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Penetrating Traumatic Brain Injury Triggers Dysregulation of Cathepsin B Protein Levels Independent of Cysteine Protease Activity in Brain and Cerebral Spinal Fluid

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Abstract

Cathepsin B (CatB), a lysosomal cysteine protease, is important to brain function and may have dual utility as a peripheral biomarker of moderate-severe traumatic brain injury (TBI). The present study determined levels of pro- and mature (mat) CatB protein as well as cysteine protease activity within the frontal cortex (FC; proximal injury site), hippocampus (HC; distal injury site), and cerebral spinal fluid (CSF) collected 1–7 days after craniotomy and penetrating ballistic-like brain injury (PBBI) in rats. Values were compared with naïve controls. Further, the utility of CatB protein as a translational biomarker was determined in CSF derived from patients with severe TBI. Craniotomy increased matCatB levels in the FC and HC, and led to elevation of HC activity at day 7. PBBI caused an even greater elevation in matCatB within the FC and HC within 3–7 days. After PBBI, cysteine protease activity peaked at 3 days in the FC and was elevated at 1 day and 7 days, but not 3 days, in the HC. In rat CSF, proCatB, matCatB, and cysteine protease activity peaked at 3 days after craniotomy and PBBI. Addition of CA-074, a CatB-specific inhibitor, confirmed that protease activity was due to active matCatB in rat brain tissues and CSF at all time-points. In patients, CatB protein was detectable from 6 h through 10 days after TBI. Notably, CatB levels were significantly higher in CSF collected within 3 days after TBI compared with non-TBI controls. Collectively, this work indicates that CatB and its cysteine protease activity may serve as collective molecular signatures of TBI progression that differentially vary within both proximal and distal brain regions. CatB and its protease activity may have utility as a surrogate, translational biomarker of acute-subacute TBI.

Keywords: biomarkers; cathepsin B; clinical TBI; cysteine protease; head trauma; penetrating ballistic-like brain injury; translational rodent models; traumatic brain injury

Introduction

Traumatic brain injury (TBI) represents a significant health concern. It is a major cause of mortality, long-term hospitalization, and disability, as well as an increased risk of neurodegenerative or psychological disease diagnosis.[1] Moderate to severe TBI often occurs as a consequence of automotive accidents or falls, as well as from acts of violence emanating from domestic abuse, blunt force trauma, or gunshot wounds. Military personnel are at a particularly high risk for attaining a TBI due to use of improvised explosive devices (IEDs), which are common to recent conflicts.[2–4]

Severe TBI results in an open skull wound that breaches the cranium and meninges/dura mater, resulting in immediate hemorrhage and loss of brain tissue.[5,6] Acute damage caused by TBI leads to a secondary injury cascade characterized by inflammation and neurodegeneration associated with an increased risk of dementia.[7] A key mechanism of TBI progression during this time frame involves dysregulation of cathepsin B (CatB), a lysosomal cysteine

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protease. CatB is highly expressed in neuronal and glial populations within cortical and hippocampal regions of the mammalian brain and is involved in neuronal development.[8] CatB is a key component of the lysosomal-autophagy system involved in turnover of intracellular proteins. In healthy cells, CatB aids in basal autophagy and metabolic homeostasis, antigen processing and presentation during immune responses, degradation of misfolded proteins, in addition to cleavage of hormones and activation of other proteases.[9–11] Under pathological conditions, unchecked CatB upregulation and protease activity are associated with lysosomal-autophagy imbalance, coupled to deleterious inflammation as well as apoptotic or necrotic cell death, and has been implicated in TBI and chronic neurodegenerative diseases.[12–15] Progression of TBI or related brain trauma (e.g., ischemia/reperfusion) is dependent upon CatB activity, in addition to protein levels, in rodent models.[16,17] Notably, genetic ablation of CatB results in improvement of motor dysfunction and reduction of brain tissue lesion volume in mice with contolled cortical impact.[18]

TBI causes efflux of brain biomarker proteins into the cerebral spinal fluid (CSF). Glial fibrillary protein (GFAP) and ubiquitin carboxy-terminal hydrolase (UCH)-L1, well-studied TBI biomarkers, are elevated in the CSF during the first few hours of injury[19,20] and have been well established as indicators of primary injury.[21] CatB may be useful as a CSF biomarker directly associated with processes in the brain tissue itself. Protein levels are increased in CSF of patients with neurodegenerative lysosomal disease, Guillain-Barré syndrome, demyelinating polyneuropathy, multiple sclerosis, as well as Alzheimer's disease (AD),[22] a chronic disease for which TBI is a risk factor.[23,24] Identification of proteins, such as CatB, that simultaneously reflect the mechanistic changes in brain tissues and that also have the potential to serve as surrogate biomarkers during acute-subacute injury, are sorely needed.

CatB protein levels and its contribution to cysteine protease activity have not yet been explicitly defined in the context of variable TBI severity within different brain regions. Therefore, the present study sought to determine the temporal profile of levels and cysteine protease activity of CatB within a proximal region immediately impacted by trauma (frontal cortex; FC), a distal region that is sensitive to delayed degeneration (hippocampus; HC), and CSF as the source of translational surrogate biomarkers. This study used rodent models of craniotomy and penetrating ballistic-like brain injury (PBBI) representing moderate and severe conditions of TBI injuries, respectively, to address these goals. Results demonstrated that upregulation of CatB, particularly the mature form, is associated with TBI severity. Dysregulation of CatB occurred in both proximal and distal regions relative to the injury site, and correlated with neurological deficits. Further, investigation of CSF derived from rodent models and patients with TBI suggested that elevated CatB protein levels may serve as a post-acute surrogate biomarker of TBI.

Methods

Animals

All experiments were conducted at the Walter Reed Army Institute of Research (WRAIR) are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The experimental procedures were approved by the WRAIR Animal Care and Use Committee. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals*, 2011 edition, published by the National Research Council. Male Sprague-Dawley rats weighing 250–300 g (Charles River Labs, Raleigh, VA) were housed individually under a normal 12-h light/dark cycle (lights on at 6:00 am).

Rat model of craniotomy and penetrating ballistic-like brain injury (PBBI)

Animals ($n = 6$–10 per group) were anesthetized with 5% isoflurane delivered in oxygen for surgery while body temperature was maintained at 37.0°C using a heating blanket (Harvard Apparatus, Holliston, MA). The craniotomy and PBBI models have been extensively characterized and produce a cavity in the brain mimicking the ballistic nature of a high-velocity bullet wound.[25,26] For craniotomy alone, the scalp was incised along the midline and a 4-mm-diameter burr hole was created to expose the right frontal pole of the brain (+4.5 mm anteroposterior and +2 mm mediolateral from bregma). Induction of a 10% unilateral frontal PBBI was performed after conducting a craniotomy followed by stereotaxic insertion of a specially designed probe into the right hemisphere of the brain (Mitre Corporation, McLean, VA). The probe was inserted through the cranial space through the FC and rapid inflation/deflation of a water-filled balloon was used to create a temporary cavity in the cerebrum. For biological sample collection at each time-point, animals were anesthetized with 70 mg/kg ketamine and 6 mg/kg xylaxine. All cohorts were controlled to accommodate for the potential influence of isoflurane or ketamine treatment.

Rat neuroscore assessment

Neurological scores (NS) ($n = 6$–10 per group) were determined as described and performed blinded.[26] The experimental design is displayed (Fig. 1A). The NS was taken 30 min and prior to each terminal end-point at 1, 3, or 7 days post-injury. Neurological deficits were evaluated at 30 min, then again at 1, 3, or 7 days after PBBI using a modified battery of tests. NS were based on a 12-point sliding scale ranging from 0 (normal) to 12 (severely impaired) comprised from the following four neurological tests: 1) contralateral forelimb flexion during tail suspension; 2) shoulder adduction (body upward curling behavior) during tail suspension; 3) open-field circling behavior; and 4) impaired resistance to lateral push. The maximum score for each component = 3. The maximum composite score indicating the most severe deficit = 12.

Preparation of rat brain tissues and CSF

Brain tissue and CSF were collected 1, 3, or 7 days after either craniotomy or PBBI procedures ($n = 6$–10 per group) in the absence of protease inhibitors for western blotting or protease assay analysis (Fig. 1B). CSF isolation was conducted as previously described.[27,28] The atlanto-occipital dura mater was exposed by separating the nuchal muscles. CSF was collected with a 30-gauge syringe needle through the meninges into the cisterna magna, briefly transferred to pre-chilled heparin-coated tubes to prevent clotting of any blood cells present, and centrifuged at 1200*g* for 10 min at 4°C. The resulting cell-free CSF was immediately transferred into pre-chilled Eppendorf tubes without protease/phosphatase inhibitors, and then immediately stored at −80°C until use. Samples with hemolysis were rejected from the study. Upon first thaw, samples were maintained at 0–4°C on ice, then split into single-use micro-aliquots to ensure activity was preserved. For ipsilateral brain tissue isolation, animals were decapitated. After removal of the brain from the skull, tissues were washed briefly with ice-cold 0.9% saline, then were placed onto a sterile, ice-cooled platform. The FC, ~0–2 mm bregma and the HC were dissected from the ipsilateral hemisphere. After removal of excess

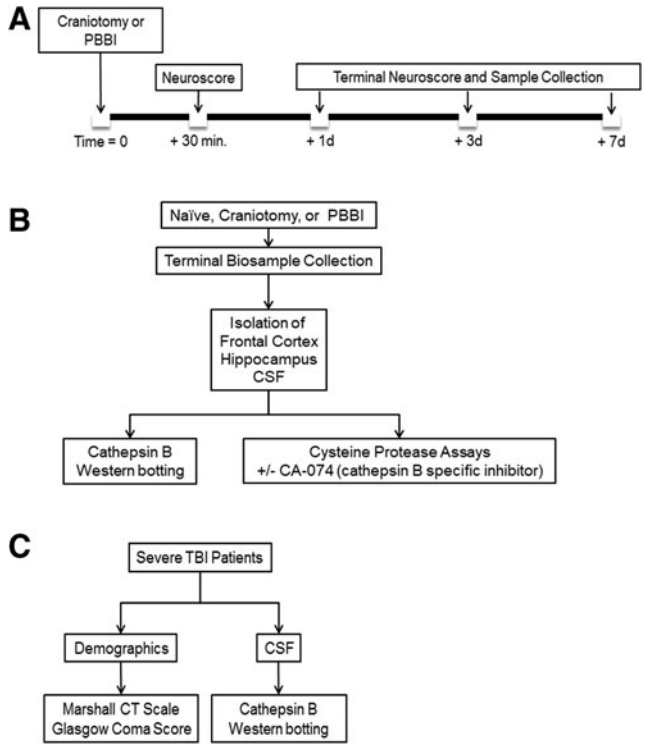

FIG. 1. Study design. **(A)** Timeline of experimental TBI in rats. Individual cohorts ($n = 6$–10 rats per group and time-point) were subjected to either craniotomy or PBBI. The composite neuroscore was determined at acute (30 min) and at terminal (1, 3, or 7 days) end-points after injury. **(B)** Schema of biochemical outcome metrics studied after experimental TBI in rats. Biological samples were derived from individual cohorts of rats subjected to craniotomy or PBBI as described in (A) or from naïve animals ($n = 6$) without surgical procedures or neuroscore assessment. Frontal cortex, hippocampus, and CSF was collected and analyzed for cathepsin B content using western blotting. Cysteine protease enzyme activity was determined in the absence or presence of CA-074 (cathepsin B-specific inhibitor). **(C)** Study design for reporting of demographic information, assessment of the Marshall CT Scale and Glasgow Coma Scale scores, and collection of CSF from patients with severe TBI compared with controls. Cathepsin B protein levels were measured in CSF using western blotting. CT, computed tomography; CSF, cerebrospinal fluid; PBBI, penetrating ballistic-like brain injury; TBI, traumatic brain injury.

saline, each brain region was immediately flash frozen in liquid nitrogen, then stored at −80°C until use.

Western blotting of rat brain tissues and CSF

Brain tissues. Brain tissues were thawed on ice in the presence of 200 μL of pre-chilled Cytobuster reagent (Millipore Sigma, Burlington, MA), gently sonicated for 1 X 10 sec, stored on ice for 10 min, then centrifuged at 10 kg, 4°C, for 10 min. Clarified supernatant was retained at 4°C and prepared as micro-aliquots and stored at −80°C until use. A single aliquot was thawed for protein concentration measurement using the MicroBCA protein assay kit (Thermo/Pierce, Rockford, IL). All samples were normalized based on total protein. Five micrograms of total protein from brain tissue supernatant were prepared in lithium dodecyl sulfate (LDS) sample buffer (final 2X LDS, 250 mM dithiothreitol [DTT], pH 8.4), heated to 95°C for 5 min, then briefly centrifuged at room temperature (RT) and was separated by one-dimensional 4–12% polyacrylamide electrophoresis (1D-PAGE) using Bis-Tris Protein NuPAGE Gels (catalog # NP0329PK2; Invitrogen/Thermo Fisher, Carlsbad, CA), followed by transfer to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Waltham, MA). Protein was transferred to PVDF membranes, which were blocked in 4% non-fat dry milk +0.01% Tween-20/1X phosphate-buffered saline (PBS), pH 7.8, washed 3 times in 1X PBS, then incubated in goat anti-mouse CatB antibody (1/500) (catalog # AF965; R and D Systems, Minneapolis, MN) in blocking solution at 4°C, overnight. Membranes were washed, incubated in donkey anti-goat-HRP secondary (catalog # sc-2020; Santa Cruz, Dallas, TX), washed, and then incubated with SuperSignal West Femtomole Reagent (Thermo/Pierce). Images were captured using the ImageQuant LAS 4000 (GE Healthcare Life Sciences, Pittsburgh, PA).

CSF. A 15-μL aliquot of CSF was diluted with an equal volume of activation buffer for 1 h at 37°C. The reaction was halted by placing samples on ice for 10 min, then supplementing with 5-μL 4X LDS sample buffer, pH 8.4, containing 250 mM DTT. Samples were denatured at 95°C for 5 min, then briefly centrifuged at RT. Thereafter, 10 μL was separated at RT by 4–12% Bis-Tris gels, then transferred to nitrocellulose membranes (BioRad, Hercules, CA), blocked with LiCOR Odyssey buffer (LiCOR Biosciences, Lincoln, NE) for 2 h at RT, then incubated with the same goat anti-mouse CatB antibody (1/500) in LiCOR blocking buffer overnight. After washing 3 times in 0.02% Tween in 1X PBS, membranes were incubated with IRDye® 800CW donkey Anti-Goat IgG Secondary Antibody (catalog # 926-32214; Li-COR Biosciences) for 1 h at RT. After washing, images were captured using the Odyssey CLx near-infrared fluorescence imaging system (Li-COR Biosciences). Recombinant 2.5- to 5-μg rat CatB was used as a loading control in each gel (catalog # 80545-R08H; Sino Biological Inc., Wayne, PA) was used as a control. For brain tissues CatB and quantitation and molecular weight estimation were conducted with ImageQuant TL software version 7.0 (GE Healthcare Life Sciences). Densitometry of CSF was conducted using Image Studio version 5.0 (Li-COR Biosciences).

Protease assays for rat brain tissues and CSF

All solutions were prepared fresh daily and stored chilled until use. A stock solution of substrate, z-Phe-Arg-amino-methyl coumarin (FR-AMC), was prepared at 1 mM in 10% dimethyl sulfoxide (DMSO)/H_2O and stored in micro-aliquots at −80°C until use per assay, per day. Protease assays were performed at 37°C unless otherwise noted. For brain tissue samples, supernatants were prepared in ice-cold Cytobuster reagent for a final concentration of 10 μg/50 μL/well. CSF samples were diluted directly into 1X assay buffer (v/v = 1/40), for a final volume of 50 μL/well. Brain tissue or CSF samples were mixed with 50 μL of 2X Assay Buffer (100 mM Na-acetate, 2 mM ethylenediaminetetraacetic acid [EDTA], 200 mM NaCl, pH 5.5) for 5 min and warmed to 37°C. The mixtures were supplemented with 25 μL of 2X Activation Buffer (assay buffer supplemented with 8 mM DTT) for 10 min without (0 nM) or supplemented with CA-074 (2S)-1-[(2S,3S)-3-methyl-2-[[(3S)-3-(propylcarbamoyl)oxirane-2-carbonyl]amino]pentanoyl] pyrrolidine-2-carboxylic acid) inhibitor. Thereafter, 25 μL of 25 μM FR-AMC (catalog # C9521; Sigma-Aldrich, St. Louis, MO) in 1X Assay Buffer was added to the reaction mixture.

Generation of the AMC product was monitored every 5 min up to 30 min (brain tissue) or 60 min (CSF) (excitation = 370 nm, emission = 450 nm) acquired with Gen5 using the Synergy plate reader (BioTek, Winooski, VT). All samples and standards were tested in triplicate. Fluorescent units were converted to concentration (μM) based on comparison to the standard curve generated by free AMC (range: 0–20 μM). Recombinant human CatB (catalog # 953-CY; R and D Systems, Minneapolis, MN) was used as a positive control in all assays. Baseline values of AMC were derived from the assay

performed in the absence of cathepsin B. The resulting value is used as a ''blank,'' which is subtracted from all values prior to extrapolation from the AMC standard curve.

Western blotting of human CSF

CSF samples were derived from patients diagnosed with blunt trauma to the head, who presented to the emergency department of Ben Taub General Hospital, Baylor College of Medicine, Houston, TX (study protocol, Baylor College of Medicine, IRB Protocol # H-13606) ($n = 20$) and were compared with non-TBI controls ($n = 10$). CSF was collected until a ventriculostomy catheter was no longer clinically indicated. CSF samples were diverted to 15-mL conical polypropylene centrifuge tubes (BD Falcon, Bedford, MA) and centrifuged at 4000*g* at RT for 5 min to remove cellular debris. Clarified CSF was transferred into cryogenic tubes, snap-frozen in liquid nitrogen, and stored at −80°C until use. Control CSF samples were purchased from BioreclamationIVT (Baltimore, MD).

Western blotting was conducted as follows. Recombinant human CatB (100 ng/lane) was loaded as a positive control. Twenty microliters of human CSF was mixed with a sample loading buffer (final, 8X sodium dodecyl sufate [SDS] sample buffer, 25 mM DTT), then centrifuged for 1 min, and resolved using 1D-PAGE (4–20% Tris-glycine; Invitrogen). Samples were then transferred onto PVDF membranes, blocked with 5% non-fat milk in TBST (50 mM Tris, 138 mM NaCl, 2.7 mM KCl, pH 8.0, 0.05% Tween-20) for 1 h at RT. After washing in 3X TBST, membranes were incubated with goat anti-mouse CatB antibody (1/500) (Catalog # AF965, R and D Systems) in blocking solution at 4°C overnight, washed, then incubated for 1 h at RT with anti-goat alkaline phosphatase-conjugated secondary antibodies at 1/5000 (Millipore Sigma). Images were captured using a high-resolution flatbed scanner (Epson, Long Beach, CA) using UN-SCAN-IT software (version 6.1, Silk Scientific Software, Orem, UT). Band quantitation of original non-manipulated images was conducted with ImageJ (version 1.5, Research Services Branch, National Institutes of Mental Health, Bethesda, MD). For representative western blot images, contrast was enhanced for visualization purposes only.

Statistical analysis

All data were analyzed using Prism (version 6; GraphPad, La Jolla, CA). Outliers were removed by the ROUT method prior to statistical analysis (rat Q = 1%, human Q = 5%).

Rat brain tissue and CSF normalization. For brain tissue, subsets of naïve, craniotomy, or PBBI samples were analyzed on the same gel to allow for direct comparison and normalization. For brain tissue, samples were normalized for total protein prior to loading. CSF was normalized to the intensity of proCatB in the 5-μg loading control per gel. Thereafter, proCatB and matCatB data were normalized to the average value of proCatB (37 kDa) detected in naïve controls. All values shown were derived based on proCatB derived from naïve controls, such that the normalized average level of proCatB mean = 1 for each brain region studied.

Brain tissue and CSF western blotting. Data were analyzed by analysis of variance (ANOVA) with the Tukey multiple comparisons post-test (#$p \le 0.05$ injury vs. naïve control, *$p \le 0.05$ craniotomy vs. PBBI).

Protease assays. The rate AMC generation ([μM]/min) was determined to be linear for brain tissue within 0–30 min and for CSF between 20 and 120 min. Therefore the reaction rate was extrapolated from data derived from the 10-min (brain tissue) or 60-min (CSF) reaction times. For protease activity assays conducted with CA-074, activity is displayed compared with samples tested without inhibitor (0% inhibition). Assays were compared using ANOVA and Tukey post-test (#$p \le 0.05$, injury vs. naïve control; *$p \le 0.05$, craniotomy vs. PBBI).

Human CSF western blotting. Results were analyzed using the two-tailed *t*-test with Welch's correction (*$p \le 0.05$). Correlations between protein levels and time were determined using the one-tailed, Pearson test.

Results

CatB protein levels in rat brain

To determine if CatB was affected by craniotomy or PBBI, pro- (proCatB) and mature (matCatB) levels were determined using semi-quantitative western blotting of ipsilateral brain tissue lysates (Fig. 2). Exemplary images of CatB in the FC are shown for proCatB (37 kDa) and matCatB (20–25 kDa), respectively (Fig. 2A, top). Quantitation of CatB immunoreactivity is indicated (Fig. 2A, bottom). Compared with naïve cohorts (dotted line = 1.0 AU), proCatB increased 1 day after craniotomy (2.5 ± 0.4 AU), but decreased to near naïve levels after 3 days (0.8 ± 0.1 AU, not significant [n.s.]). After PBBI, proCatB levels increased only at 3 days (2.3 ± 0.5 AU). MatCatB progressively increased in the FC over time compared with naïve cohorts (solid line = 2.1 AU) as a consequence of craniotomy or PBBI injury, and was much more pronounced after PBBI compared with craniotomy. MatCatB levels were generally unchanged 1–3 days after craniotomy (range: 1.3 ± 0.21 AU to 0.76 ± 0.07 AU), and were greatest at 7 days after craniotomy alone, but were found to be non-significant with the tests applied (4.7 ± 0.59 AU, n.s.). This trend was eclipsed by levels detected after PBBI, wherein values of matCatB steadily rose after 1 day (1.5 ± 0.51 AU), 3 days (3.3 ± 0.5 AU), then, more sharply, at 7 days (25 ± 2.3 AU).

Western blot analysis of HC lysates indicated moderate proCatB, and robust matCatB abundance (Fig. 2B, top). Similar to the trends in FC, proCatB was generally unchanged, but matCatB increased in a stepwise manner over time, increasing at 3 days and 7 days, in the HC (Fig. 2B, bottom). Craniotomy had little effect on proCatB levels (range: 1.0–2.04, n.s.) compared with naïve controls (dotted line = 1) and there was little or no robust difference 1–7 days after PBBI (range: 1.8–2.4, n.s.). Levels of matCatB derived from naïve cohorts are indicated (solid line = 5.489). The average matCatB level 1 day after craniotomy (5.1 ± 0.2 AU, n.s.) was similar to naïve cohorts. Values increased moderately, albeit non-significantly, at 3 days (7.1 ± 1.6 AU, n.s.) and at 7 days (8.8 ± 1.4 AU) after craniotomy. Similarly, little change in matCatB was observed 1 day after PBBI (5.5 ± 0.6 AU) compared with naïve cohorts, and levels were moderately increased after 3 days (8.8 ± 1.1 AU, n.s.). The most robust increase in matCatB occurred at 7 days after PBBI (13.5 ± 1.7 AU). This stepwise, progressive increase in matCatB was significant over the time-course tested.

Cysteine protease activity in rat brain

MatCatB, the active form of the enzyme, was progressively upregulated in the FC and HC after craniotomy and PBBI. However, its contribution to the milieu of protease activity is not known. Therefore, total cysteine protease activity and sensitivity to CA-074, the CatB-specific inhibitor, was determined using enzyme activity assays resulting in cleavage of the substrate z-FR-AMC typically used for cysteine proteases. First, cysteine protease activity in the FC was examined (Fig. 3A). Compared with naïve

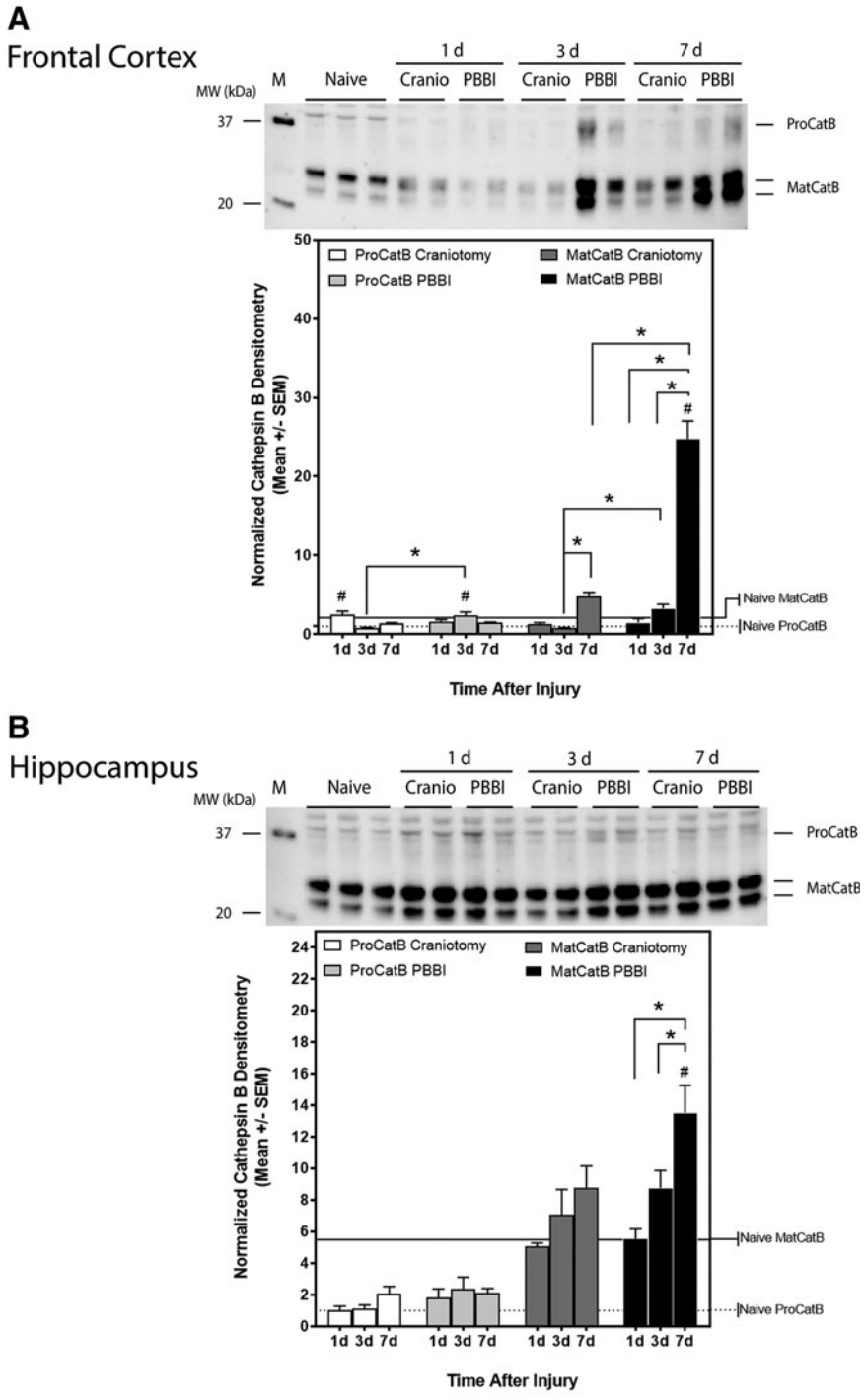

FIG. 2. Cathepsin B protein levels in rat brain after TBI. Pro- (ProCatB) and mature cathepsin B (MatCatB) protein levels were determined in **(A)** frontal cortex or **(B)** hippocampus isolated at 1, 3, or 7 days after injury. Representative western blots are indicated for each brain region and sample (5 μg/lane). ProCatB ($\sim$37 kDa) and MatCatB are (20–25 kDa). Lanes designated for the molecular weight marker are noted as ''M.'' Relative quantitation of densitometric signals is shown below each western blot image. Data are displayed as the mean $\pm$ SEM for craniotomy (ProCatB, white bars; MatCatB, light gray bars) or for PBBI (ProCatB, dark gray bars; MatCatB, black bars) at each time-point. Levels of ProCatB (dotted line, mean = 1) and MatCatB (solid line) are indicated for naïve controls. Statistically significant values are indicated (#$p \leq 0.05$ injury vs. naïve control, *$p \leq 0.05$ craniotomy vs. PBBI, ANOVA with the Tukey multiple comparisons post-test). ANOVA, analysis of variance; PBBI, penetrating ballistic-like brain injury; SEM, standard error of the mean; TBI, traumatic brain injury.

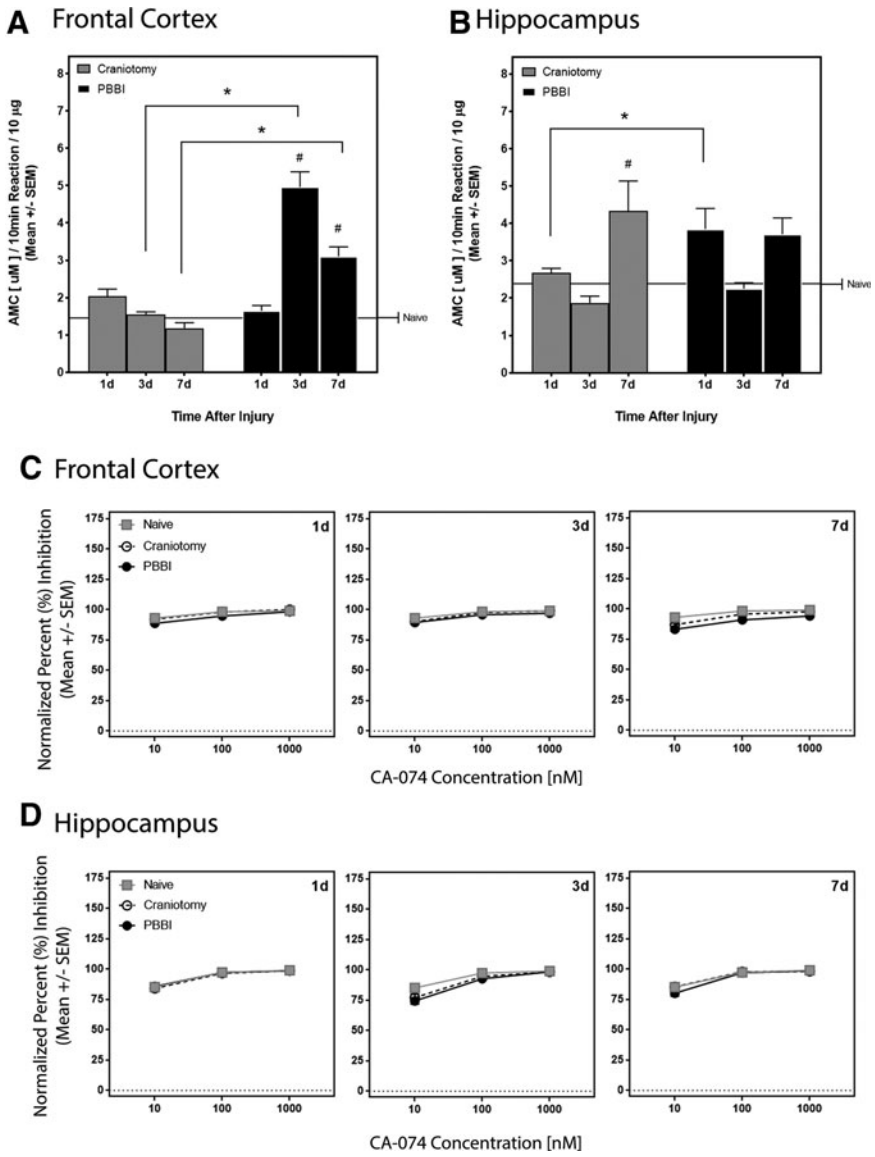

FIG. 3. Cysteine protease activity in rat brain after TBI. **(A)** Frontal cortex or **(B)** hippocampus cysteine protease activity in clarified lysates isolated from rats 1, 3, or 7 days after injury. Quantitative enzyme activity determined based on cleavage of the substrate, FR-AMC, in the absence of protease inhibitors using 10 μg total protein from clarified lysates isolated at 1, 3, or 7 days after injury. Post-injury time (x axis, days) compared with rate of AMC generated (y axis, [μM]/10 min reaction ± SEM/per 10 μg protein) is displayed for craniotomy (gray bars) and PBBI (black bars). Naïve controls are indicated (mean = solid line). Statistically significant comparisons are shown. **(C)** Frontal cortex or **(D)** hippocampal cysteine protease activity inhibition in the presence of 10 μg clarified lysates with CA-074. The percentage of inhibition was derived from AMC generated without CA-074, as in A and B. Naïve controls, craniotomy, and PBBI cohorts were analyzed independently and scaled to level of AMC generated in each reaction (no inhibitor = 0% normalized inhibition). Data are shown for samples isolated 1 day (left), 3 days (center), or 7 days (right) in samples isolated from naïve controls (closed gray squares), craniotomy (open circles), or PBBI (closed circles). The concentration of CA-074 used is displayed (x axis, range: 10–1000 nM) compared wtih the percentage (%) of inhibition of cysteine protease activity(y axis, mean ± SEM). Significant differences in % inhibition are indicated (#$p \leq 0.05$, injury vs. naïve control; *$p \leq 0.05$, craniotomy vs. PBBI, ANOVA, and Tukey post-test). ANOVA, analysis of variance; FR-AMC, z-Phe-Arg-amino-methyl coumarin; PBBI, penetrating ballistic-like brain injury; SEM, standard error of the mean; TBI, traumatic brain injury.

controls (1.46 ± 0.22 μM/min/10 μg protein) activity was relatively unchanged 1–7 days after craniotomy (range: 1.54 ± 0.08 μM to 2.03 ± 0.19 μM/min/10 μg protein, n.s.) or 1 day after PBBI (1.64 ± 0.14 μM/min/10 μg protein). In contrast, PBBI led enzyme activity to increase robustly at 3 days (4.96 ± 0.41 μM/min/10 μg protein) and at 7 days (3.11 ± 0.25 μM/min/10 μg protein). Changes in HC cysteine protease activity (Fig. 3B) were insignificant 1–3 days after craniotomy (range: 1.87 ± 0.18 to 2.67 ± 0.13 μM/min/10 μg protein) compared with naïve controls (2.38 ± 0.30 μM/min/10 μg protein). However, activity increased sharply at 7 days (4.33 ± 0.80 μM/min/10 μg protein). Interestingly, PBBI led to a bimodal increase in protease activity over time, such that values were increased at 1 day, but were not significant (3.84 ± 0.55 μM/min/10 μg protein, n.s.), then fell to near naïve levels at 3 days (2.25 ± 0.16 μM/min/10 μg protein), before increasing more prominently at 7 days (3.71 ± 0.43 μM/min/10 μg protein) post-injury.

To determine whether cysteine protease enzymatic activity was primarily due to matCatB or a mixture of cysteine proteases, assays were conducted in the presence of 10–1000 nM CA-074 (Fig. 3C,D). Values are displayed as the percent (%) of inhibited activity normalized to values determined in the absence of CA-074 (0% inhibition). In the FC (Fig. 3C), the lowest concentrations of CA-074, 10 nM, inhibited cysteine protease activity similarly in all groups collected at 1 day, 3 days, and 7 days after craniotomy and PBBI. Ten nMCA-074 inhibited cysteine protease activity in the FC (range: 83.1 ± 0.81 to 92.2 ± 0.54%) compared with naïve controls (93.0 ± 0.53%).

In the HC lysates (Fig. 3D), inhibition of protease activity by 10 nMCA-074 was high in brain lysates from the HC (range: 74.3 ± 2.60% to 85.6 ± 1.47%) compared with naïve cohorts (85.1 ± 2.10%) and showed similar profiles over time. Higher concentrations of 100–1000 nM result in 100% inhibition as expected across all time-points. These data collectively demonstrate that total cysteine protease activity in FC and HC regions is altered at 1–7 days due to craniotomy and PBBI compared with naïve controls, and that this protease activity is represented by matCatB in both brain regions over time.

CatB protein levels and cysteine protease activity in rat CSF

Using the same cohorts studied for brain tissue analysis, proCatB and matCatB content, combined with cysteine protease activity, were determined in CSF after injury (Fig. 4). Exemplary western blot images are shown and indicate pro- and matCatB in CSF collected from naïve controls compared with craniotomy or PBBI cohorts at 1 day (Fig. 4A, top), 3 days (Fig. 4A, center), and 7 days (Fig. 4A, bottom). The rat recombinant loading control (2.5–5 μg) is indicated and migration patterns for proCatB (band 1) or matCatB (band 2) are shown in each blot. Quantitation of the western blot images (Fig. 4B) indicated that average proCatB levels were more robust than those of matCatB. All bands were normalized to proCatB in naïve controls (naïve = 1, solid line). Craniotomy did not largely affect proCatB levels compared with naïve cohorts,

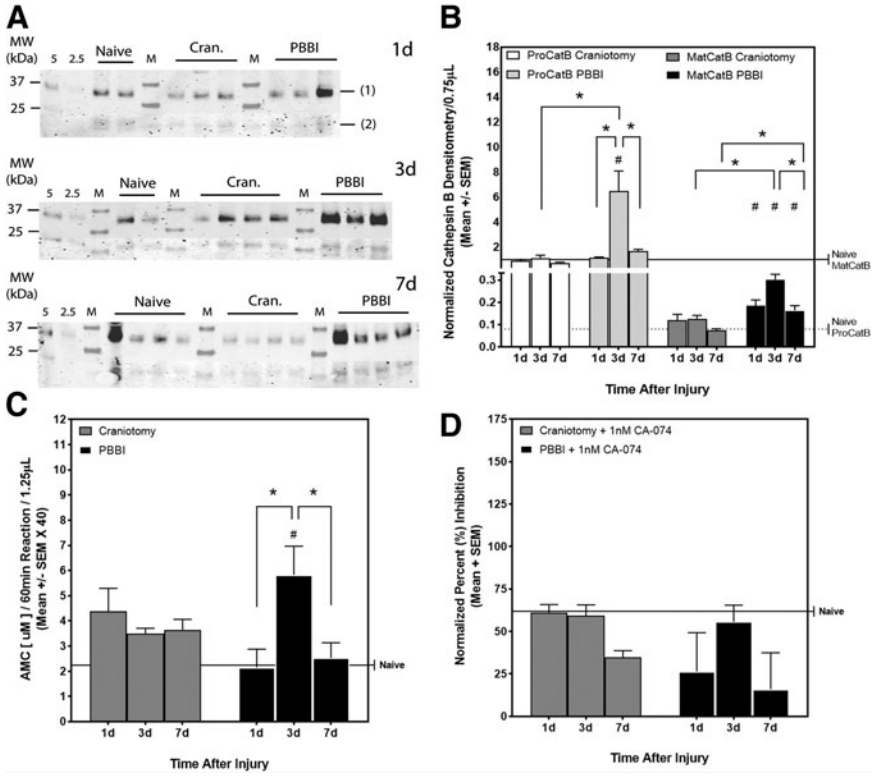

FIG. 4. Cathepsin B protein and cysteine protease activity in rat CSF after TBI. **(A)** Representative western blots of cathepsin B in CSF collected from naïve controls or from craniotomy or PBBI injured rats at 1 day (top), 3 days (center), or 7 days (bottom). Recombinant rat CatB standards [range: 2.55 ng/lane] and the molecular weight markers (M) are indicated for each blot. Numbered tick-marks signify (–1) ProCatB, (–2) MatCatB. **(B)** Quantitation of proCatB and MatCatB based on densitometry. Data are displayed as the mean ± SEM for craniotomy (ProCatB, white bars; MatCatB, light gray bars) or PBBI (ProCatB, dark gray bars; MatCatB, black bars). Naïve controls are indicated for ProCatB (dotted line, mean = 1) and MatCatB (solid line). **(C)** Quantitative enzyme activity determined in CSF isolated at 1, 3, or 7 days after injury. Post-injury time (x axis, days) compared with FR-AMC generated (y axis, mean ± SEM AMC μM/60 min reaction/1.25 μL final volume × 40 [dilution factor]) is displayed for craniotomy (gray bars) and PBBI (black bars). Activity derived from naïve controls is indicated (solid line). **(D)** Inhibition of cysteine protease activity CSF collected 1 day, 3 days, or 7 days after craniotomy (gray bars) or PBBI (black bars). Inhibition of cysteine protease activity was determined after incubation with 1 nM of CA-074 (x axis) and is displayed as the percent (%) of inhibition compared with the reaction without CA-074 (0%) as in C. Inhibition within CSF from naïve controls is indicated (solid line). Statistically significant values are indicated (#$p \leq 0.05$ injury vs. naïve control, *$p \leq 0.05$ craniotomy vs. PBBI, ANOVA with the Tukey multiple comparisons post-test). ANOVA, analysis of variance; CSF, cerebrospinal fluid; FR-AMC, z-Phe-Arg-amino-methyl coumarin; PBBI, penetrating ballistic-like brain injury; SEM, standard error of the mean; TBI, traumatic brain injury.

although it was slightly elevated at 3 days (1.13 ± 0.22 AU, n.s.) compared with 1 day (0.92 ± 0.08 AU, n.s.) and 7 days (0.74 ± 0.06 AU, n.s.). After PBBI, proCatB levels in CSF were unaffected at 1 day (1.15 ± 0.06 AU), but increased by nearly sixfold at 3 days (6.47 ± 1.60 AU), and fell to near naïve and craniotomy levels at 7 days (1.66 ± 0.16 AU). MatCatB levels were also similar among naïve controls (naïve = 0.08, dotted line) and craniotomy-injured rats (range: 0.07 ± 0.01 AU to 0.12 ± 0.03 AU, n.s.). MatCatB levels were greater after PBBI compared with naïve controls and craniotomy-injured cohorts at all time-points tested. Additionally, the temporal profile of matCatB mimicked that of proCatB such that levels were moderately increased at 1 day (0.19 ± 0.03 AU), peaked at 3 days (0.30 ± 0.02 AU), and fell at 7 days (0.16 ± 0.02 AU).

Average cysteine protease activity was similar among CSF samples collected from naïve controls (2.24 ± 0.74 μM) and those collected 1–7 days after craniotomy (range: 3.49 ± 0.22 to 4.38 ± 0.91 μM) or 1 day after PBBI (2.15 ± 0.73 μM) (Fig. 4C). After PBBI, activity levels nearly tripled at 3 days (5.83 ± 1.14 μM), yet fell to near naïve levels at 7 days (2.52 ± 0.61 μM). Next, relative levels of cysteine protease activity were determined in the presence of 1 nM CA-074, a CatB-specific inhibitor (Fig. 4D). The profile cysteine protease activity mitigation was consistent with that of matCatB protein levels and of protease activity in CSF. Here, CA-074 effectively inhibited cysteine protease activity in CSF collected from naïve controls (61.9 ± 21.3%), 1–7 days after craniotomy (range: 34.7 ± 4.02% to 61.0 ± 4.81%, n.s.). Inhibition was also robust in CSF collected after PBBI. Interestingly, inhibition by CA-074 was highest 3 days after PBBI (55.7 ± 9.80%, n.s.), compared with 1 day (26.5 ± 22.9%, n.s.) or 3 days (16.0 ± 21.5% n.s.), although these effects were not statistically significant by ANOVA. Overall, this profile indicates that CSF samples from injured rat brain are sensitive to the CatB selective inhibitor, CA-074, across time.

Association between rat neurological deficits and CatB protein levels or cysteine protease activity

Composite NS values were determined at acute (30 min) and terminal (1, 3, and 7 days) time-points after PBBI (Fig. 5) for evaluation of neurological deficits. Values were ''0'' among all craniotomy cohorts (not shown). Acute NS increased as expected among PBBI cohorts (range: 7.5 ± 0.8 to 8.9 ± 0.5 AU) and remained elevated at terminal time-points (range: 4.1 ± 0.4 AU to 6.3 ± 0.5 AU). Next, the Spearman rank correlation was used to determine if NS values had any relationship to proCatB, matCatB, or cysteine protease activity in brain tissue or CSF among PBBI cohorts (Supplementary Table S1). Among cohorts collected 1 day after PBBI, increased cysteine protease activity in the FC was associated with acute ($r = +0.48$) and terminal ($r = +0.66$) NS. In contrast, inactive proCatB levels were negatively correlated to NS values (range: $r = -0.61$ to $r = -0.67$). Among cohorts collected at 7 days after PBBI, CSF matCatB levels had an unexpected inverse relationship with composite NS values ($r = -0.76$).

Assessment of individual NS metrics revealed several relationships to CatB protein levels and cysteine protease activity (Supplementary Table S2). Acute forelimb outreach was linked to increased cysteine protease activity in the FC ($r = +0.76$). Terminal circling deficit was in line with increased matCatB levels in FC collected at 1 day ($r = +0.65$). Interestingly, increased cysteine protease activity in CSF was associated with curling ($r = +0.77$) and forelimb outreach ($r = +0.91$) deficits at 3 days after PBBI, the same time as peak CatB levels and protease activity.

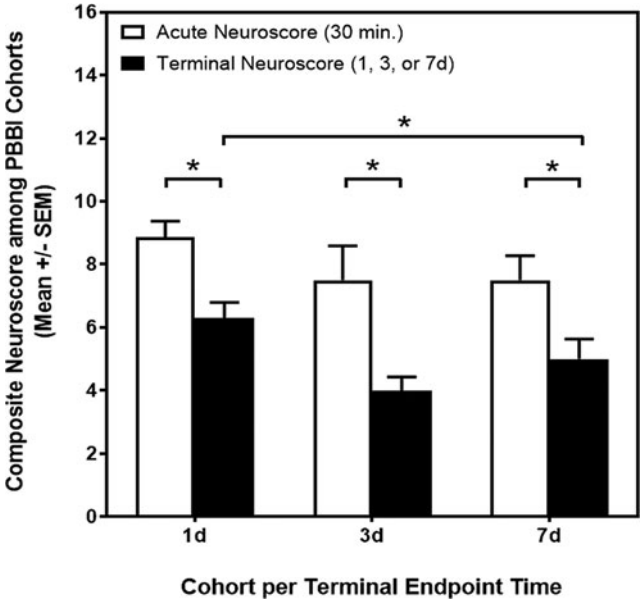

FIG. 5. Neurological deficits, cathepsin B protein levels, and protease activity in PBBI rats. The composite neuroscore determined from individual cohorts determined at acute (30 min, white bars) or terminal (1, 3, or 7 days, black bars) time-points after PBBI (mean ± SEM) (*$p \leq 0.05$, two-way ANOVA with Fisher's LSD post-test). Craniotomy-injured cohorts have a null (''0'') neuroscore value (not shown). ANOVA, analysis of variance; LSD, least significant difference; PBBI, penetrating ballistic-like brain injury; SEM, standard error of the mean.

CatB protein levels in CSF of patients with TBI

To determine if CatB may be clinically relevant, protein levels were semi-quantitatively determined using western blotting of CSF samples from patients with moderate-severe TBI (Table 1). Exemplary western blotting of control CSF samples indicated that the

Table 1. Patient Demographics

	Control (n = *10*)	*sTBI* (n = *20*)
Age years, mean (SD)	45.6 (21.5)	31.9 (10.2)
Min-Max	20-86	18-51
Male	5	14
Female	5	2
ND	NA	4
Mechanism of injury		
Motorcycle	NA	4
Assault	NA	4
Fall	NA	1
Automobile	NA	4
Auto-pedestrian	NA	2
Sports-related	NA	1
ND	NA	4
GCS [min-max]		
Initial, <24 h	NA	3-11
Follow-up, = 24 h	NA	4-9
CT Marshal Scale [min-max]	NA	II-V

Age, gender, mechanism of injury, and clinical metrics are described for each patient ($n = 20$) or control ($n = 10$).

CT, computed tomography; GCS, Glasgow Coma Scale; SD, standard deviation; sTBI, severe traumatic brain injury.

proCatB was detectable at ~38–40 kDa (Fig. 6A). The majority of patients with TBI had high levels of proCatB. Preliminary analysis of three patients detected ~25–30 kDa CSF CatB in two patients at 6–7 days post-injury that may be indicative of matCatB (Fig. 6B). To quantitate CatB levels among patients with TBI compared with controls, CSF was collected from a separate cohort of patients within 3 days of injury. Western blot quantitation indicated that average proCatB content nearly doubled (16.3 ± 2.84 AU) compared with non-TBI controls (7.16 ± 1.70 AU) (Fig. 6C). CatB levels increased with time, although these data were not significant (not shown). The small sample size among patients with TBI prohibited assessment of Glasgow Coma Scale (GCS) score, computed tomography (CT), death, survival, or disability compared with CatB levels. Evaluation of matCatB cysteine protease activity in CSF samples from patienrs with TBI could not be determined due to processing and storage conditions required to maintain sample integrity during procurement.

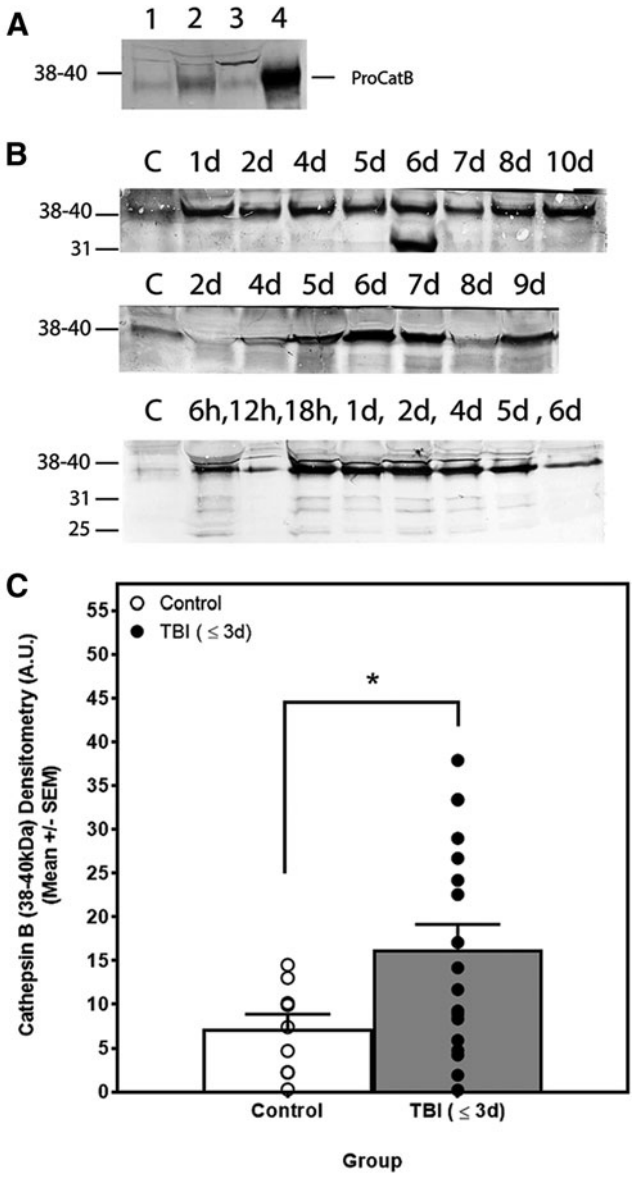

FIG. 6. CSF cathepsin B protein levels among patients with TBI. **(A)** Exemplary western blot image of non-activated procathepsin B (38–40 kDa) in CSF compared with recombinant human protein is indicated. Lane 1–3: TBI patients BYLR 2681, 2715, and 3982, respectively. Lane 4: 100 ng of recombinant human cathepsin B. **(B)** Western blots of cathepsin B detected in CSF collected from patient 1 (top), patient 2 (middle), and patient 3 (bottom) within hours (h) or days (d) after TBI. Lane ''C'' is the non-TBI control. **(C)** Semi-quantitative densitometry of procathepsin B in CSF derived from controls (open circles) or patients with penetrating TBI (closed circles) within 3 days (≤3 d) of injury (*$p \leq 0.05$ TBI vs. control, two-tailed *t*-test with Welch's correction). CSF, cerebrospinal fluid; TBI, traumatic brain injury.

Discussion

Induction of PBBI requires craniotomy followed by insertion of a probe into the brain to mimic the ballistic nature of a high-velocity bullet wound (described in the Methods section). Therefore, craniotomy alone was included as a non-PBBI injury reference. All metrics were compared with naïve controls consisting of rats that were not subjected to craniotomy or PBBI. Results of this study demonstrated that upregulation of matCatB occurs in the rat brain as a consequence of both craniotomy and PBBI. PBBI resulted in upregulation of matCatB levels, and associated cysteine protease activity was greater in the FC that contains the injury core, compared with the HC, which is distal to the injury site. Injury-induced elevation of cysteine protease activity was inhibited by CA-074, a selective inhibitor of matCatB. Several of these observations were associated with a battery of neurological deficits. Importantly, CSF derived from rats with PBBI and from patients with moderate-severe TBI had elevated levels of CatB, suggesting that this protein has utility as a surrogate biomarker of acute-subacute brain trauma.

Mature CatB and cysteine protease activity in proximal and distal brain tissues are indicators of TBI progression associated with neurological deficits

In this study, upregulation of mature, not pro-, CatB occurred in the rat brain as a consequence of craniotomy, and was elevated to a greater extent, by PBBI. This severity-based gradient, particularly in regards to proximal compared with distal effects, is consistent with previous reports of subacute immune cell activation and delayed neurodegeneration detected within cortical versus hippocampal regions within these models.[29] MatCatB is upregulated within activated microglia,[30,31] injured astrocytes,[32] and apoptotic neuronal cells.[33] Craniotomy alone does not lead to a massive lesion or permanent cavity in cortical regions. Craniotomy can result in mild contusion[34] and pro-inflammatory cytokine upregulation[35,36] within the injured cortex near the injury site, but it typically has little or no effect upon distal brain regions based on histological examination. This study shows that craniotomy is capable of eliciting distal matCatB protein elevation in a manner and time frame that is surprisingly similar to that of PBBI. Elevation of matCatB in HC after craniotomy is, to our knowledge, a novel observation that may be associated with delayed hippocampal degradation similar to that which is detected in models of mild-moderate lateral fluid percussion (LFP).[37]

Brain tissue biomarker profiling has expanded to include assessment of protein function that is aligned with propagation of known secondary injury processes. As a stand-alone observation, time-dependent cortical cysteine protease activity detected after PBBI is similar to that of widespread, progressive neuronal and glial protein degradation within lesioned cortical tissue undergoing autophagy.[38,39] Proteolytic degradation of GFAP, alpha-2 spectrin, tau, and amyloid precursor protein (APP) in the injured cortex peak

within this time frame.[40,41] Interestingly, the increase in hippocampal cysteine protease observed after craniotomy and PBBI is in accordance with delayed synaptic density loss and increased cysteine protease activity in *N*-methyl-D-aspartate (NMDA)-induced hippocampal injury.[42] Hippocampal cysteine protease activity was initially quite surprising and may be indicative of a multi-phasic response. A similar trend has been observed in rodent ischemic/reperfusion injury, wherein cysteine protease activity was elevated at 4–8 h, suppressed at 24 h, yet rebounded at 2 days post-injury.[43]

The assays used were carefully designed for favorable cleavage by CatB under acidic conditions. [44] Like CatB, cathepsin L is also enriched in brain tissues[9,45] and capable of cleaving similar substrates under reducing conditions.[46] Further, endogenous protease inhibitors, such as cystatin C, likely play a role in craniotomy or PBBI progression, and thereby may affect matCatB-dependent cysteine protease activity.[47] Assessment of specific activity or IC_{50} is difficult to accurately determine in a complex, heterogeneous mixture derived from tissue supernatants or CSF. However, this study indicated that inhibition by CA-074 was appropriately observed in brain tissue lysates and CSF at concentrations near the IC_{50} of 5 nM reported for CA-074 based on analysis of pure, recombinant cathepsin B *in vitro*.[48] The inhibition of protease activity by CA-074 confirms that matCatB is the key contributor to protease activity in both brain regions over time.

Neurological deficits are often preceded or are directly proportional to early-phase brain tissue degradation.[49] Reduction of cysteine protease activity has been shown to mitigate neurological deficits and spare tissue.[50] The highest acute and terminal NS values were observed 1 day after PBBI, whereas values observed at 3 days and 7 days were somewhat lower. As such, acute (30-min post-injury) and terminal (same-day) neurological deficits effectively preceded increased pro- and matCatB levels in brain tissues, which is a common and expected observation of this model. Individual NS components, such as curling, were associated with high levels of cysteine protease activity. Yet, the link between composite and individual NS values with proCatB, matCatB, or cysteine protease activity was mixed and may not provide the resolution for use as a direct correlate explicitly linked to these outcome metrics, particularly at 3–7 days when protein levels and protease activity are greatest.

Post-acute CatB protein levels and cysteine protease activity in CSF are novel TBI biomarkers associated with severity and time

Upregulation of CSF biomarkers is a hallmark of the acute injury response, yet assessment throughout the acute-subacute period has the power to provide evidence of pathological processes.[51] ProCatB and matCatB CSF levels peaked 3 days after PBBI in the rat models, whereas average matCatB levels increased within 3 days in the PBBI rat model and in patients with TBI. Further, detection among a subset of these patients occurred over a broad post-injury time frame, as late as 10 days post-admittance. The latter observation expands the time frame of TBI biomarker utility in humans.

Increased levels of pro- and matCatB in the CSF is generally in accordance with other biomarkers of trauma, neurodegeneration, and neurodegenerative disease. PBBI leads to acute (<12 h) efflux of GFAP and UCH-L1, acute-subacute elevation alpha-2 spectrin breakdown products (BDPs).[27] Further, tau is increased 1 week after PBBI[52] and peaks within 2 weeks after severe TBI in patients.[53,54] In contrast to initial assumptions, craniotomy led to either a null effect or a minor (non-significant) loss, rather than elevation, of matCatB levels at 7 days. Several biomarkers are decreased in response to injury or disease. For example, CSF-derived Aβ-42 is suppressed in patients diagnosed with cerebral amyloid angiopathy.[55] This inverse relationship between craniotomy and PBBI indicates that total or matCatB may be capable of differentiating relative severity. It is interesting to note that, both pro- and matCatB levels peaked at 3 days after PBBI.

CatB-dependent cysteine protease activity in rat CSF also peaks at 3 days after PBBI. Pairing cysteine protease activity with protein levels remains fairly novel in TBI-biomarker studies,[56] but is on par with biomarker analysis, inclusive of CatB, of subacute-chronic diseases. In fact, CatB is elevated in plasma derived from patients diagnosed with mild cognitive impairment as well as Alzheimer's disease compared with controls[57] and elevated cysteine protease activity has been reported in plasma from patients with septic shock polytrauma.[58] CatB activity has been reported in the CSF of patients with inflammatory neurological diseases, such as Guillain-Barré syndrome, demyelinating polyneuropathy, and multiple sclerosis.[59] This observation may indicate that efflux of pro- and matCatB into the CSF does not occur via immediate, passive, hemorrhagic leakage as it would for acute biomarkers such as GFAP and UCH-L1 or with tau and neurofilament light chain (NfL), which are relevant to subacute to chronic axonal degradation after TBI.[60,61]

At later time frames, injury-induced secretion of CatB may occur within the subacute time frame that is often characterized, in part, by reduced blood–brain barrier disruption during scar formation, but sustained inflammation and loss of neuronal integrity.[62] In fact, active matCatB is released from resident and peripheral immune cells that extravasate brain tissues or from damaged neurons during secondary TBI.[63,64] Unlike GFAP, which is frequently associated with early detection of CT- or magnetic resonance imaging (MRI)-positive findings,[65,66] delayed elevation of CSF CatB may be aligned with post-acute biomarkers such as NfL or tau and brain imaging abnormalities that remain detectable days to weeks after a brain trauma or stroke.[67,68] This work is the first to show that proCatB, matCatB, and its cysteine protease activity have utility as biomarkers for TBI that may be useful outside the 12- to 24-h window after injury.

Study limitations

This study is not without a few caveats. The delayed upregulation of proCatB and matCatB protein levels may be due to a variety of cell types, particularly inflammatory cells, which were not the current focus. Studies are in progress to determine cellular sources responsible for the temporal profile described herein. Quantitative measurement of protein levels in rodent or protease activity levels in human samples was not feasible at the time, and protease activity may vary due to presence of endogenous cysteine protease inhibitors. Lastly, assessment of CatB in the CSF is applicable to moderate-severe trauma, wherein ventriculostomy is necessary. Evaluation of blood would be highly valuable for application to a broad range of severities. These studies are currently being expanded to gain resolution regarding the role of CatB as a biomarker and facilitator of TBI progression.

Conclusion

Moderate to severe TBI caused by the craniotomy and PBBI, respectively, in the rat causes dysregulation of CatB protein levels and cysteine protease activity in the injured cortex and degenerating HC. Increases in CatB protein levels occur during and after

persistent neurological deficits caused by TBI. Further, efflux of proCatB and matCatB into CSF derived from severely injured rodents or patients with TBI indicates the potential utility of CatB as a surrogate, translational biomarker of subacute TBI.

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Author Contributions

A.M.B., B.T., G.H., V.H., and S.J. conceived the study and designed the experiments; A.M.B. and V.H. designed custom protease assays; A.M.B. performed assays, conducted data analysis and statistics; B.N.A. and B.T. designed and performed biochemical assays; G.A.S., C.S.R., Z.Y., and K.K.W. provided samples and conducted data analysis for human CSF; A.M.B., B.T., G.A.S., V.H., J.G., K.K.W., and D.A.S. prepared the article.

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Author Disclosure Statement

V. Hook, G. Hook, and S. Jacobsen have equity positions and G. Hook and S. Jacobsen are employed by American Life Science Pharmaceuticals, Inc. V. Hook's conflict has been disclosed and is managed by her employer, the University of California, San Diego. The other authors have no conflicts of interest.

Supplementary Material

Supplementary Table S1
Supplementary Table S2

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