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Calpastatin overexpression limits calpain-mediated proteolysis and behavioral deficits following traumatic brain injury

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Abstract

Traumatic brain injury (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. 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. In this study, increased 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. Naïve hCAST transgenic mice exhibited enhanced neuronal calpastatin expression and significantly reduced protease activity. Acute calpain-mediated spectrin proteolysis in the cortex and hippocampus induced by controlled cortical impact brain injury was significantly attenuated in calpastatin overexpressing mice. Aspects of posttraumatic motor and cognitive behavioral deficits were also lessened in hCAST transgenic mice compared to their wildtype littermates. However, volumetric analyses of

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neocortical contusion revealed no histological neuroprotection at either acute or long-term time points. Partial hippocampal neuroprotection observed at a moderate injury severity was lost after severe TBI. 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. In addition, the reduction in spectrin proteolysis without accompanied neocortical neuroprotection suggests the involvement of other factors that are critical for neuronal survival after contusion brain injury.

Keywords

cognition; controlled cortical impact; neuroprotection; spectrin; transgenic

INTRODUCTION

Traumatic brain injury (TBI) afflicts approximately 1.5 million individuals each year in the United States, resulting in 50,000 deaths, 235,000 hospitalizations, and 1.1 million emergency room visits (Summers et al., 2009). Efforts to reduce its high incidence are primarily preventative, and currently no pharmacological treatment has been proven to lessen the often lifelong disabilities associated with brain injury. TBI is characterized by an initial mechanical insult to the head that can result in varying amounts of damage to the brain tissue and the disruption of cellular membranes (LaPlaca et al., 2007). This primary phase is followed by a secondary, evolving neurodegenerative phase characterized by a myriad of pathogenic events including ischemia, alterations in ionic homeostasis, oxidative damage, inflammation, and the excessive release of excitatory neurotransmitters (Ray et al., 2002, LaPlaca et al., 2007). Studies using experimental models of contusion brain injury, one of the most common types of TBI, have established multiple pathways through which calcium homeostasis is dysregulated, including the excessive release of glutamate and overactivation of NMDA and AMPA receptors, widespread depolarization, voltage-gated calcium channel opening, or membrane disruption (Faden et al., 1989, Palmer et al., 1993, Arundine and Tymianski, 2003, Weber, 2004). Increases in intracellular free calcium result in altered signal transduction and the activation of calcium-dependent proteases, or calpains, that contribute to the secondary injury cascade (Palmer et al., 1993, Arundine and Tymianski, 2003, Sun et al., 2008).

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). The heterodimeric structure of calpain is composed of an 80kDa catalytic subunit and a 28kDa regulatory subunit. Calpains have been detected in all vertebrate cell types tested for their presence, with 15 isoforms of calpain identified to date, and are involved in physiological functions such as cytoskeletal remodeling, cell differentiation, cell cycle regulation, apoptosis, and long-term potentiation (Goll et al., 2003, Sorimachi et al., 2011).

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). Although no neuron-specific calpains have been identified, the highly studied ubiquitous calpains, μ - and m -calpain, have been implicated in neuronal degeneration in TBI. In rodents, posttraumatic μ -calpain autolysis precedes the accumulation of calpain-mediated α -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, Hall et al., 2005a). 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).

The endogenous, specific inhibitor of calpains, 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). Structurally, calpastatin is composed of an N-terminal leader domain followed by four inhibitory domains, each with the ability to inhibit one molecule of calpain (Goll et al., 2003, Hanna et al., 2008). This basic inhibitory structure is consistent across the several known calpastatin isoforms that result from different promoters or alternative splicing (Takano et al., 1999, Takano et al., 2000, Parr et al., 2004). 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.

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 promoter.

MATERIALS AND METHODS

Experimental Animals

Transgenic mice expressing human calpastatin (hCAST) under control of the neuron-specific calcium/calmodulin-dependent kinase II α subunit 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 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 (Institute of Laboratory Animal Resources (U.S.). Committee on Care and Use of Laboratory Animals.).

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 hours 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 minutes 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, 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 15,000 rpm for 20 minutes 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 minutes. Proteases, including calpains, present in the sample cleave the casein substrate into fluorescent cleavage products. Sample fluorescence was measured using a spectrafluorometer (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 calpain/mg of protein. All samples were run in duplicate or triplicate and the average fluorescence reading was used for analysis.

Controlled cortical impact (CCI)

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., 2005b), 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% oxygen) and subsequently maintained on 2.5% isoflurane (3.0% 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 2.5 mm diameter, rounded, steel impactor tip. Impact velocity and dwell time were set at 3.5 m/s and 500 msec, 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 14,000 rpm for 20 minutes at 4 °C and supernatant fractions were collected for protein concentration determination (Pierce BCA protein assay kit). Cortical and hippocampal samples (5 g) 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 Immunochemicals, 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 μ m 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 ipsilateral 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 μ m) were initially exposed to DAB for 5 minutes to react with endogenous peroxidases, eliminating nonspecific fluorescence due to hemorrhage. Slides were warmed at 40–45°C for 30 minutes and dried overnight at room temperature. Following rehydration in 1% NaOH and alcohol gradient and 0.06% potassium permanganate treatment for 10 minutes, tissue was stained with 0.01% Fluoro-jade B (Millipore Co.) in 0.1% acetic acid and heat-dried at 50°C for 30 minutes. Slides were immersed in xylenes 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 seconds, they were placed on the platform for 10 seconds after the first trial and 5 seconds 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 60s 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 ($68.6\text{ cm} \times 62.2\text{ cm}$) 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 minutes. 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 minutes. 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

minutes and, after a 4 h 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 seconds to the beams and rod 24 h prior to CCI injury. Upon post-injury testing, mice were allowed up to 30 seconds 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 deducted 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 2-way ANOVA (genotype × calpain). Levels of 150kDa and 145kDa spectrin breakdown products in the cortex and hippocampus were separately analyzed using a t-test (0.5mm injury) or a nested 2-way ANOVA (1.0mm injury, genotype × 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 2-way ANOVA (genotype × injury condition). Morris water maze learning data, neurological severity score, and novel object recognition tasks were analyzed using a repeated measures 1-way ANOVA. Neuroscore data was analyzed as a repeated measures 2-way ANOVA (genotype × 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 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 1A, C). In contrast, robust labeling of neurons in all cortical layers and hippocampal regions was evident in hCAST Tg mouse brains (Figure 1B, D). Within the neocortical layers, calpastatin expression was highest in layers II, III and V, appearing primarily within the neuronal cytoplasm (Figure 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 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 hCAST transgenic mice, with no detectable levels in WT littermates (n=4/genotype; Figure 1E).

In vitro calpastatin inhibitory activity

To verify the functionality of human calpastatin in 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 ($n=4-5/\text{genotype}$; Figure 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 hCAST Tg mice ($p<0.01$) (Figure 2A), calpastatin overexpression completely prevented increased protease activity in hippocampal homogenates (Figure 2B), consistent with a high expression level of calpastatin in the hippocampus of the hCAST Tg mice (Higuchi et al., 2005).

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 (Kampfll et al., 1996, Hall et al., 2005b). Levels of spectrin breakdown are undetectable or very low in naïve or sham animals (Saatman et al., 1996a, Hall et al., 2005b, 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 hCAST Tg compared to WT mice ($p<0.05$; $n=3-4/\text{genotype}$) (Figure 3A). The 145 kDa calpain-specific BDP also appeared to be reduced in hippocampal homogenates of 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 (145kDa) 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; $n=3-6/\text{time point}$) (Figure 3B). In severely injured hCAST Tg mice, cortical spectrin cleavage to the 145kDa 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 ($p<0.0005$; $n=4-6/\text{time point}$) (Figure 3C). Human calpastatin overexpression in Tg mice prevented injury-induced elevation of the 145kDa 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 150kDa spectrin BDP at both time points in the cortex and hippocampus (Figure 3B, C). However, for either moderate or severe CCI, 150kDa spectrin BDP levels were not significantly different for WT and 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 hCAST Tg mice after injury.

Assessment of acute motor function

To examine whether the inhibition of calpain-mediated spectrin proteolysis in 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 hCAST Tg mice compared to their respective sham controls (n=3/genotype; $p<0.05$) (Figure 4). Calpastatin overexpression significantly attenuated acute motor dysfunction after either moderate or severe injury ($p<0.005$ compared to WT brain-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 0.5mm or 1.0mm CCI injury, although severe CCI resulted in more extensive cortical neuron loss, with well-developed neocortical cavitation (Figure 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 5C insets). Quantitative analyses revealed a significantly larger volume of neocortical tissue damage after severe CCI compared to moderate CCI ($p<0.0005$) (Figure 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 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 24h 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 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 6C). However, no significant genotypic differences in FJB+ cell numbers were identified at a 1.0mm depth injury (Figure 6F).

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 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 (1.0mm) 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 days ($p<0.0001$, time effect) with an equivalent pattern of visuospatial learning for WT (n=29) and hCAST Tg (n= 25) mice (Figure 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 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 days post-injury ($p<0.0005$, injury effect; Figure 7C). Due to spontaneous recovery of function over the first week, only a small, but non-significant motor deficit remained at 8d ($p=0.06$, injury effect). Although neuroscores of brain-injured hCAST Tg mice were slightly higher than WT mice at 2 and 4d after injury, no significant genotype-dependent differences were detected by 2-way ANOVA (Figure 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 hCAST Tg and WT mice demonstrated an equivalent preference for the novel object (Figure 8A). After severe CCI injury (n=10–11/genotype), WT mice exhibited profound deficits in the ability to recognize the novel object at 7d ($p<0.05$ compared to pre-injury) which persisted to 14d ($p<0.01$) (Figure 8A). Calpastatin overexpression effectively prevented trauma-induced memory impairment in the NOR task. The performance of hCAST Tg mice after injury was equivalent to their pre-injury performance and significantly greater than WT littermates at both 7d and 14d post-injury ($p<0.01$) (Figure 8A). The modified NSS revealed acute motor deficits that, in both genotypes, lessened over time ($p<0.0001$, time effect; Figure 8B). However, hCAST Tg mice showed significantly improved NSS across the 7d post-injury period ($p<0.01$, genotype effect; Figure 8B). Due to spontaneous recovery of motor function, mice were not evaluated beyond 7d.

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 7d 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 protection acutely, we measured contusion volume in hCAST and WT mice 8d following severe (1.2 mm) CCI injury. The volume of the ipsilateral neocortex damaged in hCAST mice ($15.7 \pm 1.1\%$) was comparable to that in WT mice ($15.5 \pm 0.9\%$; n=12–13/genotype). The volume of neocortical contusion was similar to that observed at 24 h following severe CCI (Figure 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 colocalized 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 calcium-calmodulin-dependent kinase II α (CaMKII α) 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). 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., 2005b, 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 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, 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 inhibit 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 colocalized with other cell death markers, including those specific for apoptosis, in other injury paradigms (Oshitari et al., 2008, 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 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 are currently investigating a transgenic mouse line with a 9-fold increase in calpastatin expression.

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 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, which may have enhanced sensitivity for detecting effects of calpain inhibition. In our cognitive tests, the NOR test revealed a robust protection against posttraumatic memory dysfunction in 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. 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 address structural or functional axonal protection mediated by calpastatin overexpression.

The specific and endogenous nature of calpastatin makes it appealing for therapeutic use, in contrast to current pharmacological approaches for targeting calpains which often lack selectively, solubility, or potency. Although the action of other interventions is likely necessary to substantially reduce cell death, calpain inhibitors or calpastatin mimetics show promise in reducing the proteolysis of substrates and improving functional outcome after TBI. Investigations on endogenous protective systems will provide greater insight into the pathological responses of neurons after injury as well as provide a basis for the development of therapeutic treatments.

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Highlights

hCAST overexpression in transgenic mice effectively reduced protease activity.
Brain injury-induced spectrin proteolysis was blunted in hCAST transgenic mice.
hCAST overexpression partially protected the hippocampus but not cortex after TBI.
Motor function was improved in brain-injured mice overexpressing hCAST.
In mice with TBI, object recognition memory was improved by hCAST overexpression.

Figure 1.

Calpastatin expression in the cortex and hippocampus of wildtype (WT) and hCAST transgenic (Tg) mice. Immunohistochemical labeling using an antibody recognizing mouse and human calpastatin in the cortex (A, B) and hippocampus (C, D) of wildtype (A, C) and hCAST Tg (B, D) mice. Neocortical layers (II–V) are indicated. CAST immunoreactivity appears primarily neuronal with increased expression in hCAST Tg mice (B, inset) compared to WT mice (A, inset). Scale bars represent 100 μ m for images A–D; 20 μ m for insets. E) Immunoblot for human calpastatin in cortical and hippocampal homogenates of WT and hCAST Tg mice.

Figure 2.

Calpastatin inhibitory activity (fluorescence units/mg of protein) in cortical (A) and hippocampal (B) homogenates obtained from naïve (uninjured) wildtype (WT) and hCAST transgenic (Tg) mice. Protease activity was measured without addition of calpain and in response to addition of exogenous calpain. Data represented as mean + standard error; * $p < 0.01$, ** $p < 0.0005$ compared to homogenates without exogenous calpain; # $p < 0.0005$ compared to WT + exogenous calpain.

Figure 3.

α -Spectrin proteolysis following traumatic brain injury in wildtype (WT) and hCAST transgenic (Tg) mice. Calpains cleave α -spectrin (280kDa) into 150 and 145kDa fragments, the latter resulting exclusively from proteolysis by calpains. A) Spectrin breakdown in cortical and hippocampal homogenates obtained from WT and hCAST Tg mice at 4 h following 0.5mm depth CCI injury. Spectrin cleavage was also measured in B) cortical homogenates and C) hippocampal homogenates from WT and hCAST Tg mice at 6 and 24h after 1.0mm depth CCI injury. Data are represented as the proteolytic fragment band normalized to intact spectrin (measured as optical density units) and expressed as mean + standard error; * $p < 0.05$, ** $p < 0.0005$ compared to sham; # $p < 0.05$, ## $p < 0.0005$ compared to WT. Representative blots are shown at right.

Figure 4.

Neuroscore assessment of posttraumatic motor function 24 h following moderate (0.5mm) and severe (1.0mm) controlled cortical impact injury in wildtype (WT) and hCAST transgenic (Tg) mice. Both moderate and severe CCI brain injury resulted in significant acute motor deficits. Calpastatin overexpression significantly improved motor function in moderate and severe brain-injured groups. Neuroscores are expressed as mean + standard error; * $p < 0.05$, ** $p < 0.0005$ compared to respective sham-injured controls; # $p < 0.005$ compared to WT injured mice.

Figure 5.

Histological damage at 24 h after controlled cortical impact (CCI) brain injury. Compared to moderate (0.5mm) CCI (A, B), severe (1.0mm) CCI resulted in a larger contusion and more extensive damage to the subcortical white matter. 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 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 μ m; 20 μ m for insets. F) Quantification of tissue damage in WT and hCAST Tg mice following moderate and severe CCI brain injury. Although contusion volume increased significantly with increasing depth of impact (*p<0.0005), no genotypic differences were revealed between hCAST Tg and WT mice. Tissue damage is calculated as a percent of contralateral neocortex and expressed as mean + standard error.

Figure 6.

Fluoro-jade B staining in the dentate gyrus of the hippocampus 24h following moderate (0.5 mm) (A, B) and severe (1.0mm) (C, D) CCI in wildtype (WT) (A, D) and hCAST transgenic (Tg) (B, E) mice. Fluoro-jade B-positive (FJB+) neurons were evident in the hilus and granular layer of the dentate gyrus, which were more numerous following 1.0mm injury compared to 0.5mm injury. Calpastatin overexpression resulted in reduced FJB+ cell in the dentate gyrus compared to WT after moderate injury, but not after severe injury. Scale bar represents 100 μ m. Quantification of FJB+ neurons in hippocampal regions of WT and hCAST Tg mice at C) moderate and F) severe CCI. The number of FJB+ neurons was averaged across three sections per brain and expressed as mean + standard error; * $p < 0.005$ compared to WT.

Figure 7.

Morris water maze and neuroscore testing following severe controlled cortical impact (CCI) brain injury in wildtype (WT) and hCAST transgenic (Tg) mice. A) Prior to injury, WT and hCAST Tg mice show an equivalent ability to learn the location of the hidden platform on successive training days. B) Although CCI brain injury resulted in significant memory impairment (* $p < 0.0001$ compared to sham), no significant genotype-dependent differences were detected. C) Brain injury produced significant impairment of motor function assessed by use of the composite neuroscore at 2 d and 4 d after severe CCI (* $p < 0.0005$ compared to sham). No significant genotype-dependent differences were observed at any time point. All data are expressed as mean + standard error.

Figure 8.

Novel object recognition and modified neurological severity score (NSS) testing of wildtype (WT) and hCAST transgenic (Tg) mice after severe brain injury. A) Recognition ability of WT mice declined at 7 and 14d after severe brain injury (* $p < 0.05$ compared to pre-injury). In contrast, injured hCAST Tg mice maintained their pre-injury ability to distinguish the novel object, resulting in significantly higher scores compared to WT mice (# $p < 0.01$). Recognition index is defined as the percent interaction time spent on the novel object. B) Across the 7d post-injury period, hCAST Tg mice showed significantly improved NSS compared to WT mice (* $p < 0.01$). All data are expressed as mean + standard error.