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## ROLE OF CYCLOPHILIN D IN SECONDARY SPINAL CORD AND BRAIN INJURY

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ABSTRACT OF DISSERTATION

Jordan Mills Clark

The Graduate School  
University of Kentucky

2009

ROLE OF CYCLOPHILIN D IN SECONDARY SPINAL CORD  
AND BRAIN INJURY

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Medicine  
at the University of Kentucky

By  
Jordan Mills Clark

Lexington, Kentucky

Director: Dr. James Geddes, Professor of Anatomy and Neurobiology

Lexington, Kentucky

2009

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## ABSTRACT OF DISSERTATION

### ROLE OF CYCLOPHILIN D IN SECONDARY SPINAL CORD AND BRAIN INJURY

In the hours and days following acute CNS injury, a secondary wave of events is initiated that exacerbate spinal tissue damage and neuronal cell death. A potential mechanism driving these secondary events is opening of the mitochondrial permeability transition pore (mPTP) and subsequent release of several cell death proteins. Previous studies have shown that inhibition of cyclophilin D (CypD), the key regulating component in mPTP opening, was protective against insults that induce necrotic cell death. We therefore hypothesized that CypD-null mice would show improved functional and pathological outcomes following spinal cord injury (SCI) and traumatic brain injury (TBI). Moderate and severe spinal contusion was produced in wild-type (WT) and CypD-null mice at the T-10 level using the Infinite Horizon impactor. Changes in locomotor function were evaluated using the Basso Mouse Scale (BMS) at 3 days post-injury followed by weekly testing for 4 weeks. Histological assessment of tissue sparing and lesion volume was performed 4 weeks post SCI. Calpain activity, measured by calpain-mediated spectrin degradation, was assessed in moderate injury only by western blot 24 hours post SCI. Results showed that following moderate SCI, CypD-null mice had no significant improvement in locomotor recovery or tissue sparing compared to wild-type mice. Following severe SCI, CypD-null mice showed significantly lower locomotor recovery and decreased tissue sparing compared to WT mice. Calpain-mediated spectrin degradation was not significantly reduced in CypD-null mice compared to WT mice 24h post moderate SCI. The lack of protective effects in CypD-null mice suggests that more dominant mechanisms are involved in the pathology of SCI. In addition, CypD may have a pro-survival role that is dependent on the severity of the spinal cord injury.

**KEYWORDS:** Spinal Cord Injury, Traumatic Brain Injury, Cyclophilin D, Mitochondrial permeability, Calpain

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Jordan Mills Clark

August 5, 2009

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ROLE OF CYCLOPHILIN D IN SECONDARY SPINAL CORD  
AND BRAIN INJURY

By

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Jane Joseph

Director of Graduate Studies

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DISSERTATION

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For my wife and boys, always with me on this journey

## ACKNOWLEDGEMENTS

I would like to acknowledge Dr. James Geddes for his guidance and mentoring throughout this entire process. I would also like to acknowledge my committee members, Dr. Edward Kasarskis, Dr. Annadora Bruce-Keller, Dr. Patrick Sullivan, and Dr. Marylin Getchell for their continued support from the first project to the final dissertation. Finally, I would like to Acknowledge Dr. Subbu Apparsundaram for his early guidance and support through difficult times.

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## **Chapter One**

### **Primary and Secondary Pathology in Traumatic Spinal Cord and Brain Injury**

#### Introduction

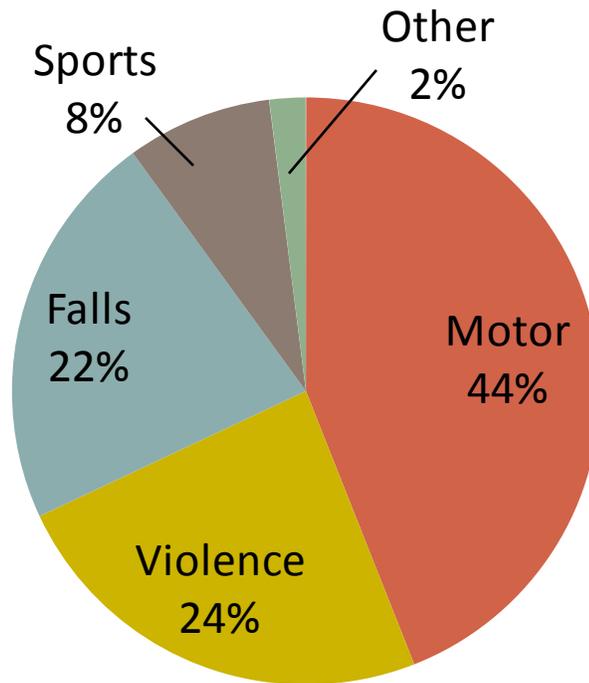
Traumatic injury that disrupts essential neurological functions can severely impact the quality of one's life. Chronic sensory deregulation, cognitive dysfunction, and motor impairment are just some of the debilitating consequences of force induced neurological damage. Two of the most common sources of neurological damage are traumatic injury to the spinal cord and the brain. The pathology of these traumatic injuries can be categorized into two major events. The first event is the primary injury, which is defined as the initial mechanical damage upon the point of impact. The second event, called secondary injury, occurs hours, days, and weeks following the primary injury. During the time course of secondary injury, there is an acceleration of tissue damage at the epicenter, or the point of injury, and at distal sites from the epicenter. Defining these pathological events is critical when considering therapeutic intervention following the injury. Unfortunately, there is little that can be done to intervene with the damage suffered during the primary injury, as the time course is too rapid for critical response. However, events during the secondary injury present a window of opportunity to attenuate and prevent further tissue damage. The ability for recovery from sensory and motor deficits following traumatic brain and spinal cord injury can be greatly enhanced by therapeutic intervention during the course of secondary injury. Thus, there is a dire need for

research into the physiological and biological mechanisms that drive secondary injury cascades. Uncovering the function of key pathways in secondary pathology can serve as the foundation for clinical treatment and recovery for those suffering from traumatic spinal cord and brain injury.

### *Pathology of Traumatic Spinal Cord Injury*

Each year, approximately 7800 people suffer from a traumatic injury to the spinal cord in the United States (NSCISC 2009). Though these injuries can occur in any age group, it is most prevalent at the ages of 16 to 30 years. Of these documented cases each, motor vehicle accidents are the leading cause of spinal cord injury. Other incidents, such as common falls, acts of violence, and sports related injuries, all contribute to spinal cord injury (Figure 1). The most common cause of spinal cord injury occurs from a compression and flexion of both the cervical and thoracic vertebra resulting in a fracturing or dislocation of the vertebral column (NSCISC 2009).

## Causes of Spinal Cord Injury



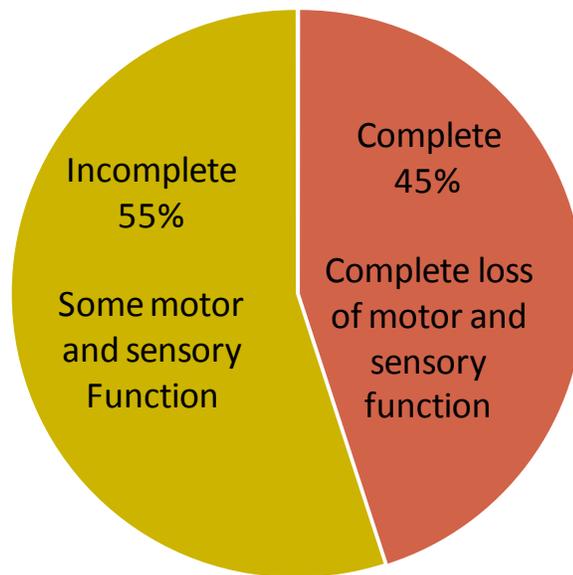
**Figure 1.1 Causes of spinal cord injury in the U.S.** The leading cause of SCI is motor accident at 44%. Violent assaults are next at 24% with injurious falls at 22%. Sports related accidents and others makeup 8% and 2% of causes of SCI. Chart modified from 2009 statistical data obtained from The National Spinal Cord Injury Association.

The impairments that follow spinal cord injuries stem from damaged axons and subsequently disruption of neurotransmission up and down the spinal cord. Perhaps the most notable deficit following spinal injury is loss of mobility from impaired neurotransmission to skeletal muscles. There is also significant impairment in autonomic functions as evident by chronic sensation of pain and bowel and bladder dysfunction. However, the severity of the spinal injury is often indicated by the extent of respiratory complications. Significant damage to the diaphragm and respiratory pathway indicates a high vertebral level injury. Spinal injuries that occur higher on the vertebral column, specifically at the cervical level, result in full or partial tetraplegia. Damage as high as the C3 level will produce complete loss of motor and sensory function from the neck down with the requirement of a ventilator for breathing. Injuries occurring at lower levels in the vertebral column, such as the thoracic level, result in paraplegia. One may still have control over head, neck, and arm function, but may suffer complete loss of lower limb mobility and autonomic control.

The degree of functional loss following spinal cord injuries is often classified as complete or incomplete injuries. Individuals diagnosed as incomplete, reported as approximately 45% of all U.S. cases, will still maintain a degree of neurological activity below the site of injury. Thus, motor and sensory function may be retained at some level, allowing for a greater recovery. Those diagnosed with complete spinal injury, reported as approximately 55% of U.S. cases, will have a complete loss of sensory and motor function below the site of

injury, often accompanied by chronic autonomic irregularities (Figure 1.2) (NSCISC 2009).

## Injury Classification



**Figure 1.2** Classification of spinal cord injuries since 1988. 55% of reported SCI cases in the U.S. result in incomplete paralysis. 45% of reported cases result in complete paralysis. Chart is modified from the 2009 statistical data obtained from The National Spinal Cord Injury Association.

Primary spinal cord damage occurs from the mechanical forces upon initial impact that cause spinal tissue to abruptly shift, shearing neuronal and endothelial cell membranes (Tator and Fehlings 1991; Profyris, Cheema et al. 2004). The shifting of tissue caudal and rostral to the epicenter, or the impact zone, places the greatest stress at the center of the cord (Blight et al. 1986; Mautes et al. 2000). This creates a unique phenomenon where vessels and axons localized near the surface of the cord experience little trauma. However, the enriched microvascular environment of the central gray matter and the axons located close to the gray matter experience severe stretching and shearing (Blight et al. 1986; Mautes et al. 2000; Young 2002).

The central hemorrhage following damage encompasses the gray area of the epicenter and radiates into the surrounding white matter (Noble et al. 1989; Noble et al. 1996; Mautes et al. 2000). Regions extending in the caudal and rostral direction from the epicenter show hemorrhagic lesion localized primarily to the dorsal columns (Mautes et al. 2000). Despite the initial formation of a hemorrhagic lesion, there is very little gross damage to the spinal cord. However, during the secondary wave of injury, the hemorrhaging that occurred during the initial injury triggers a wave of further necrosis and apoptosis causing massive tissue degeneration and cellular death at and below the site of injury (Tator et al. 1991).

In the first few hours of the secondary wave, tissue surrounding the primary lesion site appears pale due to edema response to injury. This surrounding tissue undergoes necrosis, further expanding the lesion site evolving

into a cyst cavitations bordered by glial scar tissue (Guth et al. 1999; Beattie et al. 2002). By the eighth week, the spinal lesion has localized to the dorsal columns (Guth et al. 1999; Basso et al. 2002). The cystic lesion has now extended caudal and rostral to the epicenter (Profyris et al. 2004). At the lesion site, there is a persistent neutrophil infiltration and glial response that prevents any arborization of blood vessels. However, this event in the dorsal columns attenuates over the period of a week. Macrophage presence gradually disappears by eight weeks and any cyst-like cavitation has receded to the rim of the lesion, allowing dense innervations of blood vessels and regenerated nerve fibers into the dorsal columns (Guth et al. 1999).

The histopathological changes observed in the dorsal columns may stem from Wallerian degeneration of axons around the site of injury (Dusart and Schwab 1994; Guth, Zhang et al. 1999). Following spinal trauma, axonal swelling and myelin rupturing continue for approximately 24 hours, expelling axonal debris into the extracellular space (Profyris et al. 2004). Accumulation of organelles in axonal bulb has been documented, possibly due to disruption and blockage of axoplasmic flow (Anthes et al. 1995). Rupturing of axonal membranes and leakage of organelles leads to a low intra-axonal volume. This low volume allows myelin to collapse upon itself resulting in axonal invagination (Balentine 1988; Anthes et al. 1995). Tissue compression, red blood cell damage, and blood-brain-barrier breakdown within the spinal tissue creates an ischemic environment that is conducive to cell death (Mauter et al. 2000).

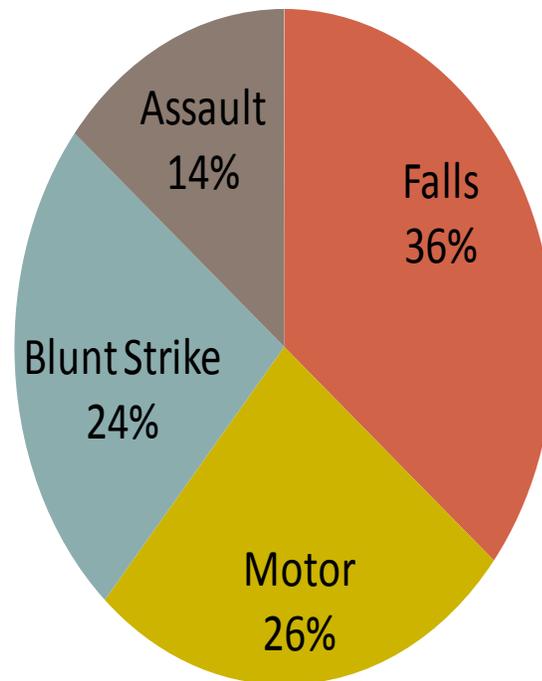
These waves of necrotic to apoptotic cascades proceed in a unique pathological track away from the site of injury. White matter, extending caudal from the impact site, becomes the primary target for apoptosis (Crowe et al. 1997). Apoptotic death not only effects neurons, but non neuronal cells, specifically oligodendrocytes, become a prime targets in the late stages of delayed death (David 2002; Profyris, Cheema et al. 2004). The loss of oligodendrocytes deprives neurons of trophic support resulting in impaired myelination of injured axons. Another devastating consequence of oligodendrocyte death is the loss of spared, healthy axons around the injury site and in remote areas (Blight et al. 1986; Crowe et al. 1997). Subsequently, both ascending and descending axons undergo Wallerian degeneration of axons, a process in which axonal cytoskeleton disintegrates and disruption of the axonal membrane (David 2002; Profyris et al. 2004).

### *Pathology of Traumatic Brain Injury*

In the United States of America, there is an estimated 1.5 million head injuries each year (braintrauma.org, CDC 2007). Traumatic brain injury is the leading cause of death in children and adults with approximately 52,000 deaths is the result from a sudden trauma to the head (Rutland-Brown et al. 2006; Prevention 2007). Some of the most common causes of head trauma are falls, motor crashes, and sports injuries (Figure 2) (Langlois et al. 2006; Prevention 2007). In addition, traumatic brain injuries have become a critical concern amongst active duty soldiers serving in war zones. It was reported by Veteran's

advocates that an estimated 10 to 20% of Iraqi veterans display some gradient of brain injury (Prevention 2007).

## Causes of Traumatic Brain Injury



**Figure 2** Causes of Traumatic Brain Injury in the United States. Approximately 36% of all TBI cases in the U.S. result from common falls. Motor accidents make up 26% of all reported TBI cases. Blunt strikes to the head make up 24% of all TBI cases while 14% of all TBI cases result from violent assaults. Graph modified from 2007 statistical data collected by the The CDC National Center for Injury Prevention and Control.

The forces that damage the brain can range from a focal contusion, directional acceleration and deceleration of the cranium, or blast-induced concussions. Focal contusion based injuries are the result of a blunt force directly to the cranium. With focal contusion, there may be a fracturing of the skull, tissue deformation, vascular disruption and subsequent hemorrhaging (Gaetz 2004). However, a direct force to the head is not necessary for producing TBI. The sheering forces from violent head movement throughout space can produce severe brain injury (Ommaya et al. 1974; Varney et al. 1995; Gieron et al. 1998). Forceful motion of the head in a rotational or forward and backward motion induces a sheering of blood vessels and neuronal components (Ommaya et al. 1974; Gaetz 2004). The initial impact produces an injury gradient with damages seen at cortical levels and, depending on severity, spreading throughout subcortical areas (Pettus et al. 1994; Gaetz 2004). Severe blasts, such as that induced by high powered weapons or explosives, produces dramatic pressure changes causing rupturing and sheering of organs with tertiary damage caused by propagation of the blast wave throughout the body (Mayorga 1997; Elder and Cristian 2009).

The observable symptoms of any trauma to the head range from mild to severe. Mild symptoms include headache, confusion, fatigue, and blurred vision. More sever symptoms includes convulsions, numbness in the extremities and loss of consciousness. The focus of this chapter will emphasize focal contusion pathology, as the model used for experimentation is contusion-induced brain deformation.

A pathological hallmark in acute brain contusion is vascular damage from deformation of the skull (Davis 2000). Hemorrhagic lesions following contusion are classified as extradural and intradural hematomas (Davis 2000). Extradural hematomas are caused by the fracturing of the skull with subsequent shearing of dural matter directly beneath the skull along with the vasculature network (Davis 2000). Intradural hematomas can occur without fracturing of the skull and are typically induced by the acceleration and deceleration of the brain from contusions and are more indicative of secondary or delayed damage (Davis 2000). Occlusion of subdural veins from dramatic shifting forces is a hallmark of an intradural injury called subdural hematoma. Another form of intradural injury is intracerebral hemaetoma, characterized as a collective swelling or clotting from extending into white matter, corpus callosum, and basal ganglia (Davis 2000). Auto regulation of cerebrovascular flow is critical for vascular dilation and constriction in response to cranial pressure (Werner et al. 2007). Primary mechanical damage often leads to impaired auto regulation of cerebrovascular flow, creating an ischemic like environment (Hermanns et al. 2001).

Vascular damage is the primary pathological outcome seen in the initial stages of brain injury. The ischemic environment created by the initial vascular damage triggers further waves of deleterious events. Peripheral damage may be initiated from peri-infarct depolarization, a global depolarization phenomenon occurring in both neuronal and glial cells in the penumbra (Nedergaard 1988; Church and Andrew 2005). Cortical spreading depression (CSD), though typically not involved in tissue damage, may actually contribute to additional cell loss

following contusion as observed in recent electrophysiological studies (Church and Andrew 2005; Fabricius, Fuhr et al. 2006; Hartings, Gugliotta et al. 2008).

In TBI, secondary neurodegeneration has been reported in several areas adjacent to and quite distal from the impact site following experimental brain injury (Hall et al. 2005; Hall et al. 2008). Depending on the severity of injury, tissue damage may be observed in sub regions of the hippocampal formation, the thalamus, and dorsal regions of the striatum over a 48 hour period (Dixon, Kochanek et al. 1999; Hall, Sullivan et al. 2005; Hall, Bryant et al. 2008).

Extensive axonal damage has also been documented in sub regions of the hippocampus, the internal capsule, thalamic nuclei, and as distal as regions in the cerebellum and brain stem (Lighthall et al. 1990; Hall et al. 2005). These observations were reported at later times, from 24 hours to 7 days, following cortical contusion suggesting a diffuse mechanism of axonal degeneration that is characteristic of secondary pathology (Lighthall, Goshgarian et al. 1990; Hall, Sullivan et al. 2005). Mechanisms contributing to these secondary events will be discussed in greater detail below.

Following TBI, the ischemic environment from vascular damage gives way to the secondary cascade of cellular damage triggered by abnormal levels of extracellular glutamate (Arundine and bTymianski 2004; Gaetz 2004). As discussed earlier, massive depolarization and subsequent  $Ca^{2+}$  homeostasis disruption propagate a cycle of secondary cell death (Choi 1988; Fiskum 2000; White, Sullivan et al. 2000; Arundine and Tymianski 2004; Gaetz 2004).

### *Cell Death in Traumatic Spinal Cord and Brain Injury*

This introduction has so far discussed much of the vascular damage from primary injury and the spatial and temporal characteristics of tissue damage from secondary damage. A more detailed discussion of the cellular events effected and contributing to secondary damage is critical as it emphasizes key mechanism targeted by current therapeutic drugs. Though, both necrotic and apoptotic events are apparent during secondary damage, the distinction is more complex than often reported as both events can occur in parallel within the same cell (Gaetz 2004).

The history of excitotoxicity by various excitatory amino acids has long been established as detrimental force in neuronal death. Glutamate, specifically, has shown to be intensely involved in acute and delayed neurological trauma (Olney 1971; Choi 1988; Goldberg, Monyer et al. 1988; Panter, Yum et al. 1990; Arundine and Tymianski 2004). Massive depolarization, often in response to the ischemic environment induced from primary damage, triggers further neuronal glutamate release, leakage of intracellular glutamate, and dysfunctional glutamate uptake (Mills, Fullwood et al. 2001; Arundine and Tymianski 2004; Gaetz 2004). Collectively, these events propagate a detrimental loop of glutamate release and autocrine-like stimulation of the same cell. Furthermore, abnormal levels of extracellular glutamate stimulate and release inhibition of glutamate-specific channels, specifically *N*-methyl-D -aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (Kwong et al.) and kainite (Doble 1999; Profyris, Cheema et al. 2004). What follows is further disruption in

ionic balance by large influxes of Na into the cell (Doble 1999; Profyris, Cheema et al. 2004) . The cell is now subjected to increased water influx and the fatal occurrence of cell lysis (Doble 1999; Profyris, Cheema et al. 2004). It is a collective effort of ion imbalance that triggers disruption of a cell's intrinsic machinery. However, elevated  $\text{Ca}^{2+}$  in both the extracellular and intracellular environment has historically been the subject of intense research in the field of brain and spinal trauma (Maynard et al. 1977; Happel et al. 1981; Banik et al. 1984; Seliger et al. 1989; Gelderd et al. 1990; Moriya et al. 1994; Kampfl et al. 1996; Rzigalinski et al. 1998; Weber et al. 1999; Bondarenko et al. 2001; Farkas et al. 2005; Nehrt et al. 2007).

The massive depolarization observed in secondary injury is accompanied by large influxes of  $\text{Ca}^{2+}$  through voltage gated  $\text{Ca}^{2+}$  channels (Profyris et al. 2004). Inside the cell, increasing  $\text{Ca}^{2+}$  concentrations actually mobilizes intracellular stores of  $\text{Ca}^{2+}$  that activate several deleterious  $\text{Ca}^{2+}$  dependent enzymes and disrupt mitochondrial function (Banik, Hogan et al. 1982; Moriya, Hassan et al. 1994; Doble 1999; Sullivan, Krishnamurthy et al. 2007). Perhaps the seminal event in death of neurons and non neuronal cells during secondary neurological injury is  $\text{Ca}^{2+}$  induced permeability of the mitochondria. The following section will address the role of mitochondria in cell vitality and how the influx of  $\text{Ca}^{2+}$  following injury can compromise mitochondria function.

### *Mitochondrial Dysfunction in Traumatic Spinal Cord and Brain Injury*

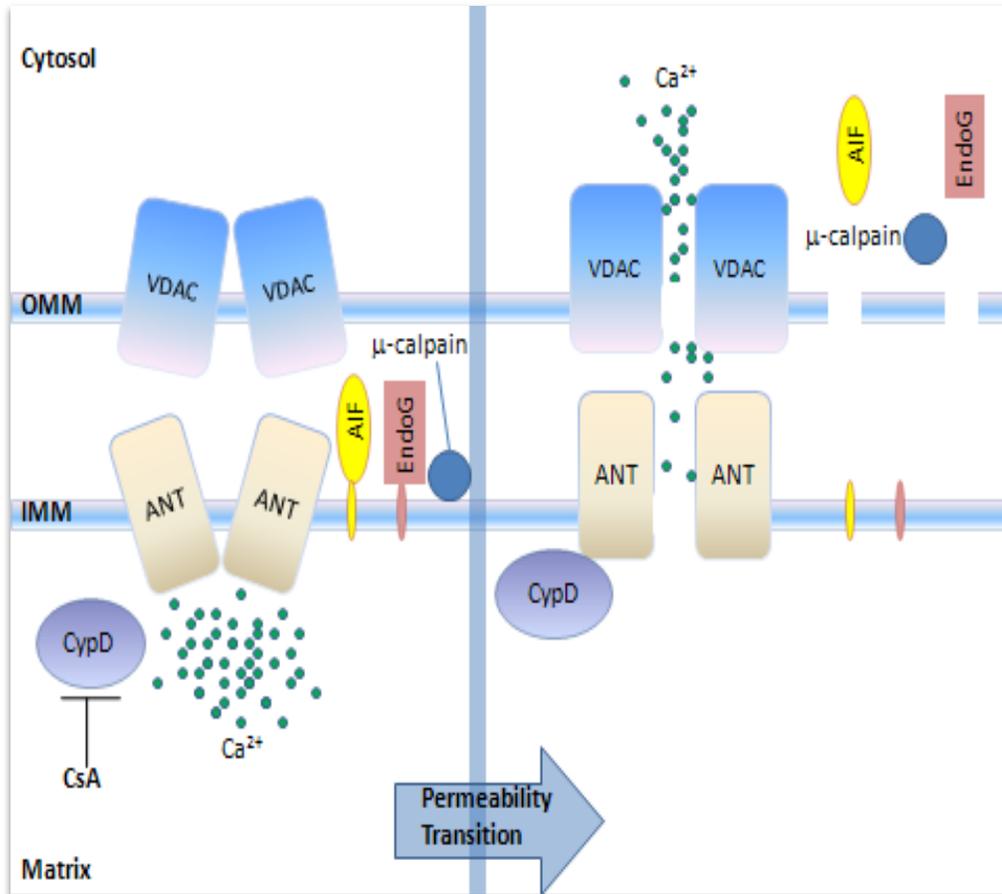
Survival and development of all cells is largely dependent on the bioenergetics of the mitochondria. These powerhouse structures are critical in the metabolism of energy sources and feature apoptotic pathways involved in development and cellular trauma (Fiskum 2000; Wieloch 2001; Sullivan, Rabchevsky et al. 2005). Much of neuronal activity, such as the demanding plasma ion pumps, is almost exclusively dependent on adenosine triphosphate (ATP). Tissue stores and anaerobic glycolysis only provide brief supplies ATP. However, the mitochondria, through breakdown of its dominant substrate pyruvate, is able to generate a rapid and abundant supply of ATP and ATP turnover is the mediating factor in mitochondrial respiration (Budd and Nicholls 1998; Nicholls and Ward 2000). Mitochondria are also critical to neuronal function due to their ability to sequester calcium ions, a function critical during neuronal hyperactivity (Ichtas and Mazat 1998; Rizzuto, Pinton et al. 1999; Nicholls and Ward 2000). Under physiological conditions, transport of  $\text{Ca}^{2+}$  into the mitochondria is dependent on the presence of an electrogenic transporter, the  $\text{Ca}^{2+}$  uniporter, as opposed to dependence on ATP hydrolysis (Ichtas and Mazat 1998; Rizzuto, Pinton et al. 1999; Bano, Young et al. 2005). The  $\text{Ca}^{2+}$  uniporter facilitates ion diffusion in the direction of its electrochemical gradient. This calcium release is facilitated by exchange with the  $\text{Na}^+ / \text{Ca}^{2+}$  antiporter. Together with the  $\text{Na}^+ / \text{H}^+$  antiporter, a slow, continuous cycling of  $\text{Ca}^{2+}$  across the mitochondrial inner membrane is established (Crompton et al. 1998; Crompton 1999; Sullivan et al. 2005). Increases in cytosolic  $\text{Ca}^{2+}$  can trigger further release

of  $\text{Ca}^{2+}$  from neuronal intracellular compartment known as the smooth endoplasmic reticulum. Ryanodine receptors, located on smooth endoplasmic reticulum, and the second messenger metabolite inositoltriphosphate<sup>3</sup> are sensitive to intracellular calcium levels and respond by releasing stored calcium (Bardo et al. 2006). This excessive rise in intracellular  $\text{Ca}^{2+}$  can compromise mitochondrial function (Nicholls 1978; Crompton 1999). Pathological conditions can facilitate large increases in extracellular  $\text{Ca}^{2+}$ , ultimately leading to mitochondrial matrix  $\text{Ca}^{2+}$  overload and subsequent increased mitochondrial permeability (mPT) (Hunter, Haworth et al. 1976; Hunter and Haworth 1979; Szabo and Zoratti 1992; Bernardi and Petronilli 1996).

#### *Mitochondrial Permeability Transitional Pore*

Mitochondrial permeability (mPT) is defined as a rapid increase in the permeability of the inner mitochondrial membrane space to solutes with molecular masses less than 1500 Daltons (Hunter and Haworth 1979; Nicolli, Basso et al. 1996; Sullivan, Rabchevsky et al. 2005). Increased permeability of the mitochondria results in a swelling phenomenon. Crompton and colleagues attributed this swelling to a high concentration of protein debris left in the inner membrane following passage of solutes (Crompton 1999; Halestrap, McStay et al. 2002). Early studies hypothesized that mitochondrial invertebrate permeability was another means of  $\text{Ca}^{2+}$  transport, specifically an ionic efflux mechanism (Gunter et al. 1990). However, extensive studies revealed this not to be the case as the increased permeability was due to the formation of a

nonselective megachannel (Bernardi and Petronilli 1996; Sullivan, Rabchevsky et al. 2005). Furthermore, unregulated opening of this megachannel, termed the mitochondrial permeability transition pore (mPTP), was found to be a driving mechanism behind apoptotic and necrotic cell death (Szabo and Zoratti 1991; Szabo and Zoratti 1992; Crompton 1999; Shimizu, Narita et al. 1999; Fiskum 2000). Formation and opening of the mPTP trigger a series of detrimental events that ultimately disrupt mitochondria bioenergetics (Figure 2). This is a pathological hallmark of necrotic cell death, such as that observed under periods of ischemia/reperfusion (Kerr, Suleiman et al. 1999; Kim, He et al. 2003; Leung, Varanyuwatana et al. 2008).



**Figure 3** Simplified illustration of mPTP complex. CypD binds to the ANT/VDAC complex initiating mPTP formation and release of  $\mu$ -calpain, AIF, and EndoG. CsA inhibits CypD binding and subsequent mPTP formation. Diagram courtesy of Dr. James Geddes.

Swelling of the mitochondrial IMM and subsequent membrane permeability results in decay of the inner membrane potential and subsequent uncoupling of the electron transport system. Dramatic increases in  $\text{Ca}^{2+}$  concentrations give rise to reactive oxygen species (ROS), exposing the electron transport chain to free radicals that disrupt oxidative phosphorylation. This breakdown of oxidative phosphorylation actually leads to hydrolyses of ATP by reversal of ATPase, the enzymatic machinery responsible for translocating protons across the inter-membrane (Szabo, Bernardi et al. 1992; Zoratti and Szabo 1995; Halestrap, Clarke et al. 2004; Forte, Gold et al. 2007). Ultimately, there is a decrease in ATP production as a result of adenine nucleotide depletion along with increased phosphate, and subsequently pH (Fiskum 2000; Halestrap, Clarke et al. 2004). These events sensitize the opening of the mPTP which mediates further cell damage by release of several degrading enzymes (Ding, Shen et al. 2002; Ferrand-Drake, Zhu et al. 2003; Kim, He et al. 2003).

mPTP formation and opening has also been implicated in apoptosis. Recent reports have suggested that mPTP can transition from necrotic induced cell death to a mediator of apoptotic pathways (Kim, He et al. 2003; Kim, He et al. 2003). Apoptotic stimuli, such as members of the pro apoptotic family of Bcl-2 stimulate opening of the mPTP (Crompton 1999; Kroemer and Reed 2000; Halestrap, McStay et al. 2002). The increased permeability of the mitochondrial outer membrane permits release of such apoptotic factors as cytochrome C, apoptosis inducing factor (AIF), and Smac/Diablo from the mitochondrial inter-

membrane (Crompton 1999; Annis, Zamzami et al. 2001; Halestrap, McStay et al. 2002).

The emerging role of mPTP in pathological events produced an intensive search for the components that constitute the megachannel. The three best characterized components to date are the adenine nucleotide translocase, the voltage dependent anion channel, and cyclophilin D. Though recent reports have uncovered a more complex makeup of the mPTP than originally documented, the following section will review the three basic components mentioned above.

*The adenine nucleotide translocase (ANT)* is a 30 kDA protein located exclusively to the inner membrane of the mitochondria (Brustovetsky and Klingenberg 1996; Crompton, Virji et al. 1998; Vieira, Haouzi et al. 2000). The ANT exhibits many operations characteristic of the mPTP. Upon exposure to high concentrations of  $Ca^{2+}$ , the ANT undergoes a conformational change from a selective antiporter to a nonselective pore (Brustovetsky and Klingenberg 1996; Crompton 1999). (Crompton, Virji et al. 1998; Crompton 1999). (Al-Nasser et al. 1986). The normal role of the ANT is the translocation of adenine nucleotides, specifically, ATP and ADP across the inner mitochondrial membrane, which serves as a proton-driven co-transport. This translocation of ATP and ADP allow for charge compensation to the calcium mediated increase in matrix volume, an effect by  $K^+$  and subsequent water entry (Haworth and Hunter 1980; Halestrap, McStay et al. 2002). Nucleotides that interact with ANT are limited to only ADP, dADP, and ATP and when bound in the c-site, induce an active channel

conformation (Woodfield, Ruck et al. 1998). Recently, it has been documented that a range of pro-apoptotic proteins are able to bind to ANT, including Bcl-2 and Bax (Marzo, Brenner et al. 1998). Previously research has shown that Bax binding cooperates in mPTP opening (Marzo, Brenner et al. 1998; Kowaltowski 2000). In addition, cells over expressing Bcl-2 allowed for greater mitochondrial  $Ca^{2+}$  capacity, attenuation in mitochondrial membrane potential, and generation of a higher reduction state, opposing the increases oxidative state induced by mPTP opening (Murphy, Bredesen et al. 1996; Vieira, Haouzi et al. 2000). While these characteristics demonstrate that ANTs are crucial in mPTP formation, recent literature has demonstrated that mPTP is still functional in ANT knock out models (Kokoszka et al. 2004). Thus, other components forming the mPTP were investigated to determine those critical in pore regulation.

*Voltage Dependent Anion Channel (VDAC)*. The release of apoptogenic factors, such as cytochrome C and AIF, from mitochondria demonstrate increased permeability of the outer membrane (Priault, Camougrand et al. 1999; Tsujimoto and Shimizu 2000). It is now widely accepted that pro-apoptotic members of the Bcl-2 family can directly control mitochondrial permeability during apoptosis (Priault, Chaudhuri et al. 1999; Shimizu, Narita et al. 1999; Tsujimoto and Shimizu 2000). Further investigations showed a highly conserved barrel protein spanning the outer membrane, the voltage dependent anion channel (VDAC), that serves as a mitochondrial highway for all entering and exiting metabolites (Lemasters et al. 2006). The channel is voltage dependent in that both positive

and negative potentials close the channel. VDAC also demonstrates ion selectivity when in an open conformation (Colombini 1983; Lemasters and Holmuhamedov 2006).

Open conformation of the VDAC is critical for release and uptake of respiratory substrates, specifically ATP and ADP that facilitate mitochondrial function (Vander Heiden, Li et al. 2001). As discussed earlier, interaction of proapoptotic factors with VDAC facilitates mitochondrial dysfunction. Recent reports revealed that interaction of pro-apoptotic Bcl members close VDAC, prohibiting mitochondrial respiration (Vander Heiden, Li et al. 2001). VDAC closure leads to increased outer membrane permeability and translocation of cytochrome c (Rostovtseva, Antonsson et al. 2004). These events can occur either independently or collectively with mitochondrial inner membrane permeability and subsequent collapse of membrane potential (Priault, Camougrand et al. 1999; Tsujimoto and Shimizu 2000). Though several reports have implicated VDAC in apoptotic induced mitochondrial dysfunction, there is still uncertainty in its involvement in mPTP formation. Similar to ANT, knock-out models of VDAC did not affect mPTP formation and VDAC closure does not attenuate  $Ca^{2+}$  flux (Basso, Fante et al. 2005; Rostovtseva, Tan et al. 2005). Further investigations into a mediating component of the mPTP unveiled a potential candidate from cyclophilin family called cyclophilin D (cypD).

*Cyclophilin D.* It was discovered that opening of the mPTP could be inhibited specifically by sub-micro molar concentrations of the immunosuppressive drug,

cyclosporin A (CsA)(Crompton, Ellinger et al. 1988; Nicolli, Basso et al. 1996; Kowaltowski, Smaili et al. 2000). Specifically, these early studies revealed that CsA inhibited the activities of peptidyl-prolyl-cis-trans-isomerase (PPIase) located in the mitochondria matrix (Crompton et al. 1988). This PPIase was identified as CypD (Crompton et al. 1988).

Cyclophilins display catalytic properties by refolding denatured proteins and importing the reformed proteins into organelles, as evident by a retarded rate of protein folding in cyclophilin mutant yeast mitochondria (Lin, Hasumi et al. 1988; Lithgow, Horst et al. 1995; Matouschek, Rospert et al. 1995). In addition, cyclophilins are recruited to fully functional protein complexes (Wu, Matunis et al. 1995; Crompton, Virji et al. 1998; Jaschke, Mi et al. 1998). Several reports showed that CypD binds to the ANT/VDAC complex, confirming CypD as a component for mPTP configuration (Crompton, Virji et al. 1998; Woodfield, Ruck et al. 1998; Halestrap, McStay et al. 2002; Leung and Halestrap 2008; Leung, Varanyuwatana et al. 2008). Evidence points to CypD's catalytic properties as a potential mechanism for proper folding of pore component aggregates, resulting in mPTP formation (Connern and Halestrap 1994; Connern and Halestrap 1996; Leung, Varanyuwatana et al. 2008). It is important to note that new research suggest that another pore component consisting of a phosphate carrier, PiC, is the primary target of CypD induced conformational change as opposed to ANT (Leung et al. 2008). This demonstrates the ever emerging complexity of the mPTP.

Both pharmaceutical inhibition of CypD and knock out of the CypD gene *Ppif* have shown modest protection in animal models of neurotrauma. CsA and its analog NIM811 showed modest improvement in tissue and behavioral assessment following traumatic brain injury and spinal cord injury (Sullivan, Rabchevsky et al. 2000; McEwen, Sullivan et al. 2007; Ravikumar, McEwen et al. 2007; Mbye, Singh et al. 2008). Other pathological states, such as muscle denervation, multiple sclerosis, and oxidative induced hippocampal damage, all illustrated the role of CypD as a potent mediator of cell damage (Khaspekov, Friberg et al. 1999; Csukly, Ascah et al. 2006; Forte, Gold et al. 2007). However, the protective effects produced from CypD inhibition are not universal to all deleterious pathways. Several reports have shown that the efficacy of targeting CypD may only be protective under necrotic events. Cell death induced by reactive oxygen species or  $Ca^{2+}$  overload, such as that observed in ischemic conditions, was attenuated by CypD ablation (Baines, Kaiser et al. 2005; Nakagawa, Shimizu et al. 2005; Schneider 2005). Apoptotic stimuli, such as members of the Bcl-2 family, induce cell death regardless of CypD inhibition (Baines, Kaiser et al. 2005; Basso, Fante et al. 2005; Nakagawa, Shimizu et al. 2005).

#### *Calpain and Traumatic Spinal Cord and Brain Injury*

The disruption of  $Ca^{2+}$  homeostasis and subsequent mitochondrial function can unleash a host of enzymatic activity that further mediates cell damage. MPTP opening is a driving force behind necrotic death, however,

closure of this channel may only divert the necrotic pathway to apoptosis (Nicotera and Leist 1997; Halestrap, McStay et al. 2002). Increased permeability of the outer membrane allow leakage of inner membrane factors such as apoptosis inducing factor (AIF), cytochrome c, and Smac/Diablo (Kroemer and Reed 2000; Loeffler, Daugas et al. 2001; Halestrap, McStay et al. 2002; Ferrand-Drake, Zhu et al. 2003). Additionally, members of the cysteine protease family are also activated and mobilized. Caspases mediate apoptosis by disruption of DNA repair, mRNA splicing, and cytoskeletal breakdown and reorganization (Kermer, Klocker et al. 1999; Kermer, Klocker et al. 1999). Other members of the cysteine protease family are  $\text{Ca}^{2+}$  activated proteolytic enzymes called calpains. The following section will discuss in greater detail the structure and physiological functions of calpains.

To date, approximately 15 calpain isoforms have been identified in humans. The typical calpains are ubiquitously expressed while atypical calpains are tissue specific (Goll et al. 2003). Many of these isoforms have prominent roles in such pathological conditions as diabetes, muscular dystrophy, and brain and spinal injury (Banik, Shields et al. 1998; Huang and Wang 2001; Zatz and Starling 2005; Deng, Thompson et al. 2007; Liu, Liu et al. 2008). Two of the most extensively characterized isoforms are the typical calpains  $\mu$ -calpain and m-calpain. The classifications are based on their large catalytic subunits calpain 1 and calpain 2 (Goll et al. 2003; Bevers et al. 2007). In addition to their distinct catalytic subunits, these two isoforms share a common regulatory small subunit (Cong et al. 1989; Bevers et al. 2007). Both isoforms are regarded as cytosolic

enzymes, though recently  $\mu$ -calpain was found to be expressed in the mitochondria inner membrane (Badugu et al. 2007). One of the distinguishing characteristics amongst  $\mu$ -calpain and m-calpain is the concentrations of  $\text{Ca}^{2+}$  required for activation (Goll et al. 2003). For example, m-calpain requires approximately 1 to 20  $\mu\text{M}$  concentration whereas 250 to 750  $\mu\text{M}$  concentration of  $\text{Ca}^{2+}$  are required for  $\mu$ -calpain (Zimmerman et al. 1984; Goll et al. 2003; Li et al. 2004).

Several proteins are substrates for calpains such as cytoskeletal structural proteins, cell motility proteins, and calcium dependent enzymes (Posmantur et al. 1994; Pang et al. 1996; Azam et al. 2001). Both  $\mu$ - and m-calpain undergo autolysis in the presence of  $\text{Ca}^{2+}$  (Cong, Goll et al. 1989; Edmunds, Nagainis et al. 1991; Saido, Nagao et al. 1992). SDS-PAGE revealed that autolysis of both calpain isoforms resulted in a reduced mass of the 80 kDa subunit to 76 kDa and the 28 kDa subunit to 18 kDa (Graham-Siegenthaler et al. 1994). As a result of calpain autolysis, specifically the large subunit, the  $\text{Ca}^{2+}$  concentration for half-maximal proteolytic activity is reduced with no consequential affect of enzyme specificity (Edmunds, Nagainis et al. 1991; Goll, Thompson et al. 2003; Li, Thompson et al. 2004). Calpains can function under a controlled state during normal physiological events. Calpain targets several substrates for enzymatic degradation such as cytoskeletal structural proteins, cell motility proteins, and other calcium dependent enzymes. For example, calpain mediated degradation of spectrin, a cytoskeleton protein, allows for synaptic

remodeling, a critical function in long term synaptic potentiation and memory storage (Luscher et al. 2000; Liu et al. 2008).

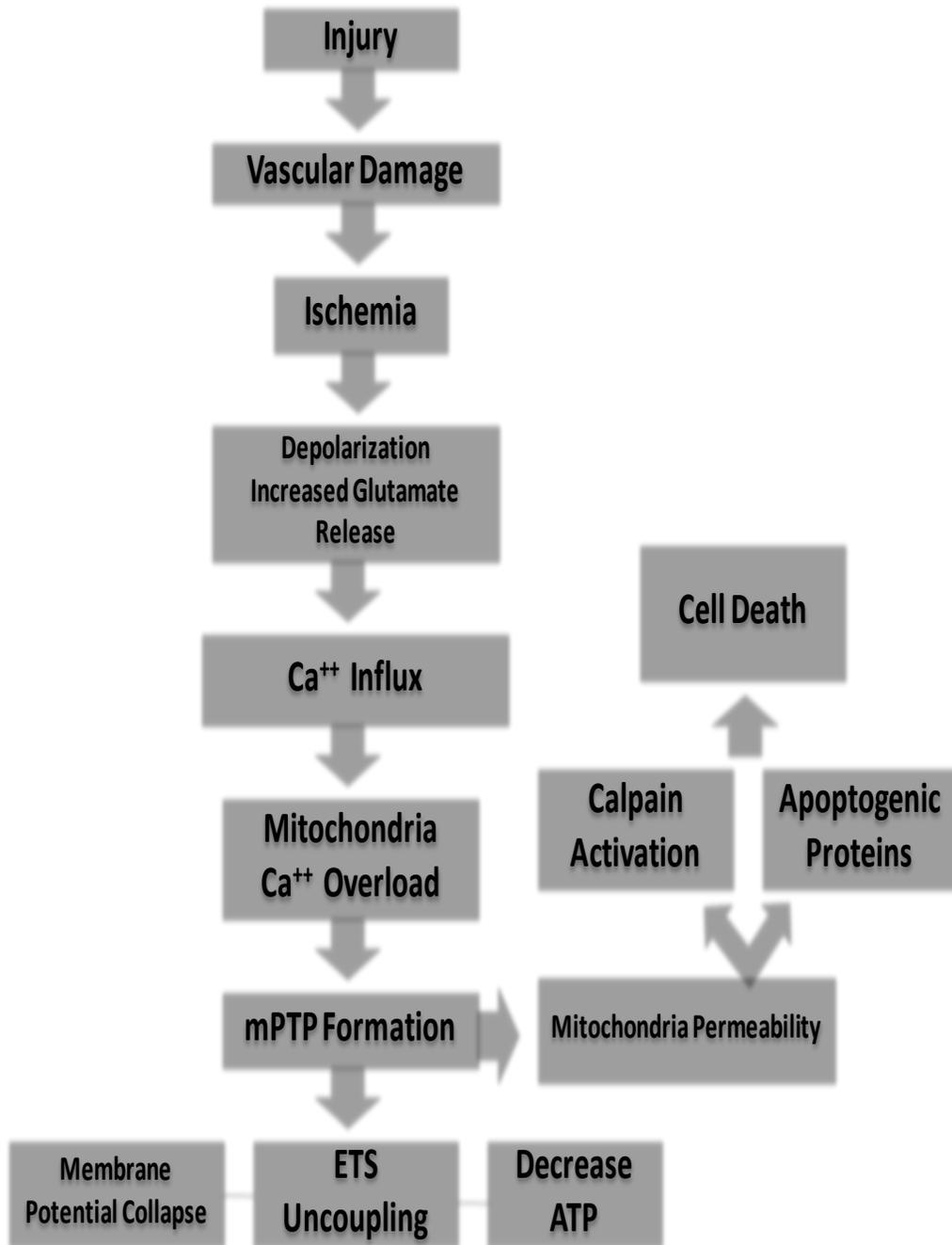
Calpain can be endogenously regulated by Calpastatin (Emori et al. 1987; Murachi 1989; Wendt et al. 2004). Calpastatin has multiple inhibitory domains and is capable of binding to several molecules of calpain in a  $\text{Ca}^{2+}$  dependent manner. (Wendt et al. 2004). The  $\text{Ca}^{2+}$  requirement for endogenous inhibition is less than that for calpain activation (Kapprell et al. 1989; Wendt et al. 2004). However, under pathological conditions, calpastatin inhibition is reversible and can lead to unregulated activation of calpain (Arrigoni et al. 1991; Springer et al. 1997; Tidball et al. 2002; Crocker et al. 2003; Bevers et al. 2007).

The dramatic increase in  $\text{Ca}^{2+}$  due to excitotoxic events may lead to a sustained activation of calpain. Neuronal cell bodies and dendrites consist of a vast network of microtubules, which maintain morphological stability. One of the critical proteins that constitute the microtubule structure is microtubule-associated protein (MAP-2). MAP-2 is highly susceptible to calpain degradation (Saatman et al. 1998; Goll et al. 2003; Liu et al. 2008). Post synaptic bulbs and axonal components are also vulnerable to calpain degradation. Alpha-II spectrin and neurofilaments (NF) are enriched in the axonal and post synapse cytoskeleton. Both of these proteins are also substrates of calpain (Dutta et al. 2002; Goll et al. 2003). Further perpetuating the detrimental cycle of excitotoxicity is calpain's targeting of glutamate receptors. Cleavage and subsequent truncation of several of the ionotropic receptors allows for further uncontested  $\text{Ca}$  entry into the cell (Minger et al. 1998; Simpkins et al. 2003).

Elevated calpain activity has been well documented as major contributor to secondary tissue damage in both spinal cord and brain injury. In spinal cord injury, early research, especially that of Banik and colleagues showed that calpain activity induces much of the structural damage in cell bodies and axons following spinal injury (Banik et al. 1998). These studies demonstrated that a calcium activated proteinase contributed to early losses in neurofilament proteins followed by granular axonal degeneration and myelin loss at later time points following spinal cord lesion (Banik, Hogan et al. 1982; Banik, Hogan et al. 1984; Banik, McAlhaney et al. 1985). Likewise, early studies characterized localization of calpain-1 and calpain-2 isoforms in brain regions that are highly susceptible to degeneration (Siman et al. 1985; Simonson et al. 1985; Hamakubo et al. 1986).

Calpain has since become a promising target for attenuation of secondary damage in spinal cord injury. A study by Springer et al., showed that pretreatment with Riluzole, an inhibitor of glutamate release, reduced loss of MAP2 levels in rats following spinal injury (Springer et al. 1997). Several classes of calpain inhibitors have provided modest protection when administered either pre injury or post injury. Calpeptin, CEP-4143, and MDL-28170, are some of the calpain inhibitors that demonstrated potential therapeutic effects.(Ray, Shields et al. 1999; Schumacher, Eubanks et al. 1999; Schumacher, Siman et al. 2000; Zhang, Bondada et al. 2003; Yu and Geddes 2007). CEP-4143 demonstrated calpain isoform specificity in its ability to target $\mu$ -calpain. Morphometric assessment of tissue sparing, survival of neurons in the red nucleus, and behavioral deficits were protected or improved when rats were pretreated with

CEP-4143 (Schumacher, Siman et al. 2000). Studies using single and sustained administration of MDL28170 showed improvements in tissue sparing and motor behavior (Hung, Hwang et al. 2005; Yu and Geddes 2007). Although not discussed here in detail, calpain has also been implemented in secondary damage following traumatic brain injury (Posmantur et al. 1994; Farkas et al. 2005; Liu et al. 2006; Thompson et al. 2006; Deng et al. 2007).



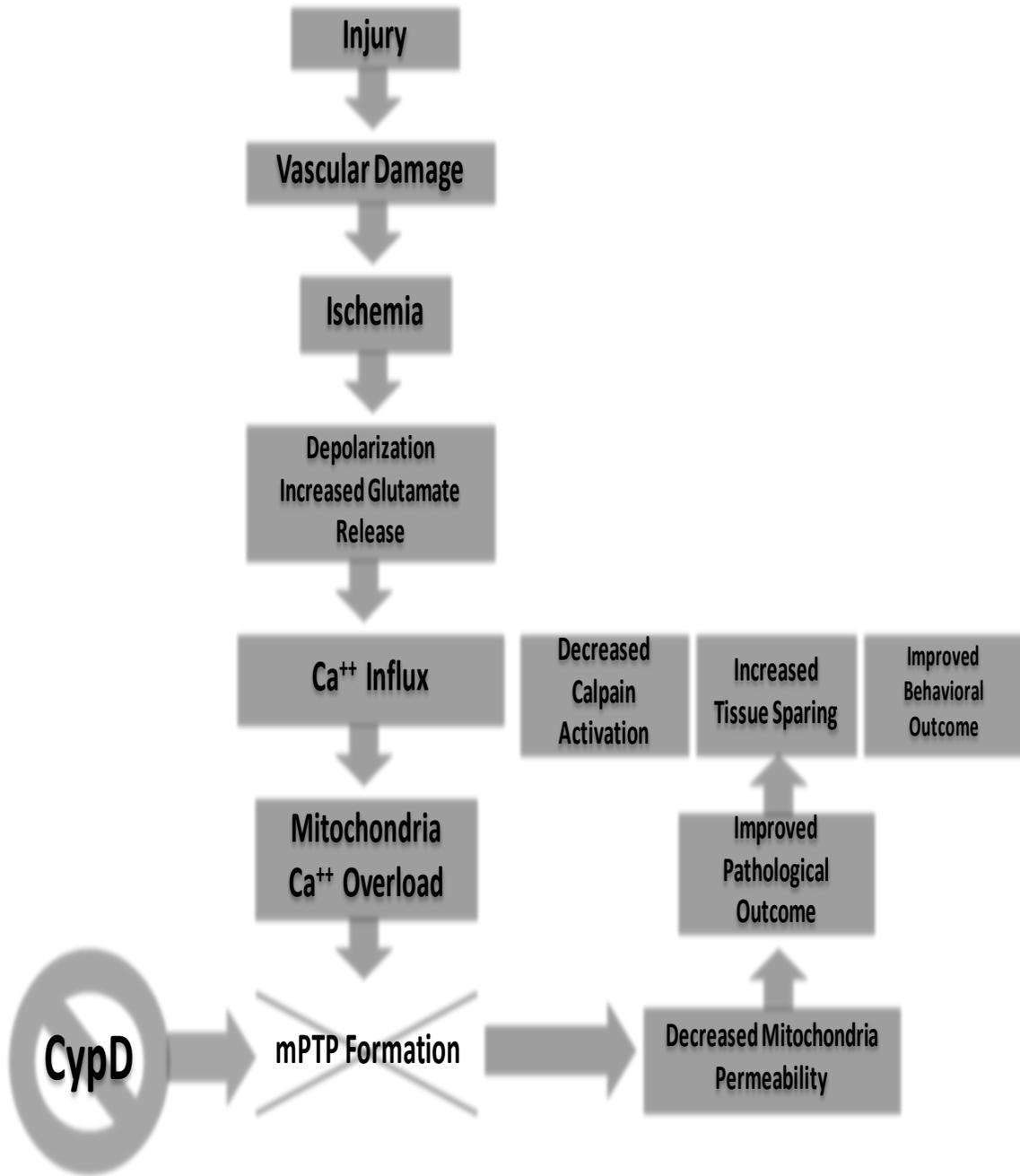
**Figure 4.1.** Simplified model of secondary injury cascade following traumatic spinal cord and brain contusion. Ischemic conditions can lead to  $\text{Ca}^{2+}$  induced mPTP formation and a compromise of mitochondrial function.

The centralized hypothesis of the current study is that cyclophilin D null mice (CypD-null) will show improved pathological outcomes following spinal cord and cortical contusion. Previous literature has shown that pharmacological inhibition of CypD improved pathological outcomes in rodent models of cortical contusion. However, the protective effects of CypD have produced confounding results following spinal cord contusion. Thus, it is uncertain if these discrepancies are due to variances in pharmacological properties or regional variations in the actions of CypD. By utilizing mice with a homozygous knockout of CypD (*encoding gene Ppif*), we can directly assess the role of CypD in secondary spinal and cortical injury. Our working model for the centralized hypothesis is that ablation of CypD will attenuate formation of mPTP formation, ultimately improving motor impairments, tissue sparing, and proteolytic enzyme activation induced by secondary damage that follows the both spinal cord and cortical contusion (Figure 4.2). To test our hypothesis, we constructed the following specific aims:

**Specific Aim One** will test the hypothesis that CypD-null mice will show improved motor function recovery following spinal contusion. The Basso Mouse Scale will be used to assess changes in open field motor function.

**Specific Aim Two** will test the hypothesis that CypD-null mice will show increased tissue sparing following spinal contusion and cortical contusion. Histological assessment of total spared tissue, white matter, grey matter, and lesion volume will be determined.

**Specific Aim Three** will test the hypothesis that CypD-null mice will show decreased calpain activity following spinal contusion and cortical contusion. Western Blot analysis of calpain-mediated spectrin metabolism will be used to determine calpain activity.



**Figure 4.2** Simplified Model of the central hypotheses. This model hypothesizes that CypD knockout attenuates Ca<sup>2+</sup> induced mPTP formation following secondary damage

## Chapter Two

### The Role of Cyclophilin D in Secondary Spinal Cord Injury

#### Introduction

The initial impact from traumatic spinal cord injury (SCI) can produce catastrophic mechanical damage. However, in the ensuing hours and days following the initial impact, a secondary wave of insults is initiated accelerating tissue destruction several segments below the epicenter. This secondary phenomenon is defined by disruption of  $\text{Ca}^{2+}$  homeostasis, free radical production, and activation of necrotic and pro apoptotic cascades (Panter et al. 1990; Moriya et al. 1994; Popovich et al. 1996). The time line for secondary injury presents a critical window for any therapeutic attempt to recover motor and sensory function. A prominent driving mechanism of secondary tissue degradation is an increase in mitochondrial permeability to any molecules  $< 1500$  daltons (Hunter et al. 1976; Al-Nasser et al. 1986). The increase in mitochondrial permeability causes massive swelling and disruption of the mitochondrial membrane, uncoupling of oxidative phosphorylation in the electron transport chain and subsequent down regulation of ATP production (Hunter et al. 1976; Bernardi et al. 1996; Crompton 1999; Halestrap et al. 2002).

Though primarily driven by large influxes of  $\text{Ca}^{2+}$ , it is the collective actions of secondary insults that initiate the formation of a regulated, non-specific megachannel within the mitochondria called the mitochondrial permeability transition pore (mPTP) (Szabo et al. 1992; Bernardi et al. 1996; Bernardi et al. 1998; Crompton 1999; Sullivan et al. 2005; Smith et al. 2008). Ultimately, it is

the formation of the mPTP that permits excessive ion permeability and disruption of mitochondrial bioenergetics (Hunter et al. 1976; Al-Nasser et al. 1986). The most characterized components of the mPTP include an outer membrane component called the voltage dependent anion channel (VDAC) and the adenine nucleotide translocase (ANT), which forms the inner membrane pore, both are integral in mPTP formation, but are not a necessity in mPTP function (Bauer et al. 1999; Gross et al. 2000; De Giorgi et al. 2002; Kokoszka et al. 2004; Baines et al. 2005). A critical finding by Crompton and colleagues was the inhibition of  $\text{Ca}^{2+}$  dependent mPTP opening by the immunosuppressive drug cyclosporine A (CsA)(1988). Cyclosporine A mediates its actions by binding to and inhibiting the mitochondrial matrix component cyclophilin D (CypD) from binding to ANT (Crompton et al. 1998). CsA inhibited pore formation and subsequent mitochondrial permeability, prompting the hypothesis that CypD is the regulating component in mPTP formation (Crompton et al. 1988; Connern et al. 1994; Crompton et al. 1998; Hansson et al. 2004).

CypD has since shown promise as a viable target for neurotrauma protection. Recent reports demonstrated the ability of cyclosporine A and its analog NIM811 to attenuate cortical damage in rodent models of TBI (Sullivan et al. 2000; Mbye et al. 2008). However, administrations of these compounds in SCI models have produced confounding results. CsA has not offered the same extent of protection in SCI as seen in TBI, except in doses that are highly toxic (Rabchevsky et al. 2001; Morota et al. 2007). NIM811 did offer moderate protection at the cellular level and in functional outcome following SCI at safe

doses (McEwen et al. 2007; Ravikumar et al. 2007). Thus, the question remains as to whether these discrepancies are due to variances in pharmacological properties or regional variations in the components and behavior of the mPTP.

The recent availability of transgenic mice with knockout of the CypD-encoding gene *Ppif* has provided a unique opportunity to directly examine the role of CypD in secondary SCI and TBI damage. We used these models to test the overall hypothesis that ablation of CypD in mice will result in attenuation of secondary injury in both spinal cord contusion and cortical contusion. Functional recovery was assessed by measuring recovery of hind limb motor skills over a 4 week period. To assess the extent of tissue sparing, we employed a modified eriochrome cyanine staining. Finally, calpain activity was measured according to the metabolism of spectrin as determined by western blot. The results of our studies show that homozygous knockout of CypD in mice offered no pathological improvements and some cases worsened outcomes following spinal cord contusion.

## Materials and Methods

*Animals.* All animals were housed in the Division of Laboratory Animal Resources sector of the University of Kentucky Medical Center, which is fully accredited by AAALAC. All experimental procedures abide by protocols approved by the University of Kentucky's Institutional Animal Care and Use Committee. These studies were performed using both WT and transgenic mice. The transgenic mice (CypD-null) features a homozygous deletion of the CypD encoding gene *Ppif* (peptidylprolyl isomerase F). CypD-null mice were generated on a chimeric strain using adult female C57BL/6 crossed to a SV129 strain (B6129) (Baines et al. 2005). The WT mice utilized were also B6129 purchased from Taconic.

*Mouse Model of Traumatic Spinal Cord Injury.* All experimental procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky. The spinal contusions administered in these studies were conducted using the commercially available Infinite Horizon (IH) spinal cord injury device (Precision Systems & Instrumentation (PSI), Lexington, KY). The IH impactor allows for user defined parameters to achieve the desired severity of injury. There are three parameters measured by the IH impactor and defined as follows: 1. *Force* as the actual force delivered by the impactor compared to the calibrated force, 2. *Velocity* as the speed of the impactor tip as it delivers the contusion, and 3. *Displacement* as the elasticity of the spinal cord required until the calibrated force is achieved. The force of the contusion, measured in kilodyns, was calibrated according to each

severity. For mild injury, a calibrated force of 30kdyn was used. For the moderate injuries, a calibrated force of 50kdyn was used. Finally, for the severe contusions, a calibrated force of 70kdyn was used. These settings were derived from a previous study characterizing injury gradients in the mouse model (Basso et al. 2006). Mice were anesthetized by an intraperitoneal injection ketamine (80mg/kg) and xylamine(10mg/kg). Laminectomies were made to expose the T10 vertebral segment. Two clamps were applied approximately at the caudal T11 segment and the rostral T9 segment. The spinal cord was then lowered and straightened until parallel with the platform. Sham animals received a laminectomy only. Following surgery, all animals were placed on a heating pad set at 37°C until consciousness and mobility (upper limbs) was regained. Postoperative care included the manual expression of bladders twice daily until recovery of bladder function, injection with 10 ml sterile saline administered subcutaneously (s.c.) immediately after surgery, 33.3 mg/kg cefazolin, administered by intramuscular injection, twice a day for 1 week then once a day for the remaining 3 weeks.

*Hind Limb Motor Function Assessment.* Motor function following spinal cord injury was measured by two raters blinded to the experimental conditions. Hind limb motor function was measured using the Basso Mouse Scale (BMS), a 9 point categorical scale that rates seven observable motor functions and motor deficits. The seven categories in the BMS included ankle extension, of hind limb joint movements, trunk stability, stepping coordination, paw placement, and tail position (Basso et al. 2006). A single score was recorded for the left and right

hind limb which was then averaged to give a total score. An 11 point subscore was obtained to assess subtle variations within each category. Each BMS test was assessed by two examiners. One examiner is the main observer while the other examiner observes and records the scores during the exam. This is to ensure that one person is observing the test for the entire duration. Each trial is 4 minutes.

*Histological Assessment of Spinal Cord Tissue Sparing.* Mice were given an overdose injection of pentobarbital followed by a cardiac perfusion of cold 1x PBS and 4% PFA. Spinal cords blocks 2 cm in length, centered at the lesion epicenter, were dissected, post-fixed in the same 4% PFA solution for 24 h at 4°C followed by submersion in 25% sucrose solution at 4°C to ensure cryoprotection. Spinal cords were cryosectioned at a thickness of 20microns and mounted onto gelatin-coated slides and stored at -20C. For histological staining, the gelatin-coated slides were allowed to thaw to room temperature. Each slide was then cleared and hydrated in Citrisolv followed by ethanol concentration gradient. Slides were then submerged in an eriochrome cyanine staining solution (10% FeCl<sub>3</sub> and .2% ECRC). The slides were submerged in a differentiation solution for 10 seconds followed by a wash in H<sub>2</sub>O then allowed to dry overnight.

Spinal cord tissue was assessed using a modified eriochrome cyanine staining protocol for myelin that stains for both white matter and neuronal cell bodies. Tissue sparing was assessed from area measurements of white matter, gray matter, and total lesion volume. Total tissue sparing is reported as a percentage calculated as follows:  $1 - (\text{Lesion area} / \text{Total cross sectional area}) \times$

100. Spared white matter was calculated as follows: Total cross sectional area - Lesion area - Gray matter area.

*Spinal Cord Processing for Western Blot Analysis of Calpain Activity.* Spinal cords segments were thawed and cut into 10mm long sections containing the injured site and weighed. The spinal sections were homogenized in 50mM 1X TBS(Tris, NaCl, H<sub>2</sub>O; pH 7.5). Spinal cord homogenates were spun at 32,000 rpm for 30 minutes to separate the supernatant and pellet. The pellets were reconstituted in protease inhibitor with 2% SDS( 50mM TBS pH 7.5, .5M EDTA pH 8.9, 100mM AEBSF, 10mM Leupeptin, 1.5mM Pepstatin) and spun at 32,000 rpm for 30 minutes and the supernatant recovered and frozen at -20°C. Brain cortex samples thawed and weighed out. Both spinal cord and brain tissue were homogenized in a 1X Tris-Saline with protease inhibitors (50mM TBS pH 7.5, .5M EDTA pH 8.9, 100mM AEBSF, 10mM Leupeptin, 1.5mM Pepstatin) and spun at 33,000 RPM for 20 minutes. Supernatant was recovered and frozen at -20°C. Samples were loaded at 20ug into a 6.5% gel followed by a transfer onto a nitrocellulose membrane. The membranes were washed in 1X TBS then blocked in 5% milk/1X TBS solution for 1 hour. For detection of spectrin in spinal cord and brain cortex samples, membranes were incubated overnight with MAS spectrin AB at 1:10,000 dilution in a 5 ml, 5% milk/1X TTBS solution (10XTBS, Tween 20, D-H<sub>2</sub>O). Membranes were washed the following day in TTBS then incubated with fluorescently conjugated secondary antibody solution at 1:5000 in for 1 hour in a 5 ml, 5% milk/1X TTBS solution (10XTBS, Tween 20, D-H<sub>2</sub>O). Membranes are then washed in 1X TTBS and visualized on Odyssey Imager.

### Statistical Analysis of Each Experimental Aim

*IH impactor parameters:* Data from the IH impactor were analyzed by two-tailed unpaired Student's t-Test. Significance was set at  $P < 0.05$ , 95% C.I. Data reported represents mean  $\pm$  STDEV. .

*Hind limb motor assessment:* Data from BMS test were analyzed by repeated measures two-way ANOVA followed by Bonferroni post hoc test. Significance was set at  $P < 0.05$ . The two independent variables in this study are the mouse type (WT and CypD-null) and time post injury. F scores are reported as F score Interaction (F score Mouse, F score Time). Data reported represents mean  $\pm$  STDEV.

*Histological assessment of tissue sparing:* Data recorded from histological assessment of spinal cord tissue was analyzed by ANOVA followed by Tukey's multiple comparison post hoc test. Significance was set at  $P < 0.05$ , 95% C.I. Data reported represents mean  $\pm$  STDEV.

*Calpain activity:* Data from WB analyses of calpain activity were analyzed by Two-way ANOVA followed by Bonferroni post hoc test. Significance was set at  $P < 0.05$ , 95% C.I. Data reported represents mean  $\pm$  STDEV. All Data were analyzed using GraphPad Prism.

*Inclusion and Exclusion Criteria:* For the current study, animals subjected to spinal cord contusion by the IH impactor must register a delivered calibrated force  $\pm$  5kdyns to ensure an appropriate contusion severity. Additionally, animals must display post operative paralysis upon recovery from anesthesia, a

time of approximately 1 hour. Animals must only display ankle extension or ankle extension and plantar or dorsal placement of the paw without weight support. This is a BMS rating of 3, though the data is not reported here. This criterion is based on our pilot study which showed CypD-null mice subjected to spinal contusion did not display an acute recovery and exhibited hind limb paralysis as observed in WT mice. After 24h of recovery, no mouse model may be excluded regardless of recovery rate. The only exception is death or illness that prevents any further testing as determined by veterinarian staff.

## Results

### *Infinite Horizon Impact Parameters.*

Table 1 displays the recorded Infinite Horizon (IH) impact parameters for all spinal cord contusion studies. Table 1A shows that for mild spinal contusions, there was no significant difference in force, displacement, or velocity of the impact tip in CypD-null mice compared to WT mice. Table 1B shows that for moderate spinal contusions, there was no significant difference in force, displacement, or velocity of the impact tip in CypD-null mice compared to WT mice. Table 1C shows that for severe spinal contusions, there was no significant difference in force, displacement, or velocity.

<i>Mouse</i>	<i>Force (kDyn)</i>	<i>Displacement(microns)</i>	<i>Velocity(ms)</i>
CypD <sup>-/-</sup>	32 <sup>+/-</sup> 2	264 <sup>+/-</sup> 25	122 <sup>+/-</sup> 1
WT	32 <sup>+/-</sup> 2	238 <sup>+/-</sup> 30	122 <sup>+/-</sup> 2

### **Mild Spinal Contusion**

<i>Mouse</i>	<i>Force (kDyn)</i>	<i>Displacement(microns)</i>	<i>Velocity(ms)</i>
CypD <sup>-/-</sup>	53 <sup>+/-</sup> 2	499 <sup>+/-</sup> 84	123 <sup>+/-</sup> 2
WT	54 <sup>+/-</sup> 2	493 <sup>+/-</sup> 143	123 <sup>+/-</sup> 3

### **Moderate Spinal Contusion**

<i>Mouse</i>	<i>Force (kDyn)</i>	<i>Displacement(microns)</i>	<i>Velocity(ms)</i>
CypD <sup>-/-</sup>	73 <sup>+/-</sup> 2	849 <sup>+/-</sup> 102	120 <sup>+/-</sup> 3
WT	73 <sup>+/-</sup> 1	811 <sup>+/-</sup> 72	124 <sup>+/-</sup> 4

### **Severe Spinal Contusion**

**Table 1** Infinite Horizon (IH) parameters for mild, moderate and severe spinal cord contusion. **A.** For mild SCI, there were no significant differences in IH parameters. **B.** For moderate SCI, there were no significant differences in IH parameters. **C.** For severe SCI, there was a significant no significant difference in IH parameters. Values=Mean <sup>+/-</sup> STDEV. Two tailed paired student's t-test.

### *Hind Limb Motor Function Assessment*

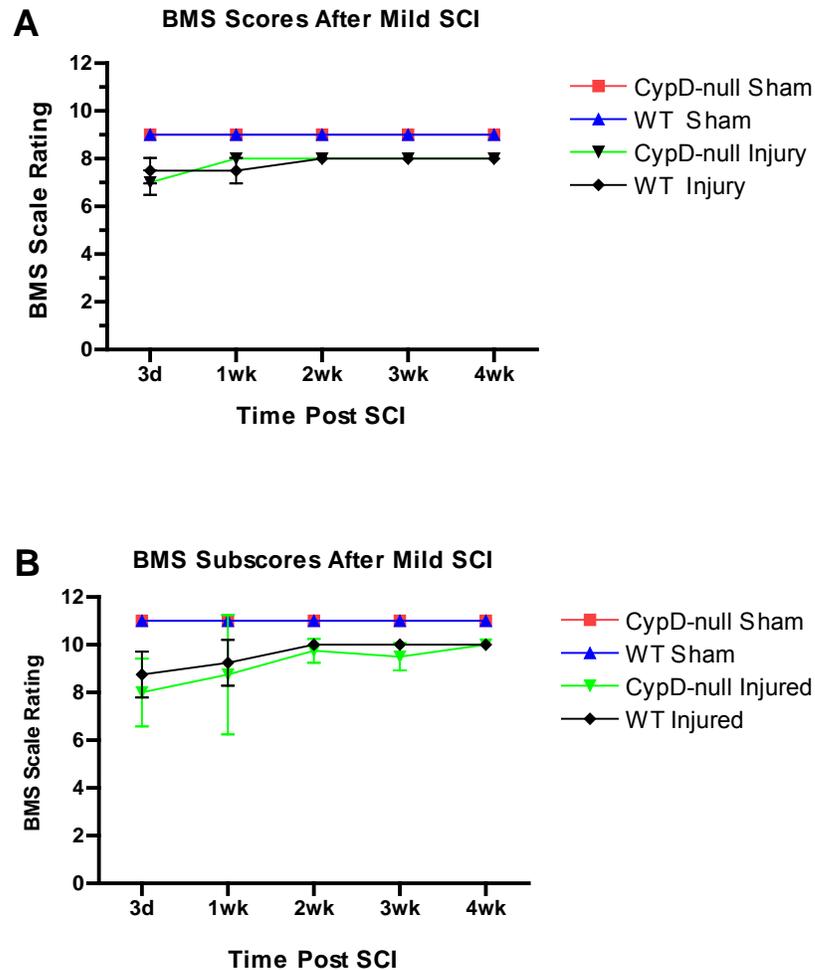
The Basso Mouse Scale (BMS) analysis, a 9 point categorical scale, was used to assess CypD-null and WT mice for open field motor function following moderate, mild and severe SCI over a period of 4 weeks. Immediately following spinal impact, both animal models displayed complete bilateral hind limb paralysis as evident by ankle extension only or ankle extension plus plantar placement without weight support for all gradients of contusion (data not shown). This immediate observation was to assess the consistency of the contusion across all models (Springer et al. 1997).

Figure 5.1A and B show that following moderate SCI, there was no significant difference in BMS scores and subscores for functional motor recovery between the two models after 4 weeks. However, both models did display gradual improvement at 3 days post spinal contusion in ankle extension and plantar placement. By the second week, both models showed further improvements in plantar placement with weight support and stepping coordination in addition to paw position and trunk stability. By week 3, both WT and CypD-null regained most coordination and showed only mild trunk instability as indicated by a side to side tail sway. The improvements observed at week 3 reached a plateau, as no greater improvements were observed in week 4.

Fig 5.2A and B show that following mild spinal contusion, there was no significant difference in BMS scores and subscores for functional motor recovery between the two models after 4 weeks. By 3 days post injury, both WT and CypD-null displayed consistent plantar stepping, regained most coordination

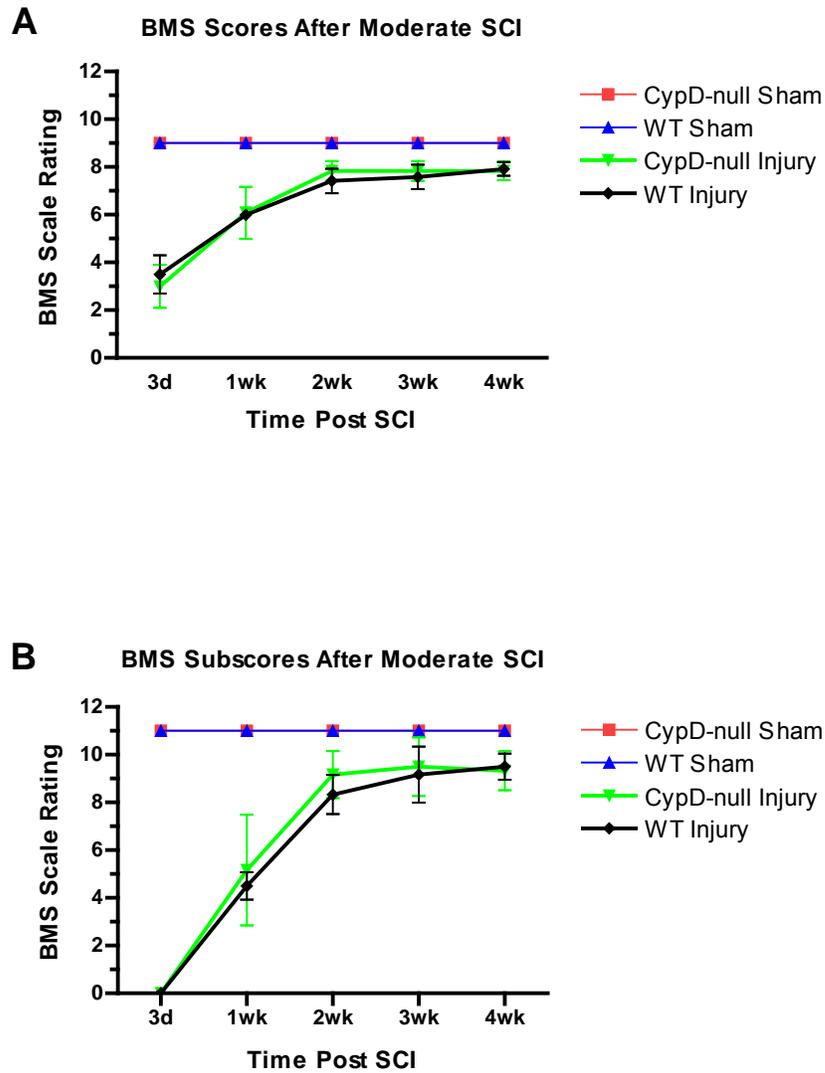
and displayed only mild trunk instability. By 1 week post injury motor recovery skills had reached a plateau as no more improvements were observed for the remaining 3 weeks.

Figure 5.3A and B show that following severe spinal contusion, there were significant differences in BMS scores and subscores for functional motor recovery between the two models at select time points after 4 weeks. Figure 5.3A shows a significant difference in BMS scores at weeks 1,2,3, and 4 [ $F=18.27(1027,44.65)$ ;  $p<.001$ ]. By 1 week post injury, WT mice regained some ability for weight supported stepping where as CypD-null showed only extensive ankle extension with some plantar placement. WT mice continued to show motor improvement with recovery of most coordination, consistent stepping, and an upgrade in trunk instability from severe to mild. CypD-null mice failed to show the same recovery over 4 weeks. CypD-null mice showed significantly lower recovery at each time point from 1 week until 4 weeks of BMS testing. By 4 weeks testing, CypD-null mice displayed only occasional to frequent weight supported stepping with recovery of some coordination. Trunk instability in CypD-null mice never improved above severe. The poor motor recovery of CypD-null mice is further illustrated by the BMS subscores in Figure 5.3B. CypD-null never achieved any subscore above 0 at any time point. WT mice also achieved subscores of 0 from day three to the second week of testing. However, at weeks 3 and 4 WT mice scored significantly higher BMS subscores as they regained consistent plantar stepping and coordination [ $F=19.11(882.7,19.11)$ ;  $p<.001$ ].

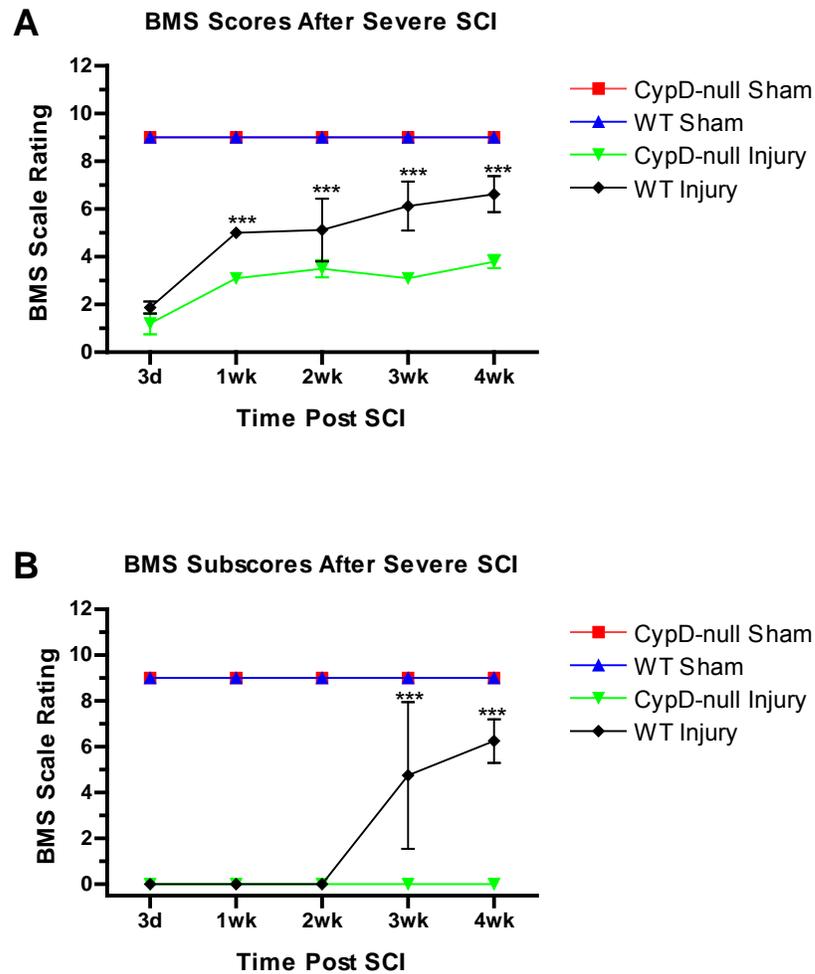


**Figure 5.1** Basso Mouse Scale(BMS) scores and subscores for WT and CypD-null sham and injured mice at 3d, 1wk, 2wk, 3wk, and 4wk post moderate injury.

**A.** No significant differences in BMS scores between WT injured and CypD-null injured mice at any time point following moderate SCI. **B.** No significant differences in BMS subscores between WT injured and CypD-null injured mice at any time point following moderate SCI. Values=Mean <sup>+/-</sup> SD. Two-way ANOVA and Bonferroni post hoc test. N=6 per injury group and 3 per sham group.



**Figure 5.2** Basso Mouse Scale(BMS) scores for WT and CypD-null sham and injured mice at 3d, 1wk, 2wk, 3wk, and 4wk post mild injury. **A and B.** No significant differences in scores or subscores for WT injured and CypD-null injured mice at any time point following mild SCI. Values=Mean  $\pm$  SD. Two-way ANOVA and Bonferroni post hoc test. N=6 per injury group and 3 per sham group.



**Figure 5.3** Basso Mouse Scale(BMS) scores for WT and CypD-null sham and injured mice at 3d, 1wk, 2wk, 3wk, and 4wk post severe injury. **A.** There was a significant difference in BMS scores between WT injured and CypD-null injured mice at select time points following severe SCI [\*\*\* $p < .001$ ,  $F = 18.27(1027, 44.65)$  at 1wk, 2wk, 3wk, and 4wk]. **B.** There was a significant difference in BMS subscores between WT injured and CypD-null2 injured mice at select time points [\*\*\* $p < .001$ ,  $F = 19.11(882.7, 19.11)$  at 3wk and 4wk]. Values=Mean  $\pm$  SD. Two-way ANOVA and Bonferroni post hoc test. N=6 per injury group and 3 per sham group.

### *Histological Assessment of Spinal Cord Tissue Sparing*

Following the 4th week of BMS analysis, all mice were euthanized and volumetric measurements were conducted by histological assessment for total spared tissue, white matter, and grey matter following moderate and severe spinal cord injury. Histological staining showed that following moderate injury, both CypD-null and WT injured mice had the greatest spinal tissue loss at the impact site, or the epicenter, with damage extending primarily in the caudal direction with some injury extending in the rostral direction (Figure 6.1). The epicenter of injury in both groups, approximated at the thoracic level of T-10, was characterized by a dense fibrous matrix encompassing the cross sectional area of the tissue (Figure 6.1). This lesion was not present in WT or CypD-null sham mice (Figure 6.1).

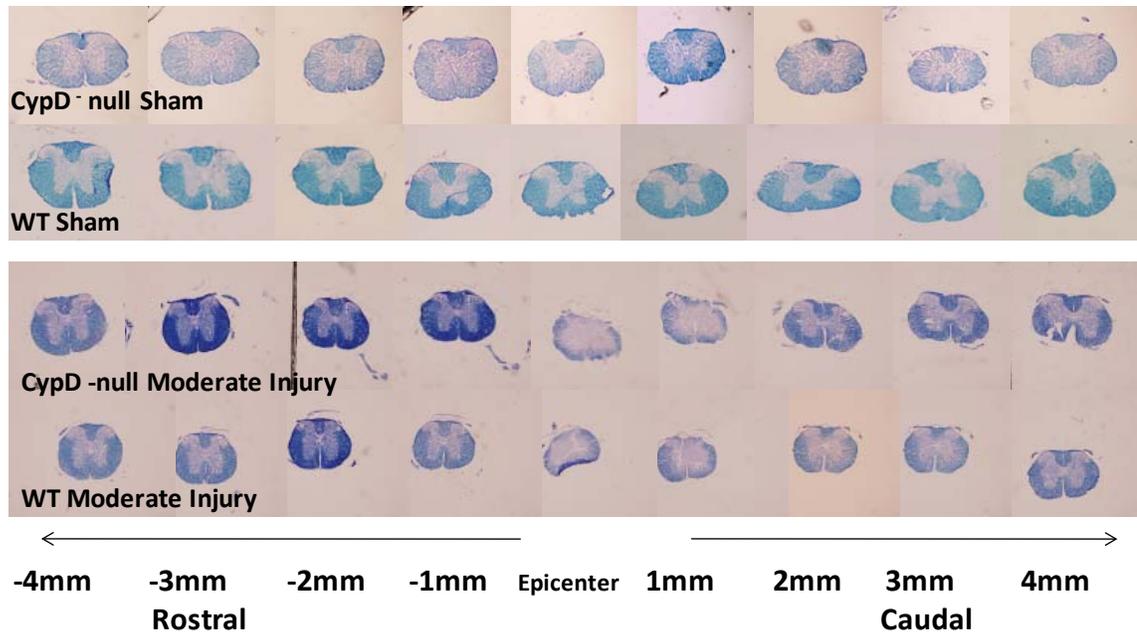
Figure 6.2 shows the histological assessment for spared tissue following moderate SCI. There was no significant difference in the volume of spared tissue between CypD-null injured and WT injured mice (Figure 6.2A). Both injured mice showed a significant reduction in spared tissue compared to both sham mice [ $p < .01$ ,  $F(24.61)$  for all injury vs shams]. A rostral to caudal expansion of the percentage of spared tissue is represented in figure 6.2B. There was no significant difference in the percentage of spared tissue at any spinal segment. Figure 6.3 shows no significant reduction in lesion volume in CypD-null injured mice compared to WT injured mice. Measurements of spared white matter showed no significant improvements in CypD-null mice compared to WT mice as measured by ANOVA (6.4A). A rostral to caudal expansion of spared white matter area is represented in figure 6.4B. There was a statistical significant

difference in white matter area at the most rostral section for CypD-null mice compared to WT mice [ $p < .05$ ,  $F = 1.763(4.450, 42.01)$ ]. However, at this rostral section, there is no measurable tissue damage and the increase white matter is likely from compression of the rostral most segments during tissue mounting. Measurements of spared gray matter showed no significant improvements in CypD-null injured mice compared to WT mice (Figure 6.5A). A rostral to caudal expansion of gray matter area is represented in Figure 6.5B. CypD-null mice showed a statistically significant increase in grey matter area compared to WT injured mice [ $p < .001$ ;  $F = 6.613(12.01, 104.7)$ ]. Again, at this rostral section there was no measurable tissue damage.

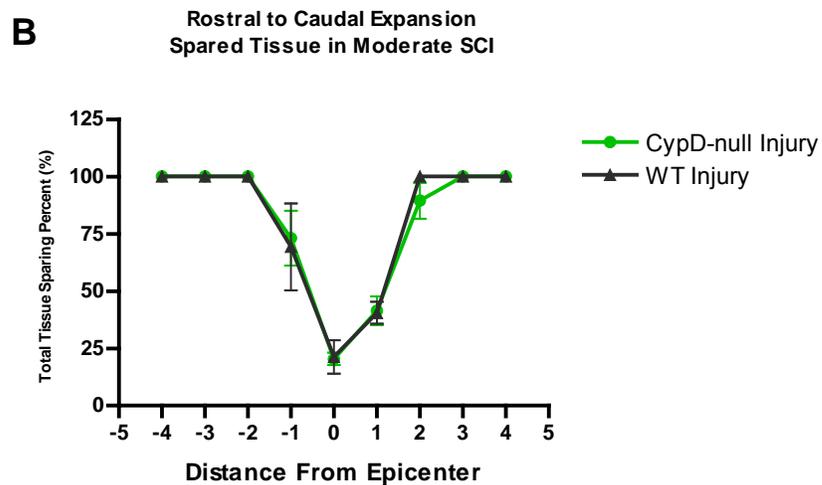
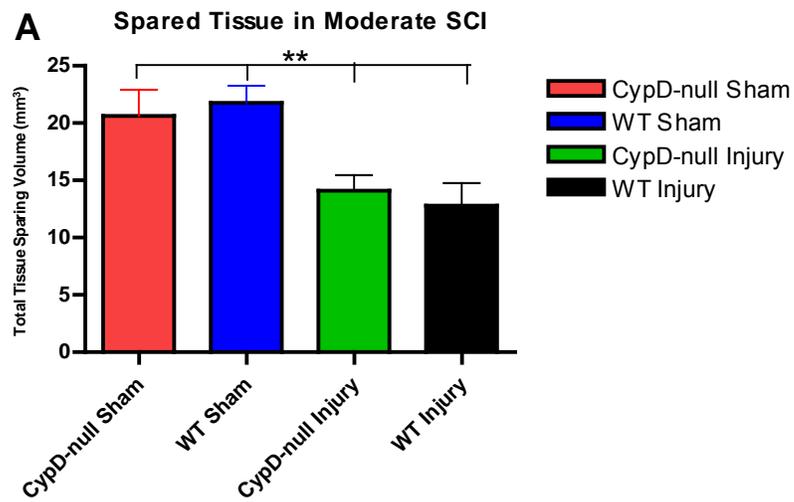
Figure 7.1 shows histological assessment for spared tissue following severe SCI. Figure 7.2A shows a significant reduction in the volume of spared tissue in CypD-null injured mice compared to WT injured mice [ $p < .01$ ;  $F(86.80)$ ; for CypD-null injury vs sham;  $p < .001$  all injury vs all sham]. A rostral to caudal expansion of the percentage of spared tissue is represented in Figure 7.2B. CypD-null injured mice showed a significant reduction in the percentage of spared tissue at select sections both caudal and rostral from the epicenter compared to WT injured mice [ $p < .01$ ;  $F(10.73(67.20, 182.2))$  at 1mm and -1mm;  $p < .001$  at 2mm and -2mm].

Figure 7.3 shows no significant difference in lesion volume in CypD-null injured mice compared to WT injured mice. Measurements of spared white matter showed that CypD-null injured mice had a significant reduction compared to WT injured mice (Figure 7.4A) [ $p < .01$ ;  $F = (59.25)$ ]. A rostral to caudal expansion of the spared white matter area is represented in Figure 7.4B. CypD-null injured

mice showed significant reductions in spared white matter at select sections both rostral and caudal from the epicenter compared to WT injured mice [ $p < .01$  at 1mm, 4mm, 6mm;  $p < .001$  at 2mm, 3mm;  $F = 4.744(108, 36.63)$ ]. Figure 7.5A shows that gray matter was also significantly reduced in CypD-null injured mice compared to WT mice [ $p < .001$ ;  $F = (71.87)$ ]. A rostral to caudal expansion of spared grey matter is represented in Figure 7.5B. CypD-null mice showed a significant reduction in gray matter sparing at select sections both rostral and caudal to the epicenter [ $p < .01$  at -3mm, 5mm;  $p < .001$  at 2mm, 3mm, 4mm, 6mm;  $F = 7.73(159.5, 89.14)$ ].

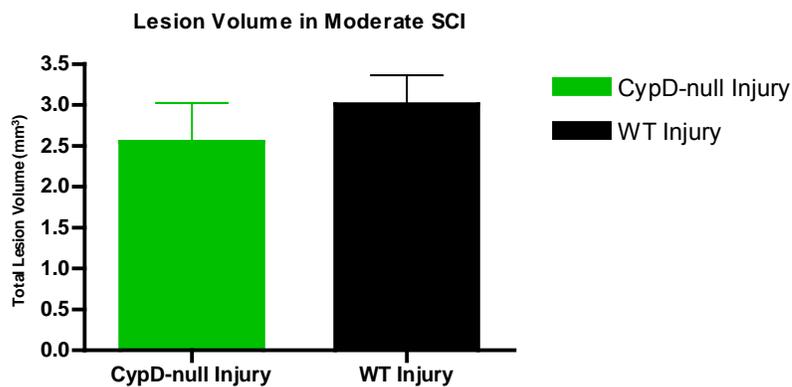


**Figure 6.1** Histological assessment showing rostral(-mm) to caudal(mm) expansion of moderate injured spinal cord sections or laminectomy only (sham models) in CypD-null and WT mice. The epicenter is the point of impact as evident by large lesion volume. Lesions in the injured animal extend into the caudal section below the epicenter.

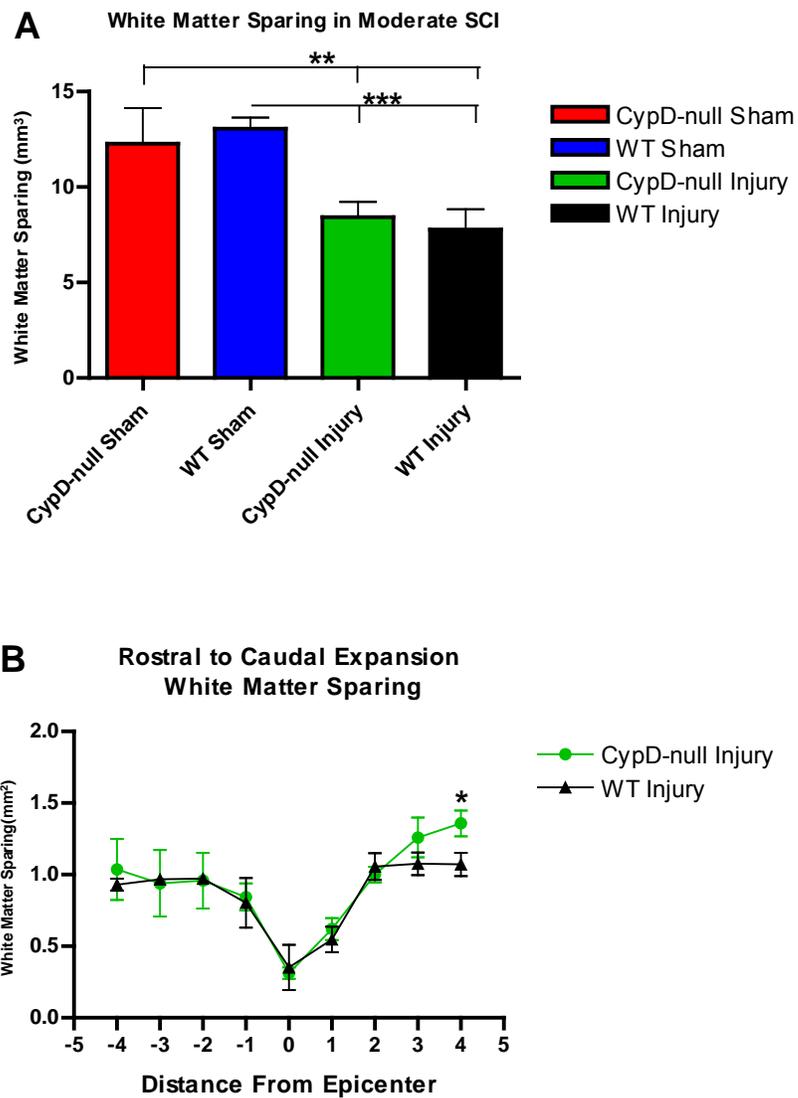


**Figure 6.2** Total tissue sparing for WT and CypD-null mice 4weeks post moderate spinal contusion. **A.** The volume of total tissue sparing. No significant difference in the volume of total tissue sparing was recorded in CypD-null and WT injured mice. Values=Mean <sup>+/-</sup> STDEV. ANOVA and Tukey's multiple

comparison post hoc test. N=6 for injured group and N=3 for sham group. **B.** The percentage of total tissue sparing represented in a caudal to rostral expansion. No significant difference in the percentage of total tissue sparing was recorded at any section at both caudal and rostral direction from the epicenter in CypD-null and WT injured mice. Values=Mean <sup>+/-</sup> STDEV. Two-way ANOVA and Bonferroni post hoc test. N=6 for injured group and N=3 for sham group.

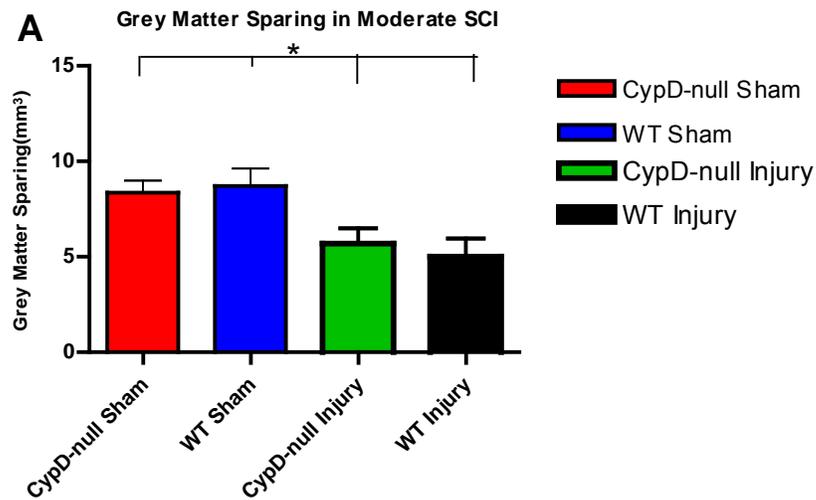


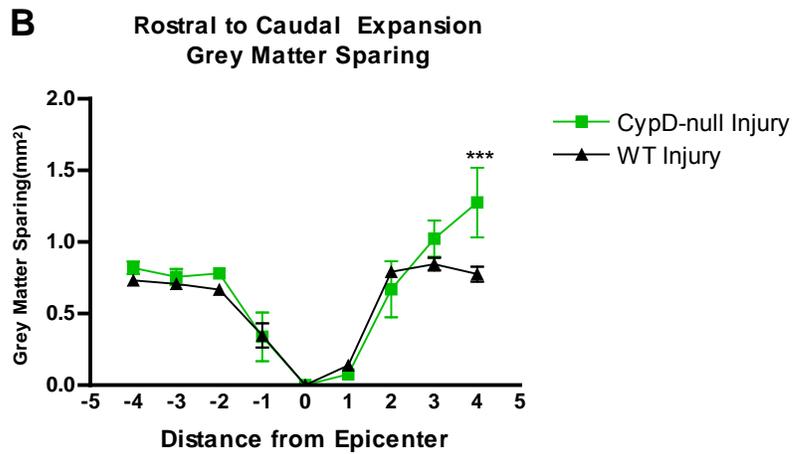
**Figure 6.3.** Lesion volume for WT and CypD-null mice 4 weeks post moderate spinal contusion. No significant difference was recorded in lesion volume between WT and CypD-null injured mice. Values=Mean <sup>+/-</sup> STDEV. ANOVA and Tukey's multiple comparison post hoc test. N=6 for injured group and N=3 for sham group.



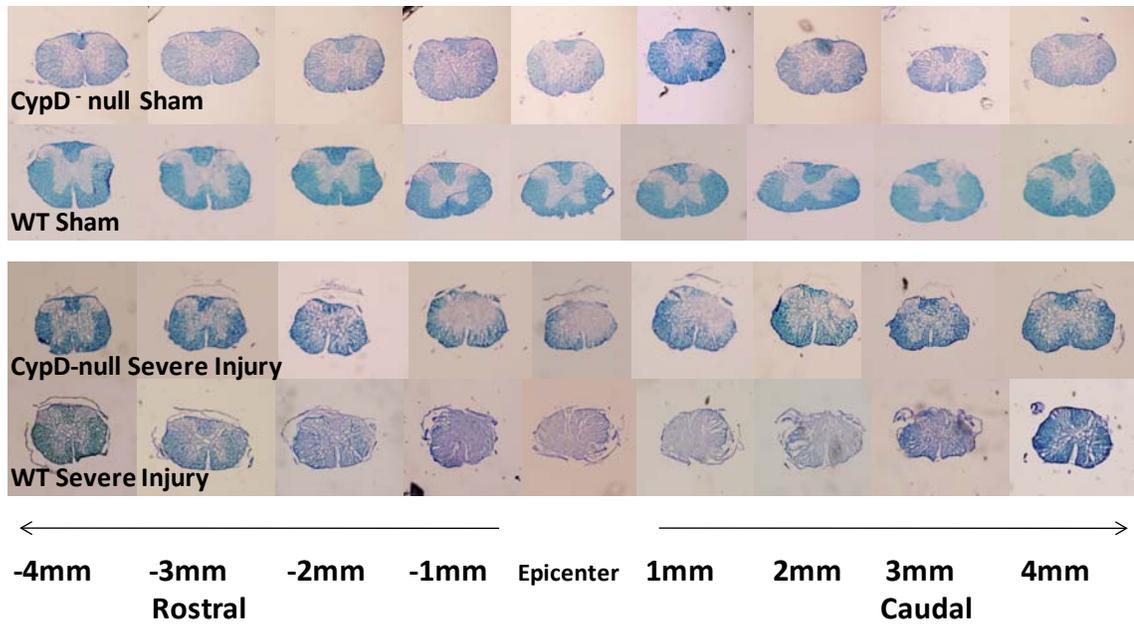
**Figure 6.4** White matter sparing for WT and CypD-null mice 4 weeks post moderate spinal contusion. **A.** Quantification of white matter sparing. No

significant difference was recorded in the white matter sparing in CypD-null injured mice compared to WT injured mice. Values=Mean  $\pm$  STDEV. ANOVA and Tukey's multiple comparison post hoc test. N=6 for injured group and N=3 for sham group. **B.** Rostral to caudal expansion of white matter sparing. White matter sparing was significantly increased in CypD-null injured mice compared to WT injured mice at rostral section -4mm from the epicenter. Values=Mean  $\pm$  STDEV. \*p<.05; Two-way ANOVA and Bonferroni post hoc test. N=6 for injured group and N=3 for sham group.

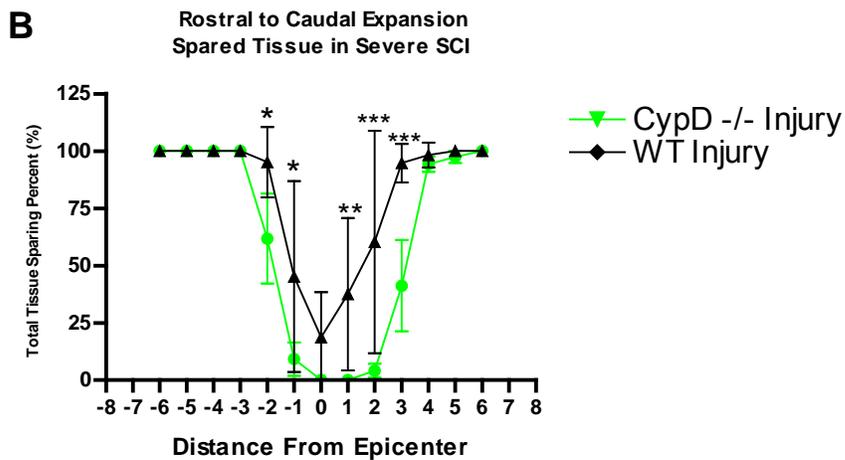
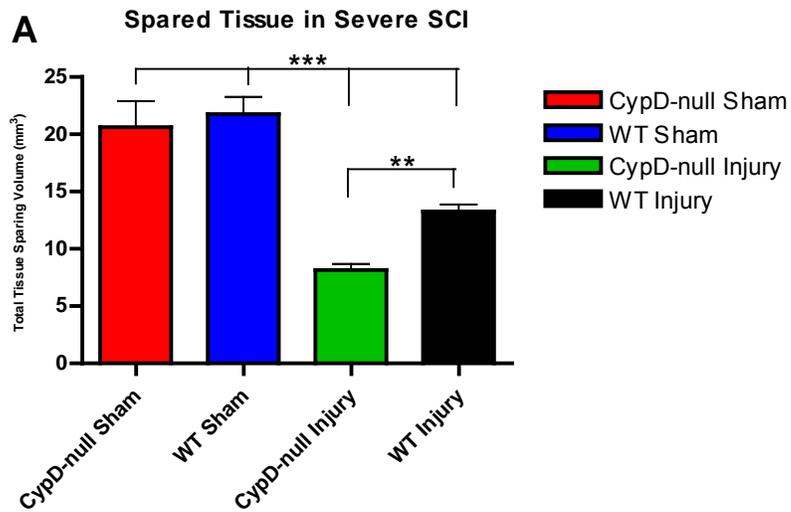




**Figure 6.5** Grey matter sparing for WT and CypD-null mice 4 weeks post moderate spinal contusion. **A.** Quantification of grey matter sparing. No significant difference in total tissue sparing in CypD-null injured mice compared to WT injured mice. Values=Mean <sup>+/-</sup> STDEV. ANOVA and Tukey's multiple comparison post hoc test. N=6 for injured group and N=3 for sham group. **B.** Rostral to caudal expansion of grey matter sparing. Grey matter sparing was significantly increased in CypD-null injured mice compared to WT injured mice at the caudal section -4mm from the epicenter. Values=Mean <sup>+/-</sup> STDEV. \*\*\*p<.01; Two-way ANOVA and Bonferroni post hoc test. N=6 for injured group and N=3 for sham group.



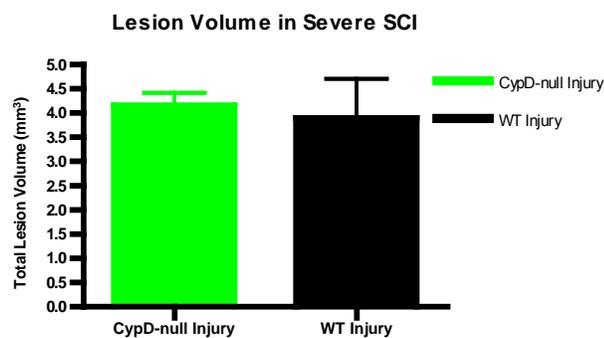
**Figure 7.1** Histological assessment showing rostral(-mm) to caudal(mm) expansion of severe injured spinal cord sections or laminectomy only (sham models) in CypD-null and WT mice. The epicenter is the point of impact as evident by large lesion volume. Lesions in the injured animal extend into the caudal section below the epicenter.



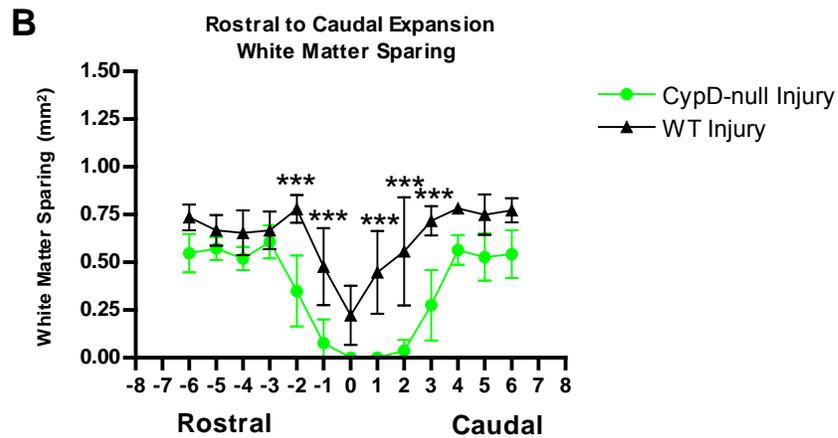
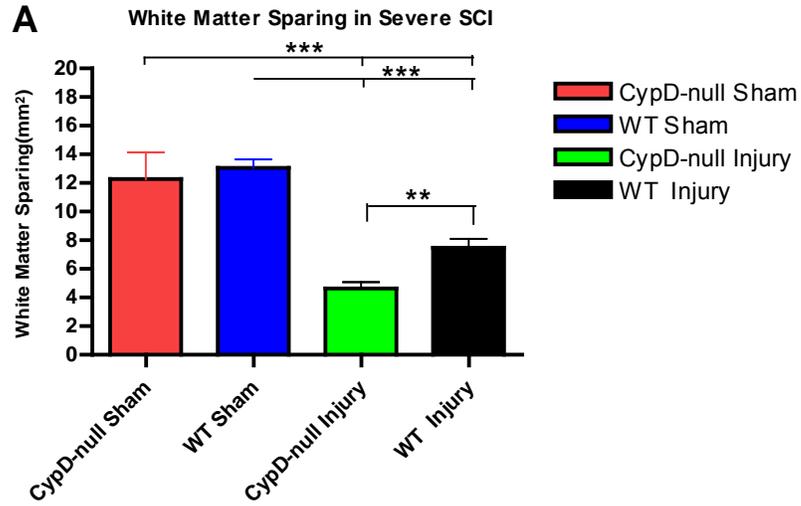
**Figure 7.2** Total tissue sparing for WT and CypD-null mice 4 weeks post severe spinal contusion. **A.** Quantification of total tissue sparing. Total tissue sparing was significantly decreased in both CypD-null and WT injured mice compared to both sham models. Total tissue sparing was a significantly decreased in CypD-null injured mice compared to WT injured mice. Values=Mean <sup>+/-</sup> STDEV.

\*\*\*p<.01; ANOVA and Tukey's multiple comparison post hoc test. N=6 for injured

group and N=3 for sham group. **B.** Rostral to caudal expansion of white matter sparing expansion. There was a significant decrease in CypD-null injured mice compared to WT injured mice at the rostral section of -1mm and -2mm and at the caudal sections 1mm, 2mm, and 3mm from the epicenter. Values=Mean <sup>+/-</sup> STDEV. \*p<.05, \*\*p<.01, \*\*\*p<.001; Two-way ANOVA and Bonferroni post hoc test. N=6 for injured group and N=3 for sham group.

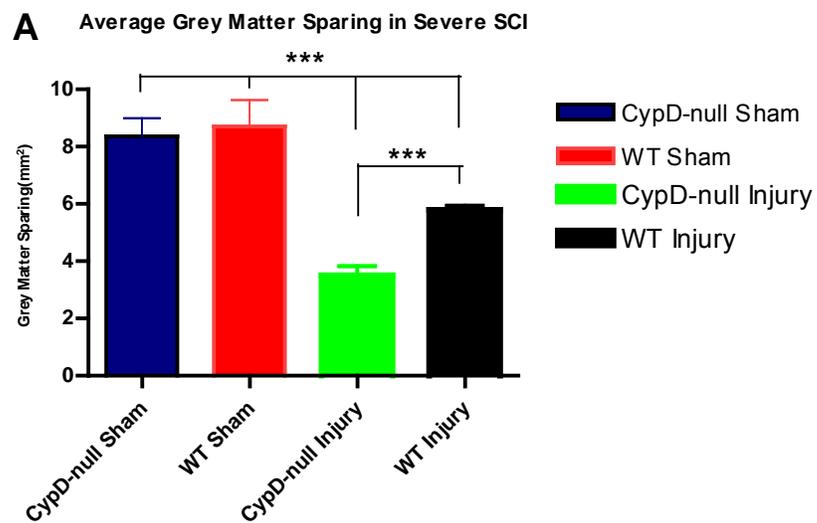


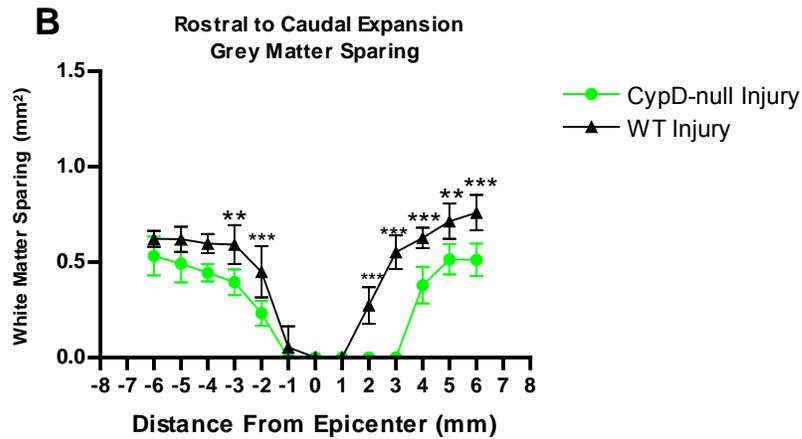
**Figure 7.3** Lesion volume for WT and CypD-null mice 4 weeks post severe spinal contusion. No significant difference was recorded in lesion volume between WT and CypD-null injured mice. Values=Mean <sup>+/-</sup> SD. N=6 for injured group and N=3 for sham group.



**Figure 7.4** White matter sparing for WT and CypD-null mice 4 weeks post severe spinal contusion. **A.** Quantification of white matter sparing volume. White matter was significantly decreased in CypD-null injured mice compared to WT injured mice. Values=Mean <sup>+/-</sup> STDEV. \*\*p<.01, \*\*\*p<.001. Data by ANOVA and

Tukey's multiple comparison post hoc test. N=6 for injured group and N=3 for sham group. **B.** Rostral to caudal expansion of white matter sparing. White matter sparing was significantly reduced in CypD-null injured mice compared to WT injured mice (\*\*p<.01 at the caudal section at 1mm, 4mm; \*\*\*p<.001 at 2mm, 3mm, 6mm). Data by Two-way ANOVA and Bonferroni post hoc test. N=6 for injured group and N=3 for sham group. Values=Mean +/- STDEV.



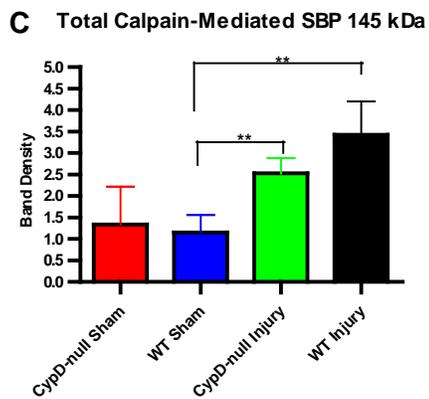
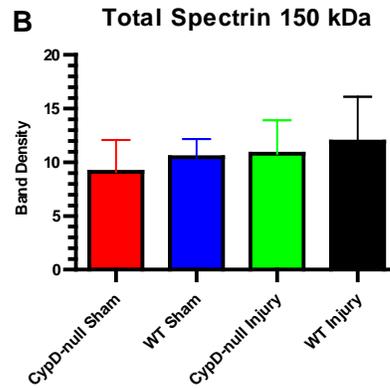
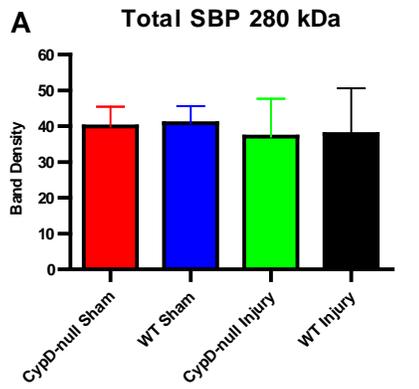
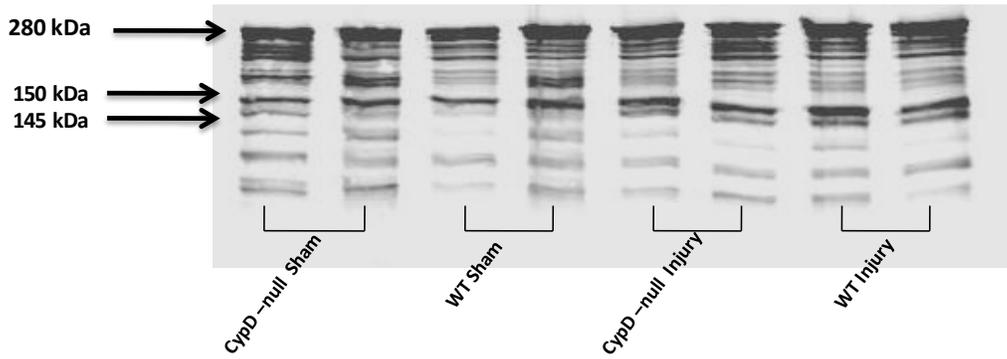


**Figure 7.5** Grey matter sparing for WT and CypD-null mice 4 weeks post severe spinal contusion. **A.** Quantification of grey matter sparing volume. Grey matter was significantly decreased in CypD-null injured mice compared to WT injured mice. Grey matter was significantly decreased in CypD-null and WT injured mice compared to CypD-null and WT sham mice ( $***p < .001$  for all shams vs all injured and CypD-null injured vs WT injured). Data by ANOVA and Tukey's multiple comparison post hoc test. N=6 for injured group and N=3 for sham group. Values=Mean +/- STDEV. **B.** Rostral to caudal expansion of grey matter sparing. Grey matter sparing was significantly reduced in CypD-null injured mice compared to WT injured mice ( $**p < .01$  at -3mm, 5mm;  $***p < .001$  at -2mm, 2mm, 4mm, 6mm). Data by 2way ANOVA and Bonferroni post hoc test. N=6 for injured group and N=3 for sham group. Values=Mean +/- STDEV.

### *Western Blot Analyses of Calpain activity following Moderate SCI*

Calpain activity, as measured by calpain mediated spectrin metabolism was assessed 24 hours following moderate spinal cord contusion. Western blot analysis of SBP produces three distinct bands. Total intact spectrin, defined as spectrin levels before enzyme catalysis, produces a specific band at 280kDa. Total spectrin breakdown product (tSBP), defined as combined calpain and caspase mediated metabolism of spectrin, produces a band at 150kDa. Calpain mediated spectrin (cSBP), defined as metabolism of spectrin exclusively by calpain, and produces a specific band at 145 kDa.

Figure 8.1A shows western blot analysis of total spectrin levels 24 hours following moderate SCI. Figure 8.1B shows that CypD-null injured mice had no significant decrease in total spectrin levels mice compared to injured WT mice or compared to both mouse sham models. Figure 8.2A shows western blot analysis of tSBP 24 hours following moderate SCI. There was no significant decrease in calpain mediated SBP in injured CypD-null mice compared to injured WT mice. Figure 8.2 B shows no significant difference in cSBP in CypD-null injured mice compared to WT injured mice. There was a significant difference in calpain specific SBP in injured injured WT mice compared to both mouse sham models [ $p < .001$ ;  $F(9.595)$ ].



**Figure 8** Western blot and quantitative analysis of total spectrin and SBP in CypD-null and WT sham and injured mice 24h post SCI. **A.** Quantitative analysis of total spectrin levels (280kDa) shows no significant difference in CypD-null and WT sham and injured mice. **B.** Quantitative analysis of total SBP (150kDa) shows no significant difference in CypD-null and WT sham and injured mice. **C.** Quantitative analysis of calpain-mediated SBP (145kDa) shows a significant difference in WT injury compared to CypD-null and WT sham (\*\*p > .01). Data measured by ANOVA with Tukeys Multiple Comparison post hoc test. N=4 per group. Values=Mean <sup>+/-</sup> STDEV.

## Discussion

The principal finding in our study was that mice with a homozygous knockout of CypD, the regulatory component in mPTP formation, showed no significant improvement in motor function, tissue sparing, or calpain activity following spinal contusion. The purpose of this study was to assess the role of CypD in neuroprotection following spinal cord and brain injury. Recent reports have shown confounding results in the efficacy of CypD as a therapeutic target in SCI (Rabchevsky et al. 2001; Ravikumar et al. 2007). Following brain injury, treatment with the CypD inhibitor cyclosporin A improved behavioral function and cortical tissue sparing (Sullivan et al. 2000; Sullivan et al. 2004). However, the same dose regiment of cyclosporine A failed to provide protection following SCI (Rabchevsky et al. 2001). The only protection following SCI observed from cyclosporin A was elicited at toxic doses. In contrast, a recent study using NIM811, a non immunosuppressive cyclosporin derivative, recorded decreased lesion size, preserved mitochondrial bioenergetics, and improved tissue sparing and motor function following spinal cord injury (McEwen et al. 2007; Ravikumar et al. 2007). Thus, the role of CypD in neuroprotection following spinal cord injury remains uncertain.

In the current study, we directly addressed the role of role of CypD in SCI by utilizing mice deficient for CypDI. The first assessment for improved outcome following injury was hind limb motor function using the Basso Mouse Scale (BMS) for 4 weeks following different severities of SCI. The mild contusion produced very minimal motor deficits in both CypD-null and WT mice. This rapid

recovery of motor function in both models would make any protective effects of CypD-null difficult to assess. Following a moderate contusion, both WT and CypD-null mice displayed complete hind limb paralysis immediately following the injury. However, both WT and CypD-null mice showed similar recovery from the moderate contusion. These results may question the sensitivity and selectivity of the BMS as a means for measuring motor function following spinal contusion. Some motor deficits, such as those resulting from gray matter damage, may be masked by white matter damage and go undetected by BMS measurement. However, this seems unlikely since spinal contusions were administered at the lower thoracic level of T10. Motor neurons and interneurons of the grey matter at this level primarily innervate axial muscles, having minor contributions to hind limb motor function. Thus, lesions around the T-10 area produce motor deficits that are mostly exclusive to white matter damage (Magnuson et al. 1999). It is possible that a moderate contusion does not produce a strong enough motor deficit to truly assess subtle changes. Within the first week following the moderate spinal contusion, WT mice demonstrated an approximate 30% recovery in function. WT mice were able to regain full weight support, most of their coordination with only minor irregular paw placement. Such extensive recoveries may make assessment of discrete motor improvements difficult.

To address this concern, we administered a severe spinal contusion. Surprisingly, CypD-null mice failed to achieve the same level of motor recovery as WT mice. Though, both mice did display complete hind limb paralysis following contusion, only WT mice showed gradual recovery of motor function

over the 4 weeks. CypD-null mice never achieved the same level of motor recovery as WT from the first week until the final fourth week. These data suggest that a basic physiological level of functional CypD is necessary for cell survival. These data clearly show that CypD knockout in mice offers no improved motor recovery following spinal cord injury regardless of the severity.

Impairments in locomotor function are indicative of axonal damage and to an extent, gray matter damage. Based on our results from the BMS assessment, it is not surprising that CypD-null mice showed no further improvement in any of the tissue measurements following moderate injury. Both WT and CypD-null mice displayed epicenter lesions consistent with previous measurements of gradient contusions in various mouse strains (Kuhn et al. 1998; Jakeman et al. 2000; Ghasemlou et al. 2005). However, the extension of the lesion was mostly exclusive to sections caudal to the epicenter. Again, these results are contradictory to previous findings in rodent models of spinal cord injury (McEwen et al. 2007; Ravikumar et al. 2007).

A possible explanation for these discrepancies, at least in spared tissue assessment, may stem from species specific inflammatory responses following SCI that mediate degeneration and healing (Zhang et al. 1996; Guth et al. 1999; Zhang et al. 2003; Sroga et al. 2003). The fibrous matrix of connective tissue, as shown at the epicenter, is in sharp contrast to the cyst-like fluid cavitation of the epicenter observed in rats (Sroga et al. 2003). Mice follow a unique secondary response compared to rats in which delayed inflammatory cell infiltration may contribute more towards wound healing instead of secondary

neurodegeneration (Guth et al. 1999; Sroga et al. 2003). Any protective effects of CypD ablation may be masked by a more universal healing mechanism in the mouse model. Though, rats do display cellular markers of wound repair, these events are largely overwhelmed by destructive inflammatory reaction (Sroga et al. 2003). Thus, the effects that CypD ablation or other therapeutic strategies may have on tissue sparing may be more evident in rats at earlier stages of secondary response.

If this were the case, the unique healing response in mice would be expected for all severities of spinal contusion. When we administered a severe contusion, WT mice did display a similar epicenter lesion as measured during moderate contusion. In CypD-null mice, however, the entire epicenter was encompassed by a lesion that persisted in sections above and below the site of injury. In moderate contusions, lesion reduction was seen in the immediate sections below the site of injury. Furthermore, the decreased tissue sparing in CypD-null mice compared to WT mice suggest that CypD may be necessary for cellular support in the spinal cord following a severe injury.

Cellular alterations following spinal cord injury may offer a more discrete assessment of the mechanisms involved in secondary damage, particularly in the acute intermediate stages of injury. The secondary pathophysiological events that follow spinal injury activate and mobilize deleterious catalytic proteins (Anderson et al. 1993; Lynch et al. 1994; Moriya et al. 1994). A selection of these catalytic proteins may be activated following mPTP formation. Of particular interest is the sustained activation of  $Ca^{2+}$  dependent calpains. Extensive

studies of calpain have demonstrated their involvement in cell structural damage and mediation of apoptotic and necrotic death (Wingrave et al. 2004; Momeni et al. 2006; Deng et al. 2007; Momeni et al. 2007). Two recent studies showed that onset of mPTP activated calpain, an effect that was blocked by administering cyclosporine A (Ding et al. 2002; Ferrand-Drake et al. 2003). Increased activation of the calpain isoform Calpain 1 has also been documented within 1h and as far out as 24h following spinal injury in rats (Springer et al. 1997). These reports demonstrate that calpain activation can result from mPTP opening.

Thus, we hypothesized that CypD ablation would mediate protective effects by decreasing calpain activity, as measured by spectrin metabolism (cSBP). However, our data showed that cSBP was not reduced in CypD-null mice compared to WT mice. Though, cSBP was measured at 24h post injury, other reports have shown that calpain is still elevated at this time (Springer et al. 1997). Additional time points closer to the time of injury may produce stronger effects, calpain activity at 24h is still a viable time point. It is unlikely that calpain has a minor role in secondary damage, as cSBP is elevated in both WT and CypD-null injured mice compared to sham models. Thus, calpain activity may be a critical mediator of secondary damage in spinal cord injury. However, the metabolism of spectrin may not be indicative of the extent of calpain-induced cell death. Additional substrates of calpain may provide more sensitive measurements.

The potential for CypD as an effective target in spinal cord injury remains uncertain. The results reported here and in previous studies have been

conflicting and difficult to interpret. Several scenarios exist that may explain why protection from targeting CypD is still elusive.

Spinal mitochondria have an inherent vulnerability even under physiological conditions. This is evident by alterations in spinal mitochondrial intrinsic properties, such as a basic lowered calcium retention, reduced mPTP threshold, and a hampered  $\text{Ca}^{2+}$  transport and buffering system (Sullivan et al. 2004; Sullivan et al. 2005; Morota et al. 2007). Spinal neurons also show increased ROS and subsequent lipid peroxidation and DNA oxidation compared to cortical neurons (Sullivan et al. 2004). Thus, spinal neurons are readily susceptible to insult making therapeutic protection difficult. The intrinsic nature of spinal mitochondria becomes a greater factor when considering the complex pathology of spinal cord injury. Though, cypD can induce both necrotic and apoptotic cell death, it is the collective actions of several degenerative pathways that may illicit the greatest damage (Schneider 2005; Forte et al. 2007)).

Apoptosis is a major inducer of secondary pathology following spinal cord injury and is suggested to be the primary mediating cause of glial cell death (Beattie et al. 2000). For example, oligodendrocytes, supportive glial cells that are fundamental to maintaining axonal integrity, readily undergo apoptosis following spinal cord injury. Inhibition of FAS and p75, receptor targets for apoptotic stimuli, has shown to reduce cell death and improve axonal preservation in rodent models of spinal cord injury (Casha et al. 2001; Casha et al. 2005; Ackery et al. 2006). Additional studies have shown that attenuation of other apoptotic stimuli, such as BAX, Bcl-2, and Fas ligand, resulted in improved

behavioral outcome and increased tissue sparing (Genovese et al. 2008; Genovese et al. 2008; Nocentini et al. 2008; Xu et al. 2009). In contrast, a recent study showed that mitochondria isolated from CypD-null mice were not resistant to BAX and Bcl-2 release of cytochrome C, an apoptotic-inducing agent, from the mitochondrial inner membrane (Nakagawa et al. 2005). Thus, increased mitochondrial permeability following spinal cord injury, as induced by apoptotic factors, may occur even in the absence of CypD.

Interestingly, several of the studies mentioned above were using pharmacological agents targeting multiple inflammatory processes involved in secondary spinal cord injury. For example, a study from Genovese et al. showed that inhibition of the MAPK signaling cascade reduced key inflammatory mediators, such as neutrophil infiltration, pro inflammatory cytokine expression, and nuclear factor-kappaB activation, in addition to apoptotic markers (Genovese et al. 2008). Another study from Xu et.al, used a combinational therapeutic approach of anti-inflammatory agents and showed similar reduction of apoptotic and inflammatory markers in compression models of spinal cord injury in rodents (Xu et al. 2009). The effectiveness of these treatments suggests that inflammatory responses following spinal cord injury may be the prominent mediator in secondary damage. Inflammatory responses can induce excitotoxicity and subsequent Ca<sup>2+</sup> increase, which presents a favorable mechanisms for CypD mediated cell death (Beattie et al. 2000). However, these studies, in addition to our findings, suggest that other inflammatory factors,

particularly apoptosis, may largely be responsible for secondary damage independent of CypD-mediated pathways.

Though, multiple secondary cascades may overwhelm CypD mechanisms, the failed protective effects of targeting CypD may concern the anatomical makeup of the spinal cord. CypD inhibition has shown to offer modest protection in animal models of brain injury (Sullivan et al. 2000; Mbye et al. 2008). The underlying cortical regions in the brain consist of vast gray matter enriched in neuronal cell bodies. In these cortical regions, CypD has been reported to be highly localized within neuronal mitochondria and to a greater extent the post synaptic mitochondria (Naga et al. 2007; Hazelton et al. 2009; Wang et al. 2009). In contrast, the lower thoracic segments, as targeted in our model of spinal cord contusion, consist largely of axonal tracts with scarce neuronal cell bodies. Contusions in regions of the spinal cord where there is a greater area of gray matter, such as the cervical enlargement or the lumbar sections containing the central pattern generator, may show a more pronounced role for CypD following injury. Other regional variations may stem from mPTP core components. Some exciting new studies in the identity and characteristics of the mPTP structure may provide clues to the discrepancies seen in SCI protection. Basso et al., revealed that the inhibitory effects of cyclosporine A and NIM811 are dependent on the binding of phosphates to inhibitory regions on the mPTP (Basso et al. 2008). In addition, Leung et al., revealed that an additional mPTP component, the phosphate carrier, can induce mPTP opening in a  $\text{Ca}^{2+}$  dependent manner (Leung et al. 2008).

The data presented here showed that CypD-null mice showed no improved outcome following spinal cord injury. However, this is not to say that CypD has no role in the pathology of spinal cord injury. CypD may have a pro survival role in the spinal cord, at least under severe injury. This concept has been suggested in a recent study from Wang et al. Following hypoxic-ischemic (HI) brain injury, Wang showed that immature CypD-null mice were more susceptible to injury following hypoxic-ischemic (HI) brain injury. Furthermore, induction of HI-induced brain injury in immature CypD-null mice was largely dependent on apoptotic mechanisms. In adult CypD-null mice, the mechanism of HI brain injury shift to CypD-dependent pathways (Wang et al. 2009). A potential dual role for CypD in the spinal cord is intriguing. A possible model may show that spinal cord cells, particularly oligodendrocyte cells, require some active CypD to maintain function. Our models using a homozygous deletion of CypD may support this phenomenon.

Basal levels of CypD activation, under strict regulation, may contribute to the preservation of mitochondrial integrity. Uncoupling proteins have been shown to control the production of reactive oxygen species (ROS) in rodent models of brain injury (Davis et al. 2008; Davis et al. 2008). Consider that mPTP formation and the subsequent increased permeability disrupt oxidative phosphorylation. If CypD-mediated mPTP formation can occur in a brief controlled burst, this may allow for momentary reduction of any ROS production. In addition, increased lipid peroxidation can produce clusters of misfolded proteins that form unregulated pores in the mitochondria membrane (He et al.

2002). CypD, as a molecular chaperone, may properly fold these clustered proteins into a regulated channel (He et al. 2002). Thus, the protection recorded using NIM811 still permits populations of mitochondria with functional CypD. Complete ablation of CypD presents an extreme environment that increases susceptibility as the injury becomes more severe. A study using heterozygous knockout of CypD may support such a dual model of CypD.

## Conclusion

These studies show the differing role of CypD in secondary neurological injury. CypD-mediated mitochondrial permeability is a dynamic factor in cell death. Therapeutic strategies that target CypD may offer protection following neurological injury. However, these studies show that the contributions of CypD differ in selective models of injury. In the pathological time course of spinal cord injury, CypD-mediated mechanisms may have only minor involvement. In circumstances of severe spinal injury, CypD may actually be necessary for basic function of surviving neuronal and glial cells. However, CypD-mediated death may have a larger role in other neurological injuries, specifically brain injury. Previous reports have shown that targeting CypD offers modest improvements in cortical damage. Such functional diversity demonstrates that strategies aimed at single pathological mechanisms may not be sufficient. In the event of spinal cord injury, consideration must be given to several factors. Injury severity, regional and temporal factors may all reveal dual functions for CypD and CypD-mediated pathways of cell death. Further investigation is warranted concerning the regional variation of CypD properties in the spinal cord and brain. A better understanding of these regional discrepancies can lead to more effective therapeutic intervention in spinal cord injury.

## Chapter Three

### The Role of Cyclophilin D in Traumatic Brain Injury

#### Introduction

CypD has shown promise as a viable target for neurotrauma protection. Recent reports demonstrated the ability of cyclosporine A and its analog NIM811 to attenuate cortical damage in rodent models of TBI (Sullivan et al. 2000; Mbye et al. 2008). However, administrations of these inhibitors following spinal cord contusion rodent models produced confounding results. Cyclosporine A has not offered the same extent of protection in SCI as seen in TBI, except in doses that are highly toxic (Rabchevsky et al. 2001; Morota et al. 2007). NIM811 did offer moderate protection at the cellular level and in functional outcome following SCI at safe doses (McEwen et al. 2007; Ravikumar et al. 2007). Furthermore, we recently showed that mice with a homozygous deletion of CypD encoding gene *Ppif* offered no increased protection from secondary damage following spinal cord contusion. These discrepancies may be attributed to regional differences in brain and spinal cord pathologies that may subsequently alter drug efficiency.

We sought to assess if this cortical protection would be consistent in our CypD-null mouse model. We hypothesized that CypD-null mice will have improved pathological outcomes following cortical contusion. To test this hypothesis, we employed a modified eriochrome cyanine staining for histological assessment of spared cortical tissue. The results of our study show that ablation of CypD in mice is protective in tissue sparing following cortical contusion.

## Materials and Methods

*Animals.* All animals were housed in the Division of Laboratory Animal Resources sector of the University of Kentucky Medical Center, which is fully accredited by AAALAC. All experimental procedures abide by protocols approved by the University of Kentucky's Institutional Animal Care and Use Committee. These studies were performed using both WT and transgenic mice. The transgenic mice (CypD-null) features a homozygous deletion of the CypD encoding gene *Ppif* (peptidylprolyl isomerase F). CypD-null mice were generated on a chimeric strain using adult female C57BL/6 crossed to a SV129F1 strain (B6129F1) (Baines et al. 2005). The WT mice utilized were also B6129 purchased from Taconic.

*Mouse Model of Traumatic Brain Injury.* The cortical contusions administered in these studies were conducted using a commercially available impactor from Precision Systems & Instrumentation (PSI), Lexington, KY). According to PSI, the pneumatic driven IH cortical impactor uses a contact sensor that enables a more accurate determination of the cortical surface prior to contusion. There are three parameters measured by the IH cortical impactor and defined as follows: *Force (kdyn)* as the actual force delivered by the impactor compared to the calibrated force, 2. *Velocity (ms)* as the speed of the impactor tip as it delivers the contusion, and 3. *Dwell Time (mm)* as the elasticity of the cortex required until the calibrated force is achieved. The contact velocity is user controlled allowing different degrees of cortical deformation to achieve the desired injury severity. In these experiments, the contact velocity of the impactor was set at 3.5 ms to

induce a moderate cortical contusion of .5mm. Mice were initially anesthetized in a Plexiglas chamber using 4.0% isoflurane and placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA). Anesthesia was maintained throughout the entire surgery with 2.5% isoflurane delivered via a nose cone. The head was positioned on a horizontal plane with the nose bar set at zero. After a midline incision exposing the skull, an approximate 4.0 mm craniotomy was made lateral to the sagittal suture and centered between bregma and lambda. The craniotomy section was carefully removed to avoid excessive perturbation of the dura under the skull cap. The exposed cortex was injured using a pneumatically controlled impactor device with a 3.0 mm flat-tip diameter. After injury, the craniotomy was closed by placement of a small disk of saline-moistened Surgical over the dura, followed by gluing a 6.0 mm disk made of dental cement over the site with super glue. The animals were placed on a heating pad set at 37°C until consciousness and mobility was regained.

*Histological Assessment of Cortical Tissue Sparing.* At 7 days post surgery, mice were given an overdose injection of pentobarbital followed by a cardiac perfusion of cold 1x PBS and 4% PFA. Whole brain were dissected, post-fixed in the same 4% PFA solution for 24 h at 4°C followed by submersion in 25% sucrose solution at 4°C to ensure cryoprotection. Whole brains were cryosectioned at 25µm thickness and mounted onto gelatin-coated slides and stored at -20°C. For histological staining, the gelatin-coated slides were allowed to thaw to room temperature. Each slide was then cleared and hydrated in Citrisolv followed by ethanol concentration gradient. Slides were then submerged in a Cresyl stain

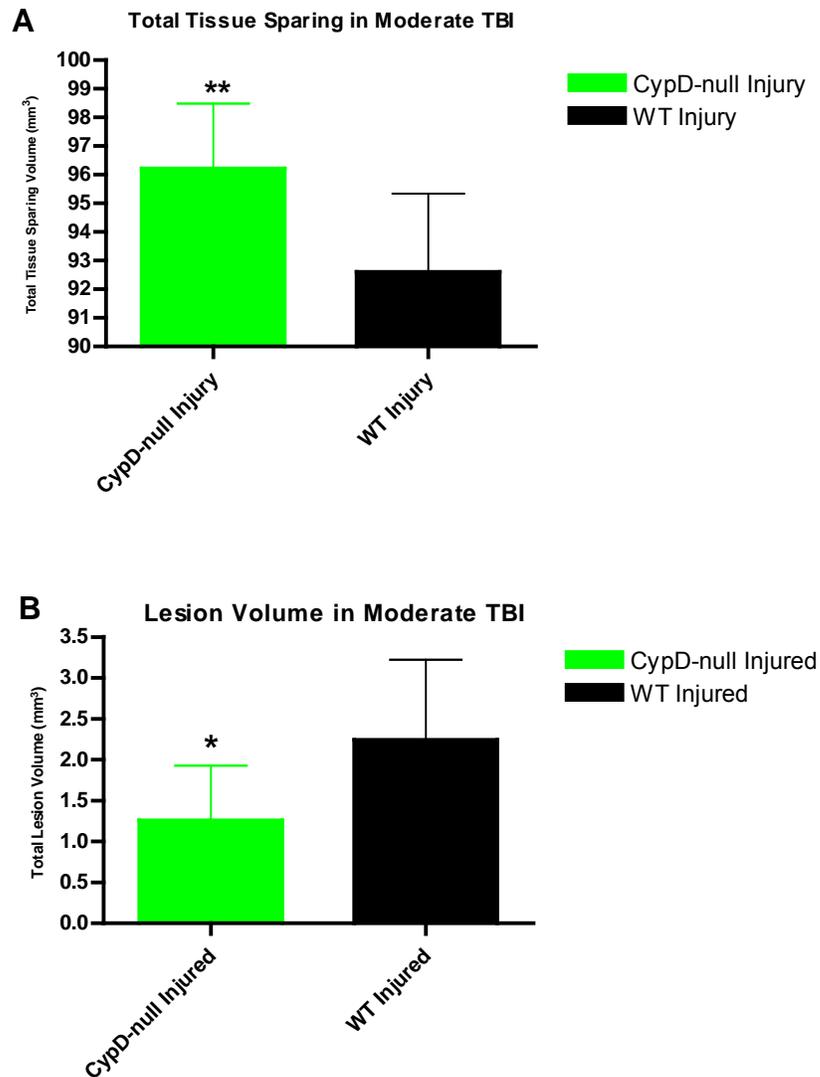
(1M acetic acid, sodium acetate, d H<sub>2</sub>O, cresyl violet acetate). The slides were submerged in d H<sub>2</sub>O followed by a reverse ethanol concentration gradient. Ipsilateral and contralateral cortical sections were assessed for percentage of spared tissue and lesion volume.

## Results

### *Histological Assessment of Spinal Cord Tissue Sparing*

At 4 week post cortical contusion or craniotomy, all mice were euthanized and volumetric measurements were conducted by histological assessment for total spared tissue and lesion volume. Histological staining showed that following moderate contusion, both CypD-null and WT injured mice had the greatest spinal tissue loss at the impact site, or the epicenter, with damage extending

Figure 9 shows spared tissue and lesion volume assessment in CypD-null and WT injured mice following moderate cortical contusion. The percentage of spared tissue was calculated from measurements taken from the contralateral cortex of each animal (Figure 9A). Analysis by two-tailed t-test showed that CypD-null mice had significantly greater tissue sparing compared to WT mice (Figure 9A)( $p < .01$ ). Lesion volume was also measured in CypD-null and WT injured mice (Figure 9B). Analysis by two-tailed t-test showed that CypD-null mice had a significant reduction in lesion volume compared to WT mice ( $p < .05$ ).



**Figure 9** Total tissue sparing for WT and CypD-null mice 1 week post moderate cortical contusion. **A.** Percentage of total tissue sparing. CypD-null injured mice had significantly greater tissue sparing compared to WT injured mice. Values=Mean <sup>+/-</sup> SD. \*\*p<.01, t=3.387. Two-tailed unpaired student's t- test. N=10,11 for injured group. **B.** Lesion volume. CypD-null injured mice had a significant reduction in lesion volume compared to WT injured mice.

Values=Mean  $\pm$  SD. \*p<.05, t=2.662. Two-tailed unpaired student's t-test.

N=10,11 for injured group.

## Discussion

The principal finding in our study was that mice with a homozygous knockout of cyclophilin D, the regulatory component in mPTP formation, showed significant improvement in cortical tissue sparing following moderate cortical contusion. The purpose of this study was to assess the role of CypD in neuroprotection following traumatic brain injury. Previous literature has shown CypD to be a viable target for neurological protection in rodent models of traumatic brain injury. Cyclosporin A and NIM811, both potent inhibitors of CypD, improved motor function, increased cortical tissue sparing, and reduction in calpain-mediated cytoskeleton degradation following cortical contusion in rats (Mbye et al. 2008; Mbye et al. 2008; Wang et al. 2009). However, following spinal cord injury, these same CypD inhibitors produced contradictory results.

Our preliminary findings support CypD as a viable target for attenuating secondary brain injury. One week following administration of a moderate cortical contusion, CypD-null mice showed improved tissue sparing and a reduction in lesion volume compared to WT mice. Interestingly, we recently showed that CypD-null mice had no improved outcome following spinal cord contusion. These findings suggest that both physiological and pathological differences in brain injury compared to spinal cord injury greatly affect the properties of CypD.

Post traumatic mitochondrial dysfunction and permeability, for example, is readily induced due to Ca<sup>2+</sup> overload following cortical injury. Mitochondrial Ca<sup>2+</sup> overload can also activate various mitochondrial free radicals that induce mPTP formation and mitochondrial dysfunction in a CypD dependent manner

(Ghafourifar et al. 1997; Richter et al. 1997; Mbye et al. 2008). Furthermore, CypD was shown to be highly localized to synaptic mitochondria in the cortex (Naga et al. 2007). These factors create a unique environment that is highly susceptible to CypD-regulated injury in cortical injury. This is not to dismiss the importance of mitochondrial dysfunction and mPTP activity in spinal cord injury. However, the complex pathology of secondary spinal cord injury may induce formation of mPTP mitochondrial dysfunction independent of CypD regulation. Likewise, other secondary cascades, such as glial apoptotic death, may be the primary contributor to secondary spinal cord injury.

The data presented in this study further supports CypD as a promising target for attenuating the secondary damage in brain injury. Future studies should assess additional pathological outcomes following brain injury using CypD-null mice. Measurements of behavioral outcomes, such as motor and cognitive function, may show additional protective effects of CypD ablation. Another intriguing study would be measurement of mitochondrial bioenergetics in CypD-null mice following brain injury. This should further confirm if the improved mitochondrial function, as demonstrated with CypD pharmacological inhibition in rats, is actually exclusive to CypD (Sullivan et al. 2000; Mbye et al. 2008).

## Chapter Four

### Summary and Conclusions

Traumatic spinal cord and brain injury pathology consist of the primary injury and secondary injury. The primary injury is the immediate damage induced by the initial mechanical contusion. In the ensuing hours, days, and weeks a secondary cascade of deleterious events accelerates the pathological damage. Intervention and attenuation of primary damage is difficult due to rapid onset following the injury. The events occurring in secondary damage, however, present a unique window of opportunity for therapeutic attention. Thus, a thorough understanding of the pathological mechanisms in secondary damage will allow for more effective pharmacological and critical treatment following traumatic spinal cord and brain injury.

Recently, cyclophilin D (CypD), the regulating component in mitochondrial permeability transitional pore formation (mPTP), has shown promise in attenuating secondary damage in brain injury. However, the role of CypD in spinal cord injury is still uncertain. In this series of studies, we utilized mice with a homozygous knockout of CypD in models of both spinal cord and brain injury. We were able to directly assess the role of CypD in spinal cord injury through measurements of motor function, tissue sparing, and calpain activation. We hypothesized that CypD-null mice would have improved pathological outcomes following spinal cord and brain injury.

Our first study utilized the Basso Mouse Scale (BMS) to measure hind limb motor function for 4 weeks following different severities of spinal contusions

CypD-null mice. Following both mild and moderate spinal contusion, CypD-null showed no greater recovery of motor function than WT mice. However, an unexpected outcome was produced when we administered a severe spinal contusion. Motor impairment was exacerbated in CypD-null compared to WT mice.

Our next study was a histological assessment of tissue sparing using eriochrome cyanine staining procedure that specifically stains white matter. This procedure was employed immediately following the 4<sup>th</sup> week of BMS testing. We measured total spared tissue, lesion volume, and white and grey matter. The results of our histological measurements were consistent with our BMS results. In our moderate spinal contusion model, CypD-null mice showed no improved sparing of tissue. Following severe spinal contusion, CypD-null mice showed a decrease in tissue sparing compared with WT mice.

We concluded this series of studies by measuring calpain activity 24 hours following moderate spinal cord contusion. Potentially, CypD-mediated death may be more prominent in the immediate stages of secondary injury. We selected calpain based on previous literature that showed elevated calpain activity following spinal cord injury. This increased calpain activity was attenuated when CypD was inhibited. However, our Western Blot analysis of calpain, measured as calpain metabolism of spectrin, showed that CypD-null mice had no reduction in calpain activity compared to WT mice.

Though pharmacological inhibition of CypD has shown to be protective following brain injury, we wanted to confirm these findings using our transgenic

model. We presented some preliminary data showing that CypD-null mice had increased cortical tissue sparing and decreased lesion volume following a moderate cortical contusion. Future studies should further assess additional improvements in brain injury pathology using CypD-null mice.

In addition to our studies with CypD-null mice, we presented some preliminary data on the protective effects of NIM811 in SCI. NIM811 was shown to have protective effects following SCI. However, those studies used rats for modeling SCI. Thus, we wanted to examine the effects of NIM811 in mice. Our results showed that NIM811 modestly improved motor function in WT mice. These improvements were observed at two weeks and three weeks following the spinal cord injury.

In summary, our studies show that CypD is a viable target for attenuating damage following cortical contusion. These protective effects were not observed in our models of SCI. We showed that CypD-null mice showed no improvements following moderate spinal cord injury. However, the exacerbated deficits observed following severe injury suggest that some basic level of functional CypD is necessary for cell survival.

This may seem contradictory as NIM811 did show moderate protection in rat models following SCI and in our study using mouse models. However, drug inhibition of CypD does not affect all populations of mitochondria. Thus, there is still functional CypD to maintain its physiological activity. It is also feasible that NIM811 interacts with additional proteins other than CypD. We did not examine the effects of NIM811 in CypD-null mice following SCI. This would be an

intriguing study as it may reveal unique characteristics of NIM811 as well as mPTP regulation.

These data warrant further investigation into the efficacy of targeting CypD in SCI. However, we provided some promising preliminary data showing another potential target for attenuating secondary spinal cord damage. We assessed the role CAPN5, a member of the calpain family, in spinal cord pathology. The results from our study showed that CAPN5 deficient mice had greater recovery in motor function following a severe spinal contusion. CAPN5 was previously shown to be ubiquitously expressed in the rat brain and spinal cord and that it has Ca<sup>2+</sup> binding domains. Thus, CAPN5 may have similar proteolytic activity as other typical mammalian calpains. Studies have shown that inhibiting calpain can attenuate some of the pathological damage following SCI and TBI. Thus, identifying calpain members that contribute to secondary cell death is critical. Our results suggest that CAPN5 may have a physiological role in spinal cord injury. Strategies aimed at multiple calpain isoforms may offer critical protection following spinal cord and brain injury.

## Appendix I

### Effects of NIM811 on Motor recovery in the Mouse Model of Spinal Cord Injury

#### Introduction

A prominent mechanism of secondary tissue degradation is an increase in mitochondrial permeability to any molecules < 1500 Daltons (Hunter et al. 1976; Al-Nasser et al. 1986). This increase in mitochondrial permeability can lead to massive swelling and disruption of the mitochondrial membrane, uncoupling of oxidative phosphorylation in the electron transport chain and subsequent down regulation of ATP production (Hunter et al. 1976; Bernardi et al. 1996; Crompton 1999; Halestrap et al. 2002).

Ultimately, it is the formation of the mitochondrial permeability transitional pore (mPTP) that permits excessive ion permeability and disruption of mitochondrial bioenergetics (Hunter et al. 1976; Al-Nasser et al. 1986). A critical finding by Crompton and colleagues was the inhibition of  $Ca^{2+}$  dependent mPTP opening by the immunosuppressive drug cyclosporine A (CsA)(1988). CsA mediates its actions by binding to and inhibiting the mitochondrial matrix component cyclophilin D(CypD) from binding to ANT (Crompton et al. 1998). CsA inhibited pore formation and subsequent mitochondrial permeability, prompting the hypothesis that CypD is the regulating component in mPTP formation.

CypD has since shown promise as a viable target for protection in rodent models of TBI (Sullivan et al. 2000; Mbye et al. 2008). However, in rodent models of SCI, CsA failed to offer any protection unless administered at highly toxic doses (Rabchevsky et al. 2001; Morota et al. 2007). Recently, a CsA analog called NIM811 did show some modest protection at the cellular level and in functional outcome in rat models of SCI (McEwen et al. 2007; Ravikumar et al. 2007). These contradictory results raise uncertainty about the role of CypD in SCI pathology.

We recently showed that mice with a homozygous knockout of *Ppif*, the CypD encoding gene, had no improvements in functional or pathological outcomes following SCI. These findings suggest that CypD has only a minor role in secondary spinal cord injury. Thus, the protective effects from NIM811 following SCI may stem from additional protein interactions that induce mPTP formation independent of CypD.

It is also feasible that there are additional variations in secondary injury response, including mPTP formation and function, between the mouse and rat model of SCI. Thus, a comparison between models is critical for translation of protective strategies across models. In this study, we administered NIM811 to wild type (WT) mice following a moderate spinal contusion. We then utilized the Basso mouse scale to assess any improvements in motor function recovery.

## Methods

*Animals.* All animals were housed in the Division of Laboratory Animal Resources sector of the University of Kentucky Medical Center, which is fully accredited by AAALAC. All experimental procedures abide by protocols approved by the University of Kentucky's Institutional Animal Care and Use Committee. These studies were performed using B6129 female mice purchased from Taconic.

*Mouse Model of Traumatic Spinal Cord Injury.* All experimental procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky. The spinal contusions administered in these studies were conducted using the commercially available Infinite Horizon (IH) spinal cord injury device (Precision Systems & Instrumentation (PSI), Lexington, KY). The IH impactor allows for user defined parameters to achieve the desired severity of injury. There are three parameters measured by the IH impactor and defined as follows: *Force* as the actual force delivered by the impactor compared to the calibrated force, 2. *Velocity* as the speed of the impactor tip as it delivers the contusion, and 3. *Displacement* as the elasticity of the spinal cord required until the calibrated force is achieved. The force of the contusion, measured in kilodyns, was calibrated according to each severity. For the severe contusions, a calibrated force of 70kdyn was used. These settings were derived from a previous study characterizing injury gradients in the mouse model (Basso et al. 2006). Mice were anesthetized by an intraperitoneal injection ketamine(mg/kg) and xylamine(mg/kg). Laminectomies

were made to expose the T10 vertebral segment. Two clamps were applied approximately at the caudal T11 segment and the rostral T9 segment. The spinal cord was then lowered and straightened until parallel with the platform. Sham animals received a laminectomy only. Following surgery, all animals were placed on a heating pad set at 37°C until consciousness and mobility (upper limbs) was regained. Postoperative care included the manual expression of bladders twice daily until recovery of bladder function, injection with 10 ml sterile saline administered subcutaneously (s.c.) immediately after surgery, 33.3 mg/kg cefazolin, administered by intramuscular injection, twice a day for 1 week then once a day for the remaining 3 weeks.

*Experimental Group and Design.* NIM811 was generously provided by Dr. Patrick Sullivan from the University of Kentucky. NIM811 (10mg/kg) was made in a solution of polyethylene glycol/ .9% sterile saline/cremaphor oil. The vehicle for NIM811 was the polyethylene glycol/sterile saline/cremaphor oil solution only. Either NIM811 or vehicle was administered by an intraperitoneally injection at 15 minutes post surgery/injury and another injection 24 hours post surgery/injury. These experiments involved four groups of mice. Shams with NIM811 2 received a laminectomy and NIM811. Shams with vehicle received a laminectomy and equal volumes of the vehicle. Injured with NIM811 received a moderate spinal contusion and NIM811, and injured with vehicle received a moderate spinal contusion and equal volumes of the vehicle. The dose of NIM811 was selected based on a previous study showing the e optimal doses for attenuating

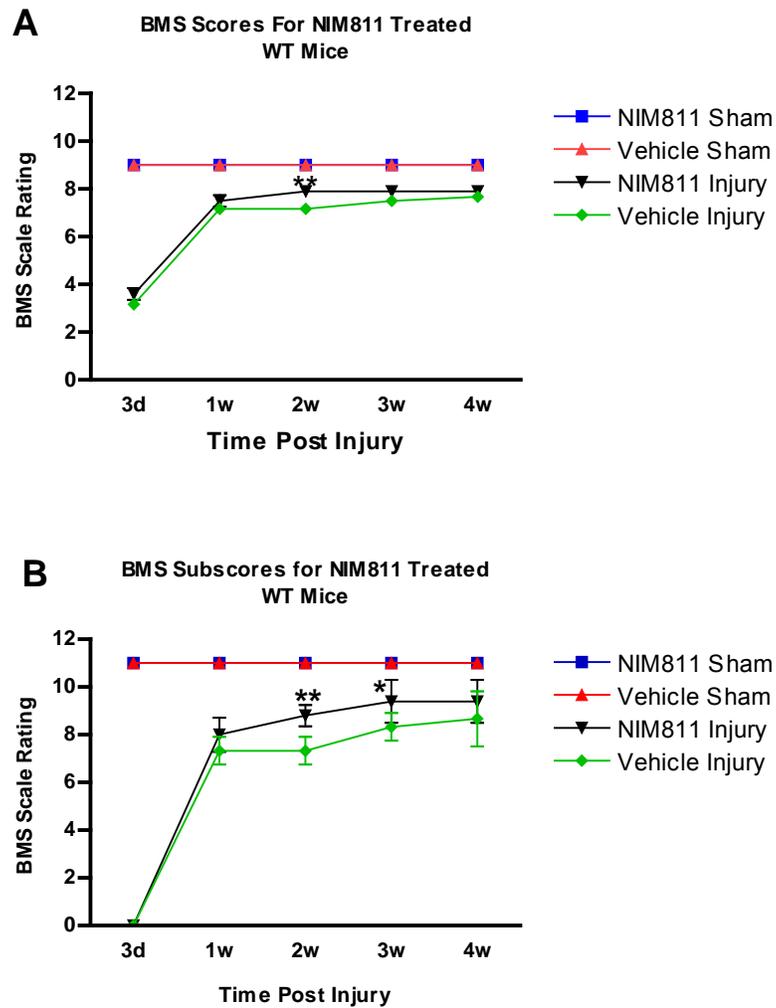
mitochondrial dysfunction in mice subjected to cortical contusion (Mbye et al. 2008).

*Hind Limb Motor Function Assessment.* Motor function following spinal cord injury was measured by two raters blinded to the experimental conditions. Hind limb motor function was measured using the Basso Mouse Scale (BMS), a 9 point categorical scale that rates seven observable motor functions and motor deficits. The seven categories in the BMS included ankle extension, of hind limb joint movements, trunk stability, stepping coordination, paw placement, and tail position (Basso et al. 2006). A single score was recorded for the left and right hind limb which was then averaged to give a total score. An 11 point subscore was obtained to assess subtle variations within each category. Each BMS test was assessed by two examiners. One examiner is the main observer while the other examiner observes and records the scores during the exam. This is to ensure that one person is observing the test for the entire duration. Each trial is 4 minutes.

## Results

The Basso Mouse Scale (BMS) analysis was used to assess open field motor function in WT mice following moderate spinal contusion. Mice were administered either NIM811 or vehicle at 15 minutes and 24 hours post injury. Immediately following spinal impact, both animals displayed complete bilateral hind limb paralysis as evident by ankle extension or only ankle extension plus plantar placement without weight support (data not shown).

Figure 10A shows that following moderate SCI, mice administered NIM811 had significantly greater improvement in functional motor recovery at the 2<sup>nd</sup> week post injury compared to mice administered vehicle only [ $F=54.28(391.6,162)$ ;  $p<.01$ ]. BMS subscores revealed additional time points of increased motor recovery (Figure 10B). Mice administered NIM811 showed significant improvements in functional motor recovery at both the 2<sup>nd</sup> and 3<sup>rd</sup> week post injury (Figure 10B)[ $F=58.51(372,173.8)$ ,  $p<.01$  at 2wk,  $p<.05$  at 3wk]. The improved motor recovery at these time points is most likely due to paw positioning upon stepping and landing. Mice receiving NIM811 maintained parallel paw positioning throughout the entire step. This motor skill was not observed in mice receiving a vehicle until the 4<sup>th</sup> week.



**Figure 10** Basso Mouse Scale(BMS) scores and subscores for WT vehicle and NIM811 sham and injured mice at 3d, 1wk, 2wk, 3wk, and 4wk post moderate injury. **A.** Mice receiving NIM811 showed significant improvement in BMS scores at 2 weeks following moderate SCI [ $**p < .01$ ,  $F = 54.28(391.6, 162)$ ]. **B.** Mice receiving NIM811 showed significant improvement in BMS subscores at 2 weeks and 3 weeks following moderate SCI [ $**p < .01$  at 2 weeks;  $*p < .05$  at 3 weeks,  $F = 58.51(372, 173.8)$ ]. Values = Mean  $\pm$  STDEV. Two-way ANOVA and Bonferroni post hoc test. N=4-5 for injury and sham groups.

## Discussion and Conclusion

The principle finding in this preliminary study was that NIM811 administered to WT mice following moderate SCI significantly improves motor function. The purpose of this study was to assess the protective effects of NIM811 in a mouse model of SCI. Previous studies have produced confounding results when administering CypD inhibitors in rat models of SCI (Rabchevsky et al. 2001)

Although NIM811-treated mice showed improvements in motor recovery at two weeks and three weeks following moderate spinal contusion, the improvements observed were very modest. Further assessment of tissue sparing and cellular alterations is needed to further characterize the protective mechanism of NIM811. Still, these preliminary data provide a critical finding in that NIM811 has protective effects in mice and rats. This may allow for more confident translation and interpretation of results across both mouse and rat models of SCI.

There is still uncertainty if CypD is exclusively targeted by NIM811, at least in the mouse model. Previously we showed that CypD null mice had no greater improvements in functional or pathological outcomes following SCI. Future studies are needed to examine the effects of NIM811 in CypD-null mice. If NIM811 does improve spinal cord injury outcome in CypD-null mice, this would suggest that NIM811 targets additional proteins involved in secondary spinal cord other than CypD.

## Appendix II

### Role of Calpain 5 in Spinal Cord Injury

#### Introduction

Calpains are  $\text{Ca}^{2+}$  dependent cysteine proteases involved in several cellular functions including cell motility, synaptic remodeling, embryonic development and (Luscher et al. 2000; Liu et al. 2008). To date, approximately 14 mammalian calpain isoforms have been identified (Goll et al. 2003). Typical calpain isoforms,  $\mu$ -calpain and m-calpain, are ubiquitously expressed while atypical isoforms are tissue specific (Goll et al. 2003). Typical mammalian calpains generally consist of a large distinctive catalytic subunit and a small common regulatory subunit (Goll et al. 2003). Several proteins are substrates for calpain such as cytoskeletal structural and cell motility proteins (Posmantur et al. 1994; Pang et al. 1996; Azam et al. 2001).

Hyper activation of calpain has been implemented in pathological events, specifically secondary cellular degradation following traumatic brain injury (TBI) and spinal cord injury (SCI) (Banik et al. 1982; Saido et al. 1994; Huang et al. 2001). (Huang et al. 2001) Several studies have shown that pharmacological inhibition of calpain attenuates motor deficits and tissue degradation in rodent models of TBI and SCI (Springer et al. 1997; Ray et al. 1999; Schumacher et al. 2000; Deng et al. 2007). Thus, there has been an extensive effort to identify additional calpains potentially involved in the pathology of TBI and SCI.

Previously, calpain-5 (CAPN5) was cloned and characterized in rats and found to have a highly homologous sequence to both human and mice (Matena et al. 1998; Waghray et al. 2004). In rats and humans, CAPN5 was shown to be ubiquitously expressed in several brain regions including the medulla oblongata, hypothalamus, thalamus, cerebellum, and frontal lobe (Waghray et al. 2004). In addition, CAPN5 was shown to have key calcium binding domains similar to the typical calcium dependent calpains. These characteristics suggest that CAPN5 may be involved in neurological injury. To assess the potential involvement of CAPN5 in SCI, we administered a severe spinal contusion to CAPN5<sup>-/+</sup> mice. Our results suggest that CAPN5 may be a key calpain isoform that exacerbates functional outcome in spinal injury.

## Methods

*Animals.* All animals were housed in the Division of Laboratory Animal Resources sector of the University of Kentucky Medical Center, which is fully accredited by AAALAC. All experimental procedures abide by protocols approved by the University of Kentucky's Institutional Animal Care and Use Committee. Mice with a heterozygous knockout of CAPN5 (Capn5<sup>tm1Dgen</sup>) were purchased from the Jackson laboratory. The WT mice utilized were B6129 purchased from Taconic.

*Mouse Model of Traumatic Spinal Cord Injury.* All experimental procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky. The spinal contusions administered in these studies were conducted using the commercially available Infinite Horizon (IH) spinal cord injury device (Precision Systems & Instrumentation (PSI), Lexington, KY). The IH impactor allows for user defined parameters to achieve the desired severity of injury. There are three parameters measured by the IH impactor and defined as follows: *Force* as the actual force delivered by the impactor compared to the calibrated force, 2. *Velocity* as the speed of the impactor tip as it delivers the contusion, and 3. *Displacement* as the elasticity of the spinal cord required until the calibrated force is achieved. The force of the contusion, measured in kilodyns, was calibrated according to each severity. For the severe contusions, a calibrated force of 70kdyn was used. These settings were derived from a previous study characterizing injury gradients in the mouse model (Basso et al. 2006). Mice were anesthetized by an

intraperitoneal injection ketamine(mg/kg) and xylamine(mg/kg). Laminectomies were made to expose the T10 vertebral segment. Two clamps were applied approximately at the caudal T11 segment and the rostral T9 segment. The spinal cord was then lowered and straightened until parallel with the platform. Sham animals received a laminectomy only. Following surgery, all animals were placed on a heating pad set at 37°C until consciousness and mobility (upper limbs) was regained. Postoperative care included the manual expression of bladders twice daily until recovery of bladder function, injection with 10 ml sterile saline administered subcutaneously (s.c.) immediately after surgery, 33.3 mg/kg cefazolin, administered by intramuscular injection, twice a day for 1 week then once a day for the remaining 3 weeks.

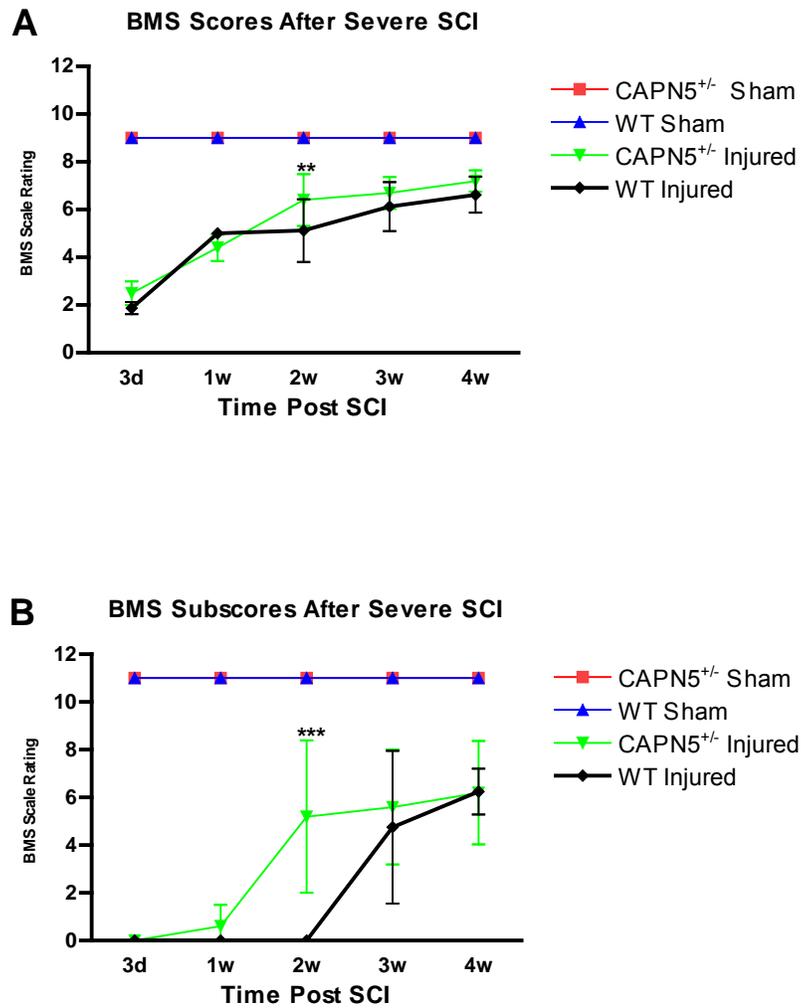
*Hind Limb Motor Function Assessment.* Motor function following spinal cord injury was measured by two raters blinded to the experimental conditions. Hind limb motor function was measured using the Basso Mouse Scale (BMS), a 9 point categorical scale that rates seven observable motor functions and motor deficits. The seven categories in the BMS included ankle extension, of hind limb joint movements, trunk stability, stepping coordination, paw placement, and tail position (Basso et al. 2006). A single score was recorded for the left and right hind limb which was then averaged to give a total score. An 11 point subscore was obtained to assess subtle variations within each category. Each BMS test was assessed by two examiners. One examiner is the main observer while the other examiner observes and records the scores during the exam. This is to

ensure that one person is observing the test for the entire duration. Each trial is 4 minutes.

## Results

The Basso Mouse Scale (BMS) analysis was used to assess CAPN5<sup>+/-</sup> and WT mice for open field motor function following severe spinal contusion. Immediately following spinal impact, both animals displayed complete bilateral hind limb paralysis as evident by ankle extension or only ankle extension plus plantar placement without weight support (data not shown).

Figure 11A. and B show that following severe SCI, CAPN5<sup>+/-</sup> mice had a significantly greater improvement in functional motor recovery at the 2<sup>nd</sup> week post injury [ $F=17.28(337.2,48.65)$ ;  $p<.01$ ]. CAPN5<sup>+/-</sup> mice showed recovery of some coordination, occasional to consistent stepping, and only mild trunk stability. While WT did eventually achieve the same level of motor recovery, this wasn't observed until the 3<sup>rd</sup> and 4<sup>th</sup> week, as shown by BMS subscores (Figure 9B)[ $F=7.861(256.2,18.03)$ ,  $p<.001$ ].



**Figure 11** Basso Mouse Scale(BMS) scores and subscores for WT and CAP5<sup>+/-</sup> sham and injured mice at 3d, 1wk, 2wk, 3wk, and 4wk post severe injury. **A-B.** CAP5<sup>+/-</sup> injured mice showed significant improvement in BMS scores and subscores compared to WT injured mice at 2 weeks following severe SCI. Values=Mean <sup>+/-</sup> STDEV. \*\*p<.01, F=17.28(337.2,48.65); \*\*\*p<.001, F=7.861(256.2,18.03). Two-way ANOVA and Bonferroni post hoc test. N=4-5 for injury and sham groups.

## Discussion

The results from this study show that CAPN5<sup>-/+</sup> mice have improved functional motor recovery following severe spinal cord contusion. Little is known about the physiological function of CAPN5<sup>-/+</sup> in mice. However, calpains have been implicated in the pathology of spinal cord injury (Banik et al. 1982; Banik et al. 1998; Ray et al. 1999). Following spinal contusion, calpain can remain elevated for days and weeks. Hyper activation of Calpain degrades several cellular proteins leading to both necrotic and apoptotic mechanisms of death (Ray et al. 1999). Substantial evidence has shown calpain to be a promising target for neurological protection following SCI. Previous studies using calpain inhibitors of calpain demonstrated decreased cellular protein degradation, increased tissue sparing and improved motor function in rat models of SCI (Springer et al. 1997; Zhang et al. 2003; Yu et al. 2007).

Here we show that CAPN-5 may also have a role in spinal cord pathology. Motor recovery was improved in CAPN5<sup>-/+</sup> mice at the second week following severe spinal cord injury. WT mice at this time point had regained only occasional stepping with some weight support. CAPN5<sup>-/+</sup>, however, showed frequent stepping with full weight support, followed by recovery of some coordination. Both models achieved the same level of recovery by the 4<sup>th</sup> week. These motor skills included consistent weight bearing and stepping, coordination, and mild trunk stability. Though, CAPN5<sup>-/+</sup> mice recovered these motor skills at an earlier time point, it did not continue to display improved function.

Recently, CAPN5 was shown to be ubiquitously expressed in the rat brain and spinal cord (Waghray et al. 2004). Further characterization showed CAPN5 contained key  $\text{Ca}^{2+}$  binding domains (Wagner et al. 1999). These properties of CAPN5 may reveal a protease function similar to  $\mu$  and m-calpain. This recent characterization and localization of CAPN5, along with our findings of motor improvements in CAPN5<sup>-/+</sup> mice, suggest that CAPN5 may have a critical role in spinal cord pathology. It is important to note that we utilized mice with a heterozygous knockout of CAPN5. This is because homozygous knockouts are embryonic lethal. Despite only partial ablation of CAPN5, we still observed functional improvements. Thus, multiple strategies using potent CAPN5-specific inhibitors in conjunction with other calpain inhibitors may greatly improve functional and pathological recovery in spinal injury.

### Appendix III

#### *Glossary of Important Terms*

AIF	apoptosis inducing factor	mPT	mitochondrial permeability
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	mPTP	mitochondrial permeability transitional pore
ANT	adenine nucleotide translocase	NMDA	<i>N</i> -Methyl-D-aspartate
ATP	adenosine triphosphate	PPIase	peptidyl-prolyl-cis-trans-isomerase
BMS	Basso Mouse Scale	ROS	reactive oxygen species
CsA	cyclosporine A	SBP	spectrin breakdown product
CypD	cyclophilin D	SCI	spinal cord injury
CypD-null	cylophilin D homozygous knockout	TBI	traumatic brain injury
MAP-2	microtubule-associated protein	VDAC	voltage dependent anionic channel
		WT	wild type

## References

- Ackery, A., S. Robins and M. G. Fehlings (2006). "Inhibition of Fas-mediated apoptosis through administration of soluble Fas receptor improves functional outcome and reduces posttraumatic axonal degeneration after acute spinal cord injury." J Neurotrauma 23(5): 604-16.
- Al-Nasser, I. and M. Crompton (1986). "The reversible Ca<sup>2+</sup>-induced permeabilization of rat liver mitochondria." Biochem J 239(1): 19-29.
- Anderson, D. K. and E. D. Hall (1993). "Pathophysiology of spinal cord trauma." Ann Emerg Med 22(6): 987-92.
- Anthes, D. L., E. Theriault and C. H. Tator (1995). "Characterization of axonal ultrastructural pathology following experimental spinal cord compression injury." Brain Res 702(1-2): 1-16.
- Arrigoni, E. and F. Cohadon (1991). "Calcium-activated neutral protease activities in brain trauma." Neurochem Res 16(4): 483-7.
- Azam, M., S. S. Andrabi, K. E. Sahr, L. Kamath, A. Kuliopulos and A. H. Chishti (2001). "Disruption of the mouse mu-calpain gene reveals an essential role in platelet function." Mol Cell Biol 21(6): 2213-20.
- Badugu, R., M. Garcia, V. Bondada, A. Joshi and J. W. Geddes (2007). "N-terminal of calpain 1 is a mitochondrial targeting sequence." J Biol Chem.
- Baines, C. P., R. A. Kaiser, N. H. Purcell, N. S. Blair, H. Osinska, M. A. Hambleton, E. W. Brunskill, M. R. Sayen, R. A. Gottlieb, G. W. Dorn, J. Robbins and J. D. Molkentin (2005). "Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death." Nature 434(7033): 658-62.
- Balentine, J. D. (1988). "Spinal cord trauma: in search of the meaning of granular axoplasm and vesicular myelin." J Neuropathol Exp Neurol 47(2): 77-92.
- Banik, N. L., E. L. Hogan, J. M. Powers and L. J. Whetstine (1982). "Degradation of cytoskeletal proteins in experimental spinal cord injury." Neurochem Res 7(12): 1465-75.
- Banik, N. L., E. L. Hogan, L. J. Whetstine and J. D. Balentine (1984). "Changes in myelin and axonal proteins in CaCl<sub>2</sub>-induced myelopathy in rat spinal cord." Cent Nerv Syst Trauma 1(2): 131-7.
- Banik, N. L., D. C. Shields, S. Ray, B. Davis, D. Matzelle, G. Wilford and E. L. Hogan (1998). "Role of calpain in spinal cord injury: effects of calpain and free radical inhibitors." Ann N Y Acad Sci 844: 131-7.
- Bardo, S., M. G. Cavazzini and N. Emptage (2006). "The role of the endoplasmic reticulum Ca<sup>2+</sup> store in the plasticity of central neurons." Trends Pharmacol Sci 27(2): 78-84.
- Basso, D. M., M. S. Beattie and J. C. Bresnahan (2002). "Descending systems contributing to locomotor recovery after mild or moderate spinal cord injury in rats: experimental evidence and a review of literature." Restor Neurol Neurosci 20(5): 189-218.

- Basso, D. M., L. C. Fisher, A. J. Anderson, L. B. Jakeman, D. M. McTigue and P. G. Popovich (2006). "Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains." J Neurotrauma 23(5): 635-59.
- Basso, E., V. Petronilli, M. A. Forte and P. Bernardi (2008). "Phosphate is essential for inhibition of the mitochondrial permeability transition pore by cyclosporin A and by cyclophilin D ablation." J Biol Chem 283(39): 26307-11.
- Bauer, M. K., A. Schubert, O. Rocks and S. Grimm (1999). "Adenine nucleotide translocase-1, a component of the permeability transition pore, can dominantly induce apoptosis." J Cell Biol 147(7): 1493-502.
- Beattie, M. S., A. A. Farooqui and J. C. Bresnahan (2000). "Review of current evidence for apoptosis after spinal cord injury." J Neurotrauma 17(10): 915-25.
- Beattie, M. S., G. E. Hermann, R. C. Rogers and J. C. Bresnahan (2002). "Cell death in models of spinal cord injury." Prog Brain Res 137: 37-47.
- Bernardi, P., R. Colonna, P. Costantini, O. Eriksson, E. Fontaine, F. Ichas, S. Massari, A. Nicolli, V. Petronilli and L. Scorrano (1998). "The mitochondrial permeability transition." Biofactors 8(3-4): 273-81.
- Bernardi, P. and V. Petronilli (1996). "The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal." J Bioenerg Biomembr 28(2): 131-8.
- Bervers, M. B. and R. W. Neumar (2007). "Mechanistic role of calpains in postischemic neurodegeneration." J Cereb Blood Flow Metab.
- Blight, A. R. and V. Decrescito (1986). "Morphometric analysis of experimental spinal cord injury in the cat: the relation of injury intensity to survival of myelinated axons." Neuroscience 19(1): 321-41.
- Bondarenko, A. and M. Chesler (2001). "Rapid astrocyte death induced by transient hypoxia, acidosis, and extracellular ion shifts." Glia 34(2): 134-42.
- bZhang, S. X., V. Bondada and J. W. Geddes (2003). "Evaluation of conditions for calpain inhibition in the rat spinal cord: effective postinjury inhibition with intraspinal MDL28170 microinjection." J Neurotrauma 20(1): 59-67.
- Casha, S., W. R. Yu and M. G. Fehlings (2001). "Oligodendroglial apoptosis occurs along degenerating axons and is associated with FAS and p75 expression following spinal cord injury in the rat." Neuroscience 103(1): 203-18.
- Casha, S., W. R. Yu and M. G. Fehlings (2005). "FAS deficiency reduces apoptosis, spares axons and improves function after spinal cord injury." Exp Neurol 196(2): 390-400.
- Cong, J., D. E. Goll, A. M. Peterson and H. P. Kapprell (1989). "The role of autolysis in activity of the Ca<sup>2+</sup>-dependent proteinases (mu-calpain and m-calpain)." J Biol Chem 264(17): 10096-103.

- Connern, C. P. and A. P. Halestrap (1994). "Recruitment of mitochondrial cyclophilin to the mitochondrial inner membrane under conditions of oxidative stress that enhance the opening of a calcium-sensitive non-specific channel." Biochem J 302 ( Pt 2): 321-4.
- Crocker, S. J., P. D. Smith, V. Jackson-Lewis, W. R. Lamba, S. P. Hayley, E. Grimm, S. M. Callaghan, R. S. Slack, E. Melloni, S. Przedborski, G. S. Robertson, H. Anisman, Z. Merali and D. S. Park (2003). "Inhibition of calpains prevents neuronal and behavioral deficits in an MPTP mouse model of Parkinson's disease." J Neurosci 23(10): 4081-91.
- Crompton, M. (1999). "The mitochondrial permeability transition pore and its role in cell death." Biochem J 341 ( Pt 2): 233-49.
- Crompton, M., H. Ellinger and A. Costi (1988). "Inhibition by cyclosporin A of a Ca<sup>2+</sup>-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress." Biochem J 255(1): 357-60.
- Crompton, M., S. Virji and J. M. Ward (1998). "Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore." Eur J Biochem 258(2): 729-35.
- Crowe, M. J., J. C. Bresnahan, S. L. Shuman, J. N. Masters and M. S. Beattie (1997). "Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys." Nat Med 3(1): 73-6.
- David, S. (2002). "Recruiting the immune response to promote long distance axon regeneration after spinal cord injury." Prog Brain Res 137: 407-14.
- Davis, A. E. (2000). "Mechanisms of traumatic brain injury: biomechanical, structural and cellular considerations." Crit Care Nurs Q 23(3): 1-13.
- Davis, L. M., J. R. Pauly, R. D. Readnower, J. M. Rho and P. G. Sullivan (2008). "Fasting is neuroprotective following traumatic brain injury." J Neurosci Res 86(8): 1812-22.
- Davis, L. M., J. M. Rho and P. G. Sullivan (2008). "UCP-mediated free fatty acid uncoupling of isolated cortical mitochondria from fasted animals: correlations to dietary modulations." Epilepsia 49 Suppl 8: 117-9.
- De Giorgi, F., L. Lartigue, M. K. Bauer, A. Schubert, S. Grimm, G. T. Hanson, S. J. Remington, R. J. Youle and F. Ichas (2002). "The permeability transition pore signals apoptosis by directing Bax translocation and multimerization." Faseb J 16(6): 607-9.
- Deng, Y., B. M. Thompson, X. Gao and E. D. Hall (2007). "Temporal relationship of peroxynitrite-induced oxidative damage, calpain-mediated cytoskeletal degradation and neurodegeneration after traumatic brain injury." Exp Neurol 205(1): 154-65.
- Ding, W. X., H. M. Shen and C. N. Ong (2002). "Calpain activation after mitochondrial permeability transition in microcystin-induced cell death in rat hepatocytes." Biochem Biophys Res Commun 291(2): 321-31.

- Dutta, S., Y. C. Chiu, A. W. Probert and K. K. Wang (2002). "Selective release of calpain produced alphaspectrin (alpha-fodrin) breakdown products by acute neuronal cell death." Biol Chem 383(5): 785-91.
- Emori, Y., H. Kawasaki, S. Imajoh, K. Imahori and K. Suzuki (1987). "Endogenous inhibitor for calcium-dependent cysteine protease contains four internal repeats that could be responsible for its multiple reactive sites." Proc Natl Acad Sci U S A 84(11): 3590-4.
- Farkas, O., B. Polgar, J. Szekeres-Bartho, T. Doczi, J. T. Povlishock and A. Buki (2005). "Spectrin breakdown products in the cerebrospinal fluid in severe head injury--preliminary observations." Acta Neurochir (Wien) 147(8): 855-61.
- Ferrand-Drake, M., C. Zhu, G. Gido, A. J. Hansen, J. O. Karlsson, B. A. Bahr, N. Zamzami, G. Kroemer, P. H. Chan, T. Wieloch and K. Blomgren (2003). "Cyclosporin A prevents calpain activation despite increased intracellular calcium concentrations, as well as translocation of apoptosis-inducing factor, cytochrome c and caspase-3 activation in neurons exposed to transient hypoglycemia." J Neurochem 85(6): 1431-42.
- Forte, M., B. G. Gold, G. Marrassi, P. Chaudhary, E. Basso, D. Johnsen, X. Yu, J. Fowlkes, M. Rahder, K. Stem, P. Bernardi and D. Bourdette (2007). "Cyclophilin D inactivation protects axons in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis." Proc Natl Acad Sci U S A 104(18): 7558-63.
- Gaetz, M. (2004). "The neurophysiology of brain injury." Clin Neurophysiol 115(1): 4-18.
- Gelder, J. B. and H. W. Sampson (1990). "Tissue calcium levels following spinal cord transection in rats." Acta Neurobiol Exp (Wars) 50(6): 601-8.
- Genovese, T., E. Esposito, E. Mazzon, R. Di Paola, R. Meli, R. Caminiti, P. Bramanti, M. P. Fink and S. Cuzzocrea (2008). "Beneficial Effects of Ethyl Pyruvate in a Mouse Model of Spinal Cord Injury." Shock.
- Genovese, T., E. Esposito, E. Mazzon, C. Muia, R. Di Paola, R. Meli, P. Bramanti and S. Cuzzocrea (2008). "Evidence for the role of mitogen-activated protein kinase signaling pathways in the development of spinal cord injury." J Pharmacol Exp Ther 325(1): 100-14.
- Ghafourifar, P. and C. Richter (1997). "Nitric oxide synthase activity in mitochondria." FEBS Lett 418(3): 291-6.
- Ghasemlou, N., B. J. Kerr and S. David (2005). "Tissue displacement and impact force are important contributors to outcome after spinal cord contusion injury." Exp Neurol 196(1): 9-17.
- Gieron, M. A., J. K. Korthals and C. D. Riggs (1998). "Diffuse axonal injury without direct head trauma and with delayed onset of coma." Pediatr Neurol 19(5): 382-4.
- Goll, D. E., V. F. Thompson, H. Li, W. Wei and J. Cong (2003). "The calpain system." Physiol Rev 83(3): 731-801.

- Graham-Siegenthaler, K., S. Gauthier, P. L. Davies and J. S. Elce (1994). "Active recombinant rat calpain II. Bacterially produced large and small subunits associate both in vivo and in vitro." J Biol Chem 269(48): 30457-60.
- Gross, A., K. Pilcher, E. Blachly-Dyson, E. Basso, J. Jockel, M. C. Bassik, S. J. Korsmeyer and M. Forte (2000). "Biochemical and genetic analysis of the mitochondrial response of yeast to BAX and BCL-X(L)." Mol Cell Biol 20(9): 3125-36.
- Gunter, T. E. and D. R. Pfeiffer (1990). "Mechanisms by which mitochondria transport calcium." Am J Physiol 258(5 Pt 1): C755-86.
- Guth, L., Z. Zhang and O. Steward (1999). "The unique histopathological responses of the injured spinal cord. Implications for neuroprotective therapy." Ann N Y Acad Sci 890: 366-84.
- Halestrap, A. P., G. P. McStay and S. J. Clarke (2002). "The permeability transition pore complex: another view." Biochimie 84(2-3): 153-66.
- Hall, E. D., Y. D. Bryant, W. Cho and P. G. Sullivan (2008). "Evolution of post-traumatic neurodegeneration after controlled cortical impact traumatic brain injury in mice and rats as assessed by the de Olmos silver and fluorojade staining methods." J Neurotrauma 25(3): 235-47.
- Hall, E. D., P. G. Sullivan, T. R. Gibson, K. M. Pavel, B. M. Thompson and S. W. Scheff (2005). "Spatial and temporal characteristics of neurodegeneration after controlled cortical impact in mice: more than a focal brain injury." J Neurotrauma 22(2): 252-65.
- Hamakubo, T., R. Kannagi, T. Murachi and A. Matus (1986). "Distribution of calpains I and II in rat brain." J Neurosci 6(11): 3103-11.
- Hansson, M. J., R. Mansson, G. Mattiasson, J. Ohlsson, J. Karlsson, M. F. Keep and E. Elmer (2004). "Brain-derived respiring mitochondria exhibit homogeneous, complete and cyclosporin-sensitive permeability transition." J Neurochem 89(3): 715-29.
- Happel, R. D., K. P. Smith, N. L. Banik, J. M. Powers, E. L. Hogan and J. D. Balentine (1981). "Ca<sup>2+</sup>-accumulation in experimental spinal cord trauma." Brain Res 211(2): 476-9.
- Hazelton, J. L., M. Petrasheuskaya, G. Fiskum and T. Kristian (2009). "Cyclophilin D is expressed predominantly in mitochondria of gamma-aminobutyric acidergic interneurons." J Neurosci Res 87(5): 1250-9.
- He, L. and J. J. Lemasters (2002). "Regulated and unregulated mitochondrial permeability transition pores: a new paradigm of pore structure and function?" FEBS Lett 512(1-3): 1-7.
- Hermanns, S. and H. Werner Muller (2001). "Preservation and detection of lesion-induced collagenous scar in the CNS depend on the method of tissue processing." Brain Res Brain Res Protoc 7(2): 162-7.
- Huang, Y. and K. K. Wang (2001). "The calpain family and human disease." Trends Mol Med 7(8): 355-62.

- Hunter, D. R., R. A. Haworth and J. H. Southard (1976). "Relationship between configuration, function, and permeability in calcium-treated mitochondria." J Biol Chem 251(16): 5069-77.
- Jakeman, L. B., Z. Guan, P. Wei, R. Ponnappan, R. Dzwonczyk, P. G. Popovich and B. T. Stokes (2000). "Traumatic spinal cord injury produced by controlled contusion in mouse." J Neurotrauma 17(4): 299-319.
- Kampf, A., R. Posmantur, R. Nixon, F. Grynspan, X. Zhao, S. J. Liu, J. K. Newcomb, G. L. Clifton and R. L. Hayes (1996). "mu-calpain activation and calpain-mediated cytoskeletal proteolysis following traumatic brain injury." J Neurochem 67(4): 1575-83.
- Kapprell, H. P. and D. E. Goll (1989). "Effect of Ca<sup>2+</sup> on binding of the calpains to fgcalpastatin." J Biol Chem 264(30): 17888-96.
- Kokoszka, J. E., K. G. Waymire, S. E. Levy, J. E. Sligh, J. Cai, D. P. Jones, G. R. MacGregor and D. C. Wallace (2004). "The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore." Nature 427(6973): 461-5.
- Kuhn, P. L. and J. R. Wrathall (1998). "A mouse model of graded contusive spinal cord injury." J Neurotrauma 15(2): 125-40.
- Kwong, L. K., M. Neumann, D. M. Sampathu, V. M. Lee and J. Q. Trojanowski (2007). "TDP-43 proteinopathy: the neuropathology underlying major forms of sporadic and familial frontotemporal lobar degeneration and motor neuron disease." Acta Neuropathol 114(1): 63-70.
- Langlois, J. A., W. Rutland-Brown and M. M. Wald (2006). "The epidemiology and impact of traumatic brain injury: a brief overview." J Head Trauma Rehabil 21(5): 375-8.
- Lemasters, J. J. and E. Holmuhamedov (2006). "Voltage-dependent anion channel (VDAC) as mitochondrial governor--thinking outside the box." Biochim Biophys Acta 1762(2): 181-90.
- Leung, A. W. and A. P. Halestrap (2008). "Recent progress in elucidating the molecular mechanism of the mitochondrial permeability transition pore." Biochim Biophys Acta 1777(7-8): 946-52.
- Leung, A. W., P. Varanyuwatana and A. P. Halestrap (2008). "The mitochondrial phosphate carrier interacts with cyclophilin D and may play a key role in the permeability transition." J Biol Chem 283(39): 26312-23.
- Li, H., V. F. Thompson and D. E. Goll (2004). "Effects of autolysis on properties of mu- and m-calpain." Biochim Biophys Acta 1691(2-3): 91-103.
- Lighthall, J. W., H. G. Goshgarian and C. R. Pinderski (1990). "Characterization of axonal injury produced by controlled cortical impact." J Neurotrauma 7(2): 65-76.
- Liu, J., M. C. Liu and K. K. Wang (2008). "Calpain in the CNS: from synaptic function to neurotoxicity." Sci Signal 1(14): re1.

- Liu, M. C., V. Akle, W. Zheng, J. Kitlen, B. O'Steen, S. F. Lerner, J. R. Dave, F. C. Tortella, R. L. Hayes and K. K. Wang (2006). "Extensive degradation of myelin basic protein isoforms by calpain following traumatic brain injury." J Neurochem 98(3): 700-12.
- Luscher, C., R. A. Nicoll, R. C. Malenka and D. Muller (2000). "Synaptic plasticity and dynamic modulation of the postsynaptic membrane." Nat Neurosci 3(6): 545-50.
- Lynch, D. R. and T. M. Dawson (1994). "Secondary mechanisms in neuronal trauma." Curr Opin Neurol 7(6): 510-6.
- Magnuson, D. S., T. C. Trinder, Y. P. Zhang, D. Burke, D. J. Morassutti and C. B. Shields (1999). "Comparing deficits following excitotoxic and contusion injuries in the thoracic and lumbar spinal cord of the adult rat." Exp Neurol 156(1): 191-204.
- Matena, K., T. Boehm and N. Dear (1998). "Genomic organization of mouse Capn5 and Capn6 genes confirms that they are a distinct calpain subfamily." Genomics 48(1): 117-20.
- Mauter, A. E., M. R. Weinzierl, F. Donovan and L. J. Noble (2000). "Vascular events after spinal cord injury: contribution to secondary pathogenesis." Phys Ther 80(7): 673-87.
- Maynard, F. M. and K. Imai (1977). "Immobilization hypercalcemia in spinal cord injury." Arch Phys Med Rehabil 58(1): 16-24.
- Mbye, L. H., I. N. Singh, K. M. Carrico, K. E. Saatman and E. D. Hall (2008). "Comparative neuroprotective effects of cyclosporin A and NIM811, a nonimmunosuppressive cyclosporin A analog, following traumatic brain injury." J Cereb Blood Flow Metab.
- Mbye, L. H., I. N. Singh, P. G. Sullivan, J. E. Springer and E. D. Hall (2008). "Attenuation of acute mitochondrial dysfunction after traumatic brain injury in mice by NIM811, a non-immunosuppressive cyclosporin A analog." Exp Neurol 209(1): 243-53.
- McEwen, M. L., P. G. Sullivan and J. E. Springer (2007). "Pretreatment with the cyclosporin derivative, NIM811, improves the function of synaptic mitochondria following spinal cord contusion in rats." J Neurotrauma 24(4): 613-24.
- Minger, S. L., J. W. Geddes, M. L. Holtz, S. D. Craddock, S. W. Whiteheart, R. G. Siman and L. C. Pettigrew (1998). "Glutamate receptor antagonists inhibit calpain-mediated cytoskeletal proteolysis in focal cerebral ischemia." Brain Res 810(1-2): 181-99.
- Momeni, H. R., S. Azadi and M. Kanje (2007). "Calpain activation and apoptosis in motor neurons of cultured adult mouse spinal cord." Funct Neurol 22(2): 105-10.
- Momeni, H. R. and M. Kanje (2006). "Calpain inhibitors delay injury-induced apoptosis in adult mouse spinal cord motor neurons." Neuroreport 17(8): 761-5.
- Moriya, T., A. Z. Hassan, W. Young and M. Chesler (1994). "Dynamics of extracellular calcium activity following contusion of the rat spinal cord." J Neurotrauma 11(3): 255-63.

- Morota, S., M. J. Hansson, N. Ishii, Y. Kudo, E. Elmer and H. Uchino (2007). "Spinal cord mitochondria display lower calcium retention capacity compared with brain mitochondria without inherent differences in sensitivity to cyclophilin D inhibition." J Neurochem 103(5): 2066-76.
- Murachi, T. (1989). "Intracellular regulatory system involving calpain and calpastatin." Biochem Int 18(2): 263-94.
- Naga, K. K., P. G. Sullivan and J. W. Geddes (2007). "High cyclophilin D content of synaptic mitochondria results in increased vulnerability to permeability transition." J Neurosci 27(28): 7469-75.
- Nakagawa, T., S. Shimizu, T. Watanabe, O. Yamaguchi, K. Otsu, H. Yamagata, H. Inohara, T. Kubo and Y. Tsujimoto (2005). "Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death." Nature 434(7033): 652-8.
- Nehrt, A., R. Rodgers, S. Shapiro, R. Borgens and R. Shi (2007). "The critical role of voltage-dependent calcium channel in axonal repair following mechanical trauma." Neuroscience 146(4): 1504-12.
- Noble, L. J., A. E. Mautes and J. J. Hall (1996). "Characterization of the microvascular glycocalyx in normal and injured spinal cord in the rat." J Comp Neurol 376(4): 542-56.
- Noble, L. J. and J. R. Wrathall (1989). "Correlative analyses of lesion development and functional status after graded spinal cord contusive injuries in the rat." Exp Neurol 103(1): 34-40.
- Nocentini, G., S. Cuzzocrea, T. Genovese, R. Bianchini, E. Mazzon, S. Ronchetti, E. Esposito, D. P. Rosanna, P. Bramanti and C. Riccardi (2008). "Glucocorticoid-induced tumor necrosis factor receptor-related (GITR)-Fc fusion protein inhibits GITR triggering and protects from the inflammatory response after spinal cord injury." Mol Pharmacol 73(6): 1610-21.
- NSCISC (2009). Spinal Cord Injury Facts and Figures at a Glance NSCISC.
- Ommaya, A. K. and T. A. Gennarelli (1974). "Cerebral concussion and traumatic unconsciousness. Correlation of experimental and clinical observations of blunt head injuries." Brain 97(4): 633-54.
- Pang, Z., G. H. Umberger and J. W. Geddes (1996). "Neuronal loss and cytoskeletal disruption following intrahippocampal administration of the metabolic inhibitor malonate: lack of protection by MK-801." J Neurochem 66(2): 474-84.
- Panter, S. S., S. W. Yum and A. I. Faden (1990). "Alteration in extracellular amino acids after traumatic spinal cord injury." Ann Neurol 27(1): 96-9.
- Pettus, E. H., C. W. Christman, M. L. Giebel and J. T. Povlishock (1994). "Traumatically induced altered membrane permeability: its relationship to traumatically induced reactive axonal change." J Neurotrauma 11(5): 507-22.
- Popovich, P. G., P. J. Horner, B. B. Mullin and B. T. Stokes (1996). "A quantitative spatial analysis of the blood-spinal cord barrier. I.

- Permeability changes after experimental spinal contusion injury." Exp Neurol 142(2): 258-75.
- Posmantur, R., R. L. Hayes, C. E. Dixon and W. C. Taft (1994). "Neurofilament 68 and neurofilament 200 protein levels decrease after traumatic brain injury." J Neurotrauma 11(5): 533-45.
- Prevention, C. f. D. C. a. (2007). Morbidity and Mortality Weekly Report 56(8).
- Profyris, C., S. S. Cheema, D. Zang, M. F. Azari, K. Boyle and S. Petratos (2004). "Degenerative and regenerative mechanisms governing spinal cord injury." Neurobiol Dis 15(3): 415-36.
- Rabchevsky, A. G., I. Fugaccia, P. G. Sullivan and S. W. Scheff (2001). "Cyclosporin A treatment following spinal cord injury to the rat: behavioral effects and stereological assessment of tissue sparing." J Neurotrauma 18(5): 513-22.
- Ravikumar, R., M. L. McEwen and J. E. Springer (2007). "Post-treatment with the cyclosporin derivative, NIM811, reduced indices of cell death and increased the volume of spared tissue in the acute period following spinal cord contusion." J Neurotrauma 24(10): 1618-30.
- Ray, S. K., D. C. Shields, T. C. Saido, D. C. Matzelle, G. G. Wilford, E. L. Hogan and N. L. Banik (1999). "Calpain activity and translational expression increased in spinal cord injury." Brain Res 816(2): 375-80.
- Ray, S. K., G. G. Wilford, D. C. Matzelle, E. L. Hogan and N. L. Banik (1999). "Calpeptin and methylprednisolone inhibit apoptosis in rat spinal cord injury." Ann N Y Acad Sci 890: 261-9.
- Richter, C., P. Ghafourifar, M. Schweizer and R. Laffranchi (1997). "Nitric oxide and mitochondrial Ca<sup>2+</sup>." Biochem Soc Trans 25(3): 914-8.
- Rutland-Brown, W., J. A. Langlois, K. E. Thomas and Y. L. Xi (2006). "Incidence of traumatic brain injury in the United States, 2003." J Head Trauma Rehabil 21(6): 544-8.
- Rzagalinski, B. A., J. T. Weber, K. A. Willoughby and E. F. Ellis (1998). "Intracellular free calcium dynamics in stretch-injured astrocytes." J Neurochem 70(6): 2377-85.
- Saatman, K. E., D. I. Graham and T. K. McIntosh (1998). "The neuronal cytoskeleton is at risk after mild and moderate brain injury." J Neurotrauma 15(12): 1047-58.
- Saido, T. C., H. Sorimachi and K. Suzuki (1994). "Calpain: new perspectives in molecular diversity and physiological-pathological involvement." Faseb J 8(11): 814-22.
- Schneider, M. D. (2005). "Cyclophilin D: knocking on death's door." Sci STKE 2005(287): pe26.
- Schumacher, P. A., R. G. Siman and M. G. Fehlings (2000). "Pretreatment with calpain inhibitor CEP-4143 inhibits calpain I activation and cytoskeletal degradation, improves neurological function, and enhances axonal survival after traumatic spinal cord injury." J Neurochem 74(4): 1646-55.

- Seliger, G., F. Cosman, G. M. Abrams and R. Lindsay (1989). "Hypercalcaemia causing declining cognitive function in a head injured patient." Brain Inj 3(3): 315-8.
- Siman, R., C. Gall, L. S. Perlmutter, C. Christian, M. Baudry and G. Lynch (1985). "Distribution of calpain I, an enzyme associated with degenerative activity, in rat brain." Brain Res 347(2): 399-403.
- Simonson, L., M. Baudry, R. Siman and G. Lynch (1985). "Regional distribution of soluble calcium activated proteinase activity in neonatal and adult rat brain." Brain Res 327(1-2): 153-9.
- Simpkins, K. L., R. P. Guttman, Y. Dong, Z. Chen, S. Sokol, R. W. Neumar and D. R. Lynch (2003). "Selective activation induced cleavage of the NR2B subunit by calpain." J Neurosci 23(36): 11322-31.
- Smith, D. J., H. Ng, R. M. Kluck and P. Nagley (2008). "The mitochondrial gateway to cell death." IUBMB Life 60(6): 383-9.
- Springer, J. E., R. D. Azbill, S. E. Kennedy, J. George and J. W. Geddes (1997). "Rapid calpain I activation and cytoskeletal protein degradation following traumatic spinal cord injury: attenuation with riluzole pretreatment." J Neurochem 69(4): 1592-600.
- Sroga, J. M., T. B. Jones, K. A. Kigerl, V. M. McGaughy and P. G. Popovich (2003). "Rats and mice exhibit distinct inflammatory reactions after spinal cord injury." J Comp Neurol 462(2): 223-40.
- Sullivan, P. G., A. G. Rabchevsky, R. R. Hicks, T. R. Gibson, A. Fletcher-Turner and S. W. Scheff (2000). "Dose-response curve and optimal dosing regimen of cyclosporin A after traumatic brain injury in rats." Neuroscience 101(2): 289-95.
- Sullivan, P. G., A. G. Rabchevsky, J. N. Keller, M. Lovell, A. Sodhi, R. P. Hart and S. W. Scheff (2004). "Intrinsic differences in brain and spinal cord mitochondria: Implication for therapeutic interventions." J Comp Neurol 474(4): 524-34.
- Sullivan, P. G., A. G. Rabchevsky, P. C. Waldmeier and J. E. Springer (2005). "Mitochondrial permeability transition in CNS trauma: cause or effect of neuronal cell death?" J Neurosci Res 79(1-2): 231-9.
- Szabo, I. and M. Zoratti (1992). "The mitochondrial megachannel is the permeability transition pore." J Bioenerg Biomembr 24(1): 111-7.
- Tator, C. H. and M. G. Fehlings (1991). "Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms." J Neurosurg 75(1): 15-26.
- Thompson, S. N., T. R. Gibson, B. M. Thompson, Y. Deng and E. D. Hall (2006). "Relationship of calpain-mediated proteolysis to the expression of axonal and synaptic plasticity markers following traumatic brain injury in mice." Exp Neurol 201(1): 253-65.
- Tidball, J. G. and M. J. Spencer (2002). "Expression of a calpastatin transgene slows muscle wasting and obviates changes in myosin isoform expression during murine muscle disuse." J Physiol 545(Pt 3): 819-28.

- Varney, N. R. and R. N. Varney (1995). "Brain injury without head injury. Some physics of automobile collisions with particular reference to brain injuries occurring without physical head trauma." Appl Neuropsychol 2(2): 47-62.
- Waghray, A., D. S. Wang, D. McKinsey, R. L. Hayes and K. K. Wang (2004). "Molecular cloning and characterization of rat and human calpain-5." Biochem Biophys Res Commun 324(1): 46-51.
- Wagner, A., W. Millesi, F. Watzinger, M. Truppe, M. Rasse, G. Enislidis, C. Kermer and R. Ewers (1999). "Clinical experience with interactive teleconsultation and teleassistance in craniomaxillofacial surgical procedures." J Oral Maxillofac Surg 57(12): 1413-8.
- Wang, X., Y. Carlsson, E. Basso, C. Zhu, C. I. Rousset, A. Rasola, B. R. Johansson, K. Blomgren, C. Mallard, P. Bernardi, M. A. Forte and H. Hagberg (2009). "Developmental shift of cyclophilin D contribution to hypoxic-ischemic brain injury." J Neurosci 29(8): 2588-96.
- Weber, J. T., B. A. Rzigalinski, K. A. Willoughby, S. F. Moore and E. F. Ellis (1999). "Alterations in calcium-mediated signal transduction after traumatic injury of cortical neurons." Cell Calcium 26(6): 289-99.
- Wendt, A., V. F. Thompson and D. E. Goll (2004). "Interaction of calpastatin with calpain: a review." Biol Chem 385(6): 465-72.
- Werner, C. and K. Engelhard (2007). "Pathophysiology of traumatic brain injury." Br J Anaesth 99(1): 4-9.
- Wingrave, J. M., E. A. Sribnick, G. G. Wilford, D. D. Matzelle, J. A. Mou, S. K. Ray, E. L. Hogan and N. L. Banik (2004). "Relatively low levels of calpain expression in juvenile rat correlate with less neuronal apoptosis after spinal cord injury." Exp Neurol 187(2): 529-32.
- Xu, W. B., G. Lv, Y. F. Wang, X. H. Lu, T. Huang, Y. Zhu and L. S. Jia (2009). "Combination of Dexamethasone and Aminoguanidine Reduces Secondary Damage in Compression Spinal Cord Injury." Cell Mol Neurobiol.
- Young, W. (2002). "Spinal cord contusion models." Prog Brain Res 137: 231-55.
- Yu, C. G. and J. W. Geddes (2007). "Sustained calpain inhibition improves locomotor function and tissue sparing following contusive spinal cord injury." Neurochem Res 32(12): 2046-53.
- Zhang, S. X., V. Bondada and J. W. Geddes (2003). "Evaluation of conditions for calpain inhibition in the rat spinal cord: effective postinjury inhibition with intraspinal MDL28170 microinjection." J Neurotrauma 20(1): 59-67.
- Zhang, Z., M. Fujiki, L. Guth and O. Steward (1996). "Genetic influences on cellular reactions to spinal cord injury: a wound-healing response present in normal mice is impaired in mice carrying a mutation (WldS) that causes delayed Wallerian degeneration." J Comp Neurol 371(3): 485-95.

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## Vita

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### PUBLICATIONS

Dimayuga FO, Wang C, **Clark JM**, Dimayuga ER, Dimayuga VM, Bruce-Keller AJ.

SOD1 overexpression alters ROS production and reduces neurotoxic inflammatory signaling in microglial cells. *J Neuroimmunol.* 2007 Jan;182(1-2):89-99.

### ABSTRACTS

- **Clark JM**, Yu C.G., Geddes J. The role of Cyclophilin D in spinal cord injury. 2009 KSchIRT symposium, Louisville, Ky.
- **Clark JM**, Yu C.G., Geddes J. Cyclophilin D knock-out does not improve pathological outcome following spinal cord injury. 2009 Society for Neuroscience, Bluegrass Chapter, Lexington, Ky.
- **Clark JM**, Naga K.K, Geddes J. The role of Cyclophilin D in Acute CNS Injury. 2008 Society for Neurotrauma, Orlando, Fla.
- **Clark JM**, Dimayuga FO, Wang C, Bruce-Keller AJ. The Role of Neuronal CD40 in Amyotrophic Lateral Sclerosis. 2006 Society for Neuroscience, Atlanta, Ga.
- **Clark JM**, Apparsundaram S, Gates J, Gerhardt G. Muscarinic regulation of the high affinity choline transporter. 2004 Society for Neuroscience, Sand Diego, CA.
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- “The role of Cyclophilin D in Spinal Cord and Brain Injury.” University of Kentucky College of Medicine, Department of Anatomy and Neurobiology Doctor of Philosophy Defense Seminar. July 2009.
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- “The Role of Neuronal CD40 in Amyotrophic Lateral Sclerosis.” University of Kentucky College of Medicine, Department of Anatomy and Neurobiology Graduate Student Seminar Series. May, 2006.
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