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Inhibition of Calpains by Calpastatin: Implications for Cellular and Functional Damage Following Traumatic Brain Injury

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INHIBITION OF CALPAINS BY CALPASTATIN:
IMPLICATIONS FOR CELLULAR AND FUNCTIONAL DAMAGE
FOLLOWING TRAUMATIC BRAIN INJURY

DISSERTATION

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
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2013

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ABSTRACT OF DISSERTATION

INHIBITION OF CALPAINS BY CALPASTATIN: IMPLICATIONS FOR CELLULAR AND FUNCTIONAL DAMAGE FOLLOWING TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is a devastating health problem based on its high incidence, economic burden, and lack of effective pharmacological treatment. Individuals who suffer an injury often experience lifelong disability. TBI results in abrupt, initial cell damage leading to delayed neuronal death. The calcium-activated proteases, calpains, are known to contribute to this secondary neurodegenerative cascade. Prolonged activation of calpains results in proteolysis of numerous cellular substrates including cytoskeletal components, membrane receptors, and cytosolic proteins, contributing to cell demise despite coincident expression of calpastatin, the specific inhibitor of calpains.

A comprehensive analysis using two separate calpastatin transgenic mouse lines was performed to test the hypothesis that calpastatin overexpression will reduce posttraumatic calpain activity affording neuroprotection and behavioral efficacy. Increased calpastatin expression was achieved using transgenic mice that overexpress the human calpastatin (hCAST) construct under control of a neuron-specific calcium-calmodulin dependent kinase II α or a ubiquitous prion protein promoter. Both transgenic lines exhibited enhanced calpastatin expression within the brain, extending into peripheral tissues under the prion protein promoter. hCAST overexpression significantly reduced protease activity confirmed by reductions in acute calpain-mediated substrate proteolysis in the cortex and hippocampus following controlled cortical impact brain injury. Aspects of posttraumatic motor and cognitive behavioral deficits were also lessened in hCAST transgenic mice compared to their wildtype littermates. However, volumetric analyses of neocortical contusion revealed no histological neuroprotection at either acute or long-term time points in either transgenic line. Partial hippocampal neuroprotection observed at a moderate injury severity in neuron-specific calpastatin overexpressing transgenic mice was lost after severe TBI. Greater levels of calpastatin under the prion protein promoter line failed to protect against hippocampal cell loss after severe brain injury.
This study underscores the effectiveness of calpastatin overexpression in reducing calpain-mediated proteolysis and behavioral impairment after TBI, supporting the therapeutic potential for calpain inhibition. However, the reduction in proteolysis without accompanied neocortical neuroprotection suggests the involvement of other factors that are critical for neuronal survival after contusion brain injury. Augmenting calpastatin levels may be an effective method for calpain inhibition and may have efficacy in reducing behavioral morbidity after TBI and neurodegenerative disorders.

KEYWORDS: Calpain, Calpastatin, Neuroprotection, Transgenic, Traumatic Brain Injury

Kathleen Marie Schoch
Student’s Signature

May 3, 2013
Date
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CHAPTER 1

Introduction

TRAUMATIC BRAIN INJURY

Prevalence and Etiology

Traumatic brain injury (TBI) is a devastating health problem that afflicts approximately 1.7 million individuals each year in the United States, resulting in 52,000 deaths, 275,000 hospitalizations, and 1.3 million emergency room visits (Faul et al., 2010). Despite these already staggering statistics, the prevalence of TBIs may be underestimated, as many individuals may be unaware of the underlying severity of the injury or unwilling to seek treatment, causing many milder injuries to go undiagnosed and untreated. TBIs most often result from falls and motor vehicle accidents (Faul et al., 2010); however, more recent attention has been given to head injuries that arise from sports-related incidents and military conflicts. The mild, repeated head trauma sustained by some athletes and individuals of the armed forces is often non-lethal but can result in progressive damage to the brain, causing persistent deficits well past the initial injuries (Stern et al., 2011, Goldstein et al., 2012).

Although brain trauma can affect any individual regardless of age, gender, or race, TBI predominates in young children and adolescents and has led to approximately 3.2-5.3 million brain injury survivors with long-term disabilities
(Coronado et al., 2011). The price of injury not only includes an estimated $11.5 billion in annual medical expenses incurred by the victims, but also indirect costs associated with lost productivity that account for an additional $64.8 billion (Coronado et al., 2012). Those patients in need of continued care may also incur extra expenses related to rehabilitation and health insurance. Due to the early age and enduring costs of injury, TBI remains a significant health problem in the United States, creating a large medical and monetary burden.

**Clinical manifestations**

TBIs are commonly categorized as a mild, moderate, or severe injury using the Glasgow Coma Scale (GCS), a globally recognized clinical standard developed to provide a consistent, objective assessment of the severity of initial injury (Teasdale and Jennett, 1974) and patient outcomes (Levin et al., 1990, Marmarou et al., 2007). The GCS separately evaluates eye opening, verbal response, and motor response and the cumulative score is used to classify injury severity upon presentation. A GCS score of 13-15 identifies a mild injury, a GCS 9-12 a moderate injury, and a GCS 8 or less a severe injury. Despite the clinical utility of the GCS in classifying the initial severity of TBI and predicting patient outcome, the heterogeneity of injury makes it difficult to accurately diagnose the underlying nature and extent of the injury, particularly following severe TBI. For instance, in a comparison of six different TBI victims receiving a GCS score of less than 8, or severe TBI, each individual’s CT scan identified different pathologies including contusion, subarachnoid hemorrhage, and diffuse axonal
injury (Saatman et al., 2008), indicating that separate or mixed pathophysiological mechanisms can be grouped under the same clinical classification. The use of additional assessments in combination with the GCS and CT scan may help to verify diagnosis and enhance predictive value (Narayan et al., 1981, Choi et al., 1994). Varied clinical presentations not only complicate the categorization of injury but also confound successful implementation of therapies for the treatment of TBI. As a result, limited intervention strategies are available for TBI patients during the early medical management of a TBI.

Standard emergency care for TBI includes computed tomography (CT) scan to diagnose any brain abnormalities, intracranial pressure monitoring, and attempts to control ischemia and inflammation events that can accompany a brain injury (Rosenfeld et al., 2012). In severe cases or with worsening condition, surgical intervention may be required.

Both motor and cognitive dysfunctions are evident after human TBI with approximately 80-90% of patients reporting persistent physical, emotional, or cognitive disability years after the injury (Lew et al., 2006). The severity and persistence of these behavioral consequences can depend on both on the cause, location, and severity of injury as well as factors associated with the health of the individual and posttraumatic rehabilitation efforts. Common physical symptoms of a TBI include headache, dizziness, nausea, and possible loss of consciousness (Silver et al., 2009, Faul et al., 2010). Changes in memory, attention, speech and language, and personality have also been reported after TBI, with clear implications toward a person’s overall health, social relationships, and job
performance. Not only can TBI disturb an individual’s physical and cognitive function closely following the injury, but may also predispose patients to long-term psychological problems, depression, epilepsy, and dementia (McAllister, 2011).

Existing strategies targeting prevention, clinical care, and management techniques have been successful in standardizing treatment for TBI, yet these approaches primarily focus on reducing intracranial pressure, controlling hematoma, and monitoring cerebral perfusion (Rosenfeld et al., 2012). To date, there is no successful pharmacological agent that can mitigate the pathology and long-term consequences of TBI. The absence of an effective treatment increases the likelihood that TBI victims will suffer acute and long-term repercussions of injury, including memory loss, emotional issues, and risk for other brain disorders such as epilepsy or Alzheimer’s disease (Thurman et al., 1999, Corrigan et al., 2004). Difficulty in mitigating pathological consequences necessitates continued research in order to understand and treat the overwhelming problem of TBI.

**Experimental models**

In an effort to understand the injury mechanisms associated with TBIs and to develop effective treatment strategies, various experimental models have been developed to recreate aspects of human TBI. Isolated, neuronal events can be closely replicated using *in vitro*, or cellular, models of injury. In these types of preparations, the pathological events associated with neuronal injury are produced by the application of chemicals or by physical stretching or tearing of
the neurons. In vitro models are effective in monitoring discrete events associated with neuronal and axonal damage, yet are limited in defining the injury within the context of the whole brain (Kumaria and Tolias, 2008).

In vivo, or whole animal, models help to more accurately recreate head injuries in an environment that includes cerebral pressure changes, vascular alterations and ischemia, inflammation, and glial interactions. In an attempt to narrow the heterogeneity of TBI, several in vivo injury models have been developed to reproduce the focal and diffuse aspects of clinical TBI. Characteristics of traumatic axonal injury, diffuse swelling or diffuse vascular injury are typically produced by impact or inertial acceleration models that mimic the head rotation and deformation of human TBI (Marmarou et al., 1994, Ross et al., 1994, Cernak et al., 2004). Focal injuries with localized damage can be recreated in the rodent using the weight drop model (Feeney et al., 1981, Chen et al., 1996); however, modifications to the severity of impact and head stabilization can produce more diffuse patterns of injury (Albert-Weissenberger and Siren, 2010). As one of the most common types of TBI, focal contusion injury is reliably reproduced using the controlled cortical impact (CCI) device, tested both in rodents and larger mammals (Morales et al., 2005) (Figure 1.1). Contact of a metal impactor tip to the animal's brain is both rapid and transient, creating a focal injury site that can range in severity depending on the depth and velocity of impact as well as the size and shape of the impactor (Lighthall, 1988, Saatman et al., 2006, Pleasant et al., 2011). Aspects of both focal contusion and diffuse brain injury can be evaluated in the fluid percussion injury (FPI) model (Dixon et al.,
FPI has been extensively characterized in the rat with much less utilization in mice. By contrast, the CCI model is widely used in mice making it a highly appropriate model for studies that rely on transgenic or knockout mice.

*In vivo* models in combination with *in vitro* paradigms have advanced understanding on the progression of pathology in human TBI, identifying cellular and molecular mechanisms that collectively result in neuronal damage and death.
Figure 1.1. Controlled cortical impact (CCI) injury model for experimental contusion brain injury. The controlled cortical impact (CCI) device is a well-documented model for the replication of contusion brain injury in small mammals. Programmed depth and velocity values determine the movement of a metal impactor tip delivered to the exposed cortical surface, producing varying degrees of cortical and hippocampal cell damage and behavioral abnormalities (Goodman et al., 1994, Smith et al., 1995). For contusion injury, the cortical surface of the brain is exposed by surgical craniotomy prior to impact and positioned below the impactor tip.
Primary and secondary injury mechanisms

TBI is characterized by an initial mechanical insult to the head that can result in varying amounts of damage to brain tissue, termed the primary phase of injury, characterized by direct rupture of membranes or increased plasma membrane permeability (LaPlaca et al., 2007). Primary damage, given its immediate and mechanical nature, is difficult to target therapeutically. Efforts to reduce primary injury have focused on injury prevention or mitigation of the magnitudes of force and acceleration associated with impact. However, the primary phase triggers a myriad of secondary pathogenic events including ischemia, alterations in ionic homeostasis, oxidative damage, mitochondrial dysfunction, inflammation, and the excessive release of excitatory neurotransmitters (Ray et al., 2002, Dash et al., 2010) (Figure 1.2). Events associated with the secondary phase can be targeted for intervention and, therefore, are important avenues of TBI research in selecting cell and molecular targets for the treatment of TBI.

The brain communicates within itself and throughout distant areas of the body via neurotransmitter signaling between neurons. The excitatory neurotransmitter, glutamate, is especially important in action potential propagation and synaptic plasticity. When released from the presynaptic neuron, glutamate binds to ligand-gated N-methyl-D-aspartate (NMDA) and AMPA receptors, resulting in the influx of sodium and calcium ions necessary to propagate signals along the postsynaptic neuron and to its target. However, under conditions of membrane damage that result from TBI, the excessive
release of excitatory neurotransmitters can create an excitotoxic environment characterized by the overactivation of glutamate receptors and overwhelming influx of sodium and calcium, potassium efflux, and prolonged membrane depolarization.
Figure 1.2. Acute neurodegenerative events associated with traumatic brain injury. Brain injury and damage to the neuron initiates a secondary neurodegenerative cascade that involves excessive glutamate release and receptor stimulation, altered calcium and ionic homeostasis, mitochondrial dysfunction, oxidative damage, and the activation of proteases, including calpains. Without intervention, these events, in combination with others, lead to neuronal death.
Calcium concentrations are highly regulated by the neuron in order to participate in the important functions of neurotransmitter release and other intracellular signaling events. Under baseline conditions, the intracellular free-calcium concentration is approximately 100 nM, compared to the much greater 2 mM concentration in the extracellular environment. Within the cell, calcium is sequestered in the endoplasmic reticulum and mitochondria and buffered by various calcium handling proteins and extrusion mechanisms. The concentration gradient of calcium can prime the cell for damage if any of these regulatory mechanisms is altered. Studies using experimental models of contusion brain injury have established multiple pathways through which calcium homeostasis becomes dysregulated, including excitotoxicity, widespread depolarization, voltage-gated calcium channel opening, or membrane disruption (Faden et al., 1989, Palmer et al., 1993, Arundine and Tymianski, 2004, Weber, 2004). As a consequence, increases in intracellular free calcium can trigger mitochondrial dysfunction and energy depletion, stimulation of proteases and enzymes, free radical production, and altered gene expression (Arundine and Tymianski, 2004, Weber, 2004). Notably, the calcium-dependent proteases, calpains, are activated by calcium dysregulation. The family of calpain proteases can function in necrotic and apoptotic cell death cascades by cleaving a variety of cellular substrates. Among these substrates are receptors or proteins involved in maintaining calcium homeostasis; thus, substrate cleavage by calpains may exacerbate calcium-induced neurotoxicity. If the neuron is unable to circumvent or recover
from the proteolysis of cellular substrates, normal cellular structure and function are at risk and cell death is imminent.

Marginal success has been achieved in clinical trials targeting NMDA receptor activation with a receptor antagonist after TBI (Bullock et al., 1999, Yurkewicz et al., 2005); however, because of the vital role these receptors play in normal synaptic function, prolonged antagonism of glutamate receptors can lead to undesirable side effects (Ikonomidou and Turski, 2002). In addition, excitotoxic events triggered by glutamate receptor overactivation may happen too quickly after neuronal damage for effective post-injury treatment. Therefore, downstream effectors, like calpains, that mediate pathological damage within the cell have become attractive targets for therapeutic intervention.

**CALPAINS**

*Biochemical structure and regulation*

Calpains are commonly defined as neutral cysteine proteases distributed within the cytosol that require calcium binding to elicit full activity (Goll et al., 2003, Croall and Ersfeld, 2007). Fifteen isoforms of calpain have been identified to date with either ubiquitous or tissue-specific localization (Sorimachi et al., 2011). The two most commonly studied isoforms are the ubiquitous \( \mu \)-calpain and m-calpain, which share a similar heterodimeric structure but are activated by differential concentrations of ionic calcium. Under *in vitro* conditions, micromolar concentrations of calcium are needed to activate \( \mu \)-calpains, while m-calpains require millimolar amounts (Goll et al., 2003).
The heterodimeric structure of vertebrate \( \mu \)- and m-calpains is composed of an 80 kDa catalytic subunit and a common 28 kDa regulatory subunit (Goll et al., 2003). The catalytic subunits of \( \mu \)- and m-calpain, termed calpain-1 and calpain-2, respectively, are distinct, but closely homologous. They are generally divided into four regions, or domains, along the protein (Figure 1.3A) with the N-terminal region of the catalytic subunit designated as Domain I. Domain II, often referred to as the catalytic domain, contains the cysteine, histidine, and asparagine amino acid residues necessary for the proteolytic function of calpains. In addition to linking the catalytic domain with the C-terminal region, domain III consists of residues that function in associating with plasma membranes and calcium binding. Finally, domain IV contains four EF hand sequences responsible for calcium binding. The small regulatory subunit (calpain-4) is common to both \( \mu \)- and m-calpain isoforms and composed of Domains V and VI containing hydrophobic residues that help to achieve membrane interaction (Domain V) and additional calcium-binding EF hand sequences (Domain VI). The catalytic and regulatory subunits remain associated under inactive conditions and likely continue to interact when activated (Goll et al., 2003).

Upon calcium binding, \( \mu \)- and m-calpain autolyze, or self-cleave, an N-terminal portion of the large and small subunits to achieve full proteolytic activity. The 80 kDa subunit is reduced to a molecular mass of 76 kDa or 78 kDa for calpain-1 or -2, respectively, and the common small subunit reduces to 18 kDa (Goll et al., 2003). Although it has been contested whether this self-cleavage is
required for activation, autolysis reduces the calcium concentration requirement for calpains’ proteolytic activity (Edmunds et al., 1991, Goll et al., 1992) thereby increasing the plausibility that calpains can become activated following physiological calcium changes. In addition, calcium binding elicits a conformational change in calpain structure, bringing the catalytic amino acid residues together for proteolytic activity (Moldoveanu et al., 2002) (Figure 1.3B).
Figure 1.3. Basic structure and activation of the ubiquitous \( \mu \)- and m-calpains. A) The ‘typical’ calpains, \( \mu \)-calpain and m-calpain, share a similar heterodimeric structure composed of a large catalytic subunit and small regulatory subunit. Domain II of the large subunit includes the amino acid residues responsible for proteolysis. Calcium binding primarily occurs at EF hand sequences located on domains IV and VI. B) Binding of calcium to calpain elicits autolysis and conformational change in the protease’s structure, positioning specific amino acid residues within domain II in an arrangement facilitating proteolytic activity.
Calpains are most notably regulated by calcium concentrations within the cell to achieve proteolytic activity. As indicated previously, micromolar range concentrations of ionic calcium are necessary to activate \( \mu \)-calpains \textit{in vitro} while millimolar calcium concentrations activate m-calpains. This calcium requirement for activation of either isoform is notably higher than the nanomolar concentrations within the cell under physiological conditions. Various mechanisms including autolysis and phospholipid interaction reduce the calcium requirement for activation and, therefore, regulate calpains’ proteolytic function (Goll et al., 2003, Campbell and Davies, 2012). In addition, calpain activity is regulated by binding of a common endogenous inhibitor, calpastatin. The calcium requirement for calpastatin binding to calpain is lower than the concentration needed for calpain activation (Goll et al., 2003), suggesting that an additional mechanism such as localization or protein modification can alter the interaction of calpain with its inhibitor to regulate proteolytic function. Once activated, calpains must have access to cellular substrates, which are shared across isoforms and identified based on the protein substrate’s conformation instead of amino acid sequence and are cleaved into large fragments (Goll et al., 2003). The proteolytic fragments are often stable and can exert physiological functions independent of their parent or full-length protein, suggesting calpains can act as a modulatory protease. This property of calpains is evident in the cleavage of substrates p35 (Hashiguchi et al., 2002), protein kinase C (Kishimoto et al., 1989), or NMDA receptor subunit (Simpkins et al., 2003) that remain constitutively active with proteolysis. Calpains can also be influenced by their chemical environment as
decreased pH or increased ionic strength or oxidation lead to a loss of proteolytic activity (Guttmann and Johnson, 1998, Geesink and Koohmaraie, 2000, Maddock et al., 2005).

**Physiological expression and function**

That the calcium requirements for µ- and m-calpain activation far exceed the normal intracellular free calcium concentration creates speculation regarding the function or necessity of each isoform under physiological conditions. Traditionally, µ-calpain was theorized to act under physiological calcium changes, while m-calpain, requiring a much greater calcium concentration for activation, mediated proteolysis under pathological calcium changes. This dogma was challenged, however, by the finding that genetic disruption of the catalytic subunit of m-calpain (calpain-2, capn2) results in embryonic lethality (Dutt et al., 2006). By contrast, mice with genetic deletion of the large subunit of µ-calpain (calpin-1, capn1) are viable, despite dysfunctional platelet aggregation (Azam et al., 2001). Disruption of the small regulatory subunit gene (calpain-4, capn4) that abolishes both µ- and m-calpain activity resulted in embryonic lethality (Arthur et al., 2000, Zimmerman et al., 2000), again indicative of calpains’ importance to normal cell function. Loss of µ-calpain alone may be compensated for by m-calpain or another of the fifteen calpain isoforms; however, due to the lethality of m-calpain knockout, the latter isoform appears to exert physiological functions independent to those of µ-calpain. Recently, a study determined the necessity of calpain-2 is due to placental cell survival and demonstrated that conditional
calpain-2 and conditional dual calpain (calpain-1 and calpain-2) knockouts survive to adulthood (Takano et al., 2011). These knockout models not only offer valuable insight into the isoform-specific functions of μ- and m-calpain but also underscore their essential role in normal cell function.

The developmental expression of both calpain-1 and calpain-2 in the rodent central nervous system (CNS) has been documented with varied results. Although one study documented early downregulation of calpain-2 protein expression but stable calpain-1 levels in the brain throughout postnatal mouse development (Zhu et al., 2005), another identified steady message and protein levels of calpain-2 with increasing calpain-1 expression in the developing rat (Li et al., 2009). In addition to these developmental discrepancies, relative levels of adult calpain-1 versus -2 have varied between studies with reports of considerably greater calpain-2 mRNA expression above calpain-1 in mouse CNS tissues (Li et al., 1996) or the opposite (Li et al., 2009). Unfortunately, differences in the probes, antibodies, or species used in these studies prevent definitive conclusions on the developmental and adult calpain expression. Regional mRNA analysis by in situ hybridization determined that calpain-1 was homogenously distributed throughout the brain while calpain-2 appeared to predominate in neuronal versus non-neuronal cell types (Li et al., 1996). Studies identifying the calpain-mediated cleavage products support a neuronal localization of calpain activity (Saatman et al., 1996a, Newcomb et al., 1997). While calpains have historically been deemed cytosolic proteases, they also have associations with specific structures within the cell including the nucleus (Mellgren and Lu, 1994),
phospholipids of the endoplasmic reticulum and Golgi apparatus (Hood et al., 2003), and the mitochondria (Garcia et al., 2005, Cao et al., 2007), perhaps indicating substrate specificities and additional regulation through subcellular interactions.

Regardless of their subcellular localization, calpains are multi-faceted regulators of physiological functions such as cytoskeletal remodeling for cell growth and motility, fibroblast migration for wound healing, cell cycle and differentiation processes, apoptosis, and long-term potentiation (Goll et al., 2003, Sorimachi et al., 2011). Calpains are known to cleave cytoskeletal proteins (spectrin, neurofilaments, tubulin, and microtubule associated proteins like MAP-2 and tau), cytosolic proteins involved in signal transduction and cell cycle (calcium-calmodulin dependent kinase, protein kinase C, calcineurin, and cyclin-dependent kinase 5), transcription factors for gene expression (c-Jun, c-Fos, p53), apoptotic proteins (caspases, Bcl-2 family proteins), and membrane channels and receptors (NMDA receptor, voltage-gated sodium channel, sodium-calcium exchanger) (Goll et al., 2003, Saatman et al., 2010). Although proteolysis of many of these substrates has been determined from in vitro or in vivo studies, the consequences of calpain activation and substrate proteolysis under different physiological stimuli may vary. It is also unclear whether the limited proteolysis of some substrates by calpains results in protein turnover or induces an alternative function in the cell.

Pathological activation
Identifying either physiological or pathological calpain activation can be technically difficult, requiring arduous purification steps or large amounts of protein. Gel electrophoresis can aid in visualization of calpain activation into its autolytic fragments but with relatively poor resolution. Alternative methods take advantage of the proteolytic function of calpains, by identifying cleavage of a casein substrate within a zymogram gel or the appearance of breakdown products associated with calpain-specific substrate cleavage. Under pathological conditions such as TBI, calpains can cleave cytoskeletal elements, membrane receptors, mitochondrial proteins, and gene regulatory elements leading to necrosis and apoptosis (Saatman et al., 2010). Events such as global ischemia, stroke, and spinal cord injury and neurodegenerative diseases including Alzheimer’s disease, Huntington’s disease, and Parkinson’s disease share several pathological secondary mechanisms with TBI, and therefore couple calpain activation and substrate proteolysis, in part, to neurodegeneration (Nixon, 2003, Ray and Banik, 2003, Bevers and Neumar, 2008).

Neurofilaments, microtubule-associated proteins, and spectrins are proteins involved in the composition or stabilization of the neuronal cytoskeleton. The cytoskeleton is necessary for axonal transport, serving as the route for the movement of proteins, mitochondria, and neurotransmitters to and from the soma. In addition, structural cytoarchitecture is responsible for protein scaffolding, anchoring of channels and receptors within the membrane, and synaptic integrity. Cytoskeleton proteins are also commonly recognized calpain substrates, especially following TBI. One of the most extensively studied calpain
substrates is α-spectrin, a submembrane cytoskeleton protein involved in structural support and protein anchoring. Spectrin is cleaved by calpain from its intact 280 kDa form into characteristic 150 and 145 kDa breakdown products, while cleavage by caspase-3 results in 150 and 120 kDa fragments. These characteristic, stable breakdown products allow definitive identification of calpain activation, especially with disease or pathological insult. Numerous studies have documented the temporal pattern of calpain-mediated spectrin breakdown into 150 kDa and 145 kDa products after TBI via immunoblot, demonstrating an early rise in spectrin proteolysis that peaks in rodents at approximately 24 h after CCI injury in the cortex and hippocampus (Kampfl et al., 1996, Pike et al., 1998, Hall et al., 2005, Thompson et al., 2006, Deng et al., 2007). Calpain-mediated spectrin breakdown products were also observed to be concomitant with compacted neurofilament protein (Buki et al., 1999) that preceded axonal transport disruption (Saatman et al., 2003), suggesting a mechanistic link between calpain proteolysis of the cytoskeleton and structural and functional demise of the axon. In addition to spectrin, significant calpain-specific cleavage of the microtubule associated protein tau was detected in the rat cortex as early as 2 h post-CCI (Liu et al., 2011). Given the integral role of the cytoskeleton in cells, posttraumatic cytoskeleton cleavage has clear implications for neuronal structure, axonal transport, and synaptic integrity.

Calpains are also known to cleave membrane proteins, which provide a multitude of functions to neurons, including binding neurotransmitters, mediating action potential propagation, ion permeability, and calcium regulation. Proteolysis
of the sodium-calcium exchanger and voltage-gated sodium channel after calcium perturbation could be limited with calpain inhibition (von Reyn et al., 2009, Brustovetsky et al., 2010). Glutamate stimulation in cell culture triggered degradation and down-regulation of the NMDA receptor that was prevented by the presence of calpastatin or pharmacological calpain inhibitors (Guttmann et al., 2002, Wu et al., 2005). Subsequent reports demonstrated that cleaved NMDA receptors remained active at the cell surface (Simpkins et al., 2003), suggesting continued contribution to excitotoxic damage. Therefore, fragmentation of membrane proteins and ion channels that control ion flux may exacerbate calcium dysregulation and lead to the loss of normal cell function, prompting cell death. Alternatively, protein breakdown products may mediate modified functions within the cell that are different from the parent protein. Under conditions of TBI, however, knowledge of the implications for certain cytoskeletal and membrane protein breakdown is relatively incomplete, yet this substrate proteolysis may ultimately contribute to neuronal dysfunction and cell death.

Strong evidence exists for calpain activation after TBI, pointing to these proteases as key mediators in cell death and dysfunction. Both lateral FPI (Saatman et al., 1996a) and CCI injury (Newcomb et al., 1997) triggered early posttraumatic calpain activity recognized by an antibody specific for calpain-mediated spectrin proteolytic fragments in association with regions of subsequent cell death. These results were supported by immunoblot data demonstrating \( \mu \)-calpain activation within 6 h of CCI injury, preceding a rise in spectrin breakdown products and neuronal degeneration (Kampfl et al., 1996). These studies and
others provide compelling evidence for a relationship between calpain activation and the neurodegenerative pathology of TBI.

Calpain inhibition by exogenous synthetic agents has helped to directly identify calpains as major mediators of pathology, making calpain inhibition a plausible strategy for therapeutic intervention. Pharmacological calpain inhibitors include several classes of peptide compounds targeted at the catalytic site of calpains as well as non-peptide inhibitors, which affect the catalytic properties of calpains by indirectly binding to target sites responsible for conformational changes of the protein (Carragher, 2006). The effectiveness of each inhibitor varies based on its cell permeability, solubility, and pharmacokinetics, requiring careful consideration in choosing the most appropriate inhibitor. Above all, the primary limitation to these inhibitors has been their lack of specificity for calpains compared to other cysteine or enzymatic proteases like cathepsins, so their results must be interpreted carefully. By contrast, calpastatin, the endogenous inhibitor of calpains, is specific for calpains.

CALPASTATIN

Biochemical structure

Calpastatin is an intracellular 110 kDa protein consisting of an N-terminal leader domain followed by four identical inhibitory domains, each able to specifically inhibit one molecule of calpain (Maki et al., 1987, Goll et al., 2003, Hanna et al., 2008) (Figure 1.4A). This basic inhibitory structure is consistent across the several known calpastatin isoforms that result from different
promoters or alternative splicing of the N-terminal region (Takano et al., 1999, Takano et al., 2000, Parr et al., 2004). Type III, or prototypical, calpastatin is ubiquitously expressed within the cytosol of mouse tissue (Goll et al., 2003).

Calpastatin reversibly binds at both sides of calpain’s active site to block its enzymatic activity (Crawford et al., 1993, Goll et al., 2003, Hanna et al., 2008, Moldoveanu et al., 2008). When free calcium levels rise and activate calpains, a conformational change in the protease allows for inhibitor binding across the active site of calpain, blocking the active site and its access to substrates (Hanna et al., 2008, Moldoveanu et al., 2008) (Figure 1.4B). Studies performed with purified calpastatin have documented its degradation into smaller-sized fragments when co-incubated with calpains (Pontremoli et al., 1991, Doumit and Koohmaraiie, 1999), suggesting calpastatin may mediate inhibition of calpains by acting as a suicide substrate. However, calpastatin degradation could be competitively inhibited by the addition of another calpain substrate (Pontremoli et al., 1991) such that calpastatin may not be a preferential substrate for calpain in the presence of additional protein substrates. In addition, calpastatin actually appears to evade proteolysis by calpains through a unique interaction with the m-calpain active site domain (Hanna et al., 2008, Moldoveanu et al., 2008). Calpastatin has also been recognized as a substrate for caspase-3 and caspase-1 during apoptosis (Wang et al., 1998) pointing to a mechanism for increased calpain activity and crosstalk between the two proteolytic families. Limited proteolysis of calpastatin, however, may not completely destroy its inhibitory activity and instead may cause the release of its repetitive inhibitory domains (De
Tullio et al., 2000). Despite these reports citing calpastatin degradation, changes in the molecular weight of calpastatin can be the product of its random coil conformation causing anomalous migration patterns during gel electrophoresis or alternative splicing (Goll et al., 2003) and, therefore, must be interpreted carefully.
Figure 1.4. Basic structure and function of calpastatin, the specific inhibitor of calpains. 

A) The structure of prototypical calpastatin consists of an N-terminal leader (L) domain, followed by four identical inhibitory domains (1-4). Each inhibitory domain contains subdomains A, B, and C, specific sequences that bind across the calpain molecule. 

B) Calpastatin’s inhibitory regions reversibly bind to calcium-bound calpain blocking its enzymatic activity and access to substrates. Calpain domain IV of the large subunit and domain VI of the small subunit are essential in mediating this close contact with calpastatin.
Physiological function and regulation

As the specific inhibitor of calpains, calpastatin is co-expressed with calpains in the cytosol of vertebrate cells (Goll et al., 2003) and is found uniformly throughout the brain (Li et al., 1996). Throughout development, protein and message levels of calpastatin were shown to parallel a progressive increase in calpain-1 between embryonic day 18 and postnatal day 90 in the rat brain (Li et al., 2009). The physiological or pathological significance of calpastatin expression data alone is difficult to interpret since various regulatory mechanisms are available to influence calpastatin’s interaction with calpain and thus its inhibitory activity. Calpastatin does not contain any predicted calcium-binding sites, yet calcium binding to calpains and accompanying autolysis reduces the calcium concentration required for calpastatin binding to calpains (Kapprell and Goll, 1989, Goll et al., 2003). Therefore, alternative mechanisms that change the location or protein interaction of calpains and calpastatin are necessary for calpain proteolysis to occur under physiological conditions. Both protein kinase A (PKA) and protein kinase C (PKC) are intracellular kinases shown to phosphorylate calpastatin, affecting its localization and function, respectively. Phosphorylation of calpastatin by PKA promotes calpastatin aggregation while phosphorylation by PKC reduces its inhibitory function (Salamino et al., 1997, Averna et al., 2001). These modifications restrict or reduce calpastatin’s interaction with calpain resulting in calpain’s proteolytic activation and substrate degradation. Several reports cite the presence of additional endogenous calpain inhibitors, Gas2 and serpins, that act to regulate apoptotic and necrotic
mechanisms, respectively (Brancolini et al., 1995, Benetti et al., 2001, Luke et al., 2007); however, limited information regarding their specificity, tissue expression, or pathological function prevents direct comparison to the unique inhibitory properties of calpastatin.

**Calpastatin in pathology**

Few studies have assessed the inhibitory or expression changes of calpastatin that could lead to increased calpain activity following neurodegeneration disease or traumatic insult. Under conditions of ischemia, a decrease in calpastatin expression concomitant with its degradation was noted in vulnerable cell populations and brain regions, suggesting dysregulated calpain activation after insult and support for endogenous calpastatin as a substrate of calpains (Saido et al., 1997, Blomgren et al., 1999). Reductions in calpastatin were also identified in the cortex and hippocampus of a transgenic mouse model of Alzheimer’s disease (Vaisid et al., 2007), later confirmed in diseased human brains coincident with increased calpain activity and tau hyperphosphorylation (Rao et al., 2008). With age, it appears that calpains become abnormally activated both by dysregulated calcium homeostasis and a decline in regulation by calpastatin that can ultimately lead to neuronal death and synaptic loss. This age-related decline in calpastatin expression was also identified in older rats, and was associated with greater pathological damage after spinal cord injury (Wingrave et al., 2004). In turn, reduced calpastatin levels due to proteolysis or transcriptional regulation may provide a trigger for increased calpain activation.
and partially explain the increased susceptibility for neurodegenerative disease or damage with age.

Despite the rapid activation of calpains after TBI, only delayed increases in calpastatin protein (Newcomb et al., 1999) and mRNA (Ringger et al., 2004) were identified in the cortex and hippocampus after CCI injury. The reciprocal relationship between calpain and calpastatin appears to be a combination of abnormal calpain activity and loss of calpastatin through proteolysis; however, the transcriptional regulation or possible feedback mechanisms within the calpain-calpastatin system is relatively unknown, especially with trauma. In a transgenic model of retinal degeneration, reductions in mRNA and protein expression of calpastatin and its supposed transcriptional activator, CREB, were coincident with increased calpain activity without transcriptional changes in calpain isoforms (Paquet-Durand et al., 2006), but it is unclear whether increased calpain activity led to decreased calpastatin or vice versa.

Despite limited understanding of pathological calpain-calpastatin regulation, the apparent decline in expression or activity of calpastatin after disease or traumatic insult in addition to the unregulated increase in calpain activity supports the participation of calpains in mediating excitotoxic and neurodegenerative pathology. Therefore, strategies targeting calpains or augmenting calpastatin may ameliorate neuronal damage and dysfunction associated with ischemia, age-related disease, and trauma.

**MANIPULATING CALPAINS AND CALPASTATIN**
Clear evidence is available to link increases in calpain activity or loss of endogenous inhibition by calpastatin to calcium-induced physiological and pathological events. Pharmacological and genetic strategies that target calpains or calpastatin have been developed to identify the distinct role calpains play in neurodegenerative diseases and trauma and investigate the therapeutic potential of calpain inhibition in these processes.

**Strategies targeted at calpains**

Support for pharmacological calpain inhibitors in the treatment of excitotoxic injury was propelled by studies in models of ischemia. Administration of calpain inhibitors such as E-64-d, AK275, AK295, MDL-28170, and SNJ-1945 were effective in reducing infarct volume after focal ischemia in the rodent (Bartus et al., 1994a, Bartus et al., 1994b, Markgraf et al., 1998, Tsubokawa et al., 2006, Koumura et al., 2008). This neuroprotective outcome was maintained even when treatment with MDL-28170 or SNJ-1945 was delayed up to 6 h post-ischemia (Markgraf et al., 1998, Koumura et al., 2008). In models of transient global ischemia, continuous pre-treatment with the calpain inhibitor leupeptin afforded protection of CA1 hippocampal cell viability and electrophysiological function (Lee et al., 1991). When treatment was initiated after ischemia, continuous intravenous administration of MDL-28170 or CEP-3453 also reduced ischemia-induced CA1 cell death (Rami et al., 2000, Frederick et al., 2008). The duration and route of administration may be important factors in achieving neuroprotection as only modest, non-significant reductions in CA1 cell death
were identified with repeated intraperitoneal doses of MDL-28170 following reperfusion (Li et al., 1998).

Early studies in spinal cord injury demonstrated that treatment with the calpain inhibitor E-64-d could reduce calpain activity and apoptosis (Ray et al., 2000, Ray et al., 2001), while treatment with CEP-4143 could further preserve axonal structure and motor function (Schumacher et al., 2000). Direct intraspinal administration or daily intraperitoneal injections over 7 d of MDL-28170 was required for improved long-term tissue sparing and locomotor function after spinal cord injury (Yu and Geddes, 2007, Yu et al., 2008), which may be mechanistically linked to reductions in calpain substrate proteolysis (Zhang et al., 2003, Hung et al., 2005). Spinal cord injured rats receiving single injections, both early and delayed, of AK295 exhibited a decrease in numbers of apoptotic cells and reduction in long-term motor deficits (Colak et al., 2009), indicating next generation inhibitors may not require continued post-injury dosing.

Despite their promise in ischemia and spinal cord injury, use of pharmacological calpain inhibitors in TBI has generated mixed results. Reductions in calpain-mediated spectrin or neurofilament breakdown were identified in studies using calpain inhibitor 2, MDL-28170, and SNJ-1945 following CCI injury (Posmantur et al., 1997, Thompson et al., 2010, Bains et al., 2012), showing promise for posttraumatic calpain inhibition in protecting against neurochemical proteolytic events. However, the histological neuroprotective effect of calpain inhibition is less clear. Treatment with calpain inhibitor 2 decreased contusion volume by qualitative analysis at 24 h post-CCI (Posmantur
et al., 1997), an outcome that was not evident after administration of the more selective inhibitors MDL-28170 or AK295 quantified at 48 h or one week after injury, respectively (Saatman et al., 2000, Thompson et al., 2010). Single dose, pre- or post-injury administration of MDL-28170 reduced axonal damage following diffuse brain injury (Buki et al., 2003, Ai et al., 2007); however, pre-injury dosing alone was ineffective after in vivo optic nerve stretch (Ma et al., 2012a) suggesting that prolonged or post-injury treatment may be necessary for sufficient calpain inhibition in the context of axonal injury. Despite this discrepancy in neuronal protection or axonal preservation with calpain inhibition, treatment with AK295 or SJA-6017 afforded behavioral improvement after lateral FPI or weight drop injury, respectively, which did not require reductions in calpain-mediated proteolysis of spectrin (Saatman et al., 1996b, Saatman et al., 2000, Kupina et al., 2001).

These studies indicate the effectiveness of pharmacological calpain inhibitors at reducing calpain activation or behavioral dysfunction after injury. Depending on the inhibitor used, timing of administration and dosing paradigm, calpain inhibition afforded structural and functional benefits in several models of excitotoxic or traumatic injury. However, properties associated with their selectivity, bioavailability, tissue penetration, and administration parameters have obvious implications for data interpretation. Strategies that achieve a more specific inhibition of calpain may have greater utility towards clearly implicating calpains in the pathogenic cascade of neurodegeneration and injury.
Due to the embryonic lethality of dual isoform and calpain-2 knockout mice, genetic models for calpain inhibition that target calpains have been restricted to calpain-1 knockout mice or viral-mediated gene knockdown. The few studies that have employed these strategies following excitotoxic pathology show compelling results. Knockdown of the catalytic subunit of m-calpain increased hippocampal neuronal survival after NMDA-mediated excitotoxicity (Bevers et al., 2009) while \( \mu \)-calpain but not m-calpain knockdown suppressed ischemia-induced CA1 hippocampal cell death and protected neuronal function (Bevers et al., 2010). Following spinal cord injury, knockdown of calpain-1 reduced both histopathological and locomotor deficits (Yu et al., 2012). Additionally, calpain-1 deletion reduced cortical cell death, neurodegeneration and apoptosis following contusion TBI (Yamada et al., 2012). These studies continue to support a neuroprotective outcome through reduction in calpains, now with more direct targeting of these proteases by genetic manipulation. Additionally, novel mouse models with conditional calpain-2 or dual isoform knockout (Takano et al., 2011) offer innovative tools to study the role of calpains in neurodegeneration and traumatic injury, especially ideal for understanding the isoform-specific roles of \( \mu \)- and m-calpains in pathology.

**Strategies targeted at calpastatin**

Manipulation of calpastatin to enhance endogenous inhibitory mechanisms enables suppression of both \( \mu \)- and m-calpain, providing a powerful research tool for understanding the role of pathological calpain proteolysis. In
addition, the specific nature of calpastatin’s inhibition of calpains is a unique and important element. Therefore, potential problems associated with the delivery and selectivity of pharmacological calpain inhibitors can be minimized or avoided.

The efficacy of transgenic calpastatin manipulation in reducing calpain activation is documented in several studies. Transgenic mice expressing calpastatin under a neuron-specific calcium-calmodulin dependent protein kinase II α (CaMKIIα) promoter exhibited potent reduction in in vitro m-calpain activity, resulting in significantly fewer apoptotic events and less hippocampal cell death in response to excitotoxic insult (Higuchi et al., 2005, Takano et al., 2005). An alternative calpastatin overexpressing mouse (Thy1.1 promoter) confirmed the therapeutic potential for calpastatin overexpression following excitotoxicity by demonstrating reductions in calpain-mediated substrate proteolysis and CA1 hippocampal cell death (Rao et al., 2008). Genetic overexpression of calpastatin in these models conferred similar neuroprotective outcomes seen in excitotoxicity studies using pharmacological calpain inhibitors (Rami et al., 1997, Volbracht et al., 2001), confirming a direct link between calpain activation and hippocampal cell death. In a model of Alzheimer’s disease, mice with calpastatin overexpression exhibited reduced β-amyloid and tau pathology and longer lifespan compared to control diseased mice (Higuchi et al., 2012), such that restoring calpastatin levels could hinder calpain-mediated formation of hallmark Alzheimer’s pathology. Constitutive calpastatin overexpression in transgenic models undoubtedly has implicated calpains in disease or traumatic pathology, but restricts manipulation of the timing and location of calpain inhibition that may
be necessary to optimize the effectiveness of treatment. Therefore, strategies that are more adaptable can increase understanding on the actions of calpains after injury and may more readily lead to translatable therapeutics.

Viral-mediated delivery of calpastatin once again takes advantage of the specificity of calpastatin and may circumvent some of the limitations associated with genetic alterations. Calpastatin overexpression through adenoviral infusion afforded neuroprotection of dopamine neurons and behavioral improvements in a model of toxin-induced Parkinson's disease in rodents (Crocker et al., 2003, Grant et al., 2009). Only one study has investigated use of viral-mediated calpastatin expression and in vivo traumatic injury, reporting partial preservation of axons with calpastatin overexpression after optic nerve stretch injury (Ma et al., 2012b). Genetic or viral-mediated alterations of calpastatin are ideal for preclinical studies since full-length calpastatin is unable to cross the blood-brain barrier for exogenous delivery. Yet, these strategies may be difficult or impractical to translate into treatments for human patients. Thus, novel peptide inhibitors or calpastatin mimetics that exploit the specific nature of calpastatin’s inhibitory interaction with calpains provide an alternate approach. Membrane-permeable peptides derived from the essential subdomains of calpastatin inhibitory sequences were shown to inhibit calpain activity in vitro (Gil-Parrado et al., 2003a, Fiorino et al., 2007) and in models of cell death and ischemia (McCollum et al., 2006, Anagli et al., 2009).

Overall, strategies aimed at reducing the activation of calpain proteases after neurodegeneration or trauma generally have been effective in reducing
calpain activity or calpain-mediated substrate proteolysis. Pharmacological, genetic and gene-knockdown strategies targeted at calpains have helped elucidate a role for calpain activity after trauma but have also generated considerable uncertainty given the apparent isoform-specific functions of μ- and m-calpains and drugs’ lack of specificity for calpains. Genetic models that overexpress calpastatin or calpastatin-like mimetics seem to be the most optimal approaches to specifically implicate calpains in posttraumatic pathology and guide further advancements in drug design for TBI patients. Given the highly specific inhibition of calpain by calpastatin, genetic overexpression of calpastatin was chosen to determine the efficacy of posttraumatic calpain inhibition. Calpastatin transgenic mice are evaluated using a comprehensive set of substrate proteolysis, neuronal degeneration and death, and behavioral outcomes in subsequent chapters.

**CELLULAR AND FUNCTIONAL OUTCOMES USED TO EVALUATE THE EFFICACY OF POSTTRAUMATIC CALPAIN INHIBITION**

*Substrate proteolysis*

An essential method of examining effective calpain inhibition is through immunohistochemical or immunoblot analysis of various calpain substrates after injury. Calpain-mediated fragmentation of substrates is validated when cleavage patterns are reproduced *in vitro* or *in situ* by incubation with purified calpains. The development of antibodies that bind to these calpain-specific breakdown products has further enhanced identification of calpain activity *in vivo*, as it may
be difficult to assess acute changes in the enzyme itself. In turn, prevention of substrate loss or its characteristic fragmentation in vivo by specific inhibition of calpains using knockout or calpastatin overexpression strategies confirms calpain-specific cleavage. Because each substrate serves an important purpose in neuronal structure or function, analysis of substrate proteolysis may indicate a mechanism by which calpains cause damage, cell death, and functional abnormalities after traumatic injury.

Substrate proteolysis is a telltale sign of calpain activation, especially if cleavage can be directly linked to calpains via production of unique fragments after incubation with the protease or detection by calpain cleavage-specific antibodies. However, it may be difficult to contextualize calpain-mediated protein cleavage within the posttraumatic environment considering the simultaneous involvement of other secondary mechanisms. Thus, a reduction in substrate proteolysis achieved by calpain inhibition is not a complete or direct measure of neuroprotection and behavioral efficacy. Additional cellular and functional measurements are necessary to draw conclusions about the protective or beneficial effects of calpain inhibition.

**Neurodegeneration and cell death**

Cell death is a principal outcome following traumatic injuries as a result of secondary neurodegenerative mechanisms that alter ion homeostasis, induce oxidative damage and drive protease activation. Common methods for analyzing cell death include terminal deoxynucleotidyl transferase dUTP nick end labeling.
(TUNEL) to identify DNA damage coupled with the assessment of necrotic and apoptotic morphology or staining with the fluorochrome, Fluoro-jade, to discern degenerating cells. These markers provide a discrete snapshot of regional and temporal posttraumatic cell death within the injured brain. Silver staining for neurodegeneration can provide a more cumulative assessment of evolving cellular and axonal pathology occurring many hours to days after injury. Alternatively, neuronal staining using hematoxylin and eosin, cresyl violet, or NeuN will identify viable neurons and thus can be used as a marker to determine the extent of neuronal survival after injury. While each marker offers its own separate advantages in identifying pathology, neuronal degeneration and death is an important outcome in evaluating brain injury-induced damage and the efficacy of calpain inhibition.

In contusion CCI injuries, neuronal death begins within hours of impact, with significant cortical cavitation and regional hippocampal cell degeneration observed up to 24 h after severe TBI in mice (Pleasant et al., 2011). Progressive neurodegeneration in the injured hemisphere was observed to peak 48-72 h after CCI (Hall et al., 2005, Hall et al., 2008), which is subsequent to the peak in spectrin breakdown (Kampfl et al., 1996, Hall et al., 2005, Deng et al., 2007). Manipulation of calpains via pharmacological inhibition post-CCI was ineffective at attenuating neuronal loss (Saatman et al., 2000, Thompson et al., 2010). However, injured calpain-1 knockout mice exhibited a significant reduction in cortical contusion size following CCI brain injury (Yamada et al., 2012). Due to limited selectivity of pharmacological calpain inhibitors in the former two studies,
more specific calpain inhibition with genetic knockout may be required to conclusively determine a role for calpains in injury-induced neurodegeneration. In addition, systemic administration of exogenous inhibitors may delay the time to reach therapeutic concentrations in vulnerable brain regions after injury, leading to incomplete conclusions on the efficacy of calpain inhibition. Difficulties in drug administration can be avoided with genetic manipulation and may provide clearer support for the neuroprotective potential of calpain inhibition.

Functional deficits may arise as a result of cell death after TBI, or alternatively from sublethal cellular changes such as axonal or synaptic injury. Pathogenic events occurring at the level of the axon or synapsee are not easily accounted for by overt neuronal labeling, yet may be modulated by calpain inhibition. Behavioral assessments can further validate the extent of injury or repair and are key indicators of the therapeutic relevance of a particular intervention.

**Behavioral assessments**

Experimental injury to particular brain regions, especially the cortex and hippocampus, can reproduce many of these behavioral outcomes and help identify pathological targets or areas involved in mediating the dysfunction. In rodents, motor behavior deficits are often characterized by reflex and balance impairment, hemiparesis with unilateral injuries, and limb weakness (Fujimoto et al., 2004, Saatman et al., 2006) and are often assessed experimentally by tasks that evaluate and score limb movement and balance. Although rodents exhibit
spontaneous recovery of function over time, the initial deficits are often large enough to allow beneficial effects of treatments targeting early secondary mechanisms of damage, including calpains, to be detected (Zhao et al., 2012). In rodent models of TBI, cognitive and emotional abnormalities manifest as spatial and recognition memory impairment, increased anxiety, and depressive behavior (Smith et al., 1995, Fujimoto et al., 2004, Saatman et al., 2006, Washington et al., 2012). Cognitive evaluations have typically relied on the Morris water maze task to assess encoding, consolidation, and retrieval aspects of learning and memory. Additional cognitive measures including the novel object recognition paradigm evaluating recognition memory also show TBI-induced deficits (Prins et al., 2010, Scafidi et al., 2010, Wakade et al., 2010). In addition to markers of neuronal damage, behavioral measurements are valuable tools for evaluating treatment strategies, like calpain inhibition, that can improve outcome after TBI.

Brain injury-induced motor and cognitive behavioral impairments in rodents are lessened with calpain inhibition. Persistent motor and memory deficits evaluated at 48 h following lateral FPI in rats were alleviated with continuous intraarterial infusion of AK295 initiated 15 min post-injury (Saatman et al., 1996b). Consistent with this improved outcome, calpain activation suppressed by compound SJA6017 administered up to 4 h after weight-drop injury was associated with improvements in forepaw gripping ability in mice (Kupina et al., 2001). Behavioral improvements identified above in diffuse or mixed focal and diffuse models show promise for calpain inhibition as a treatment strategy following injury. However, no studies prior to our work have assessed
the therapeutic potential of calpain inhibition on behavioral parameters following CCI injury, which produces a contusive, focal pathology.

SUMMARY

Given the scope of TBI, it is imperative to find an effective therapeutic agent or pharmacological treatment that can successfully mitigate the pathological responses to injury. Among these responses, the calcium-dependent proteases, calpains, are chief mediators of cellular damage, acting downstream of rapid and prolonged injury-induced calcium dysregulation. Apparent unregulated cleavage of vital cytoskeletal, membranous, and cytosolic proteins by calpains occurs despite coincident expression of calpains’ endogenous inhibitor, calpastatin. Genetic and pharmacological methods of calpain inhibition have been evaluated in a variety of neurodegenerative disease and trauma models; however, embryonic lethality with constitutive genetic deletion of calpains prevents disruption of both calpain isoforms, and problems associated with drug selectivity and delivery complicate attempts to specifically implicate calpains as central mediators of damage.

The dissertation herein describes the comprehensive studies undertaken to investigate the therapeutic potential of calpastatin following contusion injury (Figure 1.5). Inhibition of calpains by calpastatin takes advantage of the specific nature of the calpain-calpastatin interaction in order to clearly implicate calpains in cell death and behavioral dysfunction after TBI. To this end, two separate models of transgenic mice were used to test the hypothesis that calpastatin
overexpression will reduce posttraumatic calpain activity affording neuroprotection and behavioral efficacy. Neuronal-specific calpastatin expression was achieved using transgenic mice that overexpress the human calpastatin (hCAST) construct under control of a calcium-calmodulin dependent kinase II α promoter (Chapter 2). While hCAST expression in this model previously afforded hippocampal neuroprotection after excitotoxic stimulus (Higuchi et al., 2005), our studies are the first to evaluate transgenic calpastatin overexpression after experimental TBI. Subsequently, we characterized a novel calpastatin overexpressing transgenic mouse model with ubiquitous hCAST expression driven by the prion protein promoter (Chapter 3). This model exhibits robust calpastatin expression within nervous system and peripheral tissue, at levels that surpassed the previously tested neuron-specific mouse model. Significant attenuation of calpain activation observed with ubiquitous hCAST expression propelled additional investigations of posttraumatic behavior and neurodegeneration in the CCI model (Chapter 4). Collectively, our analysis of protein, histological, and behavioral data from two separate transgenic mouse lines has generated considerable speculation on calpastatin as a therapeutic approach to TBI, requiring thorough discussion of our major findings and considerations for future research directions (Chapter 5).

Although genetic alterations are not feasible for rapid translation to clinical TBI, strategies like calpastatin overexpression that are able to specifically inhibit calpains can provide valuable information on the role of calpains in the sequelae of injury and invariably link calpain activation to a multitude of traumatic and
neurodegenerative pathologies. If protective, calpastatin can be used as a model for the design of novel pharmacological inhibitors to specifically target regions of calpain activation in TBI and neurodegenerative disorders.
Figure 1.5. *Calpastatin overexpression as a therapeutic approach to traumatic brain injury.* **A)** Brain injury results in rapid, overwhelming calcium influx into the neuron leading to the activation of calcium-dependent proteases, calpains. Subsequent proteolysis of numerous cellular substrates contributes to neuronal damage and death and motor and cognitive functional impairments. **B)** Although the specific inhibitor of calpains, calpastatin, is present within neurons, normal levels of calpastatin are unable to fully prevent the damaging proteolytic activity of calpains after injury. Therefore, we hypothesize that overexpression of calpastatin will effectively inhibit calpain activation, sparing substrates and neurons from damage and ultimately mitigate behavioral deficits following severe brain injury.
CHAPTER 2

Preface

Chapter 2 is reprinted, with alterations described herein, from Experimental Neurology, Vol. 236(2), K.M. Schoch, H.N. Evans, J.M. Brelsfoard, S.K. Madathil, J. Takano, T.C. Saido, and K.E. Saatman, “Neuronal-specific calpastatin overexpression limits calpain-mediated proteolysis and behavioral deficits following traumatic brain injury,” pp. 371-382. Copyright 2012, with permission from Elsevier. Alterations to the published manuscript were made to the “Introduction” to reduce redundancies with Chapter 1. Figure legends were expanded from the original manuscript to provide greater detail. Minor additions to other sections were included for coherency.

Work in this chapter represents a collaboration among the coauthors. Specific contributions are as follows: Immunohistochemical and immunoblot characterization of calpastatin transgenic mice (Figure 2.1) was performed by K.M. Schoch with the exception of protease activity data (Figure 2.2) by S.K. Madathil. Homogenates for spectrin proteolysis were generated by S.N. Thompson (Figure 2.3A) and K.M. Schoch (Figure 2.3B, C) and analyzed by K.M. Schoch. All behavioral testing and histological analyses were performed by either H.N. Evans or J.M. Brelsfoard. Breeder pairs of mice were contributed by J. Takano and T.C. Saido. Preparation of the manuscript for publication was performed by K.M. Schoch and K.E. Saatman.
CHAPTER 2

Neuronal-specific calpastatin overexpression limits calpain-mediated proteolysis and behavioral deficits following traumatic brain injury

INTRODUCTION

Efforts to reduce the high incidence of traumatic brain injuries (TBIs) are primarily preventative, and currently no pharmacological treatment has been proven to lessen the secondary neurodegenerative pathology, indicating a critical need for an effective treatment that could mitigate these early cellular events and prevent the long-lasting deficits of injury. Various targets for treatment have been identified through extensive study of the secondary injury mechanisms that mediate cellular and functional damage after TBI and other neurodegenerative conditions. Many of these mechanisms involve calcium dysregulation associated with excitotoxicity and calcium influx following mechanical damage to the cell membrane, overactivation of membrane receptors and channels, or excessive depolarization (Faden et al., 1989, Palmer et al., 1993, Arundine and Tymianski, 2004, Weber, 2004).

Increases in intracellular free calcium or altered calcium handling mechanisms result in the activation of calcium-dependent proteases, or calpains, that contribute to the secondary injury cascade (Palmer et al., 1993, Arundine
and Tymianski, 2004, Sun et al., 2008). Calpain activation contributes to the evolution of neurodegeneration in Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis as well as damage associated with stroke, TBI, and spinal cord injury (Camins et al., 2006). The pathological activation of calpains that occurs after TBI and other traumatic insults results in the cleavage of cellular proteins. Without appropriate regulation, calpains can mediate proteolytic damage within the cell and lead to cell demise.

Although no neuron-specific calpains have been identified, the highly studied ubiquitous calpains, \( \mu \)- and m-calpain, have been implicated in neuronal degeneration in TBI. In rodents, posttraumatic \( \mu \)-calpain autolysis precedes the accumulation of calpain-mediated \( \alpha \)-spectrin breakdown products in the injured cortex (Kampfl et al., 1996, Pike et al., 1998). Calpain-mediated spectrin proteolysis appears early in soma and dendrites and, in the hours following injury, in regions closely correlating with subsequent neurodegeneration (Saatman et al., 1996a, Newcomb et al., 1997). Pharmacological inhibition of calpains results in improved motor and cognitive behavior and decreased axonal injury with little alteration in contusion size and inconsistent or incomplete attenuation of calpain-mediated spectrin breakdown (Saatman et al., 1996b, Posmantur et al., 1997, Saatman et al., 2000, Kupina et al., 2001, Buki et al., 2003, Ai et al., 2007, Thompson et al., 2010). However, limitations in the specificity, solubility, tissue penetration, and stability of these calpain inhibitors can complicate interpretation of pathological outcomes. Investigations into the endogenous, specific inhibitor of calpains, calpastatin, may be advantageous in
limiting calpain-mediated protein degradation and critical in designing future, more effective calpain inhibitor therapies.

Despite its co-expression with calpains in the cytosol, few studies have assessed the action of calpastatin in response to injury. After trauma, prolonged calpain activity suggests that the endogenous action or levels of calpastatin may be insufficient to fully inhibit the proteolytic activity of calpain. Employing a transgenic model of calpastatin overexpression allows researchers to specifically target calpains without the technical hurdles associated with proper timing and dosing regimens of a pharmacological inhibitor. Transgenic calpastatin expression previously was shown to be neuroprotective following excitotoxic insult, with reductions in both dendritic degeneration and axonal damage (Higuchi et al., 2005). Alternatively, genetic deletion of calpastatin resulted in increased calpain-mediated substrate cleavage and hippocampal neurodegeneration (Takano et al., 2005). Based on these results, calpains are clear mediators of excitotoxic injury and inhibition by calpastatin is effective in reducing cellular damage. The characteristic excitotoxicity and calpain activation that occur with TBIs may similarly be mitigated by calpastatin overexpression.

We hypothesized that calpastatin overexpression in a transgenic mouse model would reduce proteolysis after TBI, thereby attenuating cell death and behavioral dysfunction. To this end, we comprehensively assessed acute calpain-mediated spectrin breakdown, cortical and hippocampal neuroprotection, and motor and cognitive function after contusion brain injury in mice expressing...
human calpastatin under control of a neuron-specific calcium-calmodulin dependent kinase II α subunit promoter (CaMKIIα-hCAST).
METHODS

Experimental animals

Transgenic mice expressing human calpastatin (hCAST) under control of the neuron-specific CaMKIIα promoter were created on a C57Bl/6 background and characterized as previously described (Higuchi et al., 2005, Takano et al., 2005). These mice exhibit a 3-fold increase in calpastatin activity and display no adverse phenotype (Higuchi et al., 2005). A colony was established at the University of Kentucky from which CaMKIIα-hCAST transgenic (Tg) and wildtype (WT) littermates were bred for experimental studies. All mice were housed in controlled conditions under a 14:10 light:dark photoperiod and allowed food and water ad libitum. Animal husbandry and surgical procedures were performed according to standards set by the University of Kentucky Institutional Animal Care and Use Committee and federal guidelines (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al., 2011).

Tissue processing

For calpastatin inhibitory activity or immunoblot analyses of brain tissue, mice were asphyxiated with carbon dioxide gas and decapitated. Brain tissue was promptly removed and the contralateral and ipsilateral cortices and hippocampi were dissected apart. Tissue chunks were flash frozen in cold methanol and stored at -80°C until homogenization.
For histological preparation of brain tissue, mice were anesthetized (65 mg/kg sodium pentobarbital, intraperitoneally), transcardially perfused with 0.9% heparinized saline followed by 10% neutral buffered formalin, and decapitated. After overnight fixation of the head, the brain was removed from the skull and fixed an additional 24 h before being placed in 30% sucrose. Brains were frozen in cold (-30°C) isopentanes and cut in coronal sections (40 μm) on a sliding microtome (Dolbey-Jamison, Pottstown, PA). Free-floating tissue sections were stored in cryoprotectant (30% ethylene glycol, 30% glycerol) at -20°C until use.

**Immunohistochemistry**

Free-floating tissue sections were rinsed in Tris-buffered saline (TBS) and pretreated with 3% hydrogen peroxide solution for 30 min to quench endogenous peroxidases. Nonspecific antibody binding was blocked via incubation in 5% NHS/0.1% Triton X-100/TBS solution for 30 minutes. Sections were incubated at 4°C overnight in anti-calpastatin primary antibody (mouse, 1:1000, Millipore Co.) diluted in 5% NHS/0.1% Triton X-100/TBS. Incubation in secondary antibody diluted in 5% NHS/0.1% Triton X-100/TBS solution (donkey anti-mouse IgG 1:5000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was performed for 1 h at room temperature. Signal from the secondary antibody was amplified using an avidin-biotin complex solution (1:50, Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA) and developed using diaminobenzidine (DAB) (Vector Laboratories, Inc.). Negative controls were treated identically but were incubated overnight in diluent without primary
antibody. Cortical and hippocampal areas were imaged under a light microscope (Eclipse 50i, Nikon Corporation, Tokyo, Japan) to compare the presence or absence of immunoreactivity between genotypes.

Calpastatin inhibitory activity

Cortical and hippocampal tissues were homogenized via sonication in a calcium-free buffer solution (20mM Tris, 1mM EDTA, 100mM KCl, 0.1% 2-mercaptoethanol), and centrifuged at 21,500 g or 20 min at 4°C. Supernatant fractions were collected for protein concentration determination (Pierce BCA protein assay kit, Thermo Scientific, Rockford, IL). To determine calpastatin’s inhibitory activity, samples were incubated in the presence of calcium-free buffer solution and BODIPY-FL casein substrate (EnzChek® Protease Assay Kit, Invitrogen, Carlsbad, CA) with or without exogenous calpain-II (15.51 μg/ml, porcine kidney calpain-II, Calbiochem, Gibbstown, NJ) for 30 min. Proteases, including calpains, present in the sample cleave the casein substrate into fluorescent cleavage products. Sample fluorescence was measured using a spectrfluorometer (Synergy HT Multi-Mode Microplate Reader, Biotek Instruments, Winooski, VT) at 485 nm excitation and 528 nm emission wavelengths. Fluorescence readings were corrected by both EDTA- and calcium-containing blanks, normalized to soluble protein concentration (Pierce BCA protein assay kit, Thermo Scientific), and recorded in units of fluorescence/mg of protein. All samples were run in duplicate or triplicate and the average fluorescence reading was used for analysis.
Controlled cortical impact brain injury

Contusion brain injury and its secondary pathology are reproducibly modeled in mice by a controlled cortical impact (CCI) device (Smith et al., 1995, Hannay et al., 1999, Hall et al., 2005), which uses a pneumatically driven piston to deliver a rapid, focal impact injury to the exposed cortex. Operation of the CCI device is controlled by a computer program allowing for the adjustment of impact depth and velocity. At a 1.0 mm depth, CCI injury results in regionally selective hippocampal neurodegeneration and early cortical cell damage that progresses to cortical cavitation within hours to days after injury (Saatman et al., 2006, Pleasant et al., 2011). In preparation for CCI injury, adult mice were initially anesthetized with 3.0% isoflurane (3.0 L/min oxygen) and subsequently maintained on 2.5% isoflurane (3.0 L/min oxygen) throughout the surgical procedure. Anesthetized mice were placed within a stereotaxic frame (Kopf, Tujunga, CA) and a midline scalp incision was made to expose the underlying skull. A circular, 5 mm diameter craniotomy was made over the left hemisphere, centered between the bregma and lambdoidal sutures and lateral to the sagittal suture. Contusion injury was created using a CCI device (TBI-0310 Impactor, Precision Systems and Instrumentation, Fairfax Station, VA) with a 3.0 mm diameter, rounded, steel impactor tip. Impact velocity and dwell time were set at 3.5 m/s and 500 ms, respectively, while depth was varied to produce a moderate (0.5 mm) or severe (1.0-1.2 mm) injury (Saatman et al., 2006). Following impact, a small cranioplast made of dental cement was secured over the injury site and
the incision was sutured. Sham mice received identical surgical procedures without induction of CCI injury. Mice were placed on a heating pad (37°C) for maintenance of body temperature until fully recovered from anesthesia and freely ambulating.

**Immunoblot for calpastatin and α-spectrin**

Brain tissue was homogenized via sonication in a lysis buffer solution (20mM Tris, 150mM NaCl, 5mM EGTA, 10mM EDTA, 10mM HEPES, 1% Triton-X, 10% glycerol) containing protease inhibitors (Complete Mini™ Protease Inhibitor Cocktail tablet, Roche Applied Science, Indianapolis, IN). Samples were centrifuged at 19,000 g for 20 min at 4°C and supernatant fractions were collected for protein concentration determination (Pierce BCA protein assay kit). Cortical and hippocampal samples (5 µg) were run on 3-8% Tris-Acetate gels (Criterion™ XT Precast Gels, Bio-Rad Laboratories, Hercules, CA) and subsequently transferred to nitrocellulose membrane using a semi-dry electrophoretic transfer cell. Membranes were blocked in a 5% milk solution in TBS prior to overnight incubation at 4°C in primary antibody (α-spectrin 1:5000, human calpastatin 1:1000; Millipore Co., Billerica, MA), diluted in 5% milk solution in 0.05% Tween 20/TBS. Secondary antibodies were prepared in a 5% milk solution in 0.05% Tween 20/TBS (goat anti-mouse IgG IRDye800 1:5,000-10,000; Rockland Immunoochemicals, Gilbertsville, PA). Membranes were visualized using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) and band intensities quantified using Odyssey imaging software.
Neocortical tissue damage

Tissue sections at 400 µm intervals from bregma 0 to -3.5 mm (Paxinos and Franklin, 2001) were mounted on gelatin-coated slides and dried overnight. Slides were rehydrated through graded alcohols before staining with 0.5% cresyl violet (Acros Organics, Morris Plains, NJ). Stained tissue was viewed on a light microscope (BH2, Olympus America Inc.) equipped with a CCD camera. Using Bioquant software (version 8.40.20, Bioquant Life Science), the area of intact or ‘spared’ neocortex was analyzed on a live image, alternating between 2X and 10X magnifications as previously described, to separately outline the boundaries of the ipsilateral and contralateral neocortices at 2X that contained surviving neurons as verified by Nissl staining and morphological assessment at 10X magnification (Pleasant et al., 2011). The area of neocortical tissue damage was calculated as the difference between the contralateral and ipsilaterial neocortical areas, and then integrated over the inter-section distance to obtain the volume of tissue damage. Data are expressed as a percentage of contralateral neocortical volume to control for any potential variation in brain size due to age, sex, or tissue processing.

Fluoro-jade B staining

Degenerating neurons can reliably be detected in injured tissue using the fluorochrome Fluoro-jade B (Schmued et al., 1997). Prior to mounting on gelatin-coated slides, free-floating tissue sections (40 µm) were initially exposed to DAB
for 5 min to react with endogenous peroxidases, eliminating nonspecific fluorescence due to hemorrhage. Slides were warmed at 40-45°C for 30 min and dried overnight at room temperature. Following rehydration in 1% NaOH and alcohol gradient and 0.06% potassium permanganate treatment for 10 min, tissue was stained with 0.01% Fluoro-jade B (Millipore Co.) in 0.1% acetic acid and heat-dried at 50°C for 30 min. Slides were immersed in xylene and coverslipped with Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI). Tissue sections were imaged on a fluorescence microscope equipped with epifluorescence (AX80, Olympus America Inc., Melville, NY). The number of Fluoro-jade B positive cells was counted separately within the dentate gyrus, CA3/CA3c, and CA1 of the hippocampus and averaged across three sections selected at 400 μm intervals within the injury epicenter (approximately bregma level -1.4 to -2.5 mm) (Paxinos and Franklin, 2001). Counts were made by an observer blinded to both genotype and injury condition.

**Cognitive and motor behavioral measures**

*Morris water maze*

Post-injury memory retention was assessed using a modified Morris water maze (MWM) paradigm (Morris, 1984, Saatman et al., 2006), a well-established and commonly used task known to recruit the hippocampus. For all swim trials, mice were placed in a 1-m diameter water tank filled with water (19-21°C) made opaque with non-toxic white paint (Rich Art Co., Northvale, NJ) to conceal a 6.3 cm diameter platform placed 0.5 cm below the water surface. Mice underwent
two sets of five training trials (total 10 trials per day, 30 minute delay between sets) for three days prior to CCI injury with the platform in place. During each trial, mice were placed in the tank at different quadrant locations and, using externally placed visual cues, had to locate the submerged platform within the maze. Latency to the platform was recorded. If the mice did not locate the visible platform within 60 sec, they were placed on the platform for 10 sec after the first trial and 5 sec on subsequent trials. Five zones, including the submerged platform and its surrounding locations, were designated for memory score calculation (Saatman et al., 2006). Memory retention was tested 48 h post-injury by removing the submerged platform (probe trial) and recording the time spent in each zone of the maze using an aerial video camera and tracking software (EZVideo version 5.51DV, Accuscan Instruments Inc., Columbus, OH). A memory score was calculated as previously described (Saatman et al., 2006). Following each swim trial, mice were returned to a heated cage for recovery.

**Neuroscore**

Motor behavior was evaluated using a modified 12-point neuroscore assessment that is sensitive to both injury severity and treatment (Scherbel et al., 1999, Saatman et al., 2006). At 1 d or 2, 4, and 8 d following injury, mice were subjected to scored behavioral tasks from which points were deducted if the mice were unable to complete the task. During the grid walk assessment (2 points total), mice walked on a wire cage top with bars spaced 1.5 cm apart elevated 20 cm above a table. One point each was deducted for a forelimb or hindlimb footfall
during 60 sec of free exploration. A cage top task assessed flexion responses of the forelimbs (3 points) and hindlimbs (3 points) as mice were suspended by the tail above a cage top. One point deductions were given for hindlimb curling and for the absence of hindlimb extension and toe splaying. Similarly, one point deductions were each given for forelimb crossing, hyperactivity, and lack of forelimb grip strength when lowered to grasp the cage top. Finally, mice were prodded on one side and guided laterally along a ribbed plastic mat (68.6 cm x 62.2 cm) for four trials with increasing speed as determined by the evaluator. The ability of the mouse to maintain balance was observed (4 points total, -1 deduction for each trial that results in loss of balance).

**Novel object recognition**

Cognitive performance was also evaluated using a novel object recognition (NOR) paradigm (Bertaina-Anglade et al., 2006, Tsenter et al., 2008). Following an acclimation period in an empty Plexiglas cage (1 h; 1 mouse/cage), mice underwent baseline (pre-injury) recognition testing in which mice were individually introduced to two identical objects (object #1), placed in opposite corners of the cage. The amount of time spent exploring each object was recorded over 5 min. A mouse was considered to be exploring an object if its nose was positioned toward the object of interest at a distance of less than 2 cm. After a 4-h interval, one of the familiar objects was replaced with a novel object (object #2) and the time of exploration of each object was recorded for 5 min. Object recognition was assessed prior to injury to evaluate the effect of
calpastatin overexpression on normal memory function. On the following day, mice were subjected to CCI injury. At 7 d post-injury, mice were re-introduced to the initial identical objects for 5 min and, after a 4-hour interval, one object was replaced with a novel object (object #3). To evaluate the persistence of injury and transgene effects, mice were introduced to the same familiar object (object #1) and a novel object (object #4) at 14 d post-injury. Data is reported as a recognition index which represents the percentage of interaction time spent exploring the novel object.

**Neurological severity score**

The neurological severity score (NSS) composite motor function test was developed for use with a closed head injury model (Tsenter et al., 2008). We adapted this test for assessing motor deficits 1 h, 1 d, 2 d, 3 d, 5d, and 7d following CCI injury, focusing on components of coordinated motor function and balance. Mice were observed traversing Plexiglas beams of 3, 2, 1, and 0.5 cm width and a 0.5 cm diameter wooden rod (elevated 47 cm), during which deficits in their movement were recorded. All mice were acclimated for 30 sec to the beams and rod 24 h prior to CCI injury. Upon post-injury testing, mice were allowed up to 30 sec to cross the beams and rod, receiving a maximum of 14 points (3 points/beam and 2 points/rod). No points were lost when mice were successfully able to cross the beam or rod with normal limb movement. Points were deducted during beam testing for footfalls (-1 point), hanging upside-down (-1 point), or unwillingness to traverse (-1 point). A mouse that fell from the beam
received 0 points. During the rod testing, points were deduced for hanging upside-down (-1 point) and inability to cross the rod (-1 point). Two points were deducted for falling off the rod.

**Statistical analysis**

Data are expressed as mean + standard error of the mean (SEM). Analysis was performed using Statistica (StatSoft, Tulsa, OK). Calpastatin inhibitory activity was assessed using a two-way ANOVA (genotype x calpain). Levels of 150 kDa and 145 kDa spectrin breakdown products in the cortex and hippocampus were separately analyzed using a t-test (0.5 mm injury) or a nested two-way ANOVA (1.0 mm injury, genotype x injury condition) where the triplicate samples were treated as a nested variable. Fluoro-jade B-positive neurons, cortical tissue damage and Morris water maze memory data were assessed using a two-way ANOVA (genotype x injury condition). Morris water maze learning data, neurological severity score, and novel object recognition tasks were analyzed using a repeated measures one-way ANOVA. Neuroscore data was analyzed as a repeated measures two-way ANOVA (genotype x injury condition). A p value <0.05 was considered significant and Newman-Keuls post-hoc testing was performed when appropriate.
RESULTS

Calpastatin expression in transgenic mice

The regional and cellular localization of calpastatin expression was examined in brain sections from naïve CaMKIIα-hCAST Tg and WT littermate mice (n=3/genotype). Brains from WT mice were only weakly immunolabeled for calpastatin in cells that appeared neuronal (Figure 2.1A, C). In contrast, robust labeling of neurons in all cortical layers and hippocampal regions was evident in CaMKIIα-hCAST Tg mouse brains (Figure 2.1B, D). Within the neocortical layers, calpastatin expression was highest in layers II, III and V, appearing primarily within the neuronal cytoplasm (Figure 2.1B). In the hippocampus, neuronal labeling was evident in pyramidal cells of the CA1 and CA3 regions and within the granule cell layer and hilar region of the dentate gyrus (Figure 2.1D). Calpastatin labeling in cell bodies was also identified in the striatum, globus pallidus, and thalamic areas; labeling of calpastatin was lowest in white matter tracts. Immunoblot analysis using an antibody specific for hCAST confirmed expression in both cortical and hippocampal homogenates of naïve CaMKIIα-hCAST transgenic mice, with no detectable levels in WT littermates (n=4/genotype; Figure 2.1E).
Figure 2.1.  Calpastatin expression in the cortex and hippocampus of naïve wildtype and CaMKIIα-hCAST transgenic mice. Immunohistochemical labeling using an antibody recognizing mouse and human calpastatin (hCAST) was analyzed in the cortex (A, B) and hippocampus (C, D) of brain sections obtained from naïve wildtype (WT) (A, C) and CaMKIIα-hCAST transgenic (Tg) (B, D) mice. Neocortical layers (II-V) are indicated. Low calpastatin immunoreactivity was noted in wildtype tissue, indicative of basal levels of calpastatin expression. With hCAST overexpression, increased immunoreactivity was identified in CaMKIIα-hCAST Tg mice, appearing primarily neuronal (B, inset) compared to WT mice (A, inset). Scale bars represent 100 μm for images A-D; 20 μm for insets. E) Immunoblot analysis for human calpastatin in cortical and hippocampal homogenates of WT and CaMKIIα-hCAST Tg mice confirms the presence of human calpastatin exclusively in transgenic mice.
In vitro calpastatin inhibitory activity

To verify the functionality of human calpastatin in CaMKIIα-hCAST Tg mice, the ability of calpastatin to inhibit protease activity was assessed. Protease activity in cortical and hippocampal homogenates from naïve WT mice was very low, consistent with the majority of calpains being in an inactive state under physiological conditions. Overexpression of calpastatin resulted in a small reduction in endogenous protease activity in neocortical tissue that was not statistically significant (n=4-5/genotype; Figure 2.2). In order to mimic the increased calpain activation evident after traumatic injury, neocortical and hippocampal homogenates were spiked with exogenous calpain resulting in a nearly 4-fold increase in protease activity in WT mice (p<0.0005). Under this calpain challenge, homogenates from calpastatin overexpressing mice exhibited a highly significant inhibition of protease activity in compared to WT mice (p<0.0005). Although the addition of calpain resulted in a modest elevation in protease activity above endogenous levels in cortical homogenates from CaMKIIα-hCAST Tg mice (p<0.01) (Figure 2.2A), calpastatin overexpression completely prevented increased protease activity in hippocampal homogenates (Figure 2.2B), consistent with a high expression level of calpastatin in the hippocampus of the CaMKIIα-hCAST Tg mice (Higuchi et al., 2005).
Figure 2.2. **Protease activity in cortical and hippocampal homogenates of naïve wildtype and CaMKIIα-hCAST transgenic mice.** Protease activity (fluorescence units/mg of protein) was measured in cortical (A) and hippocampal (B) homogenates obtained from naïve (uninjured) wildtype (WT) and CaMKIIα-hCAST transgenic (Tg) mice. Without the addition of exogenous calpain, protease activity was low compared to the response following exogenous calpain addition in WT mice to mimic elevated calpain activation following traumatic brain injury. With a calpain challenge, calpastatin overexpression significantly inhibited protease activity in both cortical and hippocampal homogenates.

Data are represented as mean + SEM; n=4-5/genotype; two-way ANOVA (genotype x calpain condition) with Newman-Keuls post-hoc tests; *p<0.01, **p<0.0005 compared to respective homogenates without exogenous calpain; #p<0.0005 compared to WT + exogenous calpain.
Acute calpain-mediated spectrin proteolysis

Spectrin is cleaved from its intact 280 kDa form into 150 kDa and 145 kDa breakdown products (BDPs) by calpains or into 150 kDa and 120kDa BDPs by caspase-3 (Nath et al., 1996, Pineda et al., 2004). In the mouse CCI model, calpain activity peaks around 6 h while accumulation of spectrin fragments is maximal at 24 h (Kampfl et al., 1996, Hall et al., 2005, Deng et al., 2007). Levels of spectrin breakdown are undetectable or very low in naïve or sham animals (Saatman et al., 1996a, Hall et al., 2005, Aikman et al., 2006, Thompson et al., 2006, Deng et al., 2007, McGinn et al., 2009). At 4 h following a moderate injury, levels of the 145 kDa calpain-specific spectrin BDP in the cortex were significantly reduced in homogenates obtained from CaMKIIα-hCAST Tg compared to WT mice (p<0.05; n=3-4/genotype) (Figure 2.3A). The 145 kDa calpain-specific BDP also appeared to be reduced in hippocampal homogenates of CaMKIIα-hCAST Tg mice compared to WT mice; however, this did not reach statistical significance (p=0.13). Severe injury resulted in an increase in calpain-specific (145 kDa) spectrin breakdown in the cortex of WT mice at both 6 h and 24 h post-injury (p<0.0005 and p<0.05, respectively; n=3-6/time point) (Figure 2.3B). In severely injured CaMKIIα-hCAST Tg mice, cortical spectrin cleavage to the 145 kDa fragment was effectively inhibited at 6 h post-injury (p<0.0005 compared to WT) and remained at sham levels at 24 h post-injury. Brain-injured WT mice also exhibited increased calpain-specific BDP levels in the hippocampus at 6 h and 24 h following severe injury (p<0.0005; n=4-6/time point) (Figure 2.3C). Human calpastatin overexpression in Tg mice prevented injury-
induced elevation of the 145 kDa spectrin BDP in the hippocampus at both 6 and 24 h (p<0.05 and p<0.0005, respectively, compared to WT). Compared to sham injury, severe CCI produced a significant elevation in the 150 kDa spectrin BDP at both time points in the cortex and hippocampus (Figure 2.3B, C). However, for either moderate or severe CCI, 150 kDa spectrin BDP levels were not significantly different for WT and CaMKIIα-hCAST Tg mice. The 120 kDa spectrin BDP resulting from cleavage by caspase-3 was not detected in cortical and hippocampal samples from either WT or CaMKIIα-hCAST Tg mice after injury.
Figure 2.3. α-Spectrin proteolysis following graded traumatic brain injury in wildtype and CaMKIIα-hCAST transgenic mice. Spectrin breakdown in cortical and hippocampal homogenates obtained from wildtype (WT) and CaMKIIα-hCAST transgenic (Tg) mice was analyzed acutely after moderate and severe controlled cortical impact injury (CCI). A) At 4 h following moderate injury (0.5 mm depth), calpain-mediated spectrin proteolysis was evident in both the cortex and hippocampus. Calpastatin overexpression reduced cortical spectrin cleavage into the 145 kDa fragment, particularly in cortical homogenates. Spectrin cleavage was also measured in B) cortical homogenates and C) hippocampal homogenates from WT and CaMKIIα-hCAST Tg mice at 6 and 24 h after 1.0 mm depth CCI injury. Significant elevations in spectrin fragmentation were again noted in WT homogenates, which were blunted in CaMKIIα-hCAST Tg mice.

Data are represented as the proteolytic fragment band normalized to intact spectrin (measured as optical density units) and expressed as mean ± SEM; n=3-6/genotype/time point; unpaired Student’s t-test (0.5 mm injury) or nested two-way ANOVA (1.0 mm injury, genotype x injury condition) with Newman-Keuls post-hoc tests; *p<0.05, **p<0.0005 compared to sham-injured controls; #p<0.05, ##p<0.0005 compared to respective WT. Representative blots are shown at right.
Assessment of acute motor function

To examine whether the inhibition of calpain-mediated spectrin proteolysis in CaMKIIα-hCAST overexpressing mice observed within the first 24 h after brain injury resulted in early cortical neuroprotection and concomitant improvement in behavioral function, cohorts of mice were subjected to moderate or severe CCI brain injury and evaluated for motor function prior to euthanasia at 24 h. Motor function was assessed using a composite neuroscore test, similar to the test we previously used to demonstrate attenuation of posttraumatic motor dysfunction with calpain inhibitor administration in rats (Saatman et al., 1996b). Both moderate (n=9/genotype) and severe (n=8/genotype) CCI brain injury resulted in motor impairment in WT and CaMKIIα-hCAST Tg mice compared to their respective sham controls (n=3/genotype; p<0.05) (Figure 2.4). Calpastatin overexpression significantly attenuated acute motor dysfunction after either moderate or severe injury (p<0.005 compared to WT brain-injured mice).
Figure 2.4. Posttraumatic motor function assessment by neuroscore testing following graded controlled cortical impact injury in wildtype and CaMKIIα-hCAST transgenic mice. Both moderate (0.5 mm impact depth) and severe (1.0 mm impact depth) controlled cortical impact (CCI) brain injury resulted in significant acute motor deficits as revealed by neuroscore analysis 24 h following injury in wildtype (WT) mice. Calpastatin overexpression in CaMKIIα-hCAST transgenic (Tg) mice significantly improved motor function in moderate and severe brain-injured groups.

Neuroscores are expressed as mean ± SEM; shams n=3/genotype, injured n=8-9/genotype/severity; two-way ANOVA (genotype x injury severity with Newman-Keuls post-hoc tests; *p<0.05, **p<0.0005 compared to respective sham-injured controls; #p<0.005 compared to WT injured mice.
Assessment of acute cortical tissue damage

At the site of impact, widespread neuronal death with occasional intraparenchymal hemorrhage was observed 24 h following either 0.5 mm or 1.0 mm CCI injury, although severe CCI resulted in more extensive cortical neuron loss, with well-developed neocortical cavitation (Figure 2.5). Damaged but surviving neurons with a shrunken, pyknotic phenotype generally surrounded the contusion area while healthy, rounded cells populated areas distal to the impact site (Figure 2.5C insets). Quantitative analyses revealed a significantly larger volume of neocortical tissue damage after severe CCI compared to moderate CCI (p<0.0005) (Figure 2.5F). However, hCAST overexpression did not reduce neocortical tissue damage at 24 h following CCI (n=5-6/genotype/injury severity).

Severe CCI was typically associated with loss of continuity in the subcortical white matter tract beneath the contused cortex and occasionally with hippocampal distortion. The hippocampus sustained focal cellular damage, evident in areas of cell loss within the dentate gyrus granular layer and hilus, which was more extensive with increased injury severity. No qualitative differences in hippocampal cell survival at either injury severity were evident in Nissl-stained tissue sections from CaMKIIα-hCAST Tg mice compared to those from WT mice. However, Nissl staining is relatively insensitive for detecting small numbers of dying cells. Therefore, we labeled degenerating neurons with the sensitive fluorochrome marker, Fluoro-jade B (Schmued et al., 1997). At 24 h following moderate or severe CCI injury, Fluoro-jade B-positive (FJB+) cells were localized primarily in the dentate gyrus area, with fewer in the CA3/CA3c and
CA1 regions (Figure 2.6). The quantity of FJB+ neurons in all regions analyzed increased with greater injury severity (injury effect, p<0.05). Calpastatin overexpression resulted in significantly reduced numbers of FJB+ cells in the dentate gyrus of mice subjected to moderate injury (p<0.005) (Figure 2.6C). However, no significant genotypic differences in FJB+ cell numbers were identified at a 1.0 mm depth injury (Figure 2.6F).
Figure 2.5. Histological damage in wildtype and CaMKIIα-hCAST transgenic mice after graded controlled cortical impact brain injury. Compared to moderate (0.5 mm) controlled cortical impact (CCI) brain injury (A, B), severe (1.0 mm) CCI (C, D) resulted in a larger contusion and more extensive damage to the subcortical white matter at 24 h after injury. Peri-contusional areas contained shrunken, pyknotic neurons while sites more distal to the impact contained healthy neurons (C, insets). However, no genotype differences were observed between wildtype (WT) (A, C) and CaMKIIα-hCAST transgenic (Tg) (B, D) mice. E) Coronal image from the contralateral hemisphere of a WT mouse is shown for reference. Scale bar represents 500 μm; 20 μm for insets. F) Quantification of cortical tissue damage in WT and CaMKIIα-hCAST Tg mice following moderate and severe CCI brain injury. Although contusion volume increased significantly with increasing depth of impact, no genotypic differences were revealed between CaMKIIα-hCAST Tg and WT mice.

Tissue damage is calculated as a percent of the volume of the contralateral neocortex and expressed as mean + SEM; n=5-6/genotype/injury severity; two-way ANOVA (genotype x injury severity); *p<0.0005.
Figure 2.6. Hippocampal neurodegeneration following moderate and severe controlled cortical impact in wildtype and CaMKIIα-hCAST transgenic mice. The fluorochrome, Fluoro-jade B, was used to identify degenerating neurons within the hippocampus acutely following controlled cortical impact (CCI) injury in wildtype (WT) and CaMKIIα-hCAST transgenic (Tg) mice. Fluoro-jade B-positive (FJB+) neurons were particularly notable in the hilus and granular layer of the dentate gyrus of the hippocampus 24 h following moderate (0.5 mm) injury (A, B), which were more numerous following severe (1.0 mm) (C, D) CCI in WT (A, D) and CaMKIIα-hCAST Tg (B, E) mice. Quantification of FJB+ neurons in hippocampal regions of WT and CaMKIIα-hCAST Tg mice with (C) moderate and (F) severe CCI revealed reduced FJB+ cells in the dentate gyrus with calpastatin overexpression compared to WT mice after moderate injury, but not after severe injury. Scale bar represents 100 µm.

The number of FJB+ neurons was averaged across three sections per brain and expressed as mean ± SEM; n=8-9/genotype/injury severity; two-way ANOVA (genotype x injury severity); *p<0.005 compared to WT.
Assessment of posttraumatic cognitive and motor function over 1-2 weeks after TBI

While attenuation of acute calpain-mediated spectrin proteolysis by calpastatin overexpression was associated with modest hippocampal neuroprotection and no notable neocortical protection at 24 h after injury, motor function was significantly improved. Therefore, to provide a more thorough evaluation of the time course of recovery of motor function in calpastatin overexpressing transgenic mice and to determine whether elevated calpastatin levels also conferred improvements in cognitive function, two sets of WT and CaMKIIα-hCAST Tg mice were subjected to severe brain injury and evaluated using tests of cognitive and motor function over a 1-2 week period after TBI.

The first group of mice received severe (1.0 mm) CCI and was evaluated using tests traditionally used in our lab: a MWM visuospatial memory test and the neuroscore test for motor function. During pre-injury training, average latency to the platform decreased significantly over 3 d (p<0.0001, time effect) with an equivalent pattern of visuospatial learning for WT (n=29) and CaMKIIα-hCAST Tg (n= 25) mice (Figure 2.7A). These data suggest that calpastatin overexpression did not substantially alter learning ability in naïve mice. Memory retention was then evaluated at 48 h after CCI brain injury. Brain-injured mice (n=15-17/genotype) displayed significant memory impairment compared to sham-injured mice (n=10-11/genotype) (p<0.0001, injury effect; Figure 2.7B). However, calpastatin overexpression did not significantly enhance memory function in the MWM. Motor function was assessed in this same cohort at 2, 4 and 8 d post-
injury using the composite neuroscore test. Compared to sham controls which demonstrated consistently high neuroscores across all time points, mice receiving severe CCI brain injury exhibited significant motor deficits at 2 and 4 d post-injury (p<0.0005, injury effect; Figure 2.7C). Due to spontaneous recovery of function over the first week, only a small, but non-significant motor deficit remained at 8 d (p=0.06, injury effect). Although neuroscores of brain-injured CaMKIIα-hCAST Tg mice were slightly higher than WT mice at 2 and 4 d after injury, no significant genotype-dependent differences were detected by two-way ANOVA (Figure 2.7C).

Subsequent to this behavioral evaluation, we characterized two additional behavioral tests for use with mouse CCI: NOR and a modified NSS. As compared to memory testing using our MWM paradigm, the NOR paradigm has the advantage that it can be utilized at multiple intervals after injury in the same group of mice to allow assessment of the duration of memory impairment. To verify that overexpression of calpastatin did not result in a pre-existing alteration in cognitive ability, mice were tested using the NOR prior to injury. Naïve CaMKIIα-hCAST Tg and WT mice demonstrated an equivalent preference for the novel object (Figure 2.8A). After severe CCI injury (n=10-11/genotype), WT mice exhibited profound deficits in the ability to recognize the novel object at 7 d (p<0.05 compared to pre-injury) which persisted to 14 d (p<0.01) (Figure 2.8A). Calpastatin overexpression effectively prevented trauma-induced memory impairment in the NOR task. The performance of CaMKIIα-hCAST Tg mice after injury was equivalent to their pre-injury performance and significantly greater
than WT littermates at both 7 d and 14 d post-injury (p<0.01) (Figure 2.8A). The modified NSS revealed acute motor deficits that, in both genotypes, lessened over time (p <0.0001, time effect; Figure 2.8B). However, CaMKIIα-hCAST Tg mice showed significantly improved NSS across the 7 d post-injury period (p<0.01, genotype effect; Figure 2.8B). Due to spontaneous recovery of motor function, mice were not evaluated beyond 7 d.
Figure 2.7. Cognitive and motor behavioral analyses using the Morris water maze and neuroscore tests following severe controlled cortical impact brain injury in wildtype and CaMKIIα-hCAST transgenic mice. A) Prior to injury, wildtype (WT) and CaMKIIα-hCAST transgenic (Tg) mice show an equivalent ability to learn the location of the hidden platform on successive training days, expressed as latency time to the platform within the Morris water maze. Thus, overexpression of the calpastatin transgene did not negatively interfere with normal learning ability. B) Although controlled cortical impact (CCI) brain injury resulted in significant memory impairment, no significant genotype-dependent differences were detected at 48 h post-CCI. C) Brain injury also produced significant impairment of motor function assessed by use of the composite neuroscore at 2 d and 4 d after severe CCI. No significant genotype-dependent differences were observed at any time point.

All data are expressed as mean ± SEM; shams n=10-11/genotype, injured n=15-17/genotype; repeated measures one-way ANOVA (A) or two-way ANOVA (genotype x injury condition, B, C); *p<0.0001, **p<0.0005 compared to sham-injured controls.
Figure 2.8. Additional cognitive and motor function analyses using the novel object recognition and modified neurological severity score paradigms with wildtype and CaMKIIα-hCAST transgenic mice after severe brain injury.

A) Recognition ability of a novel object by wildtype (WT) mice declined at 7 and 14 d after severe brain injury. In contrast, injured CaMKIIα-hCAST transgenic (Tg) mice maintained their pre-injury ability to distinguish the novel object, resulting in significantly higher scores compared to WT mice. Recognition index is defined as the percent interaction time spent on the novel object.

B) Across the 7 day post-injury period, CaMKIIα-hCAST Tg mice showed significantly improved neurological severity scores compared to WT mice.

All data are expressed as mean ± SEM; n=10-11/genotype; repeated measures one-way ANOVA with Newman-Keuls post-hoc tests; *p<0.05 compared to pre-injury, #p<0.01 compared to WT mice.
Assessment of long-term cortical tissue damage

Although in our current model, the majority of cortical neuronal damage occurs within the contusion site by 24 h after CCI brain injury in mice (Pleasant et al., 2011), other reports have suggested that neuron loss in the contusion may continue for as much as 7 d after injury (Fox et al., 1998, Hannay et al., 1999, Saatman et al., 2006). Therefore, to evaluate whether calpastatin overexpression may have protected against delayed neuronal loss in the cortex despite a lack of acute protection, we measured contusion volume in hCAST and WT mice 8 d following severe (1.2 mm) CCI injury. The volume of the ipsilateral neocortex damaged in CaMKIIα-hCAST mice (15.7 ± 1.1%) was comparable to that in WT mice (15.5 ± 0.9%; n=12-13/genotype). The volume of neocortical contusion was similar to that observed at 24 h following severe CCI (Figure 2.5F), suggesting little expansion of the contusion after 24 h in mice.
DISCUSSION

Numerous studies in multiple models of TBI have demonstrated that calpains are activated in the first minutes to hours after injury and that their persistent activation is associated with neurodegenerative changes (Saatman et al., 2010). Although calpastatin is co-localized with calpains in neurons, the persistent activation of calpains after traumatic injury argues that endogenous calpastatin levels are insufficient to prevent proteolytic activity of calpains in the acute phase of injury. Although relatively little is known of the effects of TBI on calpastatin, TBI appears to elicit only delayed increases in calpastatin protein (Newcomb et al., 1999) and mRNA (Ringger et al., 2004) levels on the order of 1 day to 1 week after CCI brain injury. Therefore, enhancing calpastatin expression early after TBI may attenuate posttraumatic calpain activation and subsequent neurodegeneration. Here we provide the first evidence that, in a model of TBI, overexpression of calpastatin reduces calpain-mediated spectrin proteolysis and improves motor and cognitive behavioral performance. Behavioral efficacy was observed without reductions in neocortical contusion size and with modest hippocampal neuroprotection.

Overexpression of human calpastatin under the CaMKIIα promoter resulted in enhanced calpastatin immunoreactivity and expression specifically within the neocortex and hippocampus, areas vulnerable after CCI injury. The functionality of calpastatin was verified by an in vitro fluorogenic assay, demonstrating reduced proteolytic activity in response to a calpain challenge.
These results are consistent with previous studies with this hCAST transgenic mouse line, reporting robust expression in the neocortex and hippocampus and an approximate 3-fold greater calpain inhibition compared to non-transgenic mice (Higuchi et al., 2005). CaMKIIα-hCAST Tg mice exhibited no overt phenotype, and uninjured calpastatin overexpressing mice performed similarly to their WT counterparts in motor tasks as well as in a visuospatial, hippocampal-dependent learning task and a novel object recognition memory task. These findings suggest that calpastatin overexpression in this transgenic line does not produce notable changes in baseline learning and memory functions, despite evidence implicating calpains in hippocampal long-term potentiation (LTP). Application of calpain inhibitors has been shown to reduce or block hippocampal LTP (del Cerro et al., 1990, Denny et al., 1990, Suzuki et al., 1992, Farkas et al., 2004), although some inhibitors tested were not selective. Conversely, rats with a genetic deficiency in calpastatin exhibited enhanced LTP (Muller et al., 1995). Recent reports, however, cite no effect on LTP in calpain-1 knockout mice (Grammer et al., 2005) and normal hippocampal-dependent memory formation in calpastatin knockout mice (Nakajima et al., 2008).

Calpain-mediated proteolysis of spectrin has been well documented in experimental models of TBI, with peak breakdown during the first days after injury (Kampfl et al., 1996, Saatman et al., 1996a, Newcomb et al., 1997, Pike et al., 1998, Kupina et al., 2003, Hall et al., 2005, McGinn et al., 2009, Thompson et al., 2010). Our results extend these findings, demonstrating that calpastatin overexpression effectively blunts or prevents early accumulation of calpain-
specific breakdown products in the cortex and hippocampus. The efficacy of exogenously administered calpain inhibitors in reducing calpain-mediated spectrin proteolysis has been inconsistent in models of TBI. Treatment with calpain inhibitor II or MDL28170 decreased spectrin breakdown (Posmantur et al., 1997, Thompson et al., 2010). However, two other calpain inhibitors, SJA6017 (Kupina et al., 2001) and AK295 (Saatman et al., 2000), did not reduce cortical or hippocampal spectrin breakdown despite positive effects on behavioral outcomes. Differences in the calpain inhibitor used, the dose, duration, frequency and route of administration, and the injury model and species employed make it difficult to draw generalized conclusions about the effectiveness of calpain inhibitor treatment after TBI. The improved reduction in calpain-mediated spectrin proteolysis achieved in the CaMKIIα-hCAST Tg mice in comparison to previous studies utilizing calpain inhibitor administration may be related to the specificity of calpastatin for calpains, its overexpression specifically within neurons in the vulnerable neocortex and hippocampus, the level of calpain inhibitor present in the brain, or the early and continued elevation of calpastatin achieved through genetic overexpression.

Despite acute protection against spectrin degradation in the cortex after both moderate and severe CCI injury, CaMKIIα-hCAST Tg mice showed no genotypic differences in neuronal death in the contused cortex. These data suggest that inhibition of spectrin proteolysis by calpains is not sufficient to prevent cortical neuron death after contusion TBI. This result is in accordance with previous TBI studies, in which short- or long-term administration of
MDL28170 or AK295 produced no reduction in hemispheric or cortical lesion size 48 h following injury (Saatman et al., 2000, Thompson et al., 2010). Although contusion TBI is associated with substantial excitotoxic injury and may involve degrees of hypoxia and ischemia (Palmer et al., 1993, Kochanek et al., 1995, Clark et al., 1997), calpain inhibition has been more successful in reducing localized neuronal death in models of hippocampal excitotoxicity (Higuchi et al., 2005, Takano et al., 2005, Bevers et al., 2009, Bevers et al., 2010), contusive spinal cord injury (Yu and Geddes, 2007, Yu et al., 2008) and cerebral ischemia (Bartus et al., 1994a, Bartus et al., 1994b, Markgraf et al., 1998, Koumura et al., 2008) than in models of contusion TBI. This differential efficacy may reflect differences in the injury pathology or the extent of calcium dysregulation. Interestingly, calpains may be required for the repair of plasma membranes (Howard et al., 1999, Mellgren et al., 2009). Thus, at sites of membrane damage within contused areas (Whalen et al., 2008), calpain inhibition may prevent membrane resealing necessary for neuron survival. To achieve neuroprotection in contusion TBI, calpain inhibitors may need to be utilized in combination with other neuroprotective agents.

Only one study, to our knowledge, has evaluated hippocampal cell death after calpain inhibitor treatment and TBI (Saatman et al., 2000). Continuous infusion of the calpain inhibitor AK295 did not reduce apoptotic cell death at 48 h after lateral fluid percussion brain injury in rats. In contrast, calpastatin overexpression resulted in partial protection of hippocampal neurons, as evidenced not only by prevention of calpain-mediated spectrin proteolysis, but
also by decreased granule neuron degeneration after moderate CCI. Although Fluoro-jade staining has been co-localized with other cell death markers, including those specific for apoptosis, in other injury paradigms (Chidlow et al., 2009, Naseer et al., 2009, Serrano et al., 2011), the mechanism of cell death accompanying Fluoro-jade positivity in TBI has not been established. A reduction in dentate gyrus granule cell death after moderate but not severe CCI may suggest a greater involvement of secondary injury cascades unrelated to calpains in severe TBI or the need for higher levels of calpastatin overexpression to protect against severe injury. The CaMKIIα-hCAST Tg mice utilized here have a 3-fold increase in calpastatin activity compared to WT mice. An alternative line of calpastatin overexpressing mice has been developed with a 7-fold greater CAST expression level compared to WT mice (Rao et al., 2008) and we have investigated a transgenic mouse line with an approximate 80-fold increase in calpastatin above endogenous levels owing to its expression under the ubiquitous prion protein promoter (Chapter 3).

Importantly, calpastatin overexpression was associated with improved functional outcome in several behavioral measures in brain-injured mice. These results support previous studies observing motor and cognitive behavioral improvements with posttraumatic administration of exogenous calpain inhibitors (Saatman et al., 1996b, Kupina et al., 2001), arguing for calpain inhibition as a potential treatment for TBI. In CaMKIIα-hCAST Tg mice, simple motor function (neuroscore) was improved early (24 h) after moderate or severe injury, but a significant benefit was not observed in repeated testing over the first week after
severe injury, perhaps due in part to the high degree of spontaneous motor recovery after CCI. Coordinated motor function assessed through beam walking (NSS test) was consistently improved in calpastatin overexpressing mice over the first week post-injury. In our hands, the modified NSS test yielded larger trauma-induced deficits as a percentage of sham scores than did the neuroscore, which may have enhanced sensitivity for detecting effects of calpain inhibition. In our cognitive tests, the NOR test revealed robust protection against posttraumatic memory dysfunction in CaMKIIα-hCAST Tg mice, but the MWM memory paradigm showed only a slight improvement in memory retention. It is possible that memory tasks such as the NOR may utilize brain regions or circuits that were better preserved through calpastatin overexpression than those mediating visuospatial MWM memory function.

Interestingly, our results demonstrate behavioral improvements after injury without accompanied cortical neuroprotection, a disconnection also reported in past studies evaluating calpain inhibitor treatment (Saatman et al., 1996b, Saatman et al., 2000, Kupina et al., 2001). Likewise, it is unclear whether behavioral effects are directly related to acute spectrin preservation. While spectrin is important in receptor localization at the membrane, sparing of other proteins such as receptors, channels, signaling proteins, or synaptic proteins may also contribute to the observed behavioral improvements. To this end, we have expanded our investigations into calpain-mediated substrate proteolysis to include the collapsin response mediator protein-2 (CRMP-2) and voltage gated sodium channel following severe CCI injury (Chapter 3). Notably, calpain inhibitor
treatments have attenuated axonal pathology and improved axonal function in
TBI (Buki et al., 2003, Ai et al., 2007) and other models of nerve injury (Araujo Couto et al., 2004, Hassen et al., 2008), implicating calpains as a mediator of
axonal degeneration. Future studies could also address structural or functional
axonal protection mediated by calpastatin overexpression.

Utilizing a transgenic mouse model of neuronal-specific calpastatin
overexpression, we have confirmed specific and robust inhibition of calpain by
calpastatin through in vitro assay and attenuations in spectrin proteolysis after
CCI injury. However, CaMKIIα-driven calpastatin expression failed to provide
overt cortical and hippocampal neuroprotection, despite improvements in
behavioral function both acutely and at later times post-injury. The limited
expression of calpastatin within a specific population of forebrain neurons may be
inadequate to effectively hinder the rapid, persistent calpain activation after injury
and significantly reduce posttraumatic cell death. Therefore, in Chapters 3 and 4,
we evaluate a novel transgenic mouse with calpastatin expression driven by the
prion protein (Prp) promoter, which is expressed ubiquitously throughout the
CNS. We hypothesize that this Prp-hCAST transgenic mouse will exhibit a
greater and more widespread calpastatin expression pattern, providing
substantial inhibition of calpain after injury and improved histological and
behavioral outcomes.
Chapter 3

Preface

Chapter 3 is reprinted, with alterations, from Journal of Neurochemistry, K.M. Schoch, C.R. von Reyn, J. Bian, G.C. Telling, D.F. Meaney, and K.E. Saatman, “Brain injury-induced proteolysis is reduced in a novel calpastatin-overexpressing transgenic mouse”, in press. Copyright 2013, with permission from Jon Wiley and Sons. Alterations to the previously published manuscript were made to the “Introduction” to reduce redundancies with Chapter 1. Figure legends were expanded from the original manuscript to provide greater detail. Minor additions to other sections were included for coherency.

Experimental data were generated, analyzed and prepared for publication by K.M. Schoch and K.E. Saatman, with the exception of the following: Methodology and data (Figure 3.1A) regarding creation of Prp-hCAST founder mice were generously provided by J. Bian and G.C. Telling. Injured brain tissue for analysis of sodium channel proteolysis was generated by K.M. Schoch; however, equipment, reagents, and antibodies to detect sodium channel proteolysis were provided by C.R. von Reyn and D.F. Meaney. Sodium channel immunoblot quantification and imaging (Figure 3.6) were performed by C.R von Reyn, with subsequent analysis as included in the manuscript by K.M. Schoch. The manuscript was written by K.M. Schoch with consultation and editing by K.E. Saatman and other coauthors.
CHAPTER 3

Brain injury-induced proteolysis is reduced in a novel calpastatin overexpressing transgenic mouse

INTRODUCTION

Calpains are multi-faceted regulators of normal physiological functions such as cytoskeletal remodeling for cell growth and motility, cell cycle and differentiation processes, apoptosis, and long-term potentiation (Goll et al., 2003, Saatman et al., 2010, Sorimachi et al., 2011), mediated by discrete alterations in intracellular calcium. Under pathological conditions and overwhelming calcium levels, altered intracellular calcium homeostasis leads to calpain activation, resulting in the cleavage of cellular substrates including cytoskeletal elements, membrane receptors, cytosolic proteins, and cell death mediators (Saatman et al., 2010). Calpain-mediated proteolysis typically is considered unregulated as its cleavage patterns are not site-specific and no known substrate preference exists in vivo (Goll et al., 2003). Many calpain substrates have been identified through in vitro experiments testing for cleavage products. The proteolytic cascade triggered by calpains in the context of TBI is much more difficult to predict and interpret.
As the most well characterized calpain substrate following TBI, the cytoskeletal component α-spectrin is a valuable surrogate marker of calpain activation and its early proteolysis may indicate the severity of cellular damage and subsequent neuronal death (Saatman et al., 1996a). Through the use of calpain inhibitors and identification of calpain-specific breakdown products (BDPs), the number of calpain substrates verified in models of TBI is expanding. Collapsin response mediator protein-2 (CRMP-2) proteolysis was detected in response to excitotoxic insult and attenuated with *in vitro* calpain inhibitor application. Identical calpain-mediated CRMP-2 cleavage patterns were identified in brain homogenates after experimental TBI (Zhang et al., 2007). Similarly, voltage-gated sodium channel cleavage, triggered by exogenous calpain activation or using an *in vitro* model of TBI, was reversed with viral-mediated calpastatin overexpression or treatment with the calpain inhibitor MDL28170 (von Reyn et al., 2009). Limited cleavage characteristic of calpains may modulate ion flux and receptor function, contributing to exacerbated calcium dysfunction, further calpain activation, and neuronal damage associated with brain injury.

Efforts to mitigate posttraumatic calpain activation and subsequent calpain-mediated proteolysis have met with mixed success. Reductions in calpain-mediated spectrin or neurofilament breakdown with use of calpain inhibitor II, MDL-28170, and SNJ-1945 following CCl injury (Posmantur et al., 1997, Thompson et al., 2010, Bains et al., 2012) were not reproduced with administration of AK295 or SJA-6017 (Saatman et al., 2000, Kupina et al., 2001). Neuronal-specific human calpastatin (hCAST) overexpression in a transgenic
mouse reduced acute spectrin proteolysis and select behavioral deficits following severe contusion TBI but did not affect cortical tissue damage (Chapter 2, Schoch et al., 2012). Calpastatin overexpression in this model was driven by the CaMKIIα promoter, limiting expression to forebrain neurons (Higuchi et al., 2005). Given the widespread activation of calpains after injury, we sought a mouse model with greater cellular distribution of calpastatin to ensure inhibition of calpains in all vulnerable regions. To this end, we entered into a collaboration with Glenn Telling, Ph.D. in the department of Microbiology, Immunology and Pathology at Colorado State University. Dr. Telling had developed a novel transgenic mouse with hCAST under constitutive control of the ubiquitous prion protein promoter (Prp) enabling our desired widespread calpastatin expression. We hypothesized that greater calpastatin expression in additional neuronal regions may produce enhanced calpain inhibition, linking reduced substrate proteolysis with acute neuroprotection. Our previous results using the CaMKIIα- hCAST mice underscore the potential for calpastatin to mitigate calpains’ damaging effects after injury; however, the lack of overt cortical and hippocampal neuroprotection despite behavioral improvements is perplexing. Characterization of an alternative hCAST overexpressing mouse model was undertaken to provide additional investigations into the role of calpains and how regional and expression level changes in calpastatin may alter outcome after TBI or other neurodegenerative conditions.

Here we demonstrate that Prp-hCAST transgenic (Tg) mice have cortical and hippocampal calpastatin levels approximately 80-fold greater than wildtype
(WT) mice and use this new transgenic tool to verify the effectiveness of calpastatin in reducing calpain-mediated damage after TBI. To this end, we subjected WT and calpastatin overexpressing Tg mice to severe contusion CCI injury and evaluated acute posttraumatic proteolysis of three proteins critical for neuronal structure and function: \( \alpha \)-spectrin, CRMP-2, and voltage-gated sodium channel 1.2 (Na\(_v\)1.2). In addition, we assessed acute regional hippocampal neurodegeneration in brain-injured WT and Prp-hCAST transgenic mice.
METHODS

Human calpastatin overexpressing transgenic mice

Human calpastatin (hCAST) cDNA (Genbank accession number D16217) in the pTicCS plasmid was obtained from Dr. Masatoshi Maki (Higuchi et al., 2005). The hCAST sequence was cloned into the unique XhoI site of the MoPrP.Xho expression vector (Borchelt et al., 1996), containing ~12 kb of the mouse prion protein (Prp) gene including the promoter, the smaller of the two 5’ introns, and the 3’ untranslated sequences. The transgene expression cassette was released from the Prp-hCAST plasmid with NotI, and the resulting purified fragment was microinjected into the pronuclei of fertilized FVB/N oocytes. Transgenic founder animals were identified by PCR screening of tail genomic DNA using primers phgPrP5’ (5’GAACTGAACCATTTCAACCGAG3’) and phgPrP3’ (5’AGAGCTACAGGTGGATAACC3’). The MoPrP.Xho vector has previously been used to produce transgenic mice expressing coding sequences under the control of mouse Prp gene transcriptional elements (Li et al., 2007, Angers et al., 2009); thus, widespread hCAST expression in the CNS of transgenic mice was expected. Calpastatin expression in the CNS of founder mice was assessed by immunoblotting and compared with previously generated transgenic mice in which the hCAST was expressed under the CaMKIIα promoter (Higuchi et al., 2005). The generation and initial immunoblot characterization of founder mice were performed in the Telling laboratory.
In the Saatman lab, mice were maintained as heterozygotes by breeding FVB/N females (Harlan Laboratories, Indianapolis, IN) with Prp-hCAST transgenic males, exhibiting normal litter sizes and survival rates with no overt phenotype. Mice were housed in controlled conditions under a 14:10 light:dark photoperiod and provided with chow diet and water *ad libitum*. For experimental procedures, young adult transgenic and WT littermates were used. For qualitative measures, both males and females were analyzed. Although no notable sex-specific expression patterns were observed, efforts were made to utilize a consistent gender (male or female) within each quantitative experiment when possible in order to minimize any potential variation. All husbandry care and surgical procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee and were consistent with federal guidelines (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al., 2011) with all possible effort made to minimize pain and discomfort of the animal.

**Controlled cortical impact injury**

Contusion brain injury was modeled using a CCI device (TBI-0310 Impactor, Precision Systems and Instrumentation, Fairfax Station, VA) as previously described (Schoch et al., 2012). In brief, mice were placed in a stereotaxic head frame, receiving inhalant isoflurane anesthesia. A midline scalp incision exposed the skull and a 5 mm craniotomy was drilled on the left hemisphere, lateral to the central suture between bregma and lambda. A
rounded, 3.0 mm diameter steel impactor, controlled by a pneumatically driven cylinder, was programmed to contact the exposed dura at a 1.0 mm depth, 3.5 m/s velocity, and 500 ms dwell time. These parameters result in a severe injury, defined by progressive cortical and hippocampal cell losses over the first 24 h after insult (Pleasant et al., 2011). Following the impact, a small cranioplast made of dental acrylic was placed over the craniotomy site and secured to the skull. The incision was sutured and the animal was placed on a 37°C heating pad until ambulating. Sham-injured mice received all surgical procedures except the impact injury.

**Tissue preparation**

Tissue designated for calpastatin inhibitory assays or immunoblots was obtained following carbon dioxide asphyxiation and immediate decapitation. Contralateral and ipsilateral cortical and hippocampal tissues were separately dissected and rapidly frozen in cold (-80°C) methanol. Samples were stored at -80°C until use.

For immunochemistry and Fluoro-jade C experiments, mice were perfused transcardially with 0.9% heparinized sterile saline and 10% neutral buffered formalin under anesthesia (65 mg/kg sodium pentobarbital, intraperitoneally). Heads were placed in formalin for 24 h after which the brain was removed for additional overnight fixation. Following cryoprotection in 30% sucrose solution, the brain was frozen in cold isopentanes (-25°C to -35°C). Brain tissue was cut on a sliding microtome (Dolbey-Jamison, Pottstown, PA) in the coronal plane at a
thickness of 40 μm. Unused sections were stored in a cryoprotectant solution (30% ethylene glycol, 30% glycerol) at -20°C.

**Calpastatin inhibitory assay in naïve mice**

Frozen cortical tissue was sonicated in a calcium-free buffer solution (20 mM Tris, 1 mM EDTA, 100 mM KCl, 0.1% 2-mercaptoethanol) without protease inhibitors and centrifuged at 21,500 g for 20 min at 4°C. Supernatants were assayed for calpastatin inhibitory activity in the presence of porcine kidney m-calpain (14.06 μg/ml, Calbiochem, Gibbstown, NJ) and a fluorescently-conjugated BODIPY-FL casein calpain substrate (EnzChek® Protease Assay Kit, Invitrogen, Carlsbad, CA). In the presence of proteases, the casein substrate is cleaved to emit a fluorescent signal, which is read on a spectrofluorometer (Synergy HT Multi-Mode Microplate Reader, Biotek Instruments, Winooski, VT) at 485 nm excitation and 528 nm emission wavelengths. Fluorescence values were normalized to the amount of protein in the sample as determined by BCA assay (Pierce BCA protein assay kit, Thermo Scientific, Rockford, IL) and reported as units/mg of total protein. An average fluorescent reading was obtained from cortical samples run in duplicate.

**Calpastatin immunohistochemistry in naïve mice**

Tissue sections were rinsed in Tris-buffered saline (TBS) and treated with 3% hydrogen peroxide solution in methanol and water for 30 min to quench endogenous peroxidases associated with blood-brain barrier breakdown and
normal vasculature. Following additional TBS washes, the tissue was blocked in 5% normal horse serum in TBS/0.1% Triton X-100 for 30 min. Tissue was incubated overnight in 4°C with primary antibody recognizing calpastatin of both mouse and human origin (Table 3.1). Biotinylated donkey anti-rabbit IgG secondary antibody (1:5000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied for 1 h at room temperature, subsequently amplified by avidin and biotin complex (1:50, Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA), and developed in diaminobenzidine (DAB). Negative control tissue was treated identically but without addition of primary antibody. Tissue was viewed under a light microscope (Eclipse 50i, Nikon Corporation, Japan).
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<td>Rabbit</td>
<td>1:1000</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

Table 3.1. **Primary antibodies used in immunohistochemistry and immunoblot experiments.** Primary antibody dilutions used for experiments within Chapter 3 were recommended by supplier specifications and previously optimized. Host species and commercial source are listed for reference.
**Immunoblot of proteins from naïve and injured mice**

Tissue was homogenized by sonication in a lysis buffer (20 mM Tris, 150 mM NaCl, 5 mM EGTA, 10 mM EDTA, 10 mM HEPES, 1% Triton-X, 10% glycerol) containing protease inhibitors (Complete Mini™ Protease Inhibitor Cocktail tablet, Roche Applied Science, Indianapolis, IN). Samples were centrifuged at 19,000 g for 20 min at 4°C and the soluble fraction collected for protein concentration determination. For analysis, equal amounts of protein were run on polyacrylamide gels (3-8% Tris-Acetate, 4-20% Tris-HCl, or 10% Tris-HCl Criterion™ Precast Gels, Bio-Rad Laboratories, Hercules, CA), transferred to polyvinylidene difluoride or nitrocellulose membrane, and blocked in a 5% dry milk/TBS/0.05% Tween 20 solution for 1 h. Membranes were incubated in primary antibody (Table 3.1) diluted in 5% dry milk/TBS/0.05% Tween 20 overnight at 4°C. Secondary antibodies conjugated to either horseradish peroxidase (goat anti-mouse IgG 1:3000; Bio-Rad Laboratories) or infrared dye (goat anti-mouse IgG, goat anti-rabbit IgG 1:5,000-10,000, Rockland Immunochemicals, Gilbertsville, PA) were similarly prepared in 5% dry milk/TBS/Tween 20 solution and applied for a 1 h incubation at room temperature. Membranes probed for α-spectrin were visualized on an Odyssey LI-COR imaging station (LI-COR Biosciences, Lincoln, NE) and quantified by Odyssey imaging software. All other membranes were visualized by enhanced chemiluminescence on a Kodak imager (Kodak 1D Image Analysis, Eastman Kodak Company, Rochester, NY) and quantified by ImageJ software (National Institutes of Health, Bethesda, MD). Membranes were subsequently probed for β-
actin, β-tubulin, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 3.1) to verify equal protein loading across lanes.

**Fluoro-jade C staining and analysis of injured tissue**

The fluorochrome Fluoro-jade C (FJC) was used to detect neuronal degeneration using methodology previously described (Chapter 2, Schoch et al., 2012). Fluoro-jade B (Chapter 2) and FJC are derivatives of the same fluorescent compound and can generally be used interchangeably to identify degenerating neurons; however, FJC exhibits slightly greater resolution and contrast labeling (Schmued et al., 2005). In brief, three 40 μm tissue sections were selected at 400 μm intervals between approximate bregma level -1.4 to -2.5 mm corresponding to the injury epicenter (Paxinos and Franklin, 2001). Tissue sections were initially exposed to DAB to quench endogenous peroxidases, thereby eliminating nonspecific fluorescence associated with hemorrhage, and were subsequently mounted on gelatin-coated slides. Following overnight drying, tissues were rehydrated in 1% NaOH and alcohol gradient, treated with 0.06% potassium permanganate, and stained with 0.01% Fluoro-jade C (Millipore Co., Billerica, MA) in 0.1% acetic acid. Tissue sections were dried on a slide warmer before immersion in xylenes and covered with Cytoseal XYL (Richard-Allen Scientific, Kalamazoo, MI).

Image analysis was performed by an observer blinded to genotype using a microscope equipped with epifluorescence (AX80, Olympus America Inc., Melville, NY). Fluoro-jade C-positive cells (FJC+) were counted separately within
the dentate gyrus, CA3/CA3c, and CA1 regions of the hippocampus and averaged across the three selected tissue sections. Although degenerating neurons were also evident in the cortex of injured tissue, these FJC+ cells were not quantified.

**Statistical analysis**

All data are presented as mean ± standard error of the mean (SEM) and analyzed using Statistica software (StatSoft, Tulsa, OK). Calpastatin inhibitory activity was evaluated by one-way ANOVA. α-spectrin BDPs, 150 kDa and 145 kDa, were analyzed by nested (triplicate samples) two-way ANOVA (genotype x injury condition). Quantification of CRMP-2 BDPs was evaluated with a two-way ANOVA (genotype x injury condition). Full-length sodium channel and its proteolytic fragments were compared by nested (duplicate samples) one-way ANOVA. Numbers of Fluoro-jade C-positive cells within each hippocampal region were analyzed by unpaired t-test. A value of p<0.05 was considered significant and Newman-Keuls post-hoc testing performed when appropriate.
RESULTS

Calpastatin expression in wildtype and Prp-hCAST transgenic mice

Three Prp-hCAST transgenic founders were mated with FVB/N mice to produce lines hemizygous for the transgene array, referred to as 7472+/-, 7473+/-, and 7474+/-.

Using an antibody that detects both mouse and human calpastatin, expression levels of calpastatin in whole brains of 7474+/- mice were estimated to be ~1.8-fold higher than levels in the brains of previously characterized calpastatin overexpressing transgenic mice (CaMKIIα promoter) which express hCAST exclusively in forebrain regions (Higuchi et al., 2005) (Figure 3.1A).

Calpastatin was not detectable in the CNS of mice derived from the two remaining transgenic founders.

Regional calpastatin expression levels were assessed in cortical and hippocampal homogenates from Prp-hCAST transgenic and WT littermates (n=4/genotype). Immunoblots performed with equal protein loading (5 μg) and developed to visualize the strong calpastatin signal in hCAST transgenic mice produced no discernible calpastatin signal in WT homogenates (Figure 3.1B). To compare endogenous levels of calpastatin in WT mice to the much higher levels in Prp-hCAST transgenic mice, differential amounts of protein from WT and Prp-hCAST cortical homogenates (n=4/genotype) were loaded for immunoblot analysis. With a 40-fold increase in protein loading of WT homogenates compared to transgenic homogenates, the signal in Prp-hCAST mice was approximately double the signal from WT samples, indicating a nearly 80-fold increase.
human calpastatin expression above endogenous, mouse calpastatin levels (Figure 3.1C). Blots also revealed a slight difference in the molecular weight of mouse calpastatin (mCAST) and hCAST, allowing differentiation of the two proteins. In addition to cortical and hippocampal brain regions, various central and peripheral tissues were also examined for calpastatin expression (n=4/genotype, 5 μg protein). Reactivity for hCAST was noted in the cerebellum and spinal cord of Prp-hCAST homogenates (Figure 3.1D). Consistent with previous literature identifying high levels of calpastatin within the cerebellum compared to other brain regions (Sato et al., 2011), endogenous mCAST was more readily detectable in cerebellar homogenates from WT mice. In peripheral tissue such as the gastrocnemius and heart muscle, calpastatin reactivity was observed exclusively in samples from Prp-hCAST mice (Figure 3.1D), again indicative of a many-fold overexpression of calpastatin in the transgenic mice compared to WT. Calpastatin was not evident in the liver, lung, kidney, and spleen (data not shown).

Immunohistochemical labeling (n=3/genotype) using an antibody that recognizes both mouse and human calpastatin demonstrated a low level of reactivity in gray matter regions of WT mouse brain (Figure 3.2A), with faint neuronal labeling in the cortex (Figure 3.2C) and neuropil staining within the hippocampus (Figure 3.2E). Brain sections from Prp-hCAST transgenic mice exhibited a robust increase in calpastatin immunoreactivity compared to WT mice in cell bodies, neuropil, and white matter tracts (Figure 3.2B). All cortical layers, especially layers III and V, showed specific labeling within the neuronal
cytoplasm (Figure 3.2D, inset). Reactivity within neuronal cell bodies was also clearly evident in CA1, CA3, and dentate gyrus hilar regions of the hippocampus (Figure 3.2F).

Immunoblot and immunohistochemistry analyses included both male and female mice and no notable differences between genders were observed.
Figure 3.1. Calpastatin expression in tissue homogenates obtained from naïve wildtype and Prp-hCAST transgenic mice. A) Calpastatin expression in whole brain homogenates of three founder hemizygous Prp-hCAST transgenic (Tg) mice (7472+/-, 7474+/-, and 7473+/-) compared the calpastatin Tg model used in Chapter 2 (CaMKIIα-Tg). The presence of the human calpastatin (hCAST) transgene was identified in mouse 7474+/-, with robust expression mediated by the prion protein promoter. Regional calpastatin expression in cortical and hippocampal tissue of Tg and wildtype (WT) mice with B) equal amounts (5 μg) of protein loaded or C) a 40-fold difference in protein load to enhance detection of mouse calpastatin (mCAST). D) Calpastatin expression was also identified in cerebellar and spinal cord tissue and peripheral tissues, including gastrocnemius and heart tissue from Tg and WT mice (5 μg protein/region).
Figure 3.2. Calpastatin localization in naïve wildtype and Prp-hCAST transgenic mice. Immunohistochemical labeling using an antibody recognizing mouse and human calpastatin in A) wildtype (WT) and B) Prp-hCAST transgenic (Tg) mice demonstrates a robust increase in calpastatin in transgenic mice. Magnified areas of cortical layers II-V (C, D) and the hippocampus (E, F) are shown below each respective genotype, illustrating the neuronal localization of human calpastatin in Prp-hCAST mice. Scale bars represent 1 mm for images A-B, 100 μm for images C-F and 20 μm for insets.
In vitro calpastatin inhibitory activity

The functionality of the hCAST construct within Prp-hCAST transgenic mice was assessed using an in vitro fluorogenic assay. Under baseline physiological conditions, calpain activity is low, consistent with its normally inactive conformation in the neuron. With the addition of exogenous calpain to mimic the elevated protease activity seen with pathological insults such as TBI, contralateral (or uninjured) cortical homogenates from WT mice (n=5, male) exhibited high protease activity measured in fluorescence units per total amount of protein (Figure 3.3A). In contrast, cortical homogenates from Prp-hCAST transgenic mice (n=6, male) exhibited an approximately 7-fold decrease in protease activity compared to WT littermates (p<0.001), indicative of effective inhibition by the overexpressed calpastatin protein.

Expression of calpains and calpain substrates in naïve mice

Inhibition of calpain proteases by constitutive calpastatin overexpression may produce compensatory changes in protease or calpain substrate expression. To test whether Prp-hCAST transgenic mice exhibited alterations in protease expression, levels of calpain-1, calpain-2, and caspase-3 proteins were assessed in homogenates from Prp-hCAST and WT mice (n=4/genotype). Protease expression did not differ between WT and Prp-hCAST mice in either the cortex or hippocampus (Figure 3.3B). Similarly, selected calpain substrates including p35, β-tubulin, MAP-2, ERK, and phospho-ERK were unaltered by calpastatin overexpression in naïve Prp-hCAST mice (Figure 3.3B).
Figure 3.3. Characterization of the inhibitory activity of calpastatin and basal levels of putative calpain substrates in wildtype and Prp-hCAST transgenic mice. 

**A)** Calpastatin inhibitory activity in contralateral (uninjured) cortical homogenates following the addition of exogenous calpain to mimic a state of elevated calpain activity. Homogenates from Prp-hCAST transgenic (Tg) mice show significantly reduced protease activity compared to homogenates obtained from wildtype (WT) mice. Data are represented as mean + SEM; n=5-6/genotype; one-way ANOVA; ##p<0.001.

**B)** Expression of select proteases and calpain substrates in cortical and hippocampal homogenates obtained from naïve WT and Prp-hCAST Tg mice. Overexpression of calpastatin in Tg mice did not induce compensatory expression of calpains or caspase-3 proteases or affect the baseline proteolysis of several common calpain substrates.
Posttraumatic calpain-mediated proteolysis of $\alpha$-spectrin

To understand the action of calpastatin during in vivo activation of calpains, male WT and Prp-hCAST transgenic mice were subjected to severe CCI brain injury. Calpains are activated acutely after experimental brain injury in the mouse, resulting in early proteolysis of $\alpha$-spectrin and subsequent increase in spectrin BDPs. To correspond to the suggested peak activation of $\mu$-calpain after CCI injury (Kampfl et al., 1996) and maximal breakdown of spectrin after brain injury (Deng et al., 2007), calpain-mediated spectrin proteolysis was analyzed at both 6 and 24 h following severe CCI. Calpains cleave intact $\alpha$-spectrin (280 kDa) into 150 and 145 kDa BDPs; the 145 kDa product is specific to calpain proteolysis while the 150 kDa fragment can result from calpain and caspase activity (Pike et al., 1998). As expected, severe CCI injury resulted in calpain-mediated spectrin proteolysis evident by an increase in cortical 145 kDa BDP levels in WT mice at 6 h (n=5) and 24 h (n=7) following injury (p<0.001 compared to sham controls). In contrast, in brain-injured Prp-hCAST mice, levels of the calpain-specific proteolytic fragment (145 kDa) were maintained at sham control levels at both 6 h (n=6) and 24 h (n=6) (Figure 3.4A). The attenuation of calpain-specific spectrin proteolysis in Prp-hCAST mice relative to WT mice was statistically significant at 6 h and 24 h after injury (p<0.001).

Spectrin proteolysis in WT mice was also evident within the hippocampus following CCI, with increases in the 145 kDa fragment at 6 h and 24 h (p<0.001 compared to sham). Similar to the effect observed in the cortex, hCAST overexpression suppressed the proteolysis of spectrin to the 145 kDa fragment to
near sham levels (Figure 3.4B). Although elevated breakdown was noted at 6 h (p<0.001) and 24 h post-injury (p<0.05) compared to sham, 145 kDa spectrin BDP levels were significantly reduced in injured Prp-hCAST mice compared to injured WT mice in the hippocampus at 24 h post-injury (p<0.001) (Figure 3.4B).

Severe brain injury resulted in increased levels of the 150 kDa fragment in both cortical and hippocampal homogenates above those in sham (p<0.001; n=4-5/genotype); however, calpastatin overexpression did not significantly alter this BDP (Figure 3.4). Caspase-mediated spectrin fragments (120 kDa) were notably absent in all time points and regions analyzed.
Figure 3.4.  α-Spectrin proteolysis following severe controlled cortical impact injury in wildtype and Prp-hCAST transgenic mice. Calpain-mediated spectrin breakdown (150 kDa and 145 kDa) in A) cortical and B) hippocampal homogenates at 6 and 24 h after controlled cortical impact (CCI) injury. Severe CCI resulted in an injury-induced increase in spectrin proteolysis to the 150 kDa product in both wildtype (WT) and Prp-hCAST transgenic (Tg) homogenates compared to sham controls. The increase in cortical and hippocampal 145 kDa fragment was effectively blunted by calpastatin overexpression in Prp-hCAST Tg mice at 6 and 24 h post-CCI in the cortex and 24 h in the hippocampus.

Breakdown product (BDP) optical densities are represented as mean ± SEM; sham n=4-5/genotype, injured n=5-7/genotype; nested (triplicate samples) two-way ANOVA (genotype x injury condition) with Newman-Keuls post-hoc tests; *p<0.05, **p<0.001 vs. sham and ##p<0.001 vs. respective WT. Representative blots with β-tubulin loading control are shown to the right.
Posttraumatic calpain-mediated proteolysis of collapsin response mediator protein-2

Due to its potential role in trauma-induced neurodegeneration (Taghian et al., 2012), posttraumatic axon sprouting (Wilson et al., 2012) and NMDA trafficking (Brittain et al., 2011), CRMP-2 was investigated in WT and calpastatin overexpressing transgenic mice following TBI. From its full-length form, CRMP-2 is cleaved by calpains to a fragment of approximately 55 kDa in response to apoptotic stimuli, excitotoxic challenge, and TBI (Zhang et al., 2007). Immunoblot analysis of cortical and hippocampal homogenates from WT mice (n=3-5/condition, female) demonstrated the progressive accumulation from 6 h to 24 h following CCI brain injury of a 55 kDa CRMP-2 fragment (Figure 3.5), consistent with calpain-mediated proteolysis. Calpastatin overexpression (n=3-4/condition, female) prevented the accumulation of this BDP for up to 24 h in the cortex, resulting in a significant decrease in levels of CRMP-2 fragments in brain-injured Prp-hCAST mice relative to brain-injured WT mice (p<0.05 and p<0.0005 at 6 h and 24 h, respectively; Figure 3.5A). Within the hippocampus, CRMP-2 breakdown in Prp-hCAST mice was reduced at 6 h, and completely inhibited at 24 h post-injury (p<0.005, Figure 3.5B). These results both confirm the calpain-specific cleavage of CRMP-2 and further support the protective effect of calpastatin overexpression following in vivo calpain activation.
Figure 3.5. **Collapsin response mediator protein-2 (CRMP-2) breakdown following severe controlled cortical impact injury in wildtype and Prp-hCAST transgenic mice.**

Quantification of a 55 kDa CRMP-2 fragment in A) cortical and B) hippocampal homogenates of wildtype (WT) and Prp-hCAST transgenic (Tg) mice at 6 and 24 h after controlled cortical impact (CCI) injury. A progressive accumulation of a 55 kDa CRMP-2 fragment was identified from 6 h to 24 h post-CCI in WT mice, consistent with calpain-mediated proteolysis. Calpastatin overexpression attenuated appearance of the CRMP-2 breakdown product for up to 24 h after injury in the cortex with statistically significant reductions in fragment levels in Prp-hCAST Tg mice compared to WT mice. Similarly, CRMP-2 proteolysis within the hippocampus of Prp-hCAST Tg mice was lessened at 6 h and effectively inhibited at 24 h post-CCI.

Breakdown product (BDP) optical densities are represented as mean + SEM; n=3-5/genotype/condition; two-way ANOVA (genotype x injury condition) with Newman-Keuls post-hoc tests; **p<0.005 vs. sham and #p<0.05, ##p<0.005 vs. respective WT. Representative blots with actin loading control are shown to the right. Full-length CRMP-2 bands (bracketed) appear overexposed in representative immunoblot images in order to identify the CRMP-2 BDP.
Posttraumatic calpain-mediated proteolysis of voltage-gated sodium channel

To investigate Na\textsubscript{v}1.2 \(\alpha\) subunit proteolysis in vivo, a separate cohort of female WT and Prp-hCAST transgenic mice was subjected to severe CCI injury. Based on spectrin and CRMP-2 proteolysis data in which the most robust effect was identified in cortical homogenates, our analysis of sodium channel cleavage was restricted to the cortex. Immunoblots probed with a pan sodium channel antibody (Table 3.1) identified the full-length Na\textsubscript{v}1.2 \(\alpha\) subunit protein (260 kDa) as well as a fragment at approximately 100 kDa (Figure 3.6A). Brain injury resulted in a progressive loss in full-length protein which was statistically significant at 24 h post-injury in WT (\(p<0.001\)) and Prp-hCAST (\(p<0.05\)) cortical homogenates. WT mice (\(n=4-5\)) exhibited a pronounced (\(p<0.01\) compared to sham controls), but transient, increase in the 100 kDa BDP that was suppressed in Prp-hCAST mice (\(n=4\)). Use of an antibody that detects an epitope within the intracellular loop between domains I and II of the Na\textsubscript{v}1.2 \(\alpha\) subunit yielded fragments of 170, 111, 100, and 85 kDa (Figure 3.6B). Brain injury resulted in a statistically significant increase in the 85 kDa fragment in WT homogenates (\(p<0.01\)), which was greatly attenuated in Prp-hCAST mice at both 6 and 24 h post-CCI (\(p<0.05\) and \(p<0.01\) compared to WT, respectively).
Figure 3.6. Cleavage of the voltage-gated sodium channel (Na_v,1.2) α subunit in cortical homogenates of wildtype and Prp-hCAST transgenic mice following severe controlled cortical impact injury. A) A pan sodium channel (NaCh) antibody detected full-length and an injury-induced 100 kDa molecular weight fragment at 6 and 24 h post-CCI. Intact NaCh protein was progressively lost with severe brain injury in cortical homogenates from both wildtype (WT) and Prp-hCAST transgenic (Tg) mice. A robust but transient increase in the 100 kDa fragment appeared with injury in WT mice, but was blocked in Prp-hCAST Tg mice. B) Detection of 170, 111, 100, and 85 kDa fragment accumulation with an antibody that recognizes an epitope within the I-II loop of the Na_v,1.2 α subunit revealed a significant posttraumatic increase in the 85 kDa fragment in WT homogenates at 6 and 24 h post-CCI. Calpastatin overexpression in Prp-hCAST Tg mice attenuated the injury-induced appearance of this channel fragment.

Breakdown product (BDP) and intact optical densities are represented as mean + SEM; n=4-5/genotype/condition; nested (duplicate samples) one-way ANOVA with Newman-Keuls post-hoc tests; *p<0.05, **p<0.01 vs. sham and #p<0.05, ##p<0.01 vs. respective WT. Representative blots with GAPDH loading control are shown to the right. Black outlines delineate samples run on the same blot (i.e. sham and 6 h samples) from samples run on a separate blot (i.e. 24 h samples). Optical density analysis was performed on injured (ipsilateral, I) cortical samples. Contralateral (C) cortical samples are shown in adjacent lanes for reference only.
Acute hippocampal neurodegeneration in brain-injured mice

Reductions in calpain-mediated proteolysis of selected substrates with calpastatin overexpression may result in enhanced neuronal survival after CCI brain injury. To address this possibility, tissue sections obtained 24 h following severe CCI from male and female WT (n=9) and Prp-hCAST transgenic (n=10) mice were stained for Fluoro-jade C, identifying neuronal degeneration. Positive cellular staining was evident within the cortex and hippocampus of both WT and Prp-hCAST transgenic mice. Qualitative comparison of cortical Fluoro-jade C staining did not reveal an overt difference in acute neurodegeneration between genotypes. In the hippocampus, Fluoro-jade C-positive (FJC+) cells were found predominantly in the dentate gyrus and CA3/CA3c, with far fewer within the CA1 region. In all hippocampal regions analyzed, no statistically significant differences in numbers of degenerating neurons were identified between WT and Prp-hCAST transgenic mice (Figure 3.7).
Figure 3.7. Hippocampal neurodegeneration assessed using Fluoro-jade C staining of tissue obtained from wildtype and Prp-hCAST transgenic mice following severe controlled cortical impact injury. Numbers of Fluoro-jade C-positive (FJC+) cells were analyzed in the dentate gyrus (DG), CA3/CA3c, and CA1 regions of the hippocampus in wildtype (WT) and Prp-hCAST transgenic (Tg) tissue to identify neuronal degeneration. No quantitative differences in acute hippocampal neurodegeneration were identified between genotypes at 24 h post-CCI.

FJC+ cell counts were averaged across three sections and represented as mean + SEM; n=9-10/genotype; unpaired t-test.
DISCUSSION

Calpains are important mediators of neuronal damage and death under conditions of neurodegenerative disease and traumatic insults. Due to the risk calpain activation poses toward neuronal cell viability, we have investigated an avenue of calpain inhibition using overexpression of calpastatin in a new transgenic mouse line. Our results demonstrate that Prp-hCAST mice robustly express calpastatin throughout the brain, yielding potent inhibition of exogenous calpain. Following in vivo activation of calpains by experimental TBI, calpastatin overexpression reduced calpain-mediated proteolysis of the substrates α-spectrin, CRMP-2, and voltage-gated sodium channel. These findings confirm calpains as a pathological target and validate calpastatin as an agent for modulating calpain activity following TBI.

Prp-hCAST transgenic mice were found to express calpastatin at levels on the order of 80-fold higher than endogenous levels in WT mice in the cortex and hippocampus, with near complete inhibition of in vitro protease activity. Other transgenic models of constitutive calpastatin overexpression have also demonstrated significant elevations in calpastatin levels or inhibitory function. In the mouse line investigated in Chapter 2, neuronal expression of calpastatin via the CaMKIIα promoter produced a 3-fold greater calpastatin inhibitory activity, although hCAST and mCAST protein levels were not quantitatively compared (Higuchi et al., 2005). Use of the Prp promoter to initiate gene expression may yield a more widespread distribution throughout the brain, particularly
concentrated in axons and terminals (Barmada et al., 2004). In addition to its central distribution, calpastatin expression was also noted in peripheral tissues, making this mouse an attractive tool for many models of both central and peripheral nerve injury and other pathologies involving non-neuronal tissues. An alternative calpastatin transgenic mouse, developed using the Thy1.1 promoter, achieved an approximately 15-fold greater calpastatin expression over WT resulting in effective calpain-2 inhibition but also in changes in basal levels of calpain-1 and calpain-2 and certain calpain substrates (Rao et al., 2008). Altered basal levels of proteases or calpain substrates was not evident in the Prp- or CaMKIIα-driven models, suggesting constitutive overexpression of calpastatin did not produce compensatory upregulation of proteases or widespread changes in substrate regulation. Both transgenic models (using CaMKIIα and Thy1.1 promoters) reduced pathological activation of calpain and proteolysis of multiple calpain substrates after excitotoxic stimulus. Prp-driven hCAST overexpression was similarly successful in abating posttraumatic calpain-mediated proteolysis of structurally and functionally relevant proteins.

α-spectrin is an essential protein component of the neuronal cytoarchitecture that functions in both structural support and membrane protein anchoring. Its cleavage may contribute to neuronal pathology due to alterations in membrane stability or membrane-associated protein function. Severe TBI led to increased α-spectrin proteolysis into its characteristic 150 and 145 kDa fragments in WT mice, consistent with previous literature documenting potent and early spectrin cleavage in neurons of affected brain regions following...
Calpastatin overexpression in Prp-hCAST transgenic mice prevented appearance of the calpain-specific 145 kDa BDP in the cortex and hippocampus up to 24 h after severe CCI injury. Targeting calpain activity via administration of pharmacological calpain inhibitors has produced inconsistent results in the ability to decrease spectrin breakdown following TBI (Posmantur et al., 1997, Saatman et al., 2000, Kupina et al., 2001, Thompson et al., 2010), which may be a reflection of the differences in treatment parameters or limitations associated with the drugs themselves. Notably, some calpain inhibitors are not solely selective for calpains, have poor blood-brain barrier permeability, and are metabolically unstable (Carragher, 2006). Newer inhibitors designed to mimic calpastatin show promise in preventing calpain-specific spectrin breakdown in response to elevated intracellular calcium (McCollum et al., 2006) or ischemic insult (Anagli et al., 2009).

Calpastatin overexpression in our transgenic model was unable to inhibit spectrin proteolysis into the 150 kDa fragment. Although a 150 kDa fragment can be generated through caspase activity, which is not inhibited by calpastatin, it is unlikely that caspase-3 contributed significantly to the accumulation of a 150 kDa BDP given the absence of the signature 120 kDa fragment (Pike et al., 1998). Rather, the 150 kDa product may represent the initial cleavage product of spectrin, later cleaved to a 145 kDa fragment (Zhang et al., 2009). Why calpain inhibition would be effective in inhibiting the second, but not the initial cleavage of α-spectrin is not clear. The 145 kDa BDP has been suggested to be a more experimental contusion injury (Saatman et al., 1996a, Pike et al., 1998).
sensitive marker for calpain activation (Zhang et al., 2009) and a biomarker for injury severity in experimental TBI (Pike et al., 2001, Ringger et al., 2004) and human TBI patients (Mondello et al., 2010). Thus, inhibition of spectrin breakdown in Prp-hCAST mice may be an indication of reduced injury severity.

The collapsin response mediator proteins are a family of intracellular proteins expressed within the CNS during development and periods of axonal growth (Quinn et al., 1999). CRMP-2, in particular, is important in protein trafficking (Rahajeng et al., 2010), microtubule assembly (Fukata et al., 2002), and neurite outgrowth (Quinn et al., 2003). Calpain digestion of CRMP-2 results in generation of a 55 kDa BDP which is inhibited with SJA6017 application in cortical lysates digested with calpain-2 (Zhang et al., 2007). Here we demonstrate that CCI injury results in the appearance and subsequent accumulation of a 55 kDa CRMP-2 fragment as previously described (Zhang et al., 2007), which is attenuated by calpastatin overexpression. CRMP-2 cleavage by calpains following TBI may disrupt the protein’s interactions with axonal transport proteins (Touma et al., 2007) and act to down-regulate surface expression of NMDA receptors (Bretin et al., 2006) and voltage-gated calcium channels (Brittain et al., 2009). Thus, CRMP-2 processing may not only affect axonal function, but also modulate receptors and channels, potentiating the characteristic ionic imbalance of TBI and neurodegenerative conditions. Inhibition of CRMP-2 cleavage in the presence of the calcium channel-binding domain prevents hippocampal cell death following TBI (Brittain et al., 2011), supporting a link between CRMP-2 cleavage and neuronal death.
Multiple voltage-gated sodium channel isoforms are present within the CNS, participating in action potential generation and propagation. The Na\textsubscript{v}1.2 protein is localized to axons and terminals of neurons (Westenbroek et al., 1989) and, with injury, is cleaved by calpains (Iwata et al., 2004, von Reyn et al., 2009). Due to the sodium channel’s pivotal role in action potential generation and ion flux, injury-induced Na\textsubscript{v}1.2 damage or dysfunction may result in increased sodium influx and prolonged membrane depolarization, further exacerbating calcium dysregulation and calpain activation (Yuen et al., 2009). Severe CCI resulted in \(\alpha\)-subunit breakdown evidenced by the appearance of fragments of similar size to \textit{in vitro} findings. Calpastatin overexpression inhibited the accumulation of two distinct channel fragments (85 kDa, 100 kDa) up to 24 h post-CCI, providing strong support that these products are calpain specific. We are the first to demonstrate attenuated sodium channel proteolysis into select fragments with calpain inhibition by calpastatin \textit{in vivo}, confirming results with application of the calpain inhibitor MDL28170 after \textit{in vitro} stretch injury (von Reyn et al., 2009, von Reyn et al., 2012). Slight differences in the molecular weights of fragments between \textit{in vivo} and neuronal stretch injury were evident, likely reflecting differences in injury pathology or gel migration patterns.

In cases where multiple breakdown products were evident, calpastatin overexpression in Prp-hCAST transgenic mice inhibited the appearance of some but not all cleavage products. In particular, Na\textsubscript{v}1.2 proteolysis into various fragments was coincident with loss of full length protein expression suggesting that calpastatin overexpression is most effective in inhibiting the progressive
breakdown of the channel into smaller sized fragments. Alternatively, cleavages may indicate the activity of alternative proteolytic pathways that function independent of calpain activation and are therefore unaffected by calpastatin overexpression. It is unknown whether partial breakdown of spectrin, CRMP-2, or sodium channel imparts irreversible damage to the protein and cell or whether the fragments may exhibit independent functions in mediating cell death. Our results demonstrate an absence of acute hippocampal neuroprotection after severe CCI brain injury, consistent with findings in Chapter 2 using CaMKIIα-driven calpastatin overexpression (Schoch et al., 2012). Nevertheless, reduced calpain-mediated proteolysis may delay neuronal damage, allowing the cell to repair itself or expanding the therapeutic window for additional survival interventions. While the accumulation of calpain-mediated fragments assessed in this study was definitively reduced in Prp-hCAST mice, subacute cell survival and functional improvements will need to be investigated.

Calpastatin overexpression within Prp-hCAST mice ensures inhibition of multiple calpain isoforms thereby allowing for broad inhibition of calpains’ proteolytic activity following TBI. However, we are unable to draw conclusions about the differential roles of calpain-1 versus calpain-2 in mediating posttraumatic damage. This study introduces a novel calpastatin overexpressing transgenic mouse, and demonstrates reduced posttraumatic proteolysis of key cellular substrates, spectrin, CRMP-2, and sodium channel. Boosting endogenous calpastatin through genetic manipulation promotes inhibitory mechanisms at the initiation of damage and maintains this inhibition long past the
primary insult, a strategy that may be critical to prevent acute, overwhelming calpain activity.

Characterization of the novel Prp-hCAST transgenic mouse has confirmed greater calpastatin expression within the cortex and hippocampus compared to the alternative CaMKIIα-hCAST transgenic mouse (Chapter 2). Boosting endogenous calpastatin levels afforded acute attenuation in calpain-mediated cleavage of several vital neuronal proteins, motivating subsequent histological and behavioral studies to examine the therapeutic potential of calpain inhibition in the Prp-hCAST line. Although acute hippocampal neurodegeneration was unaffected by calpastatin overexpression in the current study, longer-term outcome was not assessed. Therefore, in Chapter 4, we evaluate the efficacy of calpastatin in mitigating cortical and hippocampal cell death at 10 d following severe CCI. While cell death is an important factor in determining the success of calpain inhibition, we also performed motor and cognitive testing to identify the functional benefit of calpastatin overexpression.
CHAPTER 4

Preface

Experiments within this chapter were developed by K.M. Schoch and K.E. Saatman. All behavioral testing was performed by J.M. Brelsfoard to ensure observation blinded with respect to genotype and injury condition. All other experimental procedures, including surgeries and immunohistochemistry, and data analyses were performed by K.M. Schoch.
CHAPTER 4

Ubiquitous calpastatin overexpression attenuates posttraumatic motor and cognitive behavioral deficits despite lack of overt neuroprotection

INTRODUCTION

Traumatic brain injuries (TBIs) affect approximately 1.7 million U.S. individuals each year (Faul et al., 2010), resulting in 3.2-5.3 million brain injury survivors with long-term disabilities (Coronado et al., 2011). Persistent symptoms of TBI include motor impairments, memory loss, anxiety, and depression, depending on the type and severity of injury sustained. Primary injury events that physically shear axons and compromise neuronal membranes not only cause cell death but also trigger secondary, neurodegenerative events that continue to mediate functional abnormalities. This cascade characterized by ion dysregulation, protease activity, reactive oxygen species, and inflammation dominates in the acute phase of injury (Ray et al., 2002, Farkas and Povlishock, 2007) and contributes to the chronic damage of TBI (Masel and DeWitt, 2010).

Increased understanding of the pathological mechanisms of injury has identified several potential therapeutic targets for the treatment of TBI. Calpains are well-characterized calcium-activated proteases that function physiologically in cell cycle regulation, cell motility, and programmed cell death (Goll et al., 2003).
Under conditions of neurodegeneration and trauma, calcium dysregulation as a result of elevated intracellular calcium or altered calcium handling mechanisms results in calpain activation and proteolysis of various structural proteins, membrane receptors, and cytosolic elements (Goll et al., 2003, Ray and Banik, 2003). Following TBI, calpains are activated early with documented cleavage of the cytoskeletal protein, α-spectrin, in areas associated with later neuronal death (Saatman et al., 2010). Despite its co-localization with calpains, the endogenous levels or activity of calpastatin appears to be inadequate to prevent the increased and prolonged activity of calpains after injury.

In order to limit the pathological effect of calpains after TBI, strategies for calpain inhibition have been studied. Treatment with pharmacological calpain inhibitors appeared to improve behavioral outcome after experimental TBI (Saatman et al., 1996b, Kupina et al., 2001) but did not consistently result in the attenuation of spectrin proteolysis or reduction in tissue damage (Posmantur et al., 1997, Saatman et al., 2000, Kupina et al., 2001, Thompson et al., 2010, Bains et al., 2012). However, issues related to drug delivery and specificity complicate interpretation of the posttraumatic action of calpains. To circumvent these complications, we pursued a more specific approach for calpain inhibition using genetic modulation of calpastatin expression. In our initial studies, neuron-specific overexpression of calpastatin (CaMKIIα promoter) demonstrated behavioral improvements similar to effects seen with pharmacological inhibitor treatment but with more robust protection against spectrin breakdown. Although reduced hippocampal cell death was evident in calpastatin overexpressing
transgenic mice exposed to excitotoxic injury (Higuchi et al., 2005), these overt neuroprotective effects could not be reproduced in a model of severe contusion brain injury (Chapter 2, Schoch et al., 2012). Interestingly, calpastatin overexpression afforded neuronal protection within the dentate gyrus with moderate injury, which was lost with severe injury. Neuroprotection may be enhanced after severe injury with greater calpastatin expression. In Chapter 3, we characterized a transgenic mouse line with human calpastatin (hCAST) expression levels well above previous models owing to the ubiquitous prion protein (Prp) promoter. Prp-hCAST mice subjected to severe contusion injury reproduced attenuations in α-spectrin proteolysis and showed significantly reduced breakdown of additional calpain substrates, voltage-gated sodium channel and collapsin-response mediator protein-2 (CRMP-2) (Chapter 3, Schoch et al., 2013). Calpastatin overexpression was unable to reduce hippocampal neurodegeneration at 24 h post-CCI injury; however, cumulative cell survival was not assessed.

Based on the enhanced protection of calpain-mediated proteolysis, increased calpastatin expression obtained in Prp-hCAST mice may exhibit behavioral recovery after injury as identified in the previous transgenic mice. While this latter mouse line exhibited modest neuronal protection in the hippocampus alone, Prp-hCAST mice may show greater or more widespread neuroprotection not evident with calpastatin overexpression limited to neuronal subpopulations under the CaMKIIα promoter. To extend our investigations on the functional and neuroprotective benefit of calpain inhibition through calpastatin
overexpression, we assessed motor and cognitive behavioral function in Prp-hCAST and wildtype mice after severe traumatic brain injury. Additionally, cortical contusion size and surviving hippocampal neurons were measured to determine the effect of calpastatin overexpression on cell survival.
METHODS

Human calpastatin overexpressing transgenic mice

Transgenic mice with human calpastatin (hCAST) under control of the ubiquitous prion protein (Prp) promoter were originally created on an FVB/N background strain and previously characterized for experimental brain injury (Schoch et al., 2013). However, previous reports have identified behavioral and visual deficits in FVB/N mice (Taketo et al., 1991, Fox et al., 1999, Mineur and Crusio, 2002), making this strain inadequate for assessing functional outcomes after TBI. To provide consistent comparisons to published data from our lab and others, Prp-hCAST transgenic mice (FVB/N) were backcrossed onto a C57Bl/6 background strain for a minimum of 10 generations. Mice were housed in a controlled environment consisting of a 14:10 light:dark photoperiod and chow diet and water *ad libitum*. For experimental procedures, male 3- to 4-month-old Prp-hCAST transgenic and wildtype littermates were used. Husbandry and surgical procedures were approved and performed in accordance with University of Kentucky Institutional Animal Care and Use Committee and federal guidelines (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al., 2011).

Controlled cortical impact brain injury

Contusion brain injury was produced using the well-established controlled cortical impact (CCI) device (TBI-0310 Impactor, Precision Systems and
Instrumentation, Fairfax Station, VA). This model of experimental brain injury is designed to transiently deliver a rapid, focal impact to the exposed brain and has previously been described in detail (Pleasant et al., 2011, Schoch et al., 2012). In brief, mice were placed under continuous 3.0% isoflurane anesthesia and immobilized in a stereotaxic frame. A midline incision was made along the scalp and the skin resected. A circular, 5 mm-diameter craniotomy was created over the left hemisphere, lateral to the sagittal suture and positioned within bregma and lambdoidal sutures. Injured transgenic and wildtype mice were subjected to a severe (0.9 mm depth) impact using a 3 mm rounded metal impactor tip at a velocity of 3.5 m/s and 500 ms dwell time. Following injury, a small cranioplast of dental acrylic was secured over the injured area and the incision was closed. Sham mice received all surgical procedures except CCI injury. Mice recovered from anesthesia on a heating pad (37°C) until ambulatory.

**Behavioral assessments**

*Morris water maze*

Post-injury learning was assessed using a modified Morris water maze (MWM) paradigm (Morris, 1984). On days 6 through 9 post-injury, mice were placed in a water-filled tank at four different locations and, for each 70-sec swim trial, were required to locate a submerged, concealed platform using externally placed visual cues. Latency time (sec) to reach the platform was recorded. If the mice did not locate the platform within the 70 sec, they were placed on the platform by the observer for 5 sec. Following final (day 9) post-injury testing, the
platform was made visible for two successive swim trials to ensure mice understood the goal of the task to find the platform. Following each swim trial, mice were placed in a heated cage for the recovery of body temperature.

Novel object recognition

Cognitive function was also evaluated using a novel object recognition (NOR) paradigm (Bertaina-Anglade et al., 2006, Tsenter et al., 2008) used previously to identify deficits in brain-injured mice (Schoch et al., 2012). Mice were acclimated to an individual empty Plexiglas cage for 1 h followed by baseline (pre-injury) recognition testing. During baseline testing, each mouse was introduced to two identical objects (object #1), placed in opposite corners of the cage. The amount of time spent exploring each object was recorded over 5 min to ensure identical preference prior to injury. Object exploration was identified when the mouse nose was positioned toward the object of interest at less than a 2 cm distance. On the following day, mice were subjected to sham or CCI injury. At 10 d post-injury, mice were re-introduced to the initial identical objects (object #1) for 5 min and, after a 4 h delay, one object was replaced with a novel object (object #2). The time of exploration of each object was recorded and reported as a recognition index, which represents the percentage of total interaction time spent exploring the novel object.

Neurological severity score
A modified 14-point neurological severity score (NSS) assessing coordinated motor function and balance was used to identify motor deficits at 1 h, 24 h, 48 h, 3 d, 5 d, and 7 d following CCI injury (Tsenter et al., 2008, Schoch et al., 2012). At each time point, mice traversed elevated beams of decreasing width (3, 2, 1, and 0.5 cm) and a 0.5 cm diameter rod, receiving a maximum of 14 points (3 points/beam and 2 points/rod). Mice were allotted 30 sec for each beam crossing, at which time points were deducted for footfalls (-1 point), hanging upside-down (-1 point), and/or unwillingness to traverse (-1 point) or falling off the beam (-3 points). Points during the 30-sec rod testing were lost for hanging upside-down (-1 point), inability to cross the rod (-1 point), or falling off the rod completely (-2 points).

**Brain tissue preparation for histological analyses**

Anesthetized mice (65 mg/kg sodium pentobarbital, intraperitoneally) were transcardially perfused with 0.9% heparinized saline followed by 10% neutral buffered formalin. Following fixation, mice were decapitated and heads post-fixed in situ overnight. Brain tissue was removed from the skull, fixed for an additional 24 h, and dehydrated in 30% sucrose. Brains were frozen in cold (-30°C) isopentanes and cut in coronal sections (40 μm) on a sliding microtome (Dolbey-Jamison, Pottstown, PA). Free-floating tissue sections were stored in cryoprotectant solution at -20°C until use.

**Neocortical contusion volume**
Free-floating tissue sections taken at 400 μm intervals from bregma 0 to -3.5 mm (Paxinos and Franklin, 2001) were mounted on gelatin-coated slides and dried overnight. Slides were rehydrated through a gradient of alcohols, stained for Nissl substance with 0.5% cresyl violet and coverslipped. Coronal sections were imaged using a light microscope (BH2, Olympus America Inc., Center Valley, PA) equipped with a CCD camera. Cortical tissue was analyzed on a live image, alternating between 2X and 10X magnification, to separately outline the ipsilateral and contralateral hemisphere boundaries containing surviving neurons using Bioquant Life Science software version 8.40.20 (Bioquant Image Analysis Corporation, Nashville, TN). Cell exclusions were determined by both the absence of Nissl staining and morphological appearance. Image analysis was performed by an investigator blinded to genotype. The volume of cortical tissue damage was calculated as the difference between contralateral and ipsilateral cortical areas for each section integrated over the inter-section distance and was expressed as a percentage of contralateral cortical volume to control for variation in brain size due to tissue processing.

**NeuN immunohistochemistry**

For hippocampal cell count estimations, neuronal cell bodies were immunostained for NeuN, a neuronal nuclear marker. Cryoprotected coronal tissue sections taken at 400 μm intervals from bregma -1.0 to -3.5 mm (Paxinos and Franklin, 2001) were rinsed in Tris-buffered saline (TBS) and pretreated in a solution of methanol and distilled water and 3% (v/v) of 30% H₂O₂ to quench
endogenous peroxidases. Sections were then blocked with 5% normal horse serum (NHS) in TBS/0.1% Triton X-100 solution and incubated overnight in primary antibody (anti-NeuN 1:1,000; Millipore Co., Billerica, MA) at 4°C. The following day, tissue was incubated in biotinylated secondary antibody (donkey anti-mouse IgG, 1:2,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at room temperature. All antibody solutions were diluted in 5% NHS/TBS/0.1% Triton X-100. Biotinylated signal from the secondary antibody was amplified using an avidin-biotin complex solution (1:50, Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA) and developed with diaminobenzidine (Vector Laboratories, Inc.). Negative controls were treated identically but without overnight incubation in primary antibody. Sections were mounted onto gelatin-coated slides and dried overnight. Slides were dehydrated through a gradient of ethanol solutions and coverslipped.

**Two-dimensional cell counting procedure**

Hippocampal subregions were delineated by cellular landmarks (Paxinos and Franklin, 2001) and imaged on light microscope (BX53, Olympus America Inc.) equipped with a CCD camera. Cell count (NeuN+) analysis was performed separately within the CA1 and CA3 pyramidal layers, dentate gyrus granular cell layer, and dentate hilus by an observer blind to both genotype and injury condition.

CA1, CA3, and granular cell subregions were analyzed in every 10th section (400 μm interval) from bregma -1.0 to -3.5 mm and outlined at 2X
magnification using Bioquant design-based stereology software (Bioquant Image Analysis Corporation). Bregma levels for the CA1 pyramidal cell layer were restricted (approximately -1.3 to -2.0 mm) to avoid poorly defined caudal regions. A counting frame was identified by random overlay of a 100 x 100 μm sample grid size. A counting frame size of 20 x 20 μm was used for CA1 and CA3 pyramidal subregions and 15 x 15 μm for the dentate gyrus granular cell layer, at randomly chosen grid intersection points. Individual NeuN+ cells were counted under a 60X 1.4 NA oil-immersion objective and used to estimate the total number of neurons (N) within each subregion by the formula: $N = \frac{1}{ssf} \times \frac{1}{asf} \times \frac{1}{hsf} \times \sum Q$ where ssf is the section sampling frequency, asf the area sampling frequency, hsf the height sampling frequency and Q the summed total of NeuN+ cells counted.

Due to the diffuse spatial arrangement and small number of cells within the dentate hilus, individual analysis of this structure does not require design-based stereology. For this reason, total counts of NeuN+ cells within the dentate hilus were performed based on densitometric threshold values using Image-Pro Plus 6.0 analysis software (Media Cybernetics, Inc., Rockville, MD). The hilus was manually outlined in each section according to conventional landmarks (Paxinos and Franklin, 2001) and both size and intensity thresholds were defined. The total number of cells identified in the injured (ipsilateral) hilus was recorded from sections at 400 μm intervals between bregma -1.3 and -3.5 mm.

**Statistical analysis**
Data are expressed as mean + standard error of the mean (SEM) and analyzed using Statistica software (StatSoft, Tulsa, OK). Morris water maze learning data and neurological severity scores were evaluated using a repeated measures two-way ANOVA (genotype x injury condition). Novel object recognition index and hippocampal cell counts were assessed using a two-way ANOVA (genotype x injury condition) while cortical tissue damage was evaluated using a t-test. A p value <0.05 was considered significant and Newman-Keuls post-hoc testing was performed when appropriate.
RESULTS

Temporal progression of post-injury motor recovery

We have previously demonstrated that neuronal-specific calpastatin overexpression resulted in improved motor function over the first week following severe contusion injury in mice using the modified neurological severity score (NSS) task (Chapter 2, Schoch et al., 2012). As compared to the motor assessment using neuroscore analysis in this study, which failed to show long-term genotypic differences in gross motor behavior, the NSS offered a more sensitive measurement to evaluate motor performance in mice. Therefore, the NSS task was selected for assessing injury-induced motor dysfunction in the current Prp-hCAST transgenic model, which shows a greater level of calpastatin overexpression (Chapter 3, Schoch et al., 2013) than neuron-specific hCAST mice. Coordinated movement was evaluated while mice traversed a beam or rod at 1 h, 24 h, 48 h, 3 d, 5 d, and 7 d post-injury to identify the temporal progression of posttraumatic motor recovery. Sham-injured mice (n=12/genotype; p<0.001, injury effect) exhibited normal motor behavior, consistently receiving near maximum scores. Slightly lower scores at 1 h likely reflect a transient effect of anesthesia in sham mice. Severe CCI resulted in a marked, early reduction in motor function in both WT and Prp-hCAST transgenic mice (n=19-20/genotype) characterized by repeated hanging of the limbs or the inability to cross the beam/rod (Figure 4.1). Initial deficits lessened as mice spontaneously recovered coordination over the 7 d post-CCI period (p<0.0001, time effect). However,
brain-injured Prp-hCAST transgenic mice exhibited significantly better motor function over the first week compared to injured wildtype mice ($p<0.005$, genotype effect; Figure 4.1).
Figure 4.1.  Coordinated motor function of wildtype and Prp-hCAST transgenic mice following severe controlled cortical impact injury. Motor function was assessed using the neurological severity score (NSS) in wildtype (WT) and Prp-hCAST transgenic (Tg) mice subjected to sham or severe controlled cortical impact injury (injured). A) Scores were obtained, in part, by point deductions for foot falls (indicated by arrow) from the beam, characteristic of hemiparesis or uncoordinated movement in injured mice. B) Brain injury resulted in an early reduction in motor function followed by spontaneous recovery over the first week post-injury. Compared to brain-injured WT mice, injured Prp-hCAST Tg mice showed significantly improved NSS over 7 d following CCI (p<0.005, genotype effect).

Motor scores are expressed as mean + SEM; shams n=12/genotype, injured n=19-20/genotype; repeated measures two-way ANOVA (genotype x injury condition) with Newman-Keuls post-hoc tests.
Subacute spatial learning behavior after severe brain injury

To assess whether long-term motor behavior improvements with ubiquitous calpastatin overexpression in Prp-hCAST mice were associated with greater cognitive recovery, post-injury spatial learning ability was evaluated by Morris water maze paradigm. On days 6, 7, 8, and 9 post-injury, WT and Prp-hCAST transgenic mice were tested for their ability to locate a hidden platform within the maze (Figure 4.2). Across all groups, latency to the platform decreased over time (p<0.0005, time effect), consistent with learning behavior; however, this effect did not depend significantly on either injury or genotype. A significant interaction was identified between injury and genotype (p<0.05). Post-hoc testing revealed an injury effect in WT mice (n=19; p<0.0005, WT injured compared to WT sham), indicative of impaired post-injury learning ability as expected following CCI injury. In contrast, calpastatin overexpression prevented injury-induced learning deficits in Prp-hCAST transgenic mice (n=20; p=0.39, hCAST injured compared to hCAST sham). Sham-injured Prp-hCAST transgenic mice unexpectedly showed increased latency over time compared to their uninjured WT counterparts (n=20/genotype; p=0.02), indicative of a slight impairment in learning ability, resulting in no statistical difference in latency between brain-injured WT and Prp-hCAST transgenic mice across the four-day testing (p=0.23).
Figure 4.2. Learning performance in a Morris water maze following severe controlled cortical impact injury in wildtype and Prp-hCAST transgenic mice. Post-injury learning performance was evaluated in wildtype (WT) and Prp-hCAST (Tg) mice beginning on day 6 following severe controlled cortical impact (CCI) injury. Decreased latency to locate the hidden platform was observed for all groups across days 6, 7, 8, and 9 post-CCI. When compared to sham-injured WT mice (○), injured WT mice (□) showed significantly impaired post-injury learning ability (p<0.0005) whereas calpastatin overexpression prevented post-injury learning deficits in Tg mice (■) (p=0.39 when compared to sham-injured Tg mice (●)). However, Tg sham mice exhibited decreased latencies over time compared to WT sham mice (p=0.02). Post-injury latency to the hidden platform was not significantly different between brain-injured WT and Tg mice.

Latency time is expressed as mean + SEM; shams n=12/genotype; injured n=19-20/genotype; repeated measures two-way ANOVA (genotype x injury condition) with Newman-Keuls post-hoc tests.
Assessment of novel object recognition ability

In addition to spatial learning, cognitive function was also evaluated using a novel object recognition task which has previously been shown to detect both a robust injury-induced deficit as well as modulation by calpastatin overexpression in CaMKIIα-hCAST mice (Chapter 2, Schoch et al., 2012). Pre-injury testing of all mice established equal preference for two identical objects, which was also confirmed after injury (data not shown). At 10 d following sham or severe CCI injury, WT and Prp-hCAST transgenic mice were evaluated for recognition ability when one familiar object was replaced with a novel object. Uninjured mice (n=12/genotype) exhibited a preference for exploring the novel object, resulting in an approximately 73% recognition index (Figure 4.3). Brain-injured WT mice (n=19) displayed significant impairment in recognition ability compared to sham controls (p<0.0005) at 10 d post-injury. WT mice failed to distinguish the new object from a previously explored object, spending equal amounts of time on both objects receiving an approximately 52% recognition index. In contrast, brain-injured Prp-hCAST transgenic mice (n=20) spent a proportionally greater time exploring the novel object, resulting in improved recognition scores (62% recognition index) compared to brain-injured WT mice (p<0.0005), although not equivalent to sham levels (p<0.0005) (Figure 4.3). Based on these results, long-term posttraumatic recognition memory is partially spared with calpastatin overexpression.
Figure 4.3. Novel object recognition assessment of wildtype and Prp-hCAST transgenic mice following controlled cortical impact injury. Ten days following injury, recognition of a novel object by wildtype (WT) and Prp-hCAST transgenic (Tg) mice was tested. Brain-injured WT mice showed significant impairment in novel object recognition compared to sham-injured mice. However, injured Tg mice exhibited improved scores compared to WT mice, demonstrating retention of memory ability after severe CCI injury, although not equivalent to sham levels at 10 d post-injury.

Recognition index is calculated as the percent of total interaction time spent on the novel object and expressed as mean ± SEM; shams n=12/genotype, injured n=19-20/genotype; two-way ANOVA (genotype x injury condition) with Newman-Keuls post-hoc tests; *p<0.0005 compared to respective sham, #p<0.0005 compared to WT injured.
Long-term evaluation of neocortical tissue and hippocampal cell damage

In Chapter 3, we demonstrated sparing of select calpain substrates, spectrin, CRMP-2, and voltage-gated sodium channel, in Prp-hCAST mice following severe brain injury that may, in turn, impart neuroprotection of damaged cells. Furthermore, improved behavioral outcome in Prp-hCAST transgenic mice may result from the prevention or delay of neuronal death. To evaluate this premise, subsets of tissue from WT and Prp-hCAST transgenic mice tested for behavioral function were analyzed for neuroprotection in both the neocortex and hippocampus, regions that mediate motor and cognitive functions and exhibit progressive neuronal damage after injury (Pleasant et al., 2011).

In the neocortex, CCI injury produced cellular pathology characteristic of a severe contusion in WT (Figure 4.4A) and Prp-hCAST transgenic (Figure 4.4B) mice. Extensive cortical cavitation was noted throughout the area of mechanical impact, indicative of a loss of tissue within days after impact compared to the contralateral hemisphere (Figure 4.4C). Staining of Nissl substance revealed distorted neuronal architecture and dense gliosis in areas bordering the contusion site. These regions were directly adjacent to areas of healthy, rounded neuronal morphology located distal to the injury epicenter. At a 0.9 mm impact depth, CCI injury consistently resulted in complete loss of underlying subcortical white matter, often associated with mild distortion of the hippocampal formation. Quantitative analysis of contusion volume revealed equivalent neocortical tissue damage in WT and Prp-hCAST transgenic mice (n=12/genotype) 10 d after severe CCI (Figure 4.4D).
Analysis of Nissl-stained tissue also identified select populations of hippocampal cells vulnerable to severe CCI within the dentate gyrus granular layer, dentate hilus, CA3 and CA1 regions. To more clearly identify and accurately quantify neurons within hippocampal subregions, tissue was stained with NeuN to label neuronal nuclei. Compared to uninjured, sham controls (Figure 4.5A, B), severe CCI injury resulted in substantial loss of NeuN staining within the hilus and CA3 regions as well as thinning of the dentate gyrus granular cell layers (Figure 4.5 C, D), irrespective of genotype. Stereological estimation of total neuron numbers revealed a significant injury-induced loss of neurons at 10 d post-CCI (n=7/genotype) within defined regions of the CA1 (p<0.05; Figure 4.5E), CA3 (p<0.05; Figure 4.5F), and dentate gyrus granular layer (p<0.001; Figure 4.5G) compared to sham controls (n=3/genotype). Automated cell counts performed within the hilus also demonstrated loss of neurons after severe injury (p<0.05 for WT injured, p<0.001 for Prp-hCAST transgenic injured; Figure 4.5H). In all areas analyzed, neuronal counts of Prp-hCAST transgenic mice were not statistically different from injured WT mice. Therefore, calpastatin overexpression did not attenuate the injury-induced neuronal loss within the hippocampus 10 d after severe CCI.
Figure 4.4. *Cortical tissue damage of wildtype and Prp-hCAST transgenic mice after severe controlled cortical impact injury*. Severe controlled cortical impact (CCI) injury resulted in extensive cortical damage and loss of the subcortical white matter in both (A) wildtype (WT) and (B) Prp-hCAST transgenic (Tg) tissue, although no differences were observed between genotypes. C) The contralateral hemisphere of a Tg mouse is shown for reference, demonstrating uninjured histological characteristics. Scale bar represents 500 μm. D) Quantification of the cortical contusion volume at 10 d following severe CCI revealed no significant genotypic differences between WT and Prp-hCAST Tg mice. Volumetric tissue damage is calculated as a percent of contralateral cortex and expressed as mean + SEM; n=12/genotype; t-test.
Figure 4.5. Hippocampal neuron loss following severe controlled cortical impact injury in wildtype and Prp-hCAST transgenic mice. NeuN (neuronal nuclei) staining in the hippocampus of wildtype (WT) and Prp-hCAST transgenic (Tg) mice clearly identifies neuronal cells within the CA1 and CA3 pyramidal layers, dentate gyrus granular cell region, and hilus. No overt losses in neuronal labeling were revealed in sham A) WT or B) Prp-hCAST Tg mice. With contusion brain injury, neuron loss was evident within all hippocampal subregions, most notably in the CA3 pyramidal layer and hilus. No differences in NeuN staining were evident between injured C) WT and D) Prp-hCAST Tg mice. Scale bar represents 250 μm. Using stereological methodology, NeuN+ cells were quantified within the E) CA1, F) CA3, and G) dentate gyrus granular cell layer (DG GL) to estimate the total number of neurons, or N, within a region. In contrast, neurons within the dentate gyrus hilar region (H) were determined by computer-automated counting with size and densitometric threshold limits to generate the total (or sum) number of NeuN+ cells. CCI injury resulted in a significant loss of NeuN+ cells at 10 d post-injury in all regions analyzed, which was not affected by calpastatin overexpression in Tg mice.

Cell counts are expressed as mean ± SEM; shams n=3/genotype; injured n=7/genotype; two-way ANOVA (genotype x injury condition) with Newman-Keuls post-hoc tests; *p<0.05, **p<0.001 compared to respective sham.
DISCUSSION

The long-term cellular consequences of traumatic injury are mediated by a myriad of secondary events, including a rise in intracellular calcium levels, calcium dysregulation, and activation of the calpain proteases. Subsequent calpain-mediated proteolysis of numerous cellular substrates is a notable contributor to the secondary pathogenesis of TBI. In Chapter 3, we characterized a novel calpastatin overexpressing transgenic mouse (Prp-hCAST) for the purpose of evaluating calpain inhibition after traumatic injury. Compared to alternative genetic models, Prp-hCAST mice exhibited robust calpastatin expression throughout the brain tissue with attenuation of α-spectrin, CRMP-2, and voltage gated sodium channel breakdown following brain injury (Schoch et al., 2013). We have continued our investigations on the therapeutic potential of calpastatin by presently evaluating posttraumatic neuroprotection and behavioral outcome in Prp-hCAST mice over the first week after injury. We conclude that, while neuron survival was unaffected, calpastatin overexpression attenuated both motor and cognitive behavioral deficits after severe CCI injury. Due to the prevalent and often sustained physical and cognitive disturbances of human TBI patients, our findings have clear implications toward establishing calpain inhibition as an effective therapeutic strategy following brain injury.

Common motor deficits including gait and balance abnormalities experienced by human TBI patients are often reproduced in animal models of experimental injury and quantified by various scored tasks to evaluate reflexes,
movement, coordination, and/or strength. In order to assess motor function following trauma, we employed a modified neurological severity score (NSS) originally developed for neurological assessments of rats subjected to closed head injury (Tsenter et al., 2008). When adapted for mice following CCI, injured mice exhibited an initial, marked deficit that lessened over the 7-d post-injury period, comparable to our previous results (Chapter 2, Schoch et al., 2012). While both WT and Prp-hCAST transgenic mice exhibited spontaneous motor improvement over the post-injury time course, Prp-hCAST mice showed significantly better recovery from injury-induced motor deficits. These results support our recent findings with an alternative transgenic model of calpastatin overexpression (CaMKIIα promoter) identifying improvements in motor function acutely following TBI (Chapter 2, Schoch et al., 2012). Similarly, our findings are consistent with numerous calpain inhibitor studies assessing motor function following traumatic injury. Continuous 48 h administration of the calpain inhibitor AK295 improved function one week after lateral fluid percussion brain injury (Saatman et al., 1996b) while acute improvements in grip score were reported following single, immediate post-injury administration of SJA6017 (Kupina et al., 2001). Similarly, treatment with calpain inhibitors CEP4143 or MDL28170 improved hindlimb locomotor performance days to weeks after spinal cord injury (Schumacher et al., 2000, Hung et al., 2005, Yu and Geddes, 2007, Yu et al., 2008). Based on these findings with pharmacological inhibitors, calpain activation appears to contribute to motor dysfunction following injury, and may necessitate early post-injury calpain inhibition for therapeutic intervention and successful
mitigation of injury-induced deficits. Constitutive overexpression of calpastatin was similarly effective in improving motor behavior after severe contusion brain injury.

Cognitive deficits including memory difficulties are particularly common in the clinical manifestation of TBI. Experimentally, cognitive deficits have been routinely studied using the Morris water maze, employing different paradigms to assess encoding, consolidation, and retrieval aspects of learning and memory. CCI-injured rodents show both learning and memory impairments compared to sham-injured animals for days to weeks following injury (Smith et al., 1995, Schoch et al., 2012, Zhao et al., 2012). As anticipated, injured WT mice showed a decline over time in latency to the platform, yet plateaued above sham levels, suggestive of learning impairment a week after injury. However, injured Prp-hCAST demonstrated preserved spatial learning behavior evidenced by learning latencies comparable to transgenic sham mice. Although no other study has evaluated the effect of calpastatin on post-injury learning, investigations on memory function show that while treatment with AK295 significantly improved memory retention in a MWM task one week following brain injury (Saatman et al., 1996b), no improvement in a similar task was identified in calpastatin transgenic mice tested 48 h post-injury (Schoch et al., 2012). In addition to variations in study methodologies, cognitive parameters measured by the maze task (e.g., post-injury learning or memory retention) may be differentially influenced by calpain inhibition.
Notably, Prp-hCAST sham mice exhibited slower latency times compared to WT sham mice, suggestive of a preexisting cognitive impairment with transgenic calpastatin overexpression. Calpain inhibitors have been shown to interfere with long-term potentiation (LTP), implicating calpains in mediating substrate proteolysis and structural reorganization necessary for learning and memory formation (Oliver et al., 1989, del Cerro et al., 1990, Denny et al., 1990, Suzuki et al., 1992, Farkas et al., 2004). Despite this evidence, naïve mice overexpressing calpastatin (CaMKIIα promoter) showed equivalent latencies to the platform compared to WT littermates during pre-injury water maze training (Schoch et al., 2012), suggesting normal learning ability in transgenic mice despite an intracellular environment primed for calpain inhibition. In accordance with this finding, calpain-1 knockout mice showed no LTP dysfunction (Grammer et al., 2005) despite results seen with pharmacological calpain inhibitor treatment. However, recent evidence suggests calpain-2 may play a predominant role in mediating plasticity changes as silencing calpain-2 or conditional disruption of the calpain small subunit demonstrated impaired LTP and cognitive behavior function in naïve mice (Amini et al., 2013, Zadran et al., 2013). Genetic deficiency in calpastatin resulted in enhanced LTP in rats (Muller et al., 1995), while calpastatin deletion in mice did not interfere with normal hippocampal-dependent memory formation (Nakajima et al., 2008). Prp-driven expression results in higher levels of calpastatin compared to CaMKII-driven expression (Schoch et al., 2013), levels that may reveal LTP and learning impairments in Prp-hCAST mice. Uninjured Prp-hCAST mice achieve equivalent latencies to WT
mice upon multiple, successive testing days indicating a possible delay in learning ability as opposed to an overt deficiency. Calpastatin overexpression was effective in preventing injury-induced learning deficits yet inducible models of calpastatin manipulation should be investigated to ensure normal memory formation.

Alternative cognitive tests that do not rely on spatial cues and navigation strategy, such as the novel object recognition (NOR) paradigm, are also useful in evaluating cortical and hippocampal function (Clark et al., 2000, Balderas et al., 2008). In the present study, brain-injured mice exhibited significant impairment in the ability to recognize a novel object at 10 d post-injury, consistent with previous reports following experimental TBI (Prins et al., 2010, Wakade et al., 2010, Schoch et al., 2012, Zhao et al., 2012). Calpastatin overexpression attenuated object recognition deficits in brain-injured mice, but had no effect on recognition ability in sham controls. These results are similar to our results in Chapter 2 reporting the maintenance of recognition ability in calpastatin overexpressing mice at 7 and 14 d post-CCI injury (Schoch et al., 2012). However, brain-injured CaMKIIα-hCAST mice maintained recognition ability similar to sham levels weeks post-injury while injured Prp-hCAST mice in the current study still exhibited a mild deficit at 10 d. This latter finding may again suggest a disadvantage to excessive calpastatin expression after injury.

The parietal or motor cortex, presumably responsible for integrating and executing many motor behavioral responses in mice, exhibits notable cell loss hours to days following CCI brain injury (Pleasant et al., 2011). Not surprisingly,
we report development of a large cortical contusion and significant hippocampal cell losses by 10 d post-CCI injury. However, cortical contusion volume measurements and regional hippocampal cell counts were statistically equivalent between genotypes following injury, despite behavioral improvements in Prp-hCAST mice. Cortical neuroprotection has not been reported after brain injury with use of pharmacological calpain inhibitors (Saatman et al., 2000, Thompson et al., 2010) or previous genetic calpastatin modification (Schoch et al., 2012). Within the hippocampus, calpain inhibitor administration did not protect against apoptosis after lateral FPI (Saatman et al., 2000); however, calpastatin overexpression showed acute, modest reductions in neurodegeneration after moderate, but not severe CCI (Schoch et al., 2012). Greater calpastatin overexpression in Prp-hCAST mice failed to produce cortical neuroprotection or enhance hippocampal cell survival days after severe CCI injury, suggesting that calpain inhibition alone may be ineffective at measurably altering cumulative neuronal death in the injured cortex and hippocampus after severe brain injury.

Despite the mixed results seen in TBI, calpain inhibition has shown improved pathological outcomes in models of ischemia and spinal cord and axonal injury. Reductions in cortical infarct produced by focal ischemia have been consistently identified with either single or continuous administration of pharmacological calpain inhibitors (Bartus et al., 1994a, Bartus et al., 1994b, Markgraf et al., 1998, Tsubokawa et al., 2006, Koumura et al., 2008). Similarly, selective CA1 cell death accompanying global ischemia was lessened with continuous calpain inhibitor administration (Lee et al., 1991, Rami et al., 2000,
Frederick et al., 2008). Disparate neuroprotective outcomes between ischemia and TBI may result from inherent differences in injury mechanisms related to glutamate release, calcium levels and entry modes, or vascular contributions. Following spinal cord and axonal injury, pharmacological calpain inhibition reduced apoptosis (Arataki et al., 2005, Sribnick et al., 2007, Colak et al., 2009), lesion size (Yu and Geddes, 2007, Yu et al., 2008), and other histological indicators of axonal damage (Buki et al., 2003, Ai et al., 2007, Ma et al., 2012a), suggesting calpain activation and substrate proteolysis more directly affect axonal structure and transport function. Therefore, calpastatin overexpression may be ameliorating subtle axonal damage without overt neuronal protection in trauma.

Calpain inhibition via pharmacological intervention has been successful at reducing posttraumatic calpain-mediated proteolysis or behavioral deficits, outcomes that were collectively achieved with genetic calpastatin overexpression in our studies (Schoch et al., 2012, Schoch et al., 2013); however, these results generally do not equate to neuroprotection within the primary injury sites. Although we speculate that the reduced breakdown of critical substrates delays cell demise, partially proteolyzed substrates may be unable to function normally within the cell. In addition, calpain activation is one of several neurodegenerative cascades responsible for injury-induced cell dysfunction. Other secondary mechanisms including blood-brain barrier breakdown, mitochondrial dysfunction, reactive oxygen species, and inflammation may also predominate after severe
contusive trauma (Ray et al., 2002), necessitating an intervention strategy involving calpain inhibition in combination with additional therapies.

Not only are calpains one of many contributors to cell damage, but they also may be activated in regions undetected by traditional analyses. Standard cortical contusion volume measurements delineate the area of damaged tissue receiving the most direct impact from the injury. Due to the direct shearing of cell membranes, immediate calcium dysregulation, and ensuing excitotoxic injury, these cells undergo excitotoxic necrotic cell death (Kochanek et al., 1995), a process a recent study determined to be unaffected by calpain inhibition (D'Orsi et al., 2012). Delayed calpain activation and neuronal protection with calpain inhibition occurred only under conditions of excitotoxic apoptosis characterized by bax-dependency, mitochondrial depolarization, and delayed calcium dysregulation (D'Orsi et al., 2012). In addition, standard contusion volume and cell count measurements are gross assessments of cell viability, unable to discern subtle changes in axonal structure or function that may be mediating functional improvements or preservation after injury. Behavioral outcomes noted with calpain inhibition may indicate spared axonal connections or synaptic structures in regions or circuitry not easily distinguished after CCI injury.

Although calpastatin inhibits both m- and \( \mu \)-calpain, the relative participation of different calpain isoforms in the pathogenesis of trauma and neurodegeneration is largely unknown. Constitutive calpastatin overexpression also prevents manipulation of the temporal activity of calpains following trauma. Due to their role in cytoskeletal reorganization, calpains are reported to be
involved in membrane repair after cell scrape damage (Mellgren et al., 2007) and, therefore, may need to remain active for a period of time to promote membrane resealing after brain injury. Thus, both the isoform- and time-specific activation of calpains may be important factors in determining the optimal therapeutic strategy for calpain inhibition after injury, achieving both neuroprotection and functional improvements. Despite limitations associated with a transgenic mouse model, calpastatin overexpression afforded motor and cognitive recovery after trauma, highlighting calpain as an important therapeutic target and the use of calpastatin as a potential intervention for the amelioration of behavioral morbidity associated with contusion brain injury.
CHAPTER 5

Discussion and Conclusions

REVIEW OF MAJOR FINDINGS

Calcium-activated calpain proteolysis appears to be a key mediator in the pathogenesis of TBI and neurodegenerative disease. Although the use of exogenously administered calpain inhibitors has achieved positive outcomes in excitotoxicity, ischemia, and spinal cord injury, studies that seek to pharmacologically manipulate calpain activity after TBI have produced inconsistent results owing, in part, to differences in the relative selectivity of inhibitors for calpains and their variable pharmacokinetic properties. Calpastatin, the endogenous inhibitor of calpains, is not known to inhibit any other protease, making it ideal for the exclusive targeting of calpains. Therefore, we hypothesized that specific calpain inhibition through calpastatin overexpression would effectively reduce calpain activity, spare protein substrates, and afford neuronal and behavioral protection in mice subjected to contusion brain injury. We confirmed robust calpastatin immunoreactivity in the cortex and hippocampus of two calpastatin overexpressing transgenic mouse lines, identifying higher expression levels with the prion protein promoter (Prp-hCAST) compared to neuronal-specific CaMKIIα promoter (CaMKIIα-hCAST). The hCAST construct in each line was equally effective in suppressing protease activity under exogenous
calpain challenge, which was validated by the attenuation of calpain-specific spectrin breakdown in calpastatin overexpressing mice within the first 24 h after CCI injury. In this respect, we confirmed our hypothesis that specific inhibition of calpain through transgenic calpastatin overexpression provided better protection of substrates compared to post-injury pharmacological calpain inhibitor treatment. We extended our studies on acute calpain-mediated substrate proteolysis to the proteins CRMP-2 and voltage-gated sodium channel, showing reduced breakdown of both substrates in injured Prp-hCAST mice. Although we originally proposed that specific inhibition of calpain by calpastatin would provide promote neuron survival, no overt cortical or hippocampal histological neuroprotection was evident in Prp-hCAST mice after severe injury. However, consistent with our hypothesis, posttraumatic motor and cognitive behavioral deficits were significantly lessened in both CaMKIIα- and Prp-hCAST transgenic mice, providing key support for the therapeutic benefit of calpastatin and calpain inhibition in trauma.

**IMPLICATIONS FOR CELLULAR DAMAGE FOLLOWING TRAUMATIC BRAIN INJURY**

Cellular damage associated with the initial impact and the secondary phase of neurodegeneration is a major facet of TBI (Ray et al., 2002). Due to the shearing of cell membranes, inappropriate channel opening, and excessive release of excitatory neurotransmitters, calcium becomes dysregulated leading to numerous consequences including the activation of calpains (Kampfl et al.,
1997). Calpains cleave a variety of substrates contributing to cellular damage and death (Saatman et al., 2010) despite the endogenous presence of calpastatin, capable of inhibiting four molecules of calpain (Goll et al., 2003). While spectrin breakdown is the most well-characterized calpain substrate after injury, calpain-mediated cleavage of proteins involved in other structural or functional aspects of the neuron could collectively influence neuronal damage. The voltage-gated sodium channel regulates ion flux and membrane depolarization and CRMP-2 has been cited for its involvement in axonal outgrowth as well as membrane protein trafficking. The calpain-specific cleavage of these proteins serves as a marker for calpain activation, but may also indicate the important or preferential cellular mechanisms affected with injury. Although limited knowledge on the relative timeline, substrate contribution, or threshold of calpain-mediated proteolysis necessary to commit a cell to death is available, early calpain activation is evident within injured neurons after trauma (Saatman et al., 1996a, Ringger et al., 2004), suggesting a role for calpains in the process of cell death. In the CCI model of contusion brain injury in rodents, areas of acute neuronal damage progress to a cortical cavity and regional hippocampal cell loss within days post-injury (Hall et al., 2005, Saatman et al., 2006, Pleasant et al., 2011). However, calpain inhibition by pharmacological intervention has been unable to reduce cell death, possibly due to limitations posed by the drugs’ solubility, penetration, and kinetics.

To specifically implicate calpains in mediating cell dysregulation and death, we investigated the effect of increased calpastatin expression to limit
pathological calpain activation and subsequent neuronal death. Calpastatin overexpression in transgenic mice effectively reduced acute posttraumatic substrate proteolysis, supporting numerous studies using pharmacological calpain inhibitors in the CCI trauma model (Posmantur et al., 1997, Thompson et al., 2010, Bains et al., 2012). Although reduction in spectrin or MAP-2 cleavage was not evident with calpain inhibitor treatment in models of FPI (Saatman et al., 2000) or weight-drop injury (Kupina et al., 2001, Haranishi et al., 2005), the inhibitor used, dosage, delivery paradigm, or treatment duration may have impacted the effectiveness of calpain inhibition. Use of calpastatin overexpressing transgenic mice in these injury models may provide substrate protection but, to date, has not been investigated. While the use of calpastatin confirms specific calpain inhibition after TBI, calpastatin overexpression did not afford histological cortical or hippocampal neuroprotection after TBI, suggesting that reductions in calpain-mediated substrate proteolysis are not enough to reduce posttraumatic neurodegeneration. Although we argue that calpastatin insufficiency allows for the prolonged activation of calpains after TBI, increasing the expression level of calpastatin overexpression did not enhance histological outcome. Thus, another factor not tested with our methods may act to perpetuate calcium dysregulation and/or pathological calpain activation following CCI injury in areas most vulnerable to the initial and immediate impact.

One calpastatin molecule can feasibly inhibit four calpains, yet an overabundance of calpastatin in CaMKIIα- and Prp-hCAST transgenic mice failed to protect against cell death after TBI. The regulation of calpains and calpastatin
by calcium concentration, subcellular localization, and oxidation or phosphorylation status collectively influence the activation or inhibition of calpains \textit{in vitro} and under physiological conditions (Salamino et al., 1997, Goll et al., 2003, Maddock et al., 2005); however, relatively little is known about the biochemical nature or relative localization of calpains and calpastatin after TBI that, in addition to sustained free calcium levels, can result in prolonged calpain activation. For instance, calpains’ catalytic residues are susceptible to oxidation, which resulted in decreased \textit{in vitro} activity of \(\mu\)-calpain (Maddock et al., 2005). Conversely, oxidative conditions and phosphorylation of calpastatin by protein kinases were shown to decrease the ability of calpastatin to inhibit calpains (Salamino et al., 1997, Averna et al., 2001, Maddock et al., 2005). While oxidative stress and alterations in kinase expression have been identified in TBI (Yang et al., 1993, Padmaperuma et al., 1996, Lewen et al., 2000, Raghupathi et al., 2003, Atkins et al., 2007), no studies to our knowledge have investigated redox state or phosphorylation status of calpains and calpastatin after brain injury. Based on these possibilities, more information on the biochemical properties of calpain and calpastatin under pathological insults is necessary to completely understand calpain-calpastatin regulation after TBI.

The subcellular localization of calpains and calpastatin has been considered a mechanism to regulate calpain activation under low, physiological intracellular free calcium concentrations. Accordingly, studies have identified hydrophobic or basic amino acid residues within calpain domains III and V (Tompa et al., 2001, Gil-Parrado et al., 2003b) that may allow for redistribution to
the membrane to facilitate proteolytic activation (Kuboki et al., 1987, Goll et al., 2003) or in association with specific subcellular organelles (Mellgren and Lu, 1994, Hood et al., 2003, Garcia et al., 2005). The different calpastatin isoforms that result from distinct promoters or alternative splicing may also affect the localization of each isoform in relation to calpains. The L domain of calpastatin, in particular, contains sequences that function in membrane binding (Mellgren et al., 1989) while additional N-terminal regions are phosphorylation sites that can induce aggregation (Averna et al., 2001) or increased membrane association (Adachi et al., 1991). Although changes in protease-inhibitor localization seem plausible in vitro or under physiological conditions to regulate calpain activity, these mechanisms have not been confirmed in neurons following pathological insult. Under conditions such as TBI, changes in the localization, aggregation or compartmentalization of calpains and calpastatin may preclude appropriate or potent calpain inhibition, resulting in continued cellular damage.

Despite possible biochemical alterations or subcellular location differences between calpains and calpastatin, calpastatin overexpression in our studies was able to specifically inhibit calpains based on in vitro assay and proteolysis data. However, calpains are one of many known contributors to cell damage after TBI. Calcium dysregulation notably triggers additional neurodegenerative mechanisms including mitochondrial dysfunction, oxidative stress, and lipid peroxidation and associated membrane disruption (Ray et al., 2002), creating a complex and multifactorial situation such that inhibition of calpains alone may not fully protect the neuron after injury. In addition, non-calcium-mediated injury
cascades such as blood-brain barrier breakdown, caspase activation, vascular leakage, and inflammation can promote neuronal damage and death (Lenzlinger et al., 2001, Ray et al., 2002, Shlosberg et al., 2010). Recent preclinical strategies that target other acute mediators of neurodegeneration including plasmalemma permeability (Mbye et al., 2012), mitochondrial dysfunction (Sullivan et al., 2000, Mbye et al., 2009, Readnower et al., 2011), oxidative stress (Zhang et al., 2012, Singh et al., 2013), and inflammation (Yi et al., 2008, Sauerbeck et al., 2011, Atkins et al., 2013) have been effective in reducing cell death or lesion size after CCI injury. Therefore, a combination of therapies targeting calpains and other secondary mechanisms may yield a superior neuroprotective outcome with both histological and functional improvements.

Finally, the relative contribution of apoptotic or necrotic cell death pathways can vary depending on the severity, duration, or mechanism of injury and aspects of both processes can occur simultaneously within the same brain region or single neuron after TBI (Raghupathi, 2004, Stoica and Faden, 2010). This heterogeneity of cell death phenotypes and participation of different intracellular executioners complicates attempts to target specific mediators, like calpains, to reduce overall posttraumatic cell death. Based on a recent in vitro study, calpain activation was not detected in neurons undergoing NMDA-induced excitotoxic necrosis characterized by immediate, irreversible calcium dysregulation and sustained depolarization of the mitochondrial membrane potential (D’Orsi et al., 2012). Calpain inhibition was more effective in achieving neuroprotection under conditions of excitotoxic apoptosis in which neurons
experienced delayed mitochondrial depolarization and subsequent calcium
dysregulation prior to calpain activation (D'Orsi et al., 2012). Perhaps the level of
calcium dysregulation and degree of cellular injury experienced by neurons within
the primary contusion site is too rapid and irreversible to respond to intervention
by calpain inhibition alone and instead, adjacent dysfunctional neurons with
delayed calcium alterations are more receptive to specific blockade of calpains
after TBI. Standard cortical contusion volume and cell death measurements that
quantify the absence of dead neurons or presence of surviving neurons cannot
distinguish cell death mechanisms and, therefore, may not be the best tool for
assessing cell death modulation by calpain inhibition. More sensitive markers of
apoptotic or necrotic pathways or the state of calcium dysregulation after brain
injury may help to delineate neurons or brain regions in which calpains are
activated in order to better target calpastatin overexpression.

IMPLICATIONS FOR BEHAVIORAL FUNCTION FOLLOWING TRAUMATIC
BRAIN INJURY

Pathological calpain activation mediates cellular substrate damage,
contributing to cell death and behavioral impairments. Although calpastatin
overexpression was unable to spare neurons after TBI, multiple motor and
cognitive assessments revealed reduced behavioral deficits in transgenic mice
following contusion brain injury. These data indicate that the pathological
activation of calpains appears to alter processes or regions involved in
posttraumatic behavioral deficits, which can be prevented through inhibition by
calpastatin. Our results are consistent with studies using pharmacological calpain inhibitors demonstrating reductions in motor and cognitive deficits in diffuse models of TBI (Saatman et al., 2000, Kupina et al., 2001). Furthermore, our results demonstrate that functional benefits do not require overt cortical or hippocampal neuroprotection, a surprising disconnection that has been reproduced in both rats and mice with calpain inhibition by pharmacological inhibitors and calpastatin overexpression. Instead, improved functional outcomes may represent spared axonal or synaptic structure and function in areas adjacent to the contused region.

Evidence from studies on axonal injury supports a role for calpains in mediating damage to axonal structure and function that can be diminished with pharmacological or viral-mediated calpain inhibition. Pretreatment with MDL-28170 decreased immunohistochemical indications of structural damage (Buki et al., 2003) and preserved axolemmal integrity (Czeiter et al., 2009) following impact acceleration injury. Calpain inhibition also reduced ischemia-induced cleavage of cytoskeleton proteins within the axon initial segment, thereby preserving ion channel clustering (Schafer et al., 2009), which may contribute to the maintenance of action potential propagation and neuronal polarity. Although treatment with the calpain inhibitor MDL-28170 did not affect stretch-induced axonal transport disruption after in vivo optic nerve stretch injury (Ma et al., 2012a), axonal transport function was partially protected with viral-mediated calpastatin overexpression (Ma et al., 2012b), suggesting that long-term or more specific calpain inhibition is required for improved functional outcome. Calpain
inhibition reduced cytoskeleton breakdown but was ineffective at preventing
electrophysiological dysfunction after optic nerve anoxia (Jiang and Stys, 2000)
or electromyography after sciatic nerve transection (Ma et al., 2013), perhaps
highlighting additional injury mechanisms that occur simultaneous to calpain
activation. Subtle changes in axonal structure or function that may be mediating
functional preservation or improvement after brain injury may not be easily
assessed by the injury model or histological measurements used in our studies.
Calpain inhibition may be more effective in diffuse or mild TBI in which axonal
pathology greatly contributes to the overall tissue damage and functional deficits;
therefore, models such as midline FPI or impact acceleration injury models could
be used to test the hypothesis that calpastatin overexpression ameliorates
calpain-induced axonal damage and dysfunction. Additional assessments
including electrophysiology, axonal and synaptic labeling, or other measure of
axonal integrity may help elucidate the mechanisms by which calpain inhibition
affords behavioral efficacy without neuronal protection.

LIMITATIONS AND ALTERNATIVE INTERPRETATIONS

Calpastatin overexpression via genetic manipulation effectively enhanced
endogenous inhibitory mechanisms, achieving robust calpain inhibition. However,
the absence of neuroprotection despite behavioral efficacy is puzzling. In addition
to our speculations above, several caveats and limitations associated with using
transgenic mouse models must also be considered. Along with these
considerations, alternative interpretations regarding calpain function and sublethal cellular injury are discussed.

Constitutive calpastatin overexpression may result in unintentional and abnormal compensatory responses associated with developmental calpain inhibition that could interfere with posttraumatic outcomes and interpretations. Although no compensatory changes in caspase-3 expression or caspase-mediated spectrin breakdown were observed in Prp-hCAST transgenic mice, we did not investigate upregulation of other caspase family members or alternative proteolytic enzymes such as cathepsins that are increased or activated after TBI (Knoblach et al., 2002, Larner et al., 2004, Ringger et al., 2004, Larner et al., 2005, Luo et al., 2010). Although evidence suggests that calpains function in LTP and memory formation (del Cerro et al., 1990, Denny et al., 1990, Suzuki et al., 1992, Muller et al., 1995, Farkas et al., 2004, Amini et al., 2013, Zadran et al., 2013), CaMKIIα-driven calpastatin overexpression did not affect normal cognitive function in naïve or uninjured transgenic mice using a hippocampal-dependent task (Schoch et al., 2012). However, we did note a slight learning impairment in uninjured Prp-hCAST mice compared to WT controls, suggesting a delay in cognitive function or plasticity with increased and more widespread calpastatin overexpression. Without additional cognitive testing or electrophysiological data to confirm possible LTP abnormalities in these transgenic mice, cognitive data obtained from calpastatin overexpressing mice must be interpreted with caution. Conditional models of calpastatin overexpression that may lessen or eliminate
problems associated with developmental and constitutive genetic manipulation should be investigated.

A major advantage to transgenic calpastatin overexpression is its specific and sustained inhibition of calpains, affording inhibition of both $\mu$- and m-calpains during the entirety of traumatic insult. However, these characteristics notably limit interpretation of the temporal and isoform-specific role of calpains. Genetic calpain knockout and knockdown strategies have suggested distinct pathological roles for each calpain isoform in mediating ischemic and posttraumatic cell death (Bevers et al., 2009, Bevers et al., 2010, Yamada et al., 2012, Yu et al., 2012). Based on these studies, $\mu$- and m-calpains may function independently or exert different functions, which can only be delineated by selective isoform targeting. Atypical calpain isoforms, including calpains 5, 7, and 10, also have ubiquitous tissue distributions but lack EF-hand domains predicted for calcium-binding (Sorimachi et al., 2011) and, thus, may contribute to proteolytic damage after injury in a manner dissimilar to $\mu$- and m-calpains; however, their precise regulation by calpastatin and role in trauma have not been previously investigated. In addition, calpastatin overexpression could compromise calpains’ physiological or reparative functions following injury. For example, in vitro studies have cited a function for calpains in proteolyzing substrates responsible for membrane repair after injury (Howard et al., 1999, Mellgren et al., 2009); therefore, sustained calpastatin overexpression during TBI may actually prolong membrane permeability after injury, resulting in greater cell death in the contused region. Calpains also participate in the recovery of dendritic structure following
sublethal NMDA-induced excitotoxicity (Faddis et al., 1997) perhaps resulting in sustained injury if calpains are inhibited. Temporal changes in calpain inhibition could verify such a function in TBI, perhaps necessitating early calpain activation to seal neuronal membranes and remodel dendrites but subsequent calpain inhibition to prevent functional impairments.

Finally, CCI injury reliably replicates focal, contusion injuries with aspects of excitotoxicity, ischemia, hypoxia, and traumatic axonal injury (Lighthall et al., 1990, Palmer et al., 1993, Kochanek et al., 1995, Clark et al., 1997). Yet, given the functional benefits of calpastatin overexpression in transgenic mice, axonal stretch or shearing injury may be more susceptible to calpain activation and thus modulation by calpastatin, but these pathologies are difficult to assess with direct contusion and tearing of axons in a CCI injury model. Investigations using alternative injury models such as FPI or axonal stretch can help to verify calpain-mediated pathology and may reveal a mechanism for spared behavioral function with calpastatin overexpression.

Therapeutic strategies for traumatic insults, including contusion brain injury, often seek to reduce cell death and behavioral dysfunction following injury. Our studies clearly demonstrate improved posttraumatic behavioral performance with calpastatin overexpression but without histological neuroprotection within the primary contusion site. Although we have historically sought a neuroprotective outcome with calpain inhibition, perhaps our focus should be shifted to surrounding areas or other cell types to identify the source of improved behavioral function after injury. Transient or sublethal neuronal dysfunction has
been identified using electrophysiological techniques (Bain et al., 2001, Reeves et al., 2005). Calpain activation may contribute to the initial functional deficits and calpastatin overexpression, therefore, may improve recovery or prevent permanent cellular damage. In accordance with this supposition, spared proteolysis of the voltage-gated sodium channel may promote the recovery of action potential generation and, in turn, lead to improved recovery of normal behavioral function.

Outcomes to assess calpain activation and its consequences have concentrated on neuronal populations; however, injury-induced alterations to subpopulations of neurons and glial cells could influence our interpretations on overall neuronal death and potential histological neuroprotection with calpain inhibition. Staining with Fluoro-jade, Nissl, or NeuN fails to distinguish between neuronal subpopulations (i.e. excitatory or inhibitory neurons) that could be differentially affected by injury or calpastatin overexpression. In addition, glial cell activation in response to neuronal death may be modulated by calpastatin overexpression, thereby meditating an improved behavioral outcome.

**FUTURE DIRECTIONS**

The mechanism by which calpastatin overexpression is able to spare posttraumatic behavioral function without overt neuroprotection in our model is still unknown. Neither the specificity of calpastatin toward calpains nor the abundance of calpastatin appears to be a key determinant in enhancing histological outcome, since data obtained from two separate transgenic models
are consistent with administration of several less selective pharmacological calpain inhibitors. Future studies should more closely identify electrophysiological properties or axonal and synaptic markers that could pinpoint calpain activation in mediating behavioral dysfunction. Methods like these may necessitate alternative injury models that more specifically involve axonal injury or that produce diffuse axonal pathology. Differences in these models compared to CCI may also involve different biochemical and cell death mechanisms to further indicate a role for calpains in mediating damage in some, but not all, types of pathologies.

In order to avoid limitations in compensatory protein responses or possible developmental abnormalities associated with constitutive calpastatin overexpression, alternative manipulation strategies should be investigated as well. Conditional calpain isoform knockouts may reveal isoform-specific roles after TBI while a conditional, dual calpain knockout strategy can confirm calpastatin inhibition of both isoforms achieved with calpastatin overexpression transgenic mice. Strategies that can manipulate the temporal expression of calpastatin, such as viral-mediated calpastatin delivery or calpastatin mimetic administration, can be used to compare to results obtained with manipulation of one or both calpain isoforms. Not only will these future directions promote enhanced understanding of calpains and calpastatin after injury, but also may lead to modified therapies to achieve both neuroprotection and behavioral improvement after injury.
CALPASTATIN OVEREXPRESSSION AS A THERAPEUTIC APPROACH

While studies of pharmacological inhibitors that lack absolute specificity for calpains complicate interpretation of pathological outcomes after TBI, calpastatin overexpression in transgenic mice has produced little doubt that calpains mediate cellular damage after traumatic insult. Furthermore, the ability of calpastatin to specifically inhibit calpains resulted in reduced posttraumatic protein breakdown and behavioral deficits, confirming the therapeutic benefit for calpain inhibition after TBI. Behavioral protection seen with calpastatin overexpression was not coincident with neuroprotection, implying that calpains are mediating damage that affects surviving but dysfunctional neurons, synaptic connections, or axonal function; yet, more information is necessary to confirm these mechanisms. Overall, our results confirm the role of calpain in mediating neuronal dysfunction after trauma, supporting the need for specific inhibitors of calpain in the treatment of TBI. However, calpain inhibition alone is insufficient to fully protect neurons from death, suggesting that combinatorial treatments may be more beneficial to provide neuroprotection and reduce behavioral deficits after TBI. Noting these considerations, calpastatin overexpression is a viable therapeutic approach toward the treatment of TBI and other neurodegenerative diseases.

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**PRESENTATION OF RESEARCH**

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- 30th Annual National Neurotrauma Symposium 2012, Phoenix AZ


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Poster presentation at:
29th Annual National Neurotrauma Symposium, 2011, Hollywood Beach, FL
17th Annual Kentucky Spinal Cord and Head Injury Research Symposium 2011, Louisville, KY
The Biology of Calpains in Health and Disease 2010, Carefree, AZ


Schoch, K.M., G.C. Telling, K.E. Saatman, "Calpastatin overexpression under the prion promoter reduces acute spectrin proteolysis following traumatic brain injury.”
Poster presentation at:
39th Annual Meeting of the Society for Neuroscience, 2009, Chicago, IL
15th Annual Kentucky Spinal Cord and Head Injury Research Symposium 2009, Louisville, KY
Bluegrass Chapter of the Society for Neuroscience 2010, Lexington, KY

Schoch, K.M., G.C. Telling, K.E. Saatman, “Reduced posttraumatic spectrin proteolysis in mice overexpressing calpastatin under the prion promoter.”
Poster presentation at:
2nd Joint Symposium of the International and National Neurotrauma Societies, 2009, Santa Barbara, CA
4th Annual Center for Clinical and Translational Science Spring Conference 2009, Lexington, KY
Physical Medicine and Rehabilitation Research Day 2009, Lexington, KY

Poster presentation at:
26th Annual National Neurotrauma Symposium, 2008, Orlando, FL
Bluegrass Chapter of the Society for Neuroscience 2008, Lexington, KY
14th Annual Kentucky Spinal Cord and Head Injury Research Symposium 2008, Lexington, KY