPC) water for future cDNA preparation.

For cDNA synthesis, 1 μg of total RNA from all samples was used for enzymatic conversion of mRNA to first strand cDNA using an oligo-dT primer (Invitrogen/Life

Technologies, Carlsbad, CA; SuperScript™ First-Strand Synthesis System for RT-PCR). DNA contamination was monitored in the RNA samples by "no reverse transcriptase" reactions that were performed in conjunction with cDNA synthesis reaction.

Primer Design

Base pair designations for rat GAPDH refer to GeneBank locus AF106860. The primers used for all GAPDH PCR reactions were: sense 5'ggtga aggtc ggtgt gaac3' corresponding to base pairs 852-870 and antisense 5'ggcat cctgg gctac actg3' corresponding to base pairs 1657-1675. Cath B primers were designed using GeneBank locus NM_022597. The sense Cath B primer recognized an upstream rat Cath B specific mRNA sequence 5'tgagg acctg cttac ctgct3' corresponding to base pairs 466-485. The antisense Cath B primer recognized a downstream rat Cath B specific mRNA sequence 5'gcagg gagtg aggca gatag3' corresponding to base pairs 1141-1160.

Real-time PCR

The Roche LightCycler and the double-stranded DNA binding dye, SYBR Green I dye™, were used to continuously monitor all PCR reactions. The LightCycler (Roche Biochemicals) is an advanced instrument that conducts rapid thermal cycling of the polymerase chain reaction. SYBR Green I dye™ preferentially binds to double-stranded DNA and emits a fluorescent signal proportional to the DNA concentration. The reaction kinetics of this PCR reaction are represented by an amplification curve. Each amplification curve (*fluorescence vs. cycle number*) is assigned a crossing point value (CPV), which is defined as the point of intersection between the amplification curve and the noise band. A lower CPV indicates a more rapid increase in the level of fluorescence and, thus a larger initial concentration of message. When comparing templates, those

with lower CPVs contain more amplified message for the gene of interest than those with higher CPVs.

For each PCR reaction the LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche) reagent was used according to manufacturer's instructions in combination with 0.5 μM primers, 10 ng cDNA template, 6% DMSO and 2 mM $MgCl_2$. After an initial 300 s, 95° C denaturation step, 40 cycles of amplification were performed (denature @ 95° C for 5 s, anneal @ 65° C for 10 s, extend @ 72° C for 40 s). SYBR Green I™ fluorescent detection of double stranded PCR products occurred at the end of each 72° C extension period. The specificity of the amplified product was confirmed through the melting curve analysis and gel electrophoresis. To generate standard curves for the Cath B primer set, contusion-injury templates were subjected to serial dilution. Linear regression analysis of the logarithm of the dilution factor vs. the CPV generated a standard curve for each specific template. All standard curves were run concomitant with segment- and time-matched unknowns (*i.e.*, template from normal and sham-injury animals). The relative amount of RNA in the unknown sample was extrapolated from its CPV in relation to the standard curve.

Analysis of Cath B Protein Expression

The level of Cath B protein was determined via immunoblotting in the spinal cord of both sham- and contusion-injured rats at 2, 6, 12, 24, 48, 72 and 168 h post-injury (n = 4 rats/time point/group). Spinal cord tissue from 4 normal rats was also assessed for the levels of Cath B protein.

Tissue Lysis and Protein Purification

Lysis buffer (1 mM EDTA, 2 mM EGTA, 1 small Protease Cocktail Pill™ (Roche Molecular Biochemicals), 0.1% SDS, 1.0% IGEPAL, 0.5% deoxycholic acid, 150 mM NaCl, 20 mM Hepes, ddH_2O, pH = 7.5) was added to each sample based on the mass of the tissue (15 μL/1 mg). The tissue was homogenized on ice with a rotary pestle, returned to eppendorf tubes and placed at 4° C for at least 30 minutes. Samples were sonicated and spun at 8000 g for 5 minutes at 4° C before the supernatant was collected and stored at – 70° C.

Immunoblotting

The protein concentration of each tissue homogenate was determined by bichinchoninic acid (BCA) assay (Pierce Inc., Rockford, IL). Unless otherwise noted, all procedures were performed at room temperature. Eighteen (18) micrograms of total protein were mixed with 2X loading buffer (1X = 125 mM Tris-HCl, 100 mM DTT, 4.0 % SDS, 0.01% Bromophenol Blue and 10.0% glycerol) and were resolved by SDS-PAGE on 10% Tris-HCl gels (Bio-Rad, Hercules, CA). The fractionated proteins were subsequently transferred to a 0.20 μM nylon membrane (Bio-Rad, Hercules, CA) in transfer buffer (192 mM glycine, 25 mM Tris HCl, 10.0% methanol). Staining with ponceau red (Sigma, St. Louis, MO) confirmed transfer of the proteins. Blots were blocked in 5.0% nonfat milk/tris buffered saline-tween (TBS-T) (20 mM Tris HCl, 150 mM NaCl, 0.005% Tween 20, pH 7.5). Membranes were washed with TBS-T and incubated overnight with a polyclonal anti-Cath B antibody (1:1000; Upstate Biotechnology Inc.). Membranes were washed and then incubated in 3.0% non-fat milk/TBS-T with an anti-rabbit IgG horseradish peroxidase conjugated antibody (1:3000;

(Bio-Rad, Hercules, CA). After additional washing, bound antibodies were visualized using the chemiluminescent developing reagent ECL$^+$ (Amersham Pharmacia Biotech, UK). The Cath B antibody recognized three bands: the inactive proenzyme (37 kDa), the active single chain form (30 kDa) and the heavy component of the double chain form (25 kDa). Representative blots were stripped and reprobed with a monoclonal anti-GAPDH antibody (gift of Dr. Gerry Shaw, University of Florida) for loading control purposes. Data were acquired as integrated densitometry values (IDVs) by computer-assisted densitometric scanning (Alpha Imager 2000 Digital Imaging System, San Leandro, CA).

Analysis of Cath B Enzymatic Activity

Enzymatic activity of Cath B was assessed at 1, 2, 3, 5 and 7 d in both sham- and contusion-injured rats (n = 3–4 rats/group/time point). Enzymatic activity of Cath B was also measured in the spinal cord of 5 normal rats.

Sample Preparation

Frozen spinal cords were crushed with a chilled mortar and pestle. Triton extraction buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EGTA, 1.0% Triton X-100, 0.2 mM DTT) was added to the crushed tissue, which was then placed on ice for 60 minutes. Samples were vortexed every 15 minutes for the next hour. At the end of the second hour, samples were centrifuged at 15,000 rpm for 10 minutes at 4° C. The supernatant was removed and placed into a clean eppendorf. Glycerol was added to stabilize the lysates. Protein concentrations of the tissue lysates were determined by bichinchoninic acid (BCA) assay (Pierce Inc., Rockford, IL). All samples were equalized to a common protein concentration for ease of handling.

Enzymatic Activity Assay

Enzymatic activity assays were carried out in 96 round-bottom well plates (Costar, Inc., Coring, NY). Lysates from spinal cord tissue were incubated with the substrate solution (100 mM MES (pH 5.5), 200 μM Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Sigma-Aldrich, St. Louis, MO), 20 mM DTT and water). Fluorescence was measured (excitation @ 355 nm, emission @ 460 nm) using the Spectra Gemini XS (Molecular Devices, Sunnydale, CA).

Immunohistochemical Localization of Cath B

Immunohistochemistry was performed in normal (n = 2) and 7 d contusion-injured rats (n = 2).

Sections were fluorescent immunolabeled with two primary antibodies (AB) in the following experiments: polyclonal AB α Cath B (1:1000; Upstate Biotechnology, Inc., New York) and monoclonal ABs against 1) glial acidic fibrilliary protein or GFAP (1:1000; Sternberger Monoclonals, Lutherville, MD) for astrocytes, 2) NeuN (1:1000; Chemicon, Temecula, CA) for mature neurons, 3) lysosomal associated membrane protein or LAMP (1:1000, Stressgen, British Columbia, Canada) for lysosomal membranes, 4) O-4 (1:1000; Chemicon, Temeculah, CA), CNPase (1:1000; Sigma, St. Louis, MO) and myelin basic protein or MBP (1:1000; Chemicon, Temeculah, CA) for oligodendrocytes, 5) OX-42 (1:1000; Serotec Inc., Raleigh, NC) for resting microglia and activated macrophages 6) OX-8 (1:1000; Serotec Inc., Raleigh, NC) and ED-2 (1:1000, Serotec Inc., Raleigh, NC) for T lymphocytes, most thymocytes and natural killer cells and macrophages. The nuclear dye DAPI (in Vectashield, Vector Laboratories, Burlingame, CA) was used to label the nuclei. The first primary antibody was incubated

at room temperature (RT) with a 10% goat serum-10% horse serum-0.2% Triton-X 100 in 0.1 M PBS (block) solution followed by overnight incubation with the second primary antibody also in block at RT. The tissue sections were then incubated in fluorescent-tagged secondary antibody (1:1000) (Molecular Probes, Eugene, OR) and cover-slipped. The sections were viewed and digitally captured with a Zeiss Axioplan 2 microscope equipped with a SPOT Real Time Slider high-resolution color CCD digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Inhibition of Cath B Activity

Inhibitor

The specific and irreversible Cath B inhibitor CA-074 [*N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline] (Sigma-Aldrich, St. Louis, MO) was used in all experiments.

In Vitro Inhibitor Assay

To test the efficacy of CA-074 as an inhibitor of Cath B activity, purified Cath B from the bovine spleen (Sigma-Aldrich, St. Louis, MO) was incubated with various concentrations (0 μM – 5000 μM) of CA-074. Enzymatic activity assays were then conducted as previously described. Furthermore, in a second set of *in vitro* experiments, CA-074 (25 μM) was added to the reaction vessel to confirm the increases in fluorescence within the tissue lysates from the injury site were due to Cath B hydrolysis rather than autofluorescence or non-specific substrate hydrolysis. In both *in vitro* inhibitor experiments, the levels of Cath B enzymatic activity levels were determined as previously described.

Two experimental paradigms were employed to inhibit Cath B *in vivo*. In the first *in vivo* inhibitor experiment, three treatment groups (*i.e.,* one saline control and two CA-

074 treated groups; n = 5-6 rats/group) were utilized for each of three different routes of administration (bolus intraperitoneal (IP), bolus intravenous (IV) and repeated dose IP). In the second *in vivo* inhibitor experiment, a bolus injection of either CA-074 or saline (n = 4-6 rats/dose of CA-074 or saline) was delivered by a single route of administration (bolus IV). All animals were sacrificed at 72 h post-injury.

***In Vivo* Inhibitor Treatment**

Animals were weighed just prior to surgery in order to determine the correct dose of inhibitor. CA-074 was reconstituted fresh daily in 0.9 % saline and the total injection volume was consistently maintained throughout the experiments.

As indicated above, two separate inhibitor treatment experiments were performed. In the first experiment, saline and CA-074 were given either as an IP or IV (femoral) injection. In order to visualize the femoral vein, the injection site was exposed prior to the laminectomy and spinal cord injury. Bolus injections were administered immediately after closing the incision exposing the spinal cord. Animals in the repeated dose IP group received a total of three IP injections including an initial injection immediately after SCI and additional injections at 24 h and 48 h post-injury. For the second experiment, CA-074 was delivered only as a single IV injection immediately after closure of the spinal cord incision.

In the first experiment, both the route of administration and the dose of CA-074 were varied. Three experimental groups were utilized: 1) saline control, 2) 12 mg/kg CA-074 and 3) 36 mg/kg CA-074. In the second experiment, only the dose of CA-074 was varied. The three experimental groups were: 1) saline control, 2) 6 mg/kg CA-074 and 3) 12 mg/kg CA-074. In both experiments, net Cath B enzymatic activity levels were measured. To generate these net enzymatic activity levels, the background fluorescence

(injury site lysates plus exogenous CA-074) was subtracted from the contusion-injury induced increase in fluorescence (injury site lysates without exogenous CA-074).

Statistical Analyses

mRNA Expression and *InVitro* Enzymatic Activity

The average level of Cath B mRNA expression and enzymatic activity for the group of normal animals was determined. The activity levels of individual sham- and contusion-injured animals were then normalized to this averaged value. All normalized levels within the sham- and contusion-injury groups were then averaged to generate a Fold Increase vs. Normal value (± SEM) for that group. All induction values are reported in terms of Fold Increase vs. Normal. A two-way ANOVA with Tukey's post-hoc test (SigmaStat Statistical Software, SPSS Inc., Chicago, IL) was used to detect statistically significant ($p < 0.05$) differences between the two groups.

Cath B Protein Expression Levels

Levels of Cath B protein in the spinal cord of normal animals ($n = 4$) were essentially the same. Thus, the protein expression of individual sham-injured and contusion-injured animals was normalized to an animal in the normal group. These normalized values were then averaged within a group (sham- or contusion-injury) to produce a Fold Increase vs. Normal (± SEM) value. These induction values were also analyzed via two-way ANOVA with Tukey's post-hoc test (SigmaStat Statistical Software, SPSS Inc., Chicago, IL). An independent analysis was completed for each of the three forms of Cath B – the 37 kDa inactive proenzyme, the active 30 kDa and 25 kDa forms.

InVivo Enzymatic Activity

Net Cath B enzymatic activity (*i.e.*, activity levels without CA-074 - activity levels with CA-074) was measured in both *in vivo* CA-074 treatment experiments. In the first experiment, a two-way ANOVA with Tukey's post-hoc test detected significant ($p < 0.05$) differences in enzymatic activity levels of the three treatment groups (saline, 12 mg/kg CA-074 and 36 mg/kg CA-074) within a particular route of administration (either bolus IV, bolus IP, repeated dose IP). Differences between routes of administration were not assessed. As only one route of administration (bolus IV) was used in the second *in vivo* experiment, a one-way ANOVA (Tukey's post-hoc test) was utilized to detect any significant ($p < 0.05$) differences among the enzymatic activity levels of the three treatment groups (saline, 6 mg/kg CA-074 and 12 mg/kg CA-074).

CHAPTER 3
RESULTS

Analysis of Cath B mRNA Expression

Contusion-Spinal Cord Injury Increased Cath B mRNA Levels

Before examining the expression of Cath B, the samples were tested for template integrity using the housekeeping gene GAPDH. Within each set of reactions, the CPVs for the three experimental groups (normal, sham- and contusion-injury) are not significantly different from one another at any of the experimental time points (data not shown). Having confirmed template integrity, standard curves were used to ascertain the level of Cath B mRNA expression of the templates within each group. In all three segments and at nearly every time point examined, Cath B mRNA expression is increased following contusion spinal cord injury (Figure 3-1, 3-2).

Effects of Sham-Injury

Sham-injury increases the expression of Cath B mRNA although to a lesser degree than the contusion-injury. At both the injury site (Figure 3-1) and the segment rostral (Figure 3-2, top panel) to the injury site, the sham-injury induced increase in Cath B mRNA expression peaks at 48 h post-injury (5.6 fold and 4.1 fold, respectively) and then returns to near normal levels by the last time point examined (168 h). While the pattern of Cath B mRNA expression is similar in the injury site and rostral segments following sham-injury, the response in the caudal (Figure 3-2, bottom panel) segment is different. Here, sham-injury induction of Cath B mRNA expression appears to be more robust than

in the other two segments. Specifically, in the caudal segment the increase in mRNA expression is greater than 5 fold at the majority of post-injury time points.

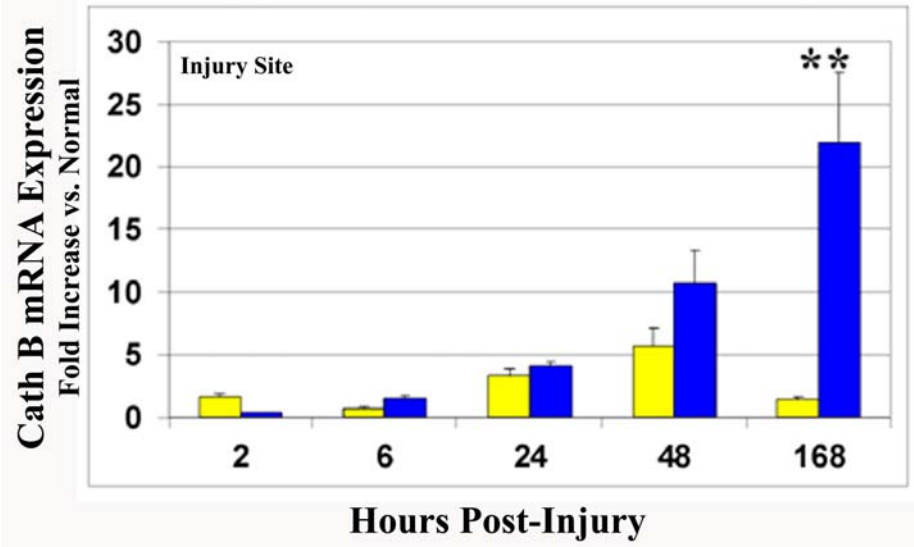

Figure 3-1: Sham- and contusion-spinal cord injury induce expression of Cath B mRNA at the injury epicenter. Increases in Cath B mRNA expression induced by sham- (yellow bars) and contusion-injury (blue bars) are presented here as Fold Increase vs. Normal. Cath B mRNA expression is transiently induced following sham-injury, returning to base line levels by 168 h post-injury. Following contusion-injury, Cath B mRNA expression increases across the experimental time line and is significantly (** $p < 0.01$) greater than the sham-injury level at 168 h post-injury, where the highest level of mRNA expression (> 20 fold) of the study is recorded.

Effects of Contusion-SCI

As indicated above, contusion-injury also induces Cath B mRNA expression. At the injury site, fold increases of 4.2, 10.7 and 19.2 are seen at 24, 48 and 168 h post-injury, respectively (Figure 3-1). The increase in Cath B mRNA expression following contusion-injury is significantly greater than sham-injury at 168 h post-injury and is the highest level of Cath B mRNA expression seen in this study. Within the rostral segment (Figure 3-2, top panel), contusion-injury significantly increases Cath B mRNA expression over that seen after sham-injury at 48 and 168 h post-injury. The maximum contusion-injury induced increase in expression occurs at 48 h post-injury (6.4 fold) and

remains elevated (6.0 fold) at 168 h post-injury. Caudal to the injury site (Figure 3-2, bottom panel), contusion-injury induces the expression of Cath B mRNA that, like the sham-injury animals, is generally more robust than observed in the rostral segment. Cath B mRNA expression is elevated at 24, 48 and 168 h post-injury (> 10 fold), although the only significant increase over the sham-injury level occurs at 48 h post-injury.

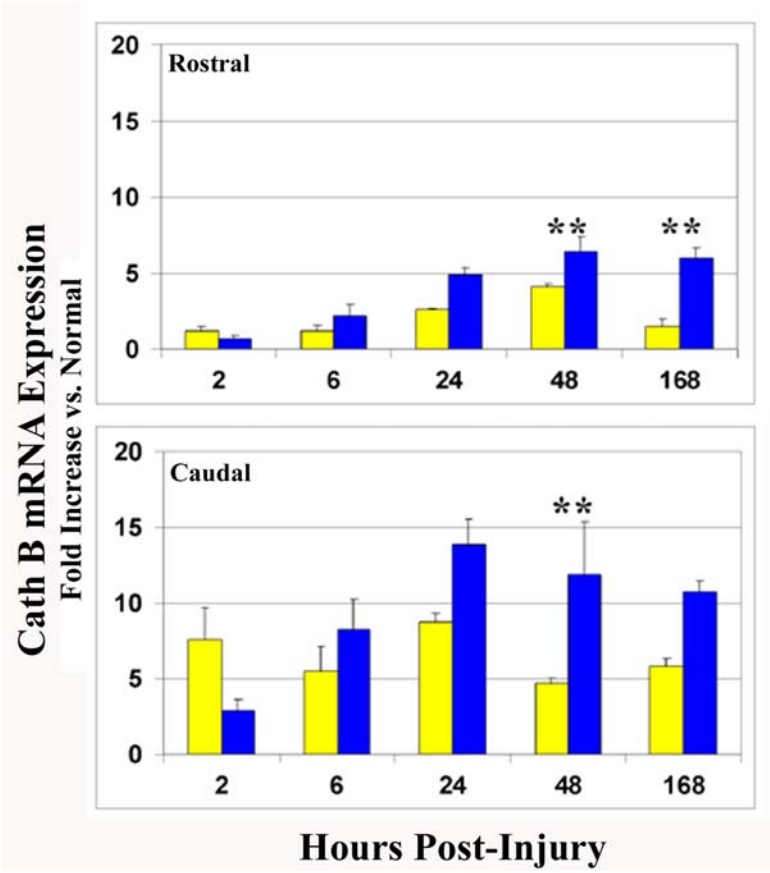

Figure 3-2: Sham- and contusion-spinal cord injury induce Cath B mRNA expression in the rostral and caudal tissue segments. Increases in Cath B mRNA expression induced by sham- (yellow bars) and contusion-injury (blue bars) are presented as Fold Increase vs. Normal. Following contusion-injury, the expression of Cath B mRNA is significantly *(** $p < 0.01$)* greater than the sham-injury level at 48 and 168 h post-injury in the rostral segment (top panel). In the caudal segment (bottom panel), Cath B mRNA expression peaks at 24 h post-injury but remains nearly as elevated at 48 and 168 h post-injury. A significant increase is present at 48 h post-injury.

Analysis of Cath B Protein Expression

Cath B Protein Expression is Elevated following Spinal Cord Injury

Figure 3-3 displays representative immunoblots (probed with anti-Cath B antibody) for the rostral (top panel), injury (middle panel) and caudal (bottom panel) segments of the spinal cord at 168 h post-injury. Also shown are the GAPDH loading controls. Normal spinal cord samples produce faint bands while sham- and contusion-injury yield broader and more intense bands for the proenzyme and the two active forms of Cath B.

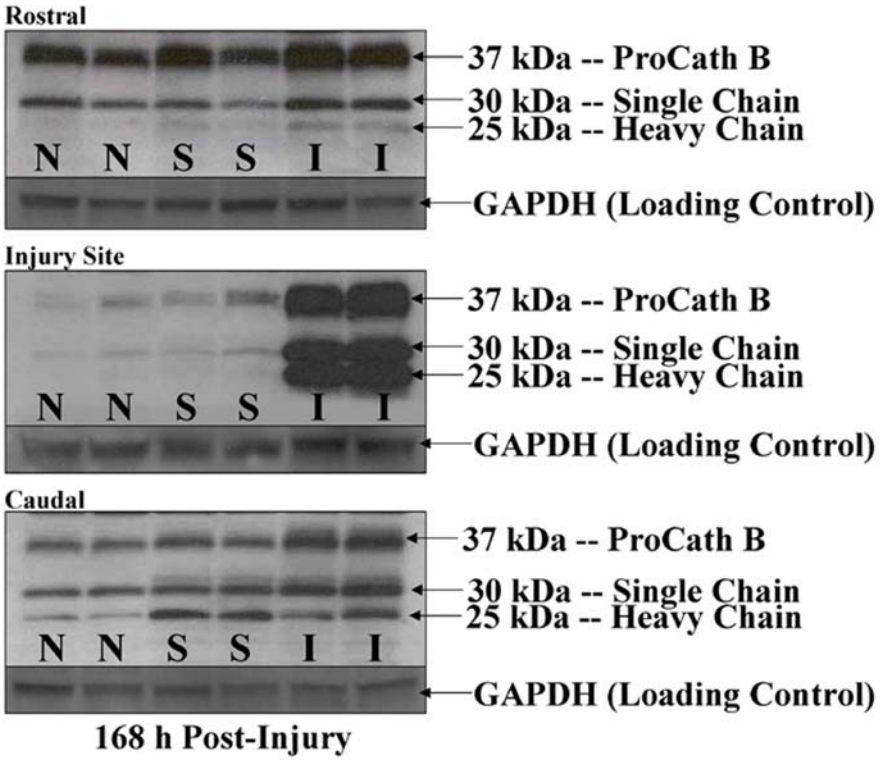

Figure 3-3: Sham- and contusion-injury increases levels of all forms of the Cath B protein. Immunoblots containing normal (N), sham- (S) and contusion-(I) injury (168 h post-injury) samples are shown. Three bands are detected using an anti-Cath B antibody: the inactive proenzyme at 37 kDa and two active mature forms, 30 kDa (single chain) and 25 kDa (double chain). Normal spinal cord samples produce very faint bands. In the injury site **(middle)**, the sham-injury animals yield only slightly more dense bands. The contusion-injury animals, however, have noticeably more intense and broad bands. These increases also extend to segments rostral **(top)** and caudal **(bottom)** to the injury site. The GAPDH loading control blots are also shown for each panel.

In all three spinal cord segments examined, sham-injury elicits a minimal increase in Cath B protein levels that rarely exceeds 2 fold. In fact, sham-injury elicits a greater than 2 fold increase (maximum value was a 2.3 fold) at only 3 of 63 total time points, two of which occur at the site of the injury. Contusion-injury, however, significantly increases Cath B protein levels in all three segments, although to different magnitudes. The level of Cath B proenzyme (37 kDa) at 48, 72 and 168 h post-injury at the injury site (Figure 3-4) and at 24, 48, 72 and 168 h post-injury in the caudal segment (Figure 3-5, bottom panel) is significantly elevated which may be indicative of ongoing protein synthesis and/or the presence of inflammatory cells containing Cath B. No significant change in the level of proenzyme is seen in the rostral segment (Figure 3-5, top panel). The expression of the 30 kDa active form of Cath B is significantly higher following contusion-injury than sham-injury at 168 h post-injury in the rostral segment (Figure 3-5), and at 72 and 168 h post-injury at both the injury site (Figure 3-4) and in the segment caudal to the injury site (Figure 3-5). The expression of the active 25 kDa form of Cath B in contusion-injured spinal cord is significantly elevated over sham-injury at 168 h post-injury in the rostral segment (Figure 3-5) and at the injury site (Figure 3-4) but not in the caudal segment. Clearly, the largest contusion-injury induced increases in all three forms of the Cath B protein occur at 168 h post-injury, *i.e.*, > 6 fold for the proenzyme, > 8 fold for the 30 kDa single chain and > 11 fold for 25 kDa double chain.

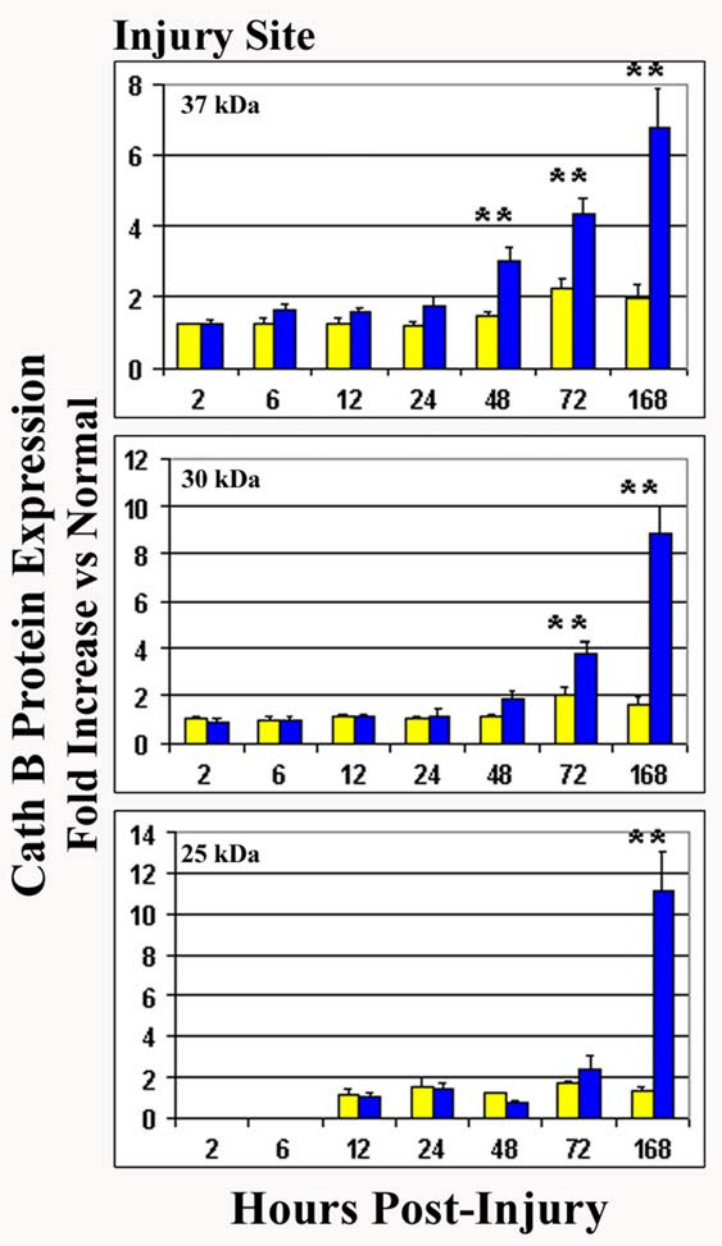

Figure 3-4: Contusion-injury increases Cath B protein levels at the injury site. The expression of all three forms of Cath B – the proenzyme (37 kDa) and two active forms of Cath B (30 and 25 kDa) – following sham- (yellow bars) and contusion- (blue bars) are presented for seven post-injury time points. Sham-injury had little effect on Cath B protein levels. Contusion-injury, however, significantly increases Cath B expression over sham-injury levels at several post-injury time points *(** $p < 0.01$)*. The largest contusion-injury induced increases in Cath B expression clearly occur at 168 h post-injury where increases are > 6 fold for the 37 kDa proenzyme, > 8 fold for the 30 kDa active and > 11 fold for the 25 kDa active forms of the protein.

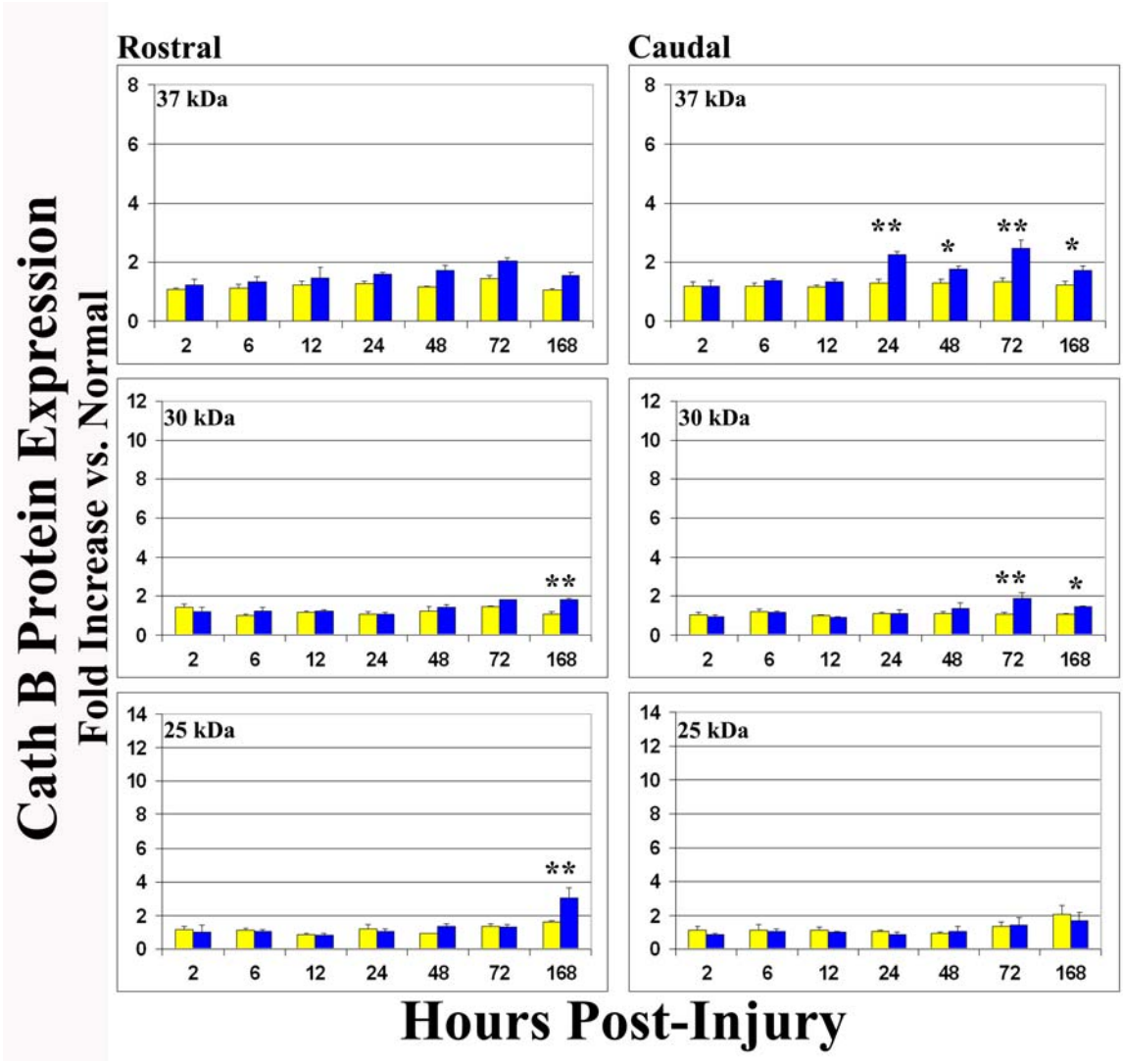

Figure 3-5: Contusion-injury increases Cath B protein levels in adjacent segments of the spinal cord. The Fold Increase vs. Normal values for the sham-injury group (yellow bars) are compared to the contusion-injury values (blue bars) at seven post-injury time points. The time course of these changes in the proenzyme (37 kDa) and two active forms of Cath B (30 and 25 kDa) are presented for the rostral (left side) and caudal segments (right side). As is the case for the injury site, sham-injury does not noticeably increase Cath B expression over normal levels. The maximum increases in expression following contusion-injury is just over 2 fold in these adjacent segments and is only observed in only three instances. The differences between the sham- and contusion-injury groups are significant *(** $p < 0.01$, * $p < 0.05$)* at several time points. No significant differences in either the proenzyme or the 25 kDa form of Cath B are present in the rostral and caudal segments, respectively.

Increases in Cath B mRNA Expression and Cath B Proenzyme Expression are Correlated

The relationship between the increases in Cath B mRNA and protein expression following contusion-injury was examined using a regression analysis. Cath B mRNA expression is the independent factor and Cath B proenzyme (37 kDa) expression is the dependent factor. Following contusion-injury, the r^2 values for the rostral, injury and caudal segments are .941, 0.971, and 0.844, respectively, suggesting that the level of Cath B proenzyme increases linearly with the increases in Cath B mRNA. This correlation does not hold for the sham-injury. Thus, while it appears that sham-injury can induce Cath B mRNA expression at the injury site to some degree, this increase does not seem to be sufficient to initiate an upregulation of Cath B protein.

Analysis of Cath B Enzymatic Activity Levels

Contusion-Spinal Cord Injury Increases Cath B Enzymatic Activity

Cath B enzymatic activity levels were determined by the production of a fluorescent product generated specifically through Cath B-mediated hydrolysis. Levels of Cath B enzymatic activity following both sham- (yellow bars) and contusion-injury (blue bars) were measured from tissue lysates of the injury site at five post-injury time points (1, 2, 3, 5 and 7 d). Cath B enzymatic activity in sham-injured animals is minimally elevated at every time point, never exceeding 1.18 fold (Figure 3-6). Following contusion-SCI, Cath B enzymatic activity begins to increase at 3 d post-injury reaching values of 5.29 and 6.57 fold above normal at 5 and 7 d post-injury, respectively. The contusion-injury induced increases at these latter two time points are significantly greater than sham-injury levels. Furthermore, Cath B activity following contusion-injury is not upregulated in the segments rostral and caudal to the injury site (Figure 3-7). This lack of

an increase in activity is likely due to the smaller increases in Cath B protein and/or the presence of a smaller number of inflammatory cells in these segments.

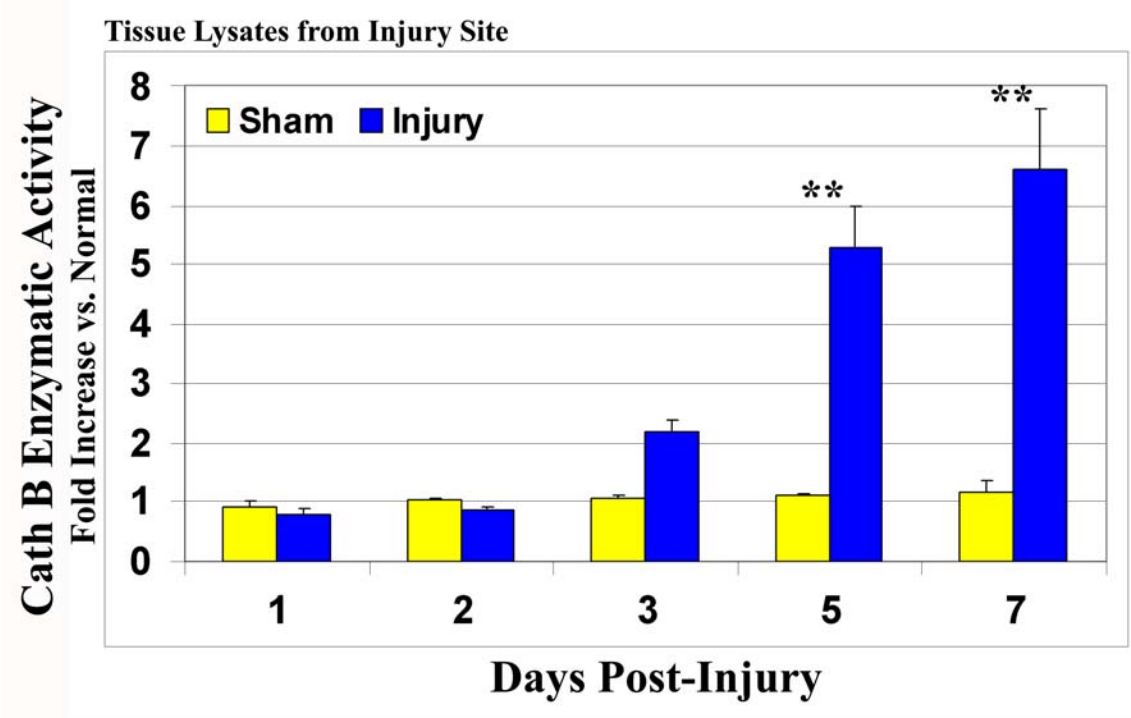

Figure 3-6: Cath B enzymatic activity levels are increased following contusion-injury. Cath B enzymatic activity levels (reported as Fold Increase vs. Normal) following both sham- (yellow bars) and contusion-injury (blue bars) were recorded from injury site lysates at five post-injury time points. Sham-injury levels are minimally elevated across the experimental timeline. The first appreciable but not significant increase in Cath B enzymatic activity following contusion-injury is detected at 3 d post-injury ($p < 0.053$). Following contusion-injury, Cath B enzymatic activity increases across the experimental timeline reaching 5.29 and 6.59 fold at 5 and 7 d post-injury, respectively. Both of these contusion-injury induced increases are significantly (** $p < 0.01$) greater than sham-injury levels.

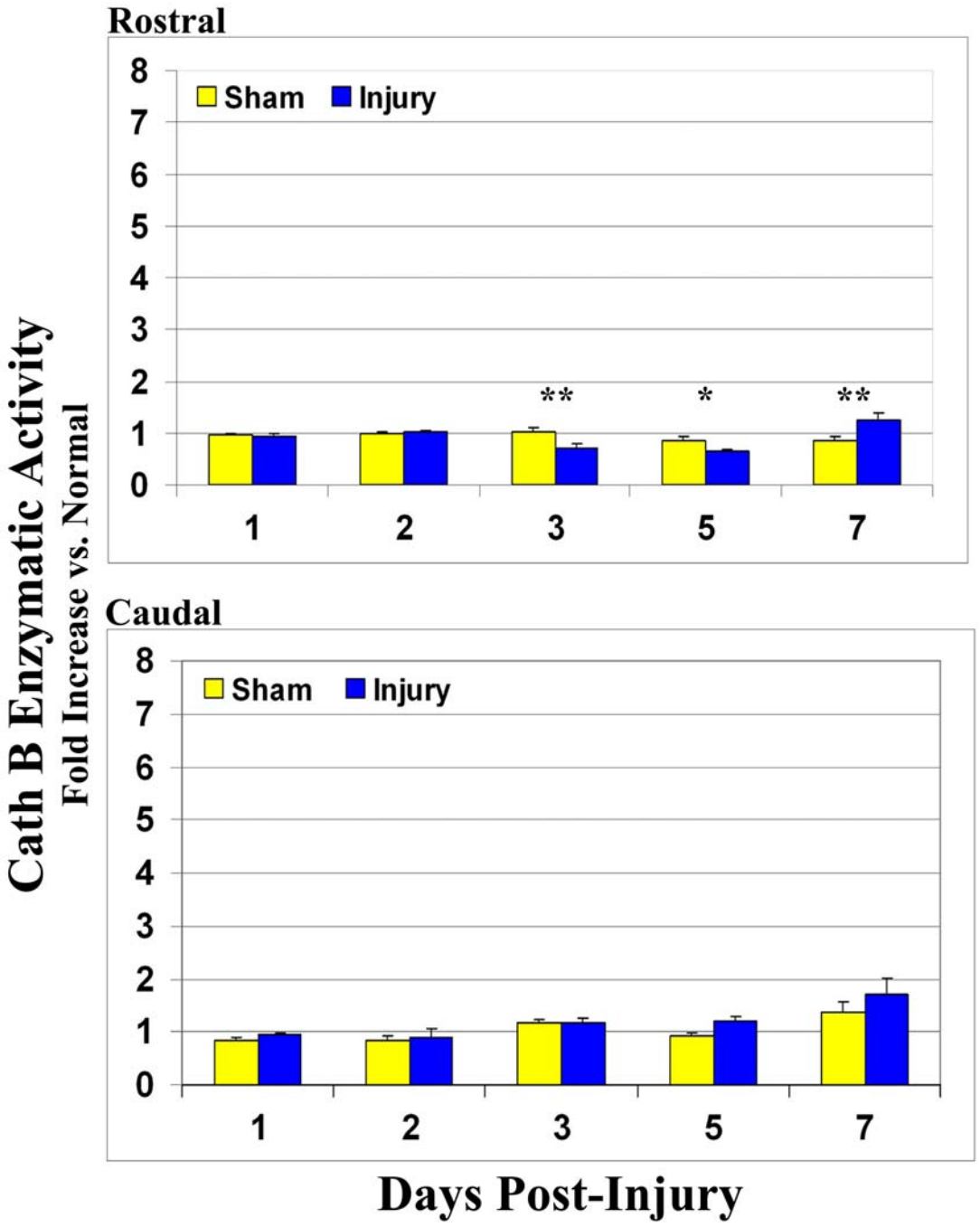

Figure 3-7: Contusion-injury does not increase Cath B enzymatic activity levels in adjacent spinal cord segments. Cath B enzymatic activity levels (reported as Fold Increase vs. Normal) are presented for lysates of tissue segments adjacent to the injury site after both sham- (yellow bars) and contusion-injury (blue bars) at five post-injury time points. Following both sham- and contusion-injury, Cath B activity levels are indistinguishable from levels of the normal spinal cord. Statistically significant differences, however, are present at 3, 5 and 7 d post-injury in the rostral segment.

Increases in Cath B Protein Expression and Cath B Activity Levels are Correlated

Regression analysis reveals the levels of the 30 kDa and 25 kDa forms of Cath B (Ellis *et al*., 2004) are positively correlated with the levels of enzymatic activity (r^2 = 0.9891 and r^2 = 0.9828, respectively) at the injury site.

Immunohistochemical Analysis of Cath B

Cath B Immunoreactivity Appears Restricted to Neurons in the Normal Spinal Cord

To investigate the cellular source of Cath B, frozen sections of adult spinal cord were double immunostained with anti-Cath B and a variety of anti-cell-specific markers. In the normal spinal cord, Cath B immunoreactivity (Figure 3-8A) is most prominent in neuronal cell bodies, which are identified by their morphology and by the neuronal marker NeuN (Figure 3-8B). The co-localization of staining for both Cath B and NeuN is presented in Figure 3-8C. These Cath B immunopositive neurons (Cath B^+) are found primarily in the spinal gray matter although an occasional Cath B^+ neuron is seen in the white matter (data not shown).

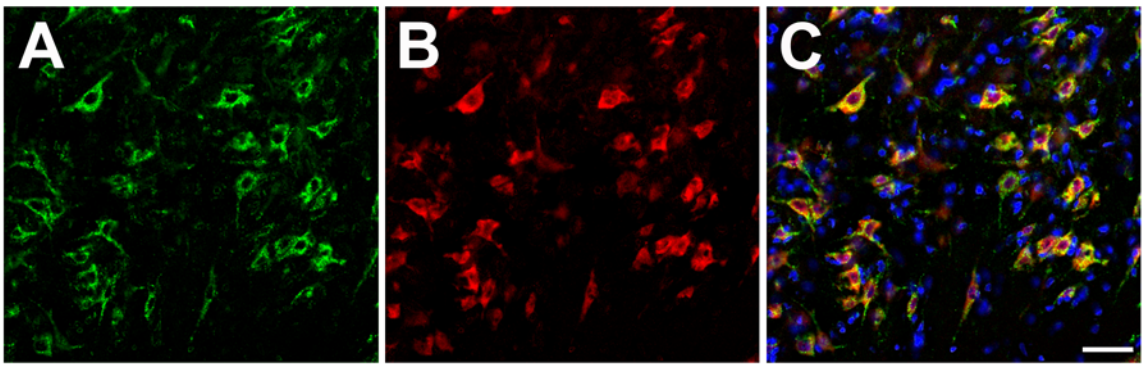

Figure 3-8: Cath B staining is localized to neurons in the gray matter of the normal spinal cord. Cath B immunoreactivity (green, A) was localized morphologically to neurons. Staining for the neuronal marker NeuN (red, B) also co-localized with Cath B staining and is presented in (C). *(The scale bar is equivalent to 50 μM)*

Cath B immunoreactivity is distinguished by its punctate granular quality (Figure 3-9A), which is characteristic of a lysosomal localization for this protease (Demchik *et al.*, 1999; Berquin & Sloane 1996). We co-localized a marker specific for lysosomes (*i.e.*, lysosomal associated membrane protein-1 or LAMP, Figure 3-9B) with Cath B immunoreactivity, thereby confirming that Cath B is contained in the lysosomes of normal healthy neurons (Figure 3-9C).

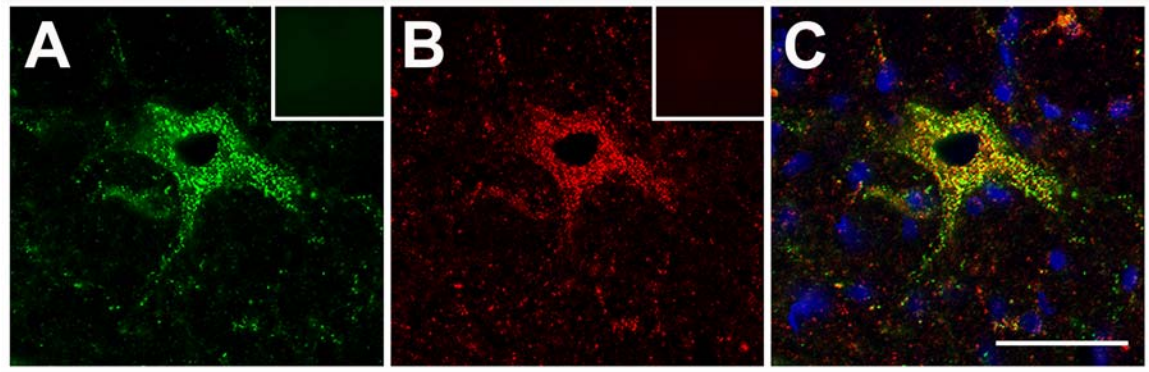

Figure 3-9: Cath B is staining is distinguished by its lysosomal localization. Cath B immunoreactivity is characterized by its punctate granular appearance (A), strongly resembling the staining pattern of lysosomal associated membrane protein-1 or LAMP (red, B). A merged image of Cath B (A) and LAMP (B) staining is shown in (C), thereby establishing a lysosomal localization for Cath B in normal healthy neurons. Negative primary antibody controls (absence of 1° antibodies) are shown in the insets of (A) and (B). *(The scale bar is equivalent to 50 μM)*

Cath B immunoreactivity does not coincide with staining of markers for astrocytes (GFAP, Figure 3-10A) or resting microglia (OX-42, Figure 3-10B) in normal spinal cord tissue. The level of Cath B immunoreactivity is also below detection in oligodendroglia in the normal spinal cord as indicated by the absence of Cath B staining with staining for MBP (Figure 3-11C). The absence of Cath B immunoreactivity in oligodendroglia is further confirmed by the lack of Cath B co-localization with other oligodendroglial markers including CNPase and O4 (data not shown). Finally, there is a low basal level of

Cath B immunoreactivity in the white matter, although it does not co-localize with any of the cell specific markers used in the study (data not shown).

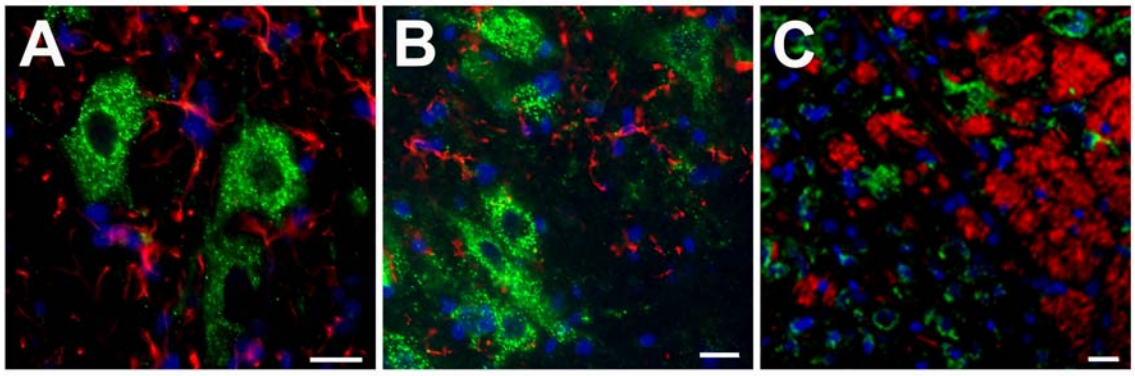

Figure 3-10: Other cell types in the normal spinal cord do not appear to express Cath B. Cath B (green) immunoreactivity is absent in non-neuronal cell types in the normal spinal cord. Cath B staining evident in neurons but does not co-localize with staining for the astrocytic marker GFAP (red, A), the resting microglial marker OX-42 (red, B) or the oligodendroglial marker MBP (red, C) in tissue sections of the normal spinal cord. *(The scale bars are equivalent to 20 μM)*

Contusion-Spinal Cord Injury Increases Cath B Immunoreactivity and Alters Cath B Localization

Although we previously detected increases in Cath B protein levels following SCI (Ellis *et al.*, 2004), the source of these increases was not identified. For the present study, we used immunohistochemical techniques to identify the potential source(s) of the elevation in Cath B at 7 d post-injury. Following contusion-injury, Cath B immunoreactivity is now evident in non-neuronal cells that essentially replace the entire spinal cord gray matter and much of the white matter (Figure 3-11B). The normal spinal cord is presented for reference in Figure 3-11A. By 7 d post-injury, these Cath B^+ cells are so numerous that few, if any, Cath B^+ neurons can be found in the injury epicenter. Often only the central canal (stained blue with DAPI) is discernible (Figure 3-11C).

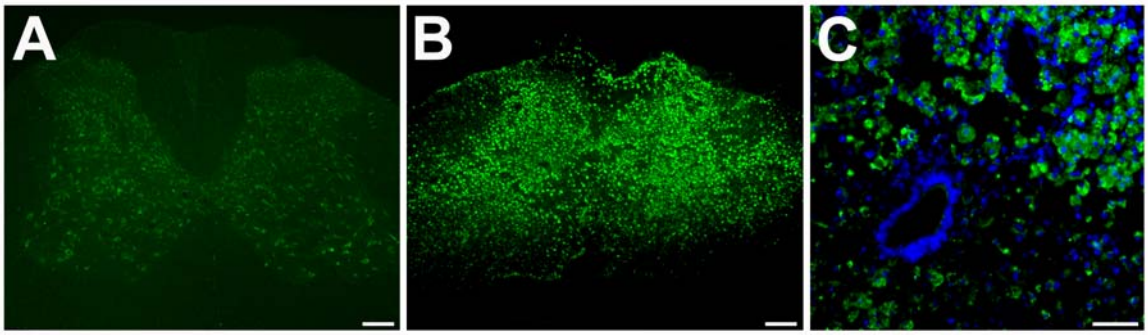

Figure 3-11: Cath B immunoreactivity is increased and altered following SCI. Cath B (green) staining is primarily restricted to gray matter neurons in normal spinal cord tissue (A). The injury site segment is characterized by the presence of Cath B immunopositive cells (B) by 7 d post-injury. The deterioration of the spinal cord is so extensive that Cath B^+ neurons are absent and only the central canal stained in blue is discernible (C). *(Scale bars represent 50 μM in panels A-B, 5 μM in panel C)*

The Cath B immunopositive cells in the injury epicenter (Figure 3-12A) stain positively for inflammatory cell markers including OX-42 (Figure 3-12B) and OX-8 and ED-2 (data not shown). The merged image of these panels demonstrating their co-localization is shown in Figure 3-12C and magnified for clarity in Figure 3-12D.

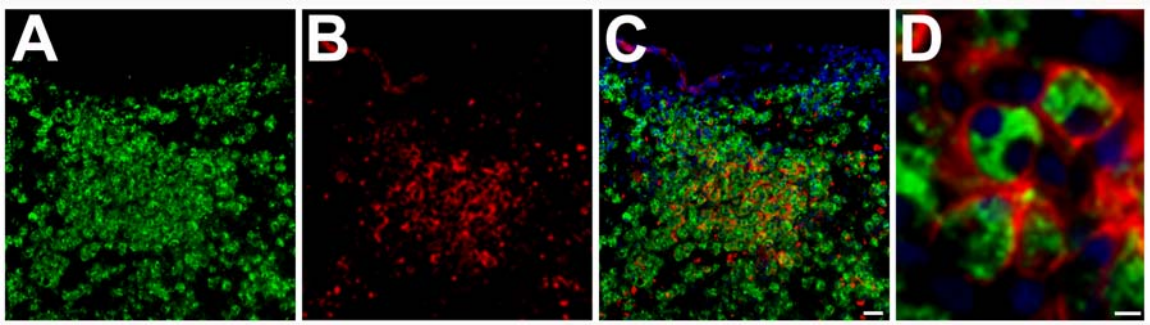

Figure 3-12: Cath B immunopositive cells (A) in the injury epicenter are inflammatory in origin. Cath B^+ cells in the injury epicenter stain positively with markers for inflammatory cells including OX-42 (B). The overlay of these images with the nuclear stain DAPI is presented in (C) and magnified in (D), thereby confirming the inflammatory source of Cath B in the post-injured spinal cord *(Scale bars represent 50 μM in panels A-C, 20 μM in panel D)*

In regions just adjacent to the injury epicenter, the number of cells immunopositive for both Cath B and inflammatory cell markers are reduced and

generally are most prominently found in the dorsal columns (Figure 3-13A). In addition, unlike that seen at the injury epicenter at 7 d post-injury, neurons can still be identified in regions that are adjacent to the injury epicenter. However, some of these neurons are more intensely Cath B immunoreactive (Figure 3-13C). In other neurons, there is a shift in the Cath B staining from its normal punctate character (Figure 3-13B) to a more uniform distribution throughout the neuronal cell body (Figure 3-13D). This shift in location of Cath B immunoreactivity with concomitant alterations in LAMP staining (Figure 3-13E) suggest that the lysosomal membrane has been compromised allowing Cath B to escape into the cytosol (Figure 3-13F). Following contusion-injury, Cath B immunoreactivity in astrocytes and oligodendroglia continues to be unremarkable.

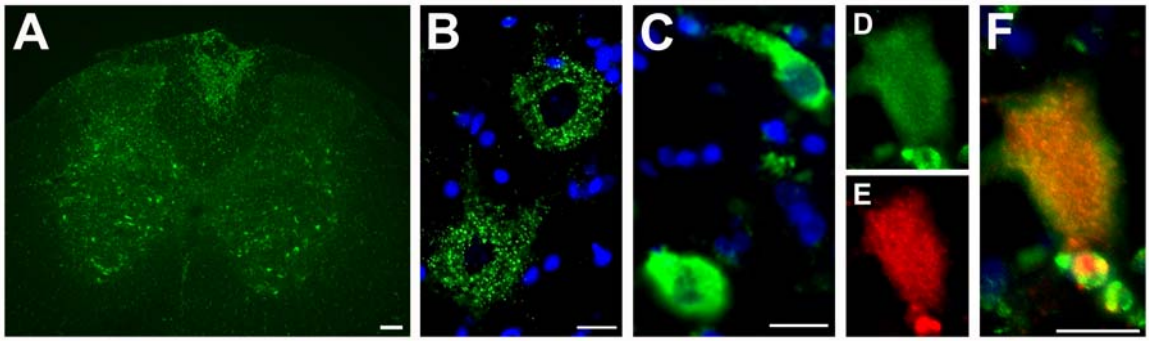

Figure 3-13: Cath B expression increases in both neuronal and non-neuronal cells in tissue segments adjacent to the injury site. Following SCI, Cath B immunoreactivity is particularly robust in a population of cells in the dorsal columns (A). Contusion-injury also alters the quality of Cath B staining from its punctate granular staining in neurons of the normal spinal cord (B). While Cath B immunoreactivity is more robust in these neurons (C), other neurons exhibit a less granular and more diffuse pattern of Cath B immunoreactivity (D). A similar change in LAMP staining (red) is also seen (E), indicating the release of Cath B from its lysosomal sequestration. An overlay of (D) and (E) is presented in (F). Morphologically, the neurons in the injured spinal cord (C, D) also differ from neurons in the normal spinal cord (B). *(Scale bars represent 50 μM in panel A and 20 μM in panels B-F)*

Inhibition of Cath B Enzymatic Activity

CA-074 was an Effective Inhibitor of Cath B Enzymatic Activity *In Vitro*

Cath B enzymatic activity levels were assessed by the production of a fluorescent product generated specifically through Cath B-mediated hydrolysis. Addition of the specific, irreversible and cell impermeable Cath B inhibitor CA-074 in a range 0-5000 μM inhibits the activity of purified Cath B from the bovine spleen in a dose-dependent manner, demonstrating the potency of CA-074 as a Cath B inhibitor *in vitro* (Figure 3-14).

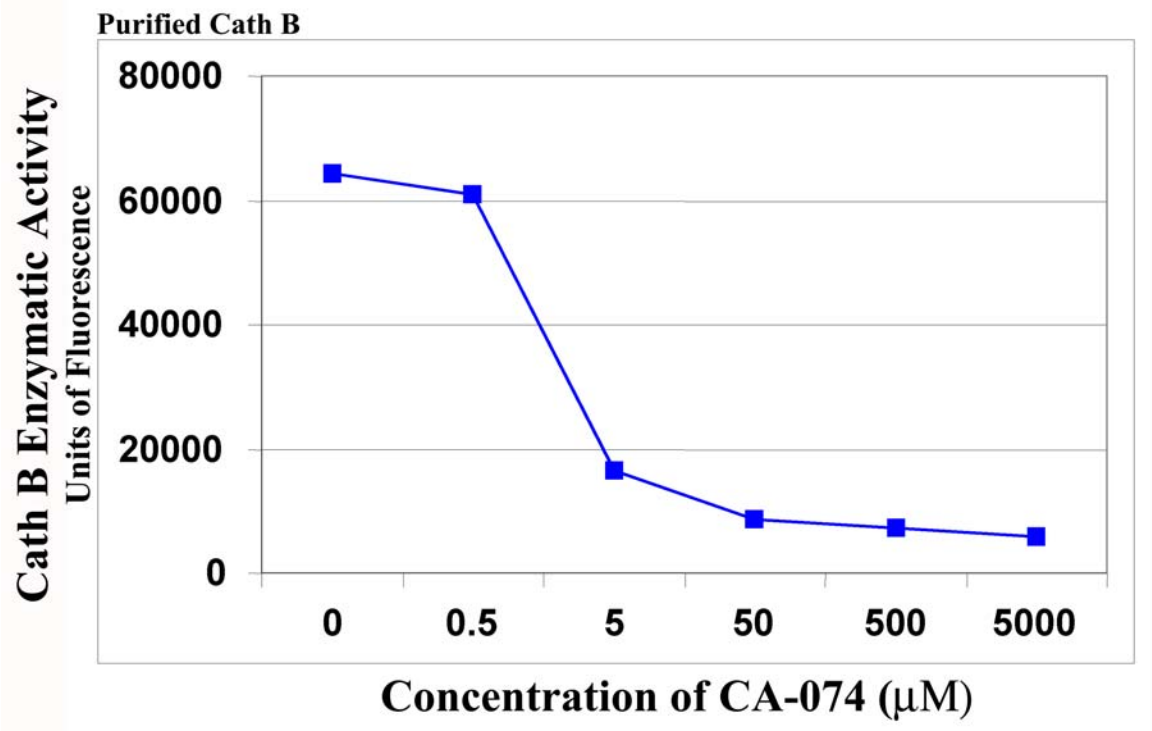

Figure 3-14: CA-074 potently inhibits Cath B enzymatic activity *in vitro*. Enzymatic activity levels of purified Cath B (bovine spleen) were assessed by the generation of a Cath B specific cleavage fluorescent product. The addition of CA-074 to the reaction vessel in increasing concentrations nearly eliminates the fluorescent signal generated through Cath B mediated hydrolysis. The level of fluorescence is ~65,000 units in the absence of CA-074 (0 μM) vs. ~6,000 units in the presence of 5000 μM CA-074.

CA-074 is an effective inhibitor of Cath B enzymatic activity *in vitro*

The addition of CA-074 to lysates of the tissue segment containing the injury epicenter (Figure 3-15) essentially eliminates the contusion-injury induced increases in Cath B enzymatic activity (previously seen in Figure 3-6). CA-074 reduces Cath B activity in the injured spinal cord to essentially the level seen in both the normal and sham-injured spinal cords at all post-injury time points (Figure 3-15), thereby confirming that the fluorescent signal is from a product of Cath B mediated hydrolysis. Despite the lack of Cath B enzymatic activity in either the sham- or contusion-injury groups following CA-074 treatment, the difference between them is statistically significantly at 5 d post-injury.

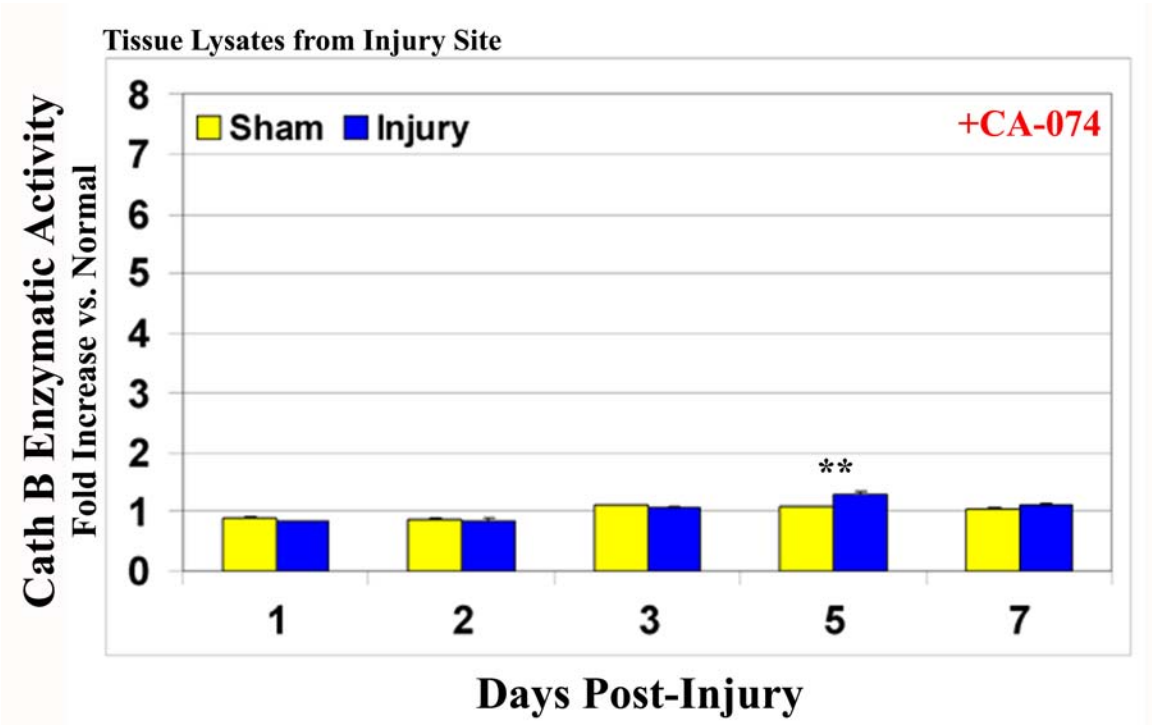

Figure 3-15: Increases in Cath B enzymatic activity are suppressed by CA-074. The addition of the specific, irreversible and cell impermeable Cath B inhibitor CA-074 (25 μM) completely inhibits the contusion-injury induced increases in Cath B enzymatic activity previously seen in Figure 3-6. In the presence of CA-074, the Cath B enzymatic activity levels in the three groups are nearly identical.

In vivo CA-074 Treatment is Ineffective at Reducing Contusion-Injury Induced Increases in Cath B Enzymatic Activity Levels

Cath B enzymatic activity levels in saline treated animals (black bars) are independent of the variations in timing and route of administration, indicating that these variables have little or no effect on Cath B enzymatic activity (Figure 3-16). Conversely, with two exceptions, treatment with CA-074 increases Cath B enzymatic activity to levels greater than the saline controls. In both IP groups, treatment with 12 mg/kg CA-074 (yellow bars) raises Cath B enzymatic activity, although not significantly over controls. Similarly, animals receiving 36 mg/kg CA-074 IV (red bars) and 36 mg/kg CA-074 IP have the highest levels of Cath B enzymatic activity in the study and are significantly greater than their respective saline controls. As for the two exceptions, the activity levels of the repeated dose 36 mg/kg CA-074 and saline groups are nearly identical and the 12 mg/kg CA-074 IV dose is the only one to show a tendency to suppress Cath B enzymatic activity *in vivo* (23% suppression, Figure 3-16).

Because 12 mg/kg of CA-074 given as a single IV dose was the only regimen that appears to suppress Cath B enzymatic activity, an additional experiment was designed to confirm this finding. A lower IV dose of CA-074 (6 mg/kg) was included to determine if CA-074 was operating on an inverse dose response curve. However, as illustrated in Figure 3-17, treatment with either 6 mg/kg CA-074 (blue bar) or 12 mg/kg CA-074 (yellow bar) does not have any significant effect on Cath B enzymatic activity. Thus, this study is unable to replicate the minimal suppression of Cath B enzymatic activity seen previously.

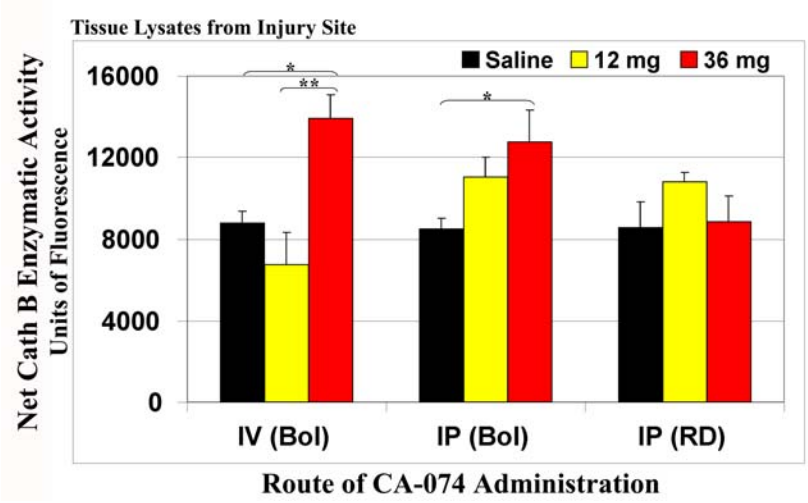

Figure 3-16: *In vivo* CA-074 does not inhibit contusion-injury induced increases in Cath B activity levels. Saline (black bars), 12 mg/kg CA-074 (yellow bars) and 36 mg/kg CA-074 (red bars) were given as a bolus (IV and IP) or a repeated dose (IP only) injection. All animals received an injection immediately after SCI while the repeated dose group received additional injections at 24 and 48 h post-injury. Saline treated animals have similar levels of Cath B enzymatic activity. Only the 12 mg/kg IV group demonstrates a tendency to suppress Cath B activity (~23%). Asterisks denote significant differences in activity levels *(* $p < 0.05$, ** $p < 0.01$)* between various treatment groups.

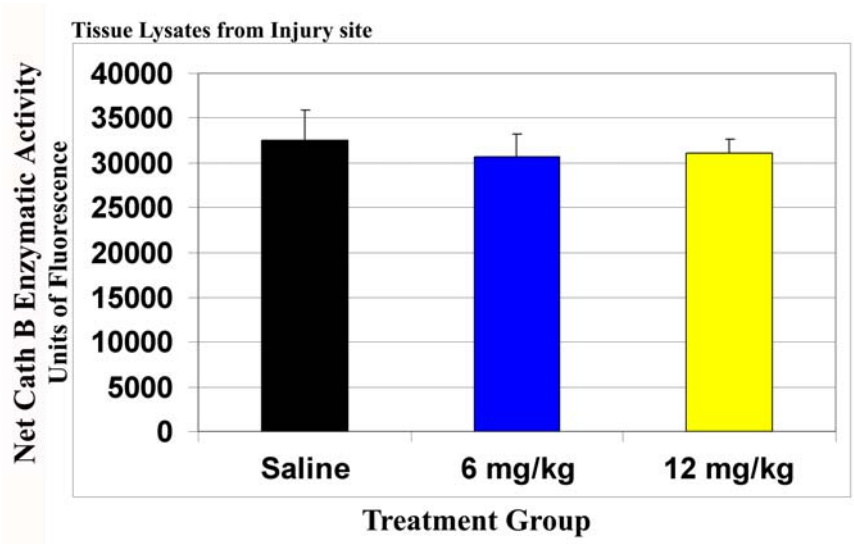

Figure 3-17: The suppression of Cath B enzymatic activity with i*n vivo* CA-074 treatment is not reproduced. A bolus IV injection of saline (black bar), 6 mg/kg CA-074 (blue bar) and 12 mg/kg CA-074 (yellow bar) was given immediately after SCI. There are no significant differences in levels of enzymatic activity following either CA-074 treatment.

CHAPTER 4
DISCUSSION

Mechanical trauma to the spinal cord initiates a complex cascade of biochemical processes that collectively contribute to neuronal and glial cell death, tissue cavitation and sensory and motor deficits. Currently, there is limited opportunity to improve and/or restore function to the individual suffering a SCI. While a number of the pathophysiological events contributing to the secondary injury have been identified, Nixon and Cataldo (1993) suggested that lysosomal leakage or rupture represents the greatest threat to neuronal cell survival. Since that report was published, research on the role of lysosomal proteases in the etiology of the secondary injury cascade following SCI has lagged and most investigations have focused on the expression and activity of other proteases such as the calpains, caspases and most recently, the matrix metalloproteinases. Consequently, very few studies have been conducted to directly examine the potent lysosomal protease, Cath B, in CNS trauma. Analyses of changes in mRNA expression following dorsal root avulsion (Hu *et al*., 2002), hemisection (Fan *et al*., 2001) and peripheral sciatic nerve crush (Fan *et al.*, 2001) suggest that Cath B may play a role in the injury cascade associated with these insults. However, to date, studies examining Cath B expression and enzymatic activity following traumatic SCI have not been carried out. The work described in this dissertation represents the first systematic examination of changes in Cath B expression, enzymatic activity and cellular localization in the spinal cord following contusion-injury. In these studies, changes in Cath B were examined at several post-injury time points, both within and adjacent to the injury site. Preliminary

experiments utilizing the Cath B inhibitor CA-074 were also conducted in an attempt to determine if Cath B contributes to the pathology of secondary SCI.

Characterization of Cath B following Contusion-Injury

mRNA Expression

Following contusion-injury, levels of Cath B mRNA were significantly higher in the injured spinal cord than in the sham-injured cord. Increased transcript levels were found in all spinal cord segments examined with the largest increase (>20 fold) occurring at the injury site itself. In general, the levels of Cath B mRNA in segments adjacent to the injury epicenter were higher in caudal segment than in the rostral segment. While the underlying conditions within the spinal cord that lead to the differences in Cath B mRNA expression between these two regions are unknown, other investigators have also noted regional differences in the response of the spinal cord to injury. For example, Yong *et al*. (1998) and Citron *et al.* (1998) have both reported increased apoptotic cell death in regions caudal to the injury site than in those rostral to it.

Currently, information regarding the regulation of transcription of the Cath B gene in the injured CNS is limited. The putative promoter of the Cath B gene contains several elements common to the promoters of housekeeping genes (*e.g.*, absence of CAAT and TATA boxes and Sp-1 binding sites) (Gong *et al*., 1993). However, unlike the ubiquitous expression patterns observed for housekeeping genes, Cath B mRNA expression varies considerably across tissues as a function of cell type and differentiation state (reviewed by Berquin & Sloane 1996; San Segundo *et al.*, 1996). The presence of variable patterns of expression suggests that additional regulatory elements control expression of Cath B. Factors that can influence Cath B mRNA expression include cytokines (Gerber *et al*.,

2000; Li & Bever 1997, 1996; Keppler *et al*., 1994), phorbol esters (Berquin et al., 1999; Phillips *et al.*, 1989) and dexamethasone (Hong & Forsberg 1995; Burnett et al., 1986).

The increase in Cath B mRNA seen following SCI may be derived from the well-characterized inflammatory response that occurs in injured spinal cord tissue. SCI induces rapid increases in the levels of transcripts encoding several proinflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-1α (Bareyre & Schwab 2003; Pan *et al*., 2002; Streit *et al*., 1998; Bartholdi & Schwab 1997; Wang *et al.*, 1996), which can lead to elevated cytokines levels in the injured tissue (Segal *et al.*, 1997). Links between increased cytokine expression and the induction of Cath B have been established experimentally, but the evidence has been generated in studies of non-CNS systems. Examples include the observations that INF-γ induces Cath B transcription in THP-1 cells (human macrophage-like cells; Li & Bever 1997), U-937 monocytes (Lah *et al*., 1995), human muscle cells (Gallardo *et al*., 2001) and in macrophages (Schmid *et al*., 2002). In addition, TNF-α, INF-γ and IL-1 have all been found to increase secretion of Cath B from synovial fibroblast-like cells in people with rheumatoid arthritis (Lemaire *et al*., 1997; Huet *et al*., 1993) while IL-1β can stimulate increases in Cath B protein in rabbit articular chondrocytes (Baici & Lang 1990). Although other regulatory mechanisms exist, it appears that the induction of Cath B mRNA expression following SCI may be due, in part, to contusion-injury mediated increases in cytokines.

Protein Expression

Levels of Cath B protein (*i.e.*, the proenzyme and both active forms) were also increased by contusion-SCI paralleling the increases seen in Cath B mRNA expression. Specifically, Cath B proenzyme increased linearly relative to the increases in Cath B

mRNA as confirmed by high r^2 values in the rostral (r^2 = .941), injury (r^2 = .971) and caudal (r^2 = .844) segments. The highest levels of Cath B protein were observed at the injury site. Significant increases in Cath B protein were also seen in the segments rostral and caudal to the injury site, but the magnitude of these increases was minimal (~2 fold). Importantly, the finding that the levels of both active forms of Cath B increased in the injured tissues indicates ongoing processing of the Cath B proenzyme in the post-injury spinal cord and/or a contribution from resident and invading inflammatory cells.

Levels of Cath B Enzymatic Activity

The observed increases in Cath B enzymatic activity at the injury site were not surprising in view of the increases in Cath B protein expression (particularly the active forms) described above. There were significant increases in Cath B enzymatic activity at the injury site on post-injury days 5 and 7. Although not significant, the increases in Cath B enzymatic activity on post-injury day 3 (>2 fold) coincided with similar increases in the expression of both active forms of Cath B protein. The increases in Cath B enzymatic activity were highly correlated with the increases in the 30 kDa (r^2 = 0.9891) and 25 kDa (r^2 = 0.9829) forms of Cath B. Interestingly, elevated Cath B enzymatic activity was not observed in the rostral or caudal segments. The absence of contusion-injury induced changes in Cath B enzymatic activity in these flanking segments likely reflects the small increases in Cath B protein (generally < 2 fold) primarily from the smaller number of inflammatory cells observed in these segments (see below).

The Importance of Inflammatory Cells

What are the cellular sources of the increases in Cath B mRNA and protein expression and activity in the injured spinal cord? Potential candidates include neurons and glia within the spinal cord parenchyma and inflammatory cells that damage tissue

following injury. Immunohistochemical analysis showed that in normal, non-injured spinal cord, Cath B was localized primarily to lysosomes of neuronal cell bodies with little, if any, Cath B staining in astrocytes, microglia or oligodendroglia. Following contusion-injury, however, intense Cath B immunoreactivity was seen in the large numbers of inflammatory cells (*e.g.*, neutrophils, macrophages, activated microglia) that essentially filled the epicenter of the injury site. Indeed, by 7 d post-injury Cath B^+ inflammatory cells were the dominant cell type and were uniformly distributed through the gray and white matter at the injury epicenter to the extent that very few, if any, Cath B^+ neurons could be found. In the segments adjacent to the injury site, smaller numbers of Cath B^+ inflammatory cells were seen and these appeared to be located primarily in the dorsal columns. While neurons could be identified in these surrounding segments, many appeared irregularly shaped with a more intense and uniform (*i.e.*, loss of punctuate character) Cath B staining. These findings suggest that the contusion-injury induced increases in Cath B expression and enzymatic activity are primarily due to both endogenous and invading inflammatory cells rather than to increased synthesis of Cath B by resident neurons and glia. Indeed, the changes seen in Cath B immunoreactivity in surviving neurons may signal the death of these neurons due to the release of Cath B from lysosomes into the cytosol. However, this does not exclude the possibility that neuronal and glial cells contribute to the increases in Cath B mRNA and protein expression that were observed immediately following injury.

The presence of large numbers of Cath B^+ inflammatory cells in the injured spinal cord and a delocalization of Cath B in injured neurons is consisted with the proposition that Cath B may be a contributor to the secondary injury cascade. This hypothesis is

supported by studies that show Cath B expression is significantly elevated in activated (but not resting) macrophages (Lah *et al.*, 1995; Kominami *et al*., 1988), and that Cath B is produced by and released from activated microglia (Reddy *et al.*, 1995; Ryan *et al.*, 1995). Furthermore, activated microglia produce and secrete various cytokines and proteases and other molecules such as oxygen species that are also likely to stimulate the production and secretion of potentially damaging proteases like Cath B (Kim & Ko 1998; Rothwell *et al.*, 1996; Giulian & Corpuz 1993; Giulian & Vaca 1993). Enzymes produced by activated microglia have been shown to play a direct role in neuronophagia (Thanos 1991; Banati *et al*., 1993), in degradation of extracellular matrix (ECM) components (Nakanishi 2003; Maeda & Sobel 1996; Gottschall *et al*., 1995) and in neuronal cell death (Gan *et al.*, 2004; Nitatori *et al.*, 1996). It is of interest that Cath B remains active despite shifts in pH (Mort *et al*., 1994; Buck *et al*., 1992; Lah *et al*., 1989), a property that would allow it to continue to hydrolyze carbohydrates, proteins, nucleic acids and ECM molecules in the post-SCI environment. Reducing or blocking the influx of neutrophils and macrophages into the injury site following SCI decreases the extent of tissue damage (Mabon *et al*., 2000; Popovich *et al*., 1999; Giulian & Robertson 1997; Hamada *et al*., 1996; Blight 1994). Thus, our data confirm the presence of a large population of inflammatory cells in, and to a lesser degree, around injury site. In addition, we show for the first time that these cells are intensely Cath B immunoreactive placing this potent protease in a position where its release could significantly amplify the secondary injury cascade.

The Effects of Sham-Injury

Previous studies from our laboratory have shown that exposing the spinal cord by laminectomy is not a benign procedure, in that it causes a decrease in spinal cord blood

flow, alters energy metabolism and modifies membrane lipid composition (Demediuk *et al*., 1987, 1985; Anderson and Means 1985; Anderson *et al*., 1980, 1978). In addition, more recent findings from our laboratory demonstrated that this surgical procedure induced expression of the MnSOD gene in spinal cord tissue (Earnhardt *et al*., 2002). While every precaution was taken to minimize damage to the dura mater and underlying spinal cord while performing the laminectomy, this procedure still cuased an elevation of Cath B mRNA expression in and around the injury site. However, by post-injury day 7, Cath B mRNA had returned to normal both at the injury site and rostral to the injury site. Cath B protein expression was also affected by sham-injury; however, the increases in protein levels were minor never becoming greater than double that seen in the normal spinal cord. Consequently, sham-injury did not increase Cath B enzymatic activity. These findings demonstrate that laminectomy alone can alter Cath B mRNA expression, but this increase is not translated into increased protein levels or enzymatic activity. Thurs, the large increases in Cath B mRNA, protein and enzymatic activity seen following SCI are due to the contusion-injury itself rather than the laminectomy procedure.

Suppression of Cath B Enzymatic Activity

The studies described thus far represent the first in-depth characterization of Cath B expression in spinal cord following SCI. However, we have not yet identified the specific post-injury substrates for Cath B nor has the role of this potentially damaging protease been defined in the secondary injury cascade. Nonetheless, because Cath B has been reported to contribute the pathobiology of ischemic brain injury (Seyfried *et al.*, 1997; Yamashima *et al.*, 1996) and other CNS (Kikuchi *et al*., 2003; Bever & Garver 1995; Mikkelsen *et al.*, 1995) and systemic (Lah *et al*., 2000; Campo *et al*., 1994; Bayliss & Ali 1978) diseases and because our studies place high concentrations of active Cath B in the

injured tissue, it remains a strong candidate for involvement in the tissue destruction that occurs following SCI. As a first step toward attempting to establish a role for Cath B in the secondary injury response, experiments were designed to determine if inhibiting Cath B activity *in vivo* could modulate the magnitude of the injury response. Our studies were based upon the previous use of Cath B inhibitors that reduced cell death and tissue loss in non-SCI models of CNS insult (Seyfried *et al*., 2001, 1997; Yoshida *et al*., 2002, Tsuchiya *et al*., 1999; Yamashima *et al*., 1998).

Based upon these previous investigations, we chose the specific Cath B inhibitor CA-074. The rate of inactivation by CA-074 for Cath B is three orders of magnitude greater than for other related proteases (Buttle *et al*., 1992). In addition, its efficacy and specificity have been demonstrated in many *in vivo* cell culture applications (Premzl *et al*., 2003; Levicar *et al.*, 2003; Montaser *et al.*, 2002; Lah *et al*., 2000) and *in vivo* whole animal models (Ohshita *et al*., 1992; Towatari *et al*., 1991).

Our experiments re-confirmed the ability of CA-074 to inhibit Cath B enzymatic activity. First, CA-074 inhibited the activity of purified bovine Cath B in a dose-dependent manner. Second, the addition of CA-074 to tissue lysates of the injury site segment eliminated the contusion-injury induced increases in Cath B enzymatic activity levels previously described. Thus, *in vitro* CA-074 reduced the levels of Cath B enzymatic activity in the injured tissue to those seen in normal and sham-injured controls.

Prompted by the success of these *in vitro* studies, we examined the *in vivo* inhibitory potential of CA-074 in our contusion model of SCI. Initially, CA-074 and saline were administered using a variety of dosing, timing and delivery strategies with a working hypothesis that levels of Cath B enzymatic activity in the spinal cord of CA-074

treated animals would be lower than those measured in the saline-treated controls. However, the degree of CA-074 inhibition of Cath B seen *in vitro* was not seen *in vivo*. Indeed, only one experimental variation produced any discernible suppression of Cath B enzymatic activity. In fact, with almost every experimental variation, the levels of Cath B enzymatic activity in the CA-074 treated animals were actually higher than those in saline-treated controls. Furthermore, the one incidence of CA-074 mediated suppression was not reproduced in a follow-up experiment. While we have no definitive explanation for these findings, it is possible that the lack of suppression is, in part, due to the negative charge of CA-074, which prevents it from passively diffusing across membranes (Bogyo *et al.*, 2000; Wilcox & Mason 1992). Additional studies are required to test this hypothesis.

Conclusion

Collectively, these studies represent the first in depth characterization of Cath B expression, enzymatic activity and cellular localization in the contusion-injured spinal cord. Our data show that contusion-injury increases Cath B expression and enzymatic activity and further suggest that these increases are due, in large measure, to the presence of inflammatory cells at the injury site. As a powerful and indiscriminate protease, Cath B may contribute to the tissue pathology of the secondary injury cascade. The attempt to confirm its role in secondary spinal cord injury through *in vivo* inhibition of its activity was not successful. With so little available information pertaining to the pharmacodynamics of CA-074, the use of alternative inhibitors coupled with a different administration protocol may prove more effective in inhibiting Cath B activity *in vivo*. Our data suggest that further inhibitory experiments are necessary to implicate Cath B in the secondary injury response following SCI.

Future Directions

Several other studies must be completed before Cath B can be considered a potential target for therapeutic intervention. First, Cath B expression should be examined over the course of a longer post-injury timeline. Second, the pharmacological properties of CA-074 and other available Cath B inhibitors should be fully characterized using appropriate *in vitro* studies. These studies would include examination of the ability of these agents to cross cell membranes. Third, the tissue distribution and clearance rates of these inhibitors must be determined in normal and contusion-injured rats for multiple routes of administration including intrathecal, direct, and intravenous infusion. Finally, the ability of CA-074 to transverse the blood-brain-barrier in both non-compromised and compromised states must be assessed. Clearly, these compounds must be able to reach the source of cellular increases in Cath B expression and activity.

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BIOGRAPHICAL SKETCH

Rebecca Catherine Ellis was born in Derby, CT, in 1976. During her years at Shelton High School, Rebecca was an active member of the study body serving as National Honor Society President, Class Secretary and Treasurer of the Spanish Honor Society. She also captained the softball and volleyball teams. After graduating with honors in June 1994, Rebecca enrolled at the College of the Holy Cross in Worcester, MA. At Holy Cross, she was a member of the Lady Crusader softball team and conducted research examining the effects of steroid treatment in multiple animal models of anxiety under the supervision of Dr. Daniel Bitran. After graduating from Holy Cross in May 1998 with a B.A. in biology, Rebecca spent the summer in the Netherlands where she coached and played for the Islanders, the hometown team of a small village south of Rotterdam. She returned in August of 1998 and entered the Interdisciplinary Program (IDP) in Biomedical Sciences at the University of Florida. In May of 1999, she joined the laboratory of Dr. Douglas K. Anderson. During her dissertation work, Rebecca has focused on characterizing the expression and activity profiles of the powerful protease cathepsin B following contusion-spinal cord injury.