

# Effects of Injury Severity on Regional and Temporal mRNA Expression Levels of Calpains and Caspases after Traumatic Brain Injury in Rats

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## ABSTRACT

Despite a preponderance of studies demonstrating gene expression and/or enzymatic activation of calpain and caspase proteases after traumatic brain injury (TBI), no studies have examined the effects of injury magnitude on expression levels of these cell death effectors after TBI. Determination of the degree to which injury severity affects specific expression profiles is critical to understanding the relevant pathways contributing to post-trauma pathology and for developing targeted therapeutics. This investigation tested the hypothesis that different injury magnitudes (1.0, 1.2, and 1.6 mm) cause alterations in the regional and temporal patterns of mRNA expression of calpain-related (calpain-1 and -2, calpastatin) and caspase-related (caspases -3, -8, -9, BID) gene products after cortical impact in rats. Quantitative RT-PCR was used to compare effects of injury severity on mRNA levels in ipsilateral (injured) cortex and hippocampus, 6 h to 5 days post-injury. TBI caused increases in mRNA expression of all proteins examined, with the highest expression detected in the cortex. Generally, injury magnitude and levels of gene expression were positively correlated. High levels of gene induction were observed with BID, caspase-3, and -8, while caspase-9 mRNA had the lowest level of induction. Interestingly, although calpains are activated within minutes of TBI, calpain mRNA expression was highest 72 h to 5 days post-TBI. This study is the first analysis of the regional and temporal expression of calpains and caspases after TBI. These data provide insight into the inter-relationship of these two protease families and on the distinct but overlapping cascades of cell death after TBI.

**Key words:** apoptosis; injury magnitude; neuronal death; proteases; RT-PCR

## INTRODUCTION

UNMITIGATED PRIMARY AND SECONDARY neuronal damage and cell death following TBI play a significant role in poor clinical outcome, and emerging evidence sug-

gests that both necrotic and apoptotic cell death contribute to TBI-induced neuropathology (Yakovlev et al., 1997; Conti et al., 1998; Newcomb et al., 1999a). Caspase-3 is a calcium-independent cysteine protease and a critical effector of apoptotic cell death that can be activated through an

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extrinsic receptor-mediated caspase-8 pathway or through an intrinsic mitochondrial caspase-9 pathway (Budihardjo et al., 1999). Calpains are calcium-dependent cysteine proteases that contribute to apoptotic and necrotic cell death (Pike et al., 1998b; Zhao et al., 1999; Newcomb-Fernandez et al., 2001). Importantly, calpain and caspase-3 have been shown to be independently and concurrently activated after TBI in different brain regions and at various times post-injury (Pike et al., 1998a). In addition, recent evidence indicates that calpains and caspases can negatively or positively modulate each other's activity depending on the model system employed (Shi et al., 2000; Rami et al., 2000; Blomgren et al., 2001; Chen et al., 2001; Witkowski et al., 2002; McGinnis et al., 1999; Wang et al., 2000). Thus, therapeutic strategies targeting both calpain and caspase proteases may prove more efficacious than selective inhibition of a single family of cysteine proteases. For instance, our laboratory has demonstrated that combined administration of calpain and caspase inhibitors in an *in vitro* model of mixed primary neuronal and glial cells provides significantly greater protection than when given separately (Pike et al., 1998b). Moreover, Rami et al., (2000) showed synergistic protection with both inhibitors in an *in vivo* model of ischemia. Importantly, while inhibition of caspase-3 and calpain protein synthesis prevents apoptotic cell death in primary neuronal cultures (Pike et al., 1998b), systematic concurrent comparison of regional and temporal assessment of *de novo* mRNA synthesis of caspase-3 and calpain related gene products after TBI has never been examined.

Increased severity of injury is associated with worse outcome and greater levels of tissue pathology and cell death (Goodman et al., 1994; Cherian et al., 1994; Raghupathi et al., 2000). For instance, in *in vivo* models of TBI, severe injury correlates with larger lesion volume, increased intracranial pressure, and decreased cortical perfusion of the contralateral cortex (Goodman et al., 1994; Cherian et al., 1994). Moderate levels of experimental TBI result in a predominately necrotic morphology (Conti et al., 1998; Newcomb et al., 1999a), while equal levels of necrotic and apoptotic morphology are observed following mild lateral fluid percussion TBI (Raghupathi et al., 2000). Factors such as the presence and duration of ischemia and increased levels of calcium, nitric oxide, and glutamate may contribute to the influence of injury magnitude on the extent and type of cell death. *In vitro*, high concentrations of A23187 (a calcium ionophore), NMDA agonists, or nitric oxide cause necrosis while lower doses are associated with apoptosis (Gwag et al., 1999; Bonfoco et al., 1995). Similarly, different injury magnitudes of mechanical stretch injury (tensile strain) to septohippocampal cell cultures result in disparate patterns of calpain and caspase-3 activation and only a moderate level of injury causes concurrent activation of cal-

pain and caspase-3 (Pike et al., 2000). In addition, magnitude of injury is correlated with temporal increases and regional distribution of intracellular calcium after controlled cortical impact TBI in rats (Fineman et al., 1993). Thus, different magnitudes of injury may trigger divergent signaling pathways that ultimately lead to calpain and/or caspase-3 mediated cell death (Wang et al., 2000). Although both calpain and caspase-3 protease activation and mRNA expression are increased in response to TBI, no study has examined the effects of different injury magnitudes on mRNA expression levels of these two major effectors of cell death. Thus, this investigation tested the hypothesis that injury magnitude would differentially affect the temporal and regional distribution of mRNA expression levels of a variety of specific genes known to be involved in cell death after TBI.

Advances in the technology of quantitative reverse transcription polymerase chain (RT-PCR) reaction in the last 5 years allow the quantitation of PCR products in real time (Gibson et al., 1996; Heid et al., 1996). Furthermore, this technology allows the simultaneous evaluation of changes in multiple transcripts. This study examined temporal and quantitative changes in caspase-3, -8, -9, BID, calpain-1, calpain-2, and calpastatin message after three magnitudes of controlled cortical impact TBI in rats. This is the first study of concurrent changes in these seven transcripts after TBI and the first study to document the dynamic relationship of injury severity with expression levels post-injury. We demonstrate that TBI increases cortical and hippocampal expression of caspase-3, caspase-8, BID, calpain-1 (mu-calpain large subunit), calpain-2 (milli-calpain large subunit), and calpastatin, and cortical caspase-9 mRNA products. Minimal induction of caspase-9 was observed in the hippocampus. Importantly, we also demonstrate that the level of injury magnitude and time are important factors affecting regional and quantitative expression of the gene products. Examining concurrent changes in message level may shed light on the inter-relationship of these two important cysteine protease families and on the distinct but overlapping cascades to cell death after TBI. The quantitative and temporal differences between mild and severe injury magnitudes may lead to a more specific, and hence, more effective therapeutic intervention.

## MATERIALS AND METHODS

### *Rat Traumatic Brain Injury Model*

As previously described (Dixon et al., 1991), a controlled cortical impact device (CCI) was used to produce traumatic brain injury (TBI) in rodents. Cortical impact TBI results in cortical deformation within the vicinity of

the impactor tip associated with contusion and neuronal and axonal damage (Gennarelli, 1994; Meaney et al., 1994). Adult male (280–300 g) Sprague-Dawley rats (Harlan, Indianapolis, IN) were initially anesthetized with 4% isoflurane in a carrier gas of 1:1 O<sub>2</sub>/N<sub>2</sub>O (4 min) followed by maintenance anesthesia of 2.5% isoflurane in the same carrier gas. Core body temperature was monitored continuously by a rectal thermistor probe and maintained at 37 ± 1°C by placing an adjustable temperature-controlled heating pad beneath the rats. Animals were mounted in a stereotactic frame in a prone position and secured by ear and incisor bars. A midline cranial incision was made, the soft tissues were reflected, and a unilateral (ipsilateral to site of impact) craniotomy (7 mm diameter) was performed adjacent to the central suture, midway between bregma and lambda. The dura mater was kept intact over the cortex. Brain trauma in rats was produced by impacting the right cortex (ipsilateral cortex) with a 5-mm-diameter aluminum impactor tip (housed in a pneumatic cylinder) at a velocity of 3.5 m/sec with a 150-msec dwell time (compression duration). Compression depth was adjusted to produce different injury magnitudes: 1.0 mm for mild injury, 1.2 mm for moderate injury, or 1.6 mm for severe injury. Velocity was controlled by adjusting the pressure (compressed N<sub>2</sub>) supplied to the pneumatic cylinder. Velocity and dwell time were measured by a linear velocity displacement transducer (Lucas Shaevitz™ model 500 HR; Detroit, MI) that produces an analogue signal by a storage-trace oscilloscope (BK Precision, model 2522B; Placentia, CA). Three naive animals did not undergo any procedures prior to euthanasia. Sham-injured animals underwent the identical surgical procedure of a craniotomy

but did not receive an injury. Sham-injured animals were euthanized at 6, 24, 72, and 120 h after craniotomy. Two rats per sham group were used at four time points (eight rats). Three rats per injury group at three injury levels were used at four time points (36 rats) of 6, 24, 72, and 120 h after TBI. Appropriate pre- and post-injury management were maintained, and these measures complied with all guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the *Guide for the Care and Use of Laboratory Animals*.

#### Quantitative-PCR

Quantitative PCR analysis was performed as previously described in our laboratory (Tolentino et al., 2002). The ipsilateral cortex and hippocampus were dissected from each rat, and snap frozen in liquid nitrogen and stored at –80°C. Tissue was homogenized in 50 mg/mL TRIzol (Invitrogen, Carlsbad, CA), and total RNA was extracted according to the manufacturer's directions. After suspension in DEPC water, concentration was calculated by A<sub>260</sub> measurement for individual rat cortex and hippocampus.

For synthesis of first strand cDNA, 3 µg RNA of each sample was incubated with 0.01 M DTT, 0.5 mM dNTP mix (each dNTP), 0.5 µg oligo (dT) primer, and 200 U Superscript II reverse transcriptase at 37°C for 1 h. The resulting cDNA products were diluted fivefold with DEPC water. cDNA product (1 µL) was used per PCR reaction. Primer and probe sets for quantitative RT-PCR were designed using Primer3 interface available online from Whitehead Institute for Biomedical Research. The primer sequences and GeneBank accession number of relevant templates are shown in Table 1.

TABLE 1. PRIMER SEQUENCES FOR ALL GENES IN THIS STUDY

Target genes	Accession no.	Primer set	Primer sequences
Caspase-3	NM012922	Forward primer	5'-ggccctgaaatcacgaagtca
	Rat	Reverse primer	5'-ggcagtagtcgcctctgaag
Caspase-9	AF286006	Forward primer	5'-ctcaggccagaggttctcac
	Rat	Reverse primer	5'-caggaaccgctcttctgtc
Caspase-8	AF279308	Forward primer	5'-gacacaggttacagctctcc
	Rat	Reverse primer	5'-atcaagcaggctcgagttgt
BID	AF259503	Forward primer	5'-accgtgattccaccaagag
	Rat	Reverse primer	5'-gcaccctcagtcctctcat
Calpain-1	U53858	Forward primer	5'-cagtttgggagtggttaga
	Rat	Reverse primer	5'-gtcctcatcttgaccctca
Calpain-2	L09120	Forward primer	5'-cttcaggatcctctctcc
	Rat	Reverse primer	5'-ggcagttgtcattccactt
Calpastatin	X56729	Forward primer	5'-aatgctgcttggatgacctg
	Rat	Reverse primer	5'-acctgtactcagcaggactg
GAPDH	AF106860	Forward primer	5'-ggctgctctctctgtgac
	Rat	Reverse primer	5'-ggccgctgctctcaccac

Quantitative PCR was carried out using a Roche LightCycler (Roche Diagnostics, Indianapolis, IN) on the cDNA products: 1 × LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN), 1.5 mM MgCl<sub>2</sub> (Roche Diagnostics, Indianapolis, IN), 6% DMSO (FisherBrand), 0.5 μM each primer (Integrated DNA Technologies). Caspase 3, 8, 9, BID, calpastatin, and calpain-1 were run at 95°C for 5 min, 1 cycle of 95°C for 5 sec, 65°C for 10 sec, 72°C for 35 sec; 1 cycle of 95°C for 5 sec, 63°C for 10 sec, 72°C for 35 sec; and 38 cycles of 95°C for 5 sec, 60°C for 10 sec, 72°C for 35 sec. Calpain-2 was run at 95°C for 5 min, followed by 40 cycles of 95°C for 5 sec, 55°C for 10 sec, and 72°C for 35 sec. Additional reactions on known dilutions of cDNA template were performed during each LightCycler run to allow construction of a standard curve. The housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as the quality control for the RNA purification and reverse transcription.

### Statistical Analysis

For each type of mRNA studied, the average expression from three naive rats was set to 100%. The mean level (± SE) of each transcript at each timepoint from rats after craniotomies and after three injury magnitudes of TBI was calculated as a percent of the average of naive expression. Two-way ANOVA was used to examine main effects and interaction effects of time and injury magnitude. One-way ANOVA with contrast to do pairwise comparisons was used to determine significance between mean levels of mRNA at individual timepoints.

## RESULTS

RT-PCR was employed to examine changes in mRNA levels for caspase-3, caspase-9, caspase-8, BID, calpain-1, calpain-2, and calpastatin in rats subjected to CCI at three different levels of injury magnitude (1.0, 1.2, 1.6 mm) or to craniotomy. Statistically significant differences were found between the injured and sham-injured animals for the majority of mRNA transcripts examined.

### Caspase-3

Injury and time interacted to significantly affect levels of caspase-3 mRNA in the ipsilateral cortex ( $p < 0.0001$ ). Across time, severe (1.6 mm) and moderate (1.2 mm) injury increased levels of cortical caspase-3 mRNA compared to levels after sham injury ( $p \leq 0.001$  and  $p \leq 0.01$ , respectively) and levels were higher after severe (1.6 mm) injury than after mild (1.0 mm) injury ( $p < 0.05$ ).

Differences in levels of cortical caspase-3 mRNA at individual timepoints compared to sham-injury are shown in Figure 1. After severe (1.6 mm) injury, caspase-3 mRNA levels peaked at 24 h (529%,  $p < 0.05$ ) and remained significantly high at 72 h (460%,  $p < 0.05$ ), decreasing at 120 h (405%,  $p < 0.05$ ). After moderate injury (1.2 mm), caspase-3 mRNA levels were significantly increased 24 h (455%,  $p < 0.05$ ) and 72 h (475%,  $p < 0.05$ ) after injury. Caspase-3 mRNA levels following mild injury (1.0 mm) were not significantly different at any timepoint from sham-injury levels.

In the ipsilateral hippocampus, injury and time interacted to significantly affect levels of caspase-3 ( $p < 0.0001$ ). Across time, severe (1.6 mm) injury increased levels of hippocampal caspase-3 mRNA compared to levels after sham injury ( $p < 0.001$ ) and levels were higher after severe (1.6 mm) injury than after mild (1.0 mm) injury ( $p < 0.001$ ).

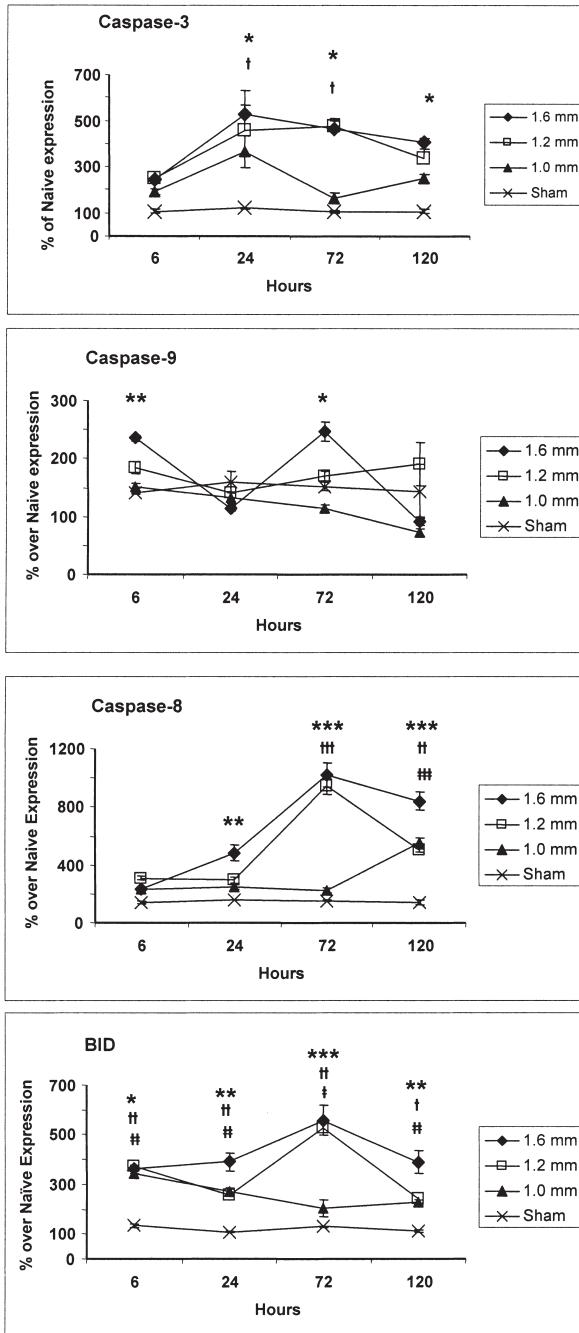
Differences in levels of hippocampal caspase-3 mRNA at individual timepoints compared to sham-injury are shown in Figure 2. Caspase-3 mRNA levels were significantly increased 24 h (207%,  $p < 0.01$ ) and 72 h (198%,  $p < 0.01$ ) after severe (1.6 mm) injury compared to sham-injury. Following moderate injury, caspase-3 mRNA levels were significantly increased at 72 h (198%,  $p < 0.01$ ) in the hippocampus. After mild injury, hippocampal caspase-3 mRNA did not increase compared to sham injury. Hippocampal caspase-3 mRNA levels were higher after severe (1.6 mm) injury than after moderate (1.2 mm) injury at 24 h ( $p < 0.01$ ) and than after mild (1.0 mm) injury at 24 h ( $p < 0.001$ ) and at 72 h ( $p < 0.01$ ). At 72 h, levels of caspase-3 mRNA were different between moderate (1.2 mm) and mild (1.0 mm) injury ( $p < 0.01$ ).

### Caspase-9

Injury and time interacted to significantly affect levels of caspase-9 mRNA in the ipsilateral cortex ( $p < 0.0001$ ). Across time, severe (1.6 mm) injury increased levels of cortical caspase-9 mRNA compared to levels after sham-injury ( $p \leq 0.05$ ) and levels were higher after severe (1.6 mm) injury than after mild (1.0 mm) injury ( $p < 0.01$ ).

Differences in levels of cortical caspase-9 mRNA at individual timepoints compared to sham-injury are shown in Figure 1. Caspase-9 mRNA levels in the ipsilateral cortex increased significantly at 6 h (235%,  $p < 0.01$ ) and 72 h (246%,  $p < 0.05$ ) after severe injury (1.6 mm). After moderate (1.2 mm) and mild (1.0 mm) injury, no significant change was noted compared to sham-injury. Cortical caspase-9 mRNA levels were significantly higher after severe (1.6 mm) injury than after mild (1.0 mm) injury ( $p < 0.05$ ) at 6 h and 72 h ( $p < 0.001$ ). At 120 h,

Ipsilateral Cortex



**FIG. 1.** Temporal expressions of caspase-3, caspase-9, caspase-8, and BID mRNA levels are affected by the severity of injury magnitude in the ipsilateral cortex. Mean levels of mRNA ( $\pm$  SEM) of caspase-3, caspase-9, caspase-8, and BID were analyzed 6, 24, 72, and 120 h following mild (1.0 mm), moderate (1.2 mm), and severe (1.6 mm) CCI injury by semi-quantitative PCR analysis. Mean levels of mRNA from rats after ( $\blacklozenge$ ) 1.6 mm injury; after ( $\square$ ) 1.2 mm injury; after ( $\blacktriangle$ ) 1.0 mm injury, or after ( $\times$ ) sham-craniotomy are expressed as a percentage of the average of mRNA from three naive rats set to 100%. One-way ANOVA with contrast to do pair-wise comparisons was used to determine significance between mean mRNA levels after sham injury compared to mean levels after injury at individual time-points. In general, greater injury severity was associated with higher relative increased expression of gene products over control levels. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.0001$  for 1.6 mm injury compared to sham-injured controls. † $p < 0.05$ , †† $p < 0.01$ , and ††† $p < 0.0001$  for 1.2-mm injury compared to sham-injured controls. ‡ $p < 0.05$ , ‡‡ $p < 0.01$ , and ‡‡‡ $p < 0.0001$  for 1.0 mm injury compared to sham-injured controls.

caspase-9 mRNA levels were higher after moderate (1.2 mm) injury than mild (1.0 mm) injury ( $p < 0.05$ ).

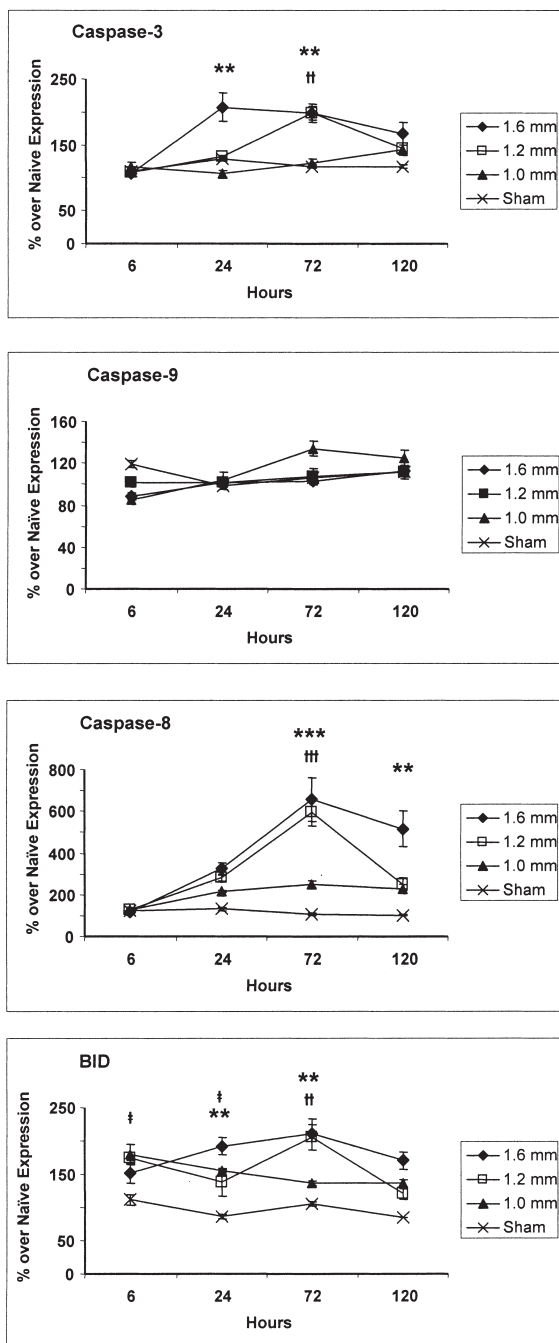
In the ipsilateral hippocampus, injury and time interacted to significantly affect levels of caspase-9 ( $p < 0.0001$ ). At individual timepoints, caspase-9 mRNA levels were significantly less at 6 h after severe (1.6 mm) and mild (1.0 mm) injury compared to sham-injury ( $p < 0.05$ ) (Fig. 2). At 72 h, caspase-9 mRNA was higher af-

ter mild (1.0 mm) injury than after moderate (1.2 mm) or severe (1.6 mm) injury ( $p < 0.05$ ).

*Caspase-8*

Injury and time interacted to significantly affect levels of caspase-8 mRNA in the ipsilateral cortex ( $p < 0.0001$ ) and across time, all treatment groups were significantly

Ipsilateral Hippocampus



**FIG. 2.** Temporal expressions of caspase-3, caspase-9, caspase-8, and BID mRNA levels are affected by the severity of injury magnitude in the ipsilateral hippocampus. Mean levels of mRNA ( $\pm$  SEM) of caspase-3, caspase-9, caspase-8, and BID were analyzed 6, 24, 72, and 120 h following mild (1.0 mm), moderate (1.2 mm), and severe (1.6 mm) CCI injury by semi-quantitative PCR analysis. Mean levels of mRNA from rats after ( $\blacklozenge$ ) 1.6 mm injury; after ( $\square$ ) 1.2 mm injury; after ( $\blacktriangle$ ) 1.0 mm injury, or after ( $\times$ ) sham-craniotomy are expressed as a percentage of the average of mRNA from three naive rats set to 100%. One-way ANOVA with contrast to do pair-wise comparisons was used to determine significance between mean mRNA levels after sham injury compared to mean levels after injury at individual timepoints. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.0001$  for 1.6 mm injury compared to sham-injured controls. † $p < 0.05$ , †† $p < 0.01$ , and ††† $p < 0.0001$  for 1.2 mm injury compared to sham-injured controls. ‡ $p < 0.05$ , ‡‡ $p < 0.01$ , and ‡‡‡ $p < 0.0001$  for 1.0 mm injury compared to sham-injured controls.

different from each other. Across time, levels of cortical caspase-8 mRNA were higher after severe (1.6 mm) injury than after moderate (1.2 mm) injury ( $p < 0.01$ ), after mild (1.0 mm) injury ( $p < 0.0001$ ) and after sham-injury ( $p < 0.0001$ ). Caspase-8 mRNA levels were higher after moderate (1.2 mm) injury than after mild (1.0 mm) injury ( $p < 0.001$ ) or after sham-injury ( $p < 0.0001$ ) and

levels were higher after mild (1.0 mm) injury than after sham-injury ( $p < 0.01$ ).

Differences in levels of cortical caspase-8 mRNA at individual timepoints compared to sham-injury are shown in Figure 1. Levels of caspase-8 mRNA were significantly increased 24, 72, and 120 h after severe injury and 72 and 120 h after moderate in the ipsilateral cortex com-

pared to after sham-injury. Following severe (1.6 mm) injury, caspase-8 mRNA levels increased at 24 h (484%,  $p < 0.01$ ), 72 h (1016%,  $p < 0.0001$ ), and 120 h (840%,  $p < 0.0001$ ). After moderate (1.2 mm) injury, caspase-8 mRNA levels were increased by 72 h (941%,  $p < 0.0001$ ), and 120 h (504%,  $p < 0.01$ ) after injury. Caspase-8 mRNA levels were significantly increased 120 h (553%,  $p < 0.0001$ ) following mild (1.0 mm) injury. Levels of caspase-8 mRNA were elevated after severe (1.6 mm) injury compared to moderate (1.2 mm) injury at 24 h ( $p < 0.05$ ) and 120 h ( $p < 0.05$ ) and compared to mild (1.0 mm) injury at 24 h ( $p < 0.05$ ), 72 h ( $p < 0.0001$ ), and 120 h ( $p < 0.01$ ). Cortical caspase-8 levels significantly differed between moderate (1.2 mm) and mild (1.0 mm) injury at 72 h ( $p < 0.0001$ ).

In the ipsilateral hippocampus, injury and time interacted to significantly affect levels of caspase-8 mRNA ( $p < 0.0001$ ). Across time, levels of hippocampal caspase-8 mRNA were higher after severe (1.6 mm) injury than after mild (1.0 mm) injury ( $p < 0.001$ ) and after sham-injury ( $p < 0.0001$ ). Caspase-8 mRNA levels were higher after moderate (1.2 mm) injury than after mild (1.0 mm) injury ( $p < 0.05$ ) or after sham-injury ( $p = 0.001$ ).

At individual timepoints, hippocampal caspase-8 mRNA levels significantly increased 72 h following both moderate (594%) and severe (657%) injury ( $p < 0.0001$ ) compared to sham-injury (Fig. 2). Levels of hippocampal caspase-8 did not differ after mild (1.0 mm) injury compared to sham-injury. At 72 h, caspase-8 mRNA was less after mild (1.0 mm) injury than after moderate (1.2 mm) ( $p < 0.01$ ) or severe (1.6 mm) injury ( $p < 0.001$ ). Hippocampal caspase-8 mRNA was higher after severe (1.6 mm) injury at 120 h than after sham injury ( $p < 0.001$ ) or after moderate (1.2 mm) injury ( $p < 0.05$ ).

## BID

Injury and time interacted to significantly affect levels of BID mRNA in the ipsilateral cortex ( $p < 0.0001$ ) and across time, all treatment groups were significantly different from each other. Across time, levels of cortical BID mRNA were higher after severe (1.6 mm) injury than after moderate (1.2 mm) injury ( $p < 0.05$ ), after mild (1.0 mm) injury ( $p < 0.0001$ ) and after sham-injury ( $p < 0.05$ ). BID mRNA levels were higher after moderate (1.2 mm) injury than after mild (1.0 mm) injury ( $p < 0.05$ ) or after sham-injury ( $p < 0.0001$ ) and levels were higher after mild (1.0 mm) injury than after sham-injury ( $p < 0.001$ ).

Differences in levels of cortical BID mRNA at the individual timepoints compared to sham-injury are shown in Figure 1. BID mRNA levels significantly increased at all three injury levels at all time points in the ipsilateral cortex. After 1.6 mm injury, BID mRNA levels significantly increased at 6 h, (360%,  $p < 0.05$ ) (at 24 h, 390%,

$p < 0.001$ ), at 72 h, (557%,  $p < 0.001$ ), and at 120 h (388%,  $p < 0.001$ ). After moderate (1.2 mm) injury, BID mRNA levels significantly increased at 6 h (372%,  $p < 0.001$ ), at 24 h (254%  $p < 0.001$ ), at 72 h (526%,  $p < 0.001$ ) and at 24 h (240%,  $p < 0.05$ ). After mild (1.0 mm) injury, BID mRNA levels increased at 6 h (341%,  $p < 0.001$ ), at 24 h (268%,  $p < 0.001$ ), at 72 h (203%,  $p < 0.05$ ), and at 120 h (227%,  $p < 0.001$ ) after injury. Cortical BID mRNA levels were significantly less after mild (1.0 mm) injury than after severe (1.6 mm) injury at 72 h ( $p < 0.0001$ ) and at 120 h ( $p < 0.05$ ) and less than after moderate (1.2 mm) injury at 72 h ( $p < 0.0001$ ).

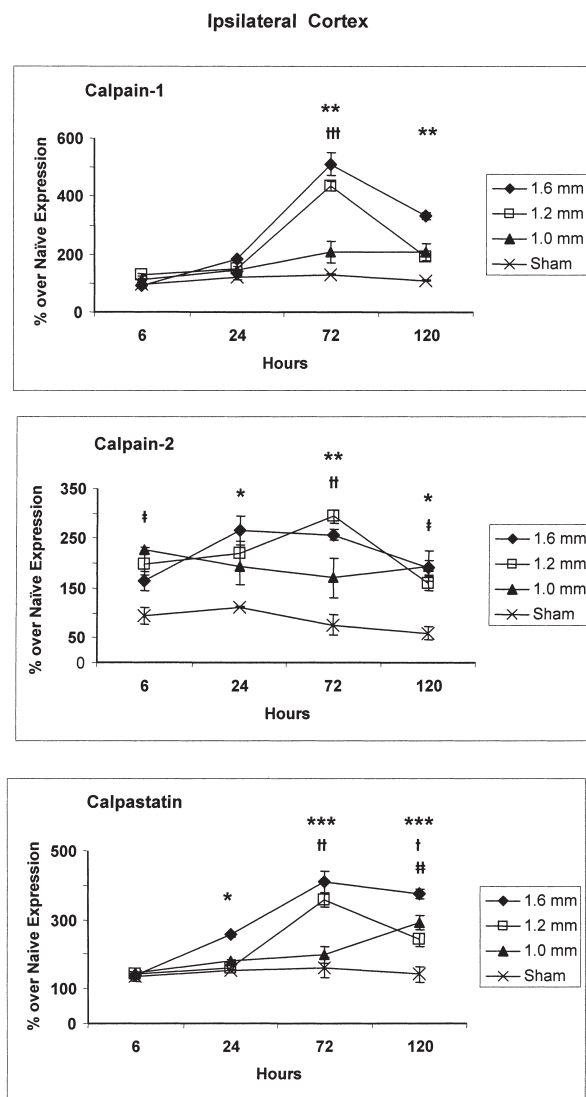
In the ipsilateral hippocampus, injury and time interacted to significantly affect levels of BID mRNA ( $p < 0.0001$ ). Across time, hippocampal BID mRNA levels were increased after severe (1.6 mm) injury ( $p < 0.0001$ ), moderate (1.2 mm) injury ( $p < 0.001$ ), and mild (1.0 mm) injury ( $p < 0.01$ ) compared to after sham injury.

At individual timepoints, BID mRNA levels in the ipsilateral hippocampus after severe (1.6 mm) injury increased significantly at 24 h (192%,  $p < 0.01$ ) and at 72 h (210%,  $p < 0.01$ ) (Fig. 2) compared to levels after sham injury. After moderate (1.2 mm) injury, BID mRNA levels increased significantly at 72 h after TBI (205%,  $p < 0.01$ ). After mild (1.0 mm) injury, BID mRNA levels increased significantly (180%,  $p < 0.05$ ) at 6 h and (155%,  $p < 0.05$ ) at 24 h. At 72 h, hippocampal BID mRNA was less after mild (1.0 mm) injury than after moderate (1.2 mm) or severe (1.6 mm) injury ( $p < 0.05$ ).

## Calpain-1

Injury and time interacted to significantly affect levels of calpain-1 (mu-calpain large subunit) mRNA in the ipsilateral cortex ( $p < 0.0001$ ) and across time, all treatment groups were significantly different from each other. Across time, calpain-1 mRNA levels were higher after severe (1.6 mm) injury than after moderate (1.2 mm) injury ( $p < 0.05$ ), after mild (1.0 mm) injury ( $p < 0.0001$ ) and after sham-injury ( $p < 0.0001$ ). Calpain-1 mRNA levels were higher after moderate (1.2 mm) injury than after mild (1.0 mm) injury ( $p < 0.05$ ) or after sham injury ( $p < 0.001$ ), and levels were higher after mild (1.0 mm) injury than after sham-injury ( $p < 0.05$ ).

Differences in levels of cortical calpain-1 mRNA at individual timepoints compared to sham-injury are shown in Figure 3. Calpain-1 mRNA levels in the ipsilateral cortex significantly increased at 72 h (511%,  $p < 0.001$ ) and at 120 h (330%,  $p < 0.001$ ) after severe (1.6 mm) injury. Calpain-1 mRNA levels increased at 72 h (434%,  $p < 0.0001$ ) after moderate (1.2 mm) injury. There was no significant change after mild (1.0 mm) injury in calpain-1 mRNA levels compared to after sham-injury. Calpain-1 mRNA levels were higher after severe (1.6 mm) injury



**FIG. 3.** Temporal expression of calpain-1, calpain-2, and calpastatin mRNA levels are affected by the severity of injury magnitude in the ipsilateral cortex. Mean levels of mRNA ( $\pm$  SEM) of calpain-1, calpain-2, and calpastatin were analyzed 6, 24, 72, and 120 h following mild (1.0 mm), moderate (1.2 mm), and severe (1.6 mm) CCI injury by semi-quantitative PCR analysis. Mean levels of mRNA from rats after ( $\blacklozenge$ ) 1.6 mm injury; after ( $\square$ ) 1.2 mm injury; after ( $\blacktriangle$ ) 1.0 mm injury, or after ( $\times$ ) sham-craniotomy are expressed as a percentage of the average of mRNA from three naive rats set to 100%. One-way ANOVA with contrast to do pair-wise comparisons was used to determine significance between mean mRNA levels after sham-injury compared to mean levels after injury at individual timepoints. As with the caspases, greater injury severity was associated with higher relative increased expression of gene products over control levels. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.0001$  for 1.6 mm injury compared to sham-injured controls. † $p < 0.05$ , †† $p < 0.01$ , and ††† $p < 0.0001$  for 1.2 mm injury compared to sham-injured controls. ‡ $p < 0.05$ , ‡‡ $p < 0.01$ , and ‡‡‡ $p < 0.0001$  for 1.0 mm injury compared to sham-injured controls.

than after mild (1.0 mm) injury at 72 h ( $p < 0.0001$ ) and 120 h ( $p < 0.05$ ).

In the ipsilateral hippocampus, injury and time interacted to significantly affect levels of calpain-1 mRNA ( $p < 0.0001$ ). Across time, hippocampal calpain-1 mRNA was significantly increased after severe (1.6 mm) and moderate (1.2 mm) injury ( $p < 0.01$ ) compared to after sham injury.

At individual timepoints, calpain-1 mRNA levels significantly increased after severe (1.6 mm) injury at 72 h (207%,  $p < 0.05$ ) in the ipsilateral hippocampus (Fig. 4). After moderate (1.2 mm) injury, calpain-1 mRNA levels significantly increased at 24 h (198%,  $p < 0.05$ ) and 72 h (190%,  $p < 0.05$ ). There was no significant change in calpain-1 mRNA levels after the mildest (1.0 mm) injury.

### Calpain-2

Injury and time interacted to significantly affect levels of calpain-2 mRNA in the ipsilateral cortex ( $p < 0.0001$ ). Across time, calpain-2 mRNA levels were increased after severe (1.6 mm) injury ( $p < 0.001$ ), moderate (1.2 mm) injury ( $p < 0.001$ ), and mild (1.0 mm) injury ( $p < 0.01$ ) compared to after sham injury.

At individual time points, cortical calpain-2 mRNA levels after severe (1.6 mm) injury increased significantly at 24 h (266%,  $p < 0.05$ ), 72 h (256%,  $p < 0.01$ ), and 120 h (190%,  $p < 0.05$ ) (Fig. 3). After moderate (1.2 mm) injury, calpain-2 levels increased significantly (294%,  $p < 0.01$ ) at 72 h. Calpain-2 mRNA levels after the mildest (1.0 mm) injury were significantly higher than after sham injury at (229.2%,  $p < 0.05$ ) 6 h and (195.2,  $p < 0.05$ ) 120 h. Calpain-2 mRNA levels differed between moderate (1.2 mm) and mild (1.0 mm) injury at 72 h ( $p < 0.05$ ).

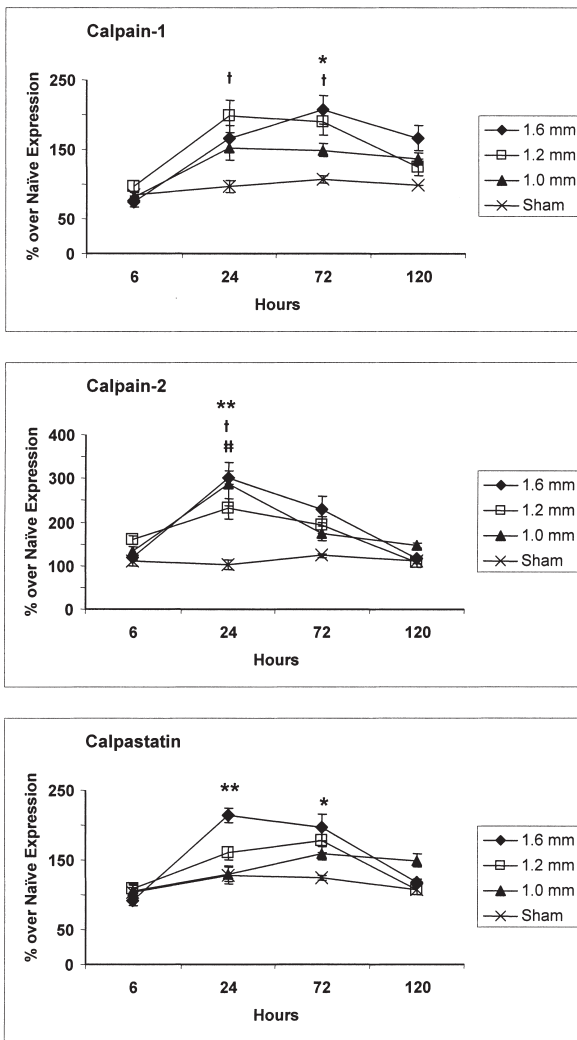
In the ipsilateral hippocampus, injury and time interacted to significantly affect levels of calpain-2 ( $p < 0.0001$ ). Across time, hippocampal calpain-2 mRNA was significantly less after sham-injury than after mild (1.0 mm) injury ( $p < 0.05$ ) or after severe (1.6 mm) injury ( $p < 0.01$ ).

At individual timepoints, hippocampal calpain-2 mRNA levels at 24 h after TBI were significantly greater after severe (1.6 mm) injury (302%,  $p < 0.001$ ), moderate (1.2 mm) injury (230%,  $p < 0.05$ ), and after mild (1.0 mm) injury (287%,  $p < 0.01$ ) compared to sham injury (Fig. 4).

### Calpastatin

Injury and time interacted to significantly effect levels of calpastatin mRNA in the ipsilateral cortex ( $p < 0.0001$ ). Across time, calpastatin mRNA levels were increased after severe (1.6 mm) injury ( $p < 0.0001$ ), mod-

Ipsilateral Hippocampus



**FIG. 4.** Temporal expression of calpain-1, calpain-2, and calpastatin mRNA levels are affected by the severity of injury magnitude in the ipsilateral hippocampus. Mean levels of mRNA ( $\pm$  SEM) of calpain-2, calpain-1 and calpastatin were analyzed 6, 24, 72, and 120 h following mild (1.0 mm), moderate (1.2 mm), and severe (1.6 mm) CCI injury by semi-quantitative PCR analysis. Mean levels of mRNA from rats after (◆) 1.6 mm injury; after (□) 1.2 mm injury; after (▲) 1.0 mm injury, or after (×) sham-craniotomy are expressed as a percentage of the average of mRNA from three naive rats set to 100%. One-way ANOVA with contrast to do pair-wise comparisons was used to determine significance between mean mRNA levels after sham-injury compared to mean levels after injury at individual timepoints. As with the caspases, greater injury severity was associated with higher relative increased expression of gene products over control levels. Relative increased expression of calpastatin, the endogenous inhibitor of calpains, generally paralleled relative increased expression of calpain-1 and -2. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.0001$  for 1.6 mm injury compared to sham-injured controls. † $p < 0.05$ , †† $p < 0.01$ , and ††† $p < 0.0001$  for 1.2 mm injury compared to sham-injured controls. ‡ $p < 0.05$ , ‡‡ $p < 0.01$ , and ‡‡‡ $p < 0.0001$  for 1.0 mm injury compared to sham-injured controls.

erate (1.2 mm) injury ( $p < 0.01$ ), and mild (1.0 mm) injury ( $p < 0.05$ ) compared to after sham injury. Calpastatin mRNA levels were significantly higher after severe (1.6 mm) injury than after moderate (1.2 mm) injury ( $p < 0.01$ ) and after mild (1.0 mm) injury ( $p < 0.0001$ ).

At individual timepoints, calpastatin mRNA levels significantly increased at 24 h (258%,  $p < 0.05$ ), at 72 h (411%,  $p < 0.0001$ ), and at 120 h (375%,  $p < 0.0001$ ) in the ipsilateral cortex after severe (1.6 mm) injury compared to after sham-injury (Fig. 3). After moderate (1.2 mm) injury, calpastatin mRNA levels significantly increased (357%,  $p < 0.001$ ) at 72 h and (243%,  $p < 0.05$ ) at 120 h. After mild (1.0 mm) injury, calpastatin mRNA levels increased (292%,  $p < 0.01$ ) at 120 h. Higher levels of calpastatin mRNA were found after severe (1.6

mm) injury than after moderate (1.2 mm) injury at 24 h ( $p < 0.05$ ) and 120 h ( $p < 0.01$ ). At 72 h, calpastatin mRNA levels were lower after mild (1.0 mm) injury than after moderate (1.2 mm) injury ( $p < 0.001$ ) or after severe (1.6 mm) injury ( $p < 0.0001$ ).

In the ipsilateral hippocampus, injury and time interacted to significantly effect levels of calpastatin mRNA ( $p < 0.0001$ ). Across time, hippocampal calpastatin mRNA levels were only elevated after severe (1.6 mm) injury ( $p < 0.01$ ).

At individual timepoints, hippocampal calpastatin mRNA levels significantly increased (214%,  $p < 0.01$ ) at 24 h and (197%,  $p < 0.05$ ) at 72 h after severe (1.6 mm) injury (Fig. 4). Calpastatin mRNA levels did not increase after moderate (1.2 mm) or mild (1.0 mm) injury. At 24 h, hippocampal calpastatin mRNA was higher af-

ter severe (1.6 mm) injury than after mild (1.0 mm) injury ( $p < 0.01$ ).

## DISCUSSION

This is the first study to concurrently examine the relative changes in gene expression of the calpain and caspase cysteine protease families after TBI. While numerous studies have shown activation of these proteins following TBI, no study has concurrently examined these proteins at a molecular level following this type of injury. We report the relative response of each gene to injury magnitude across time in the injured cortex and hippocampus after TBI. Importantly, the level of injury magnitude significantly affected the temporal and regional expression of genes that were examined. The temporal pattern of increased mRNA expression suggests that the delayed and sustained increases in mRNA expression of calpains and cysteine proteases may have an integral role in delayed neuronal cell death after TBI.

### *Caspase-3*

Caspase-3 is activated upstream by caspase-8 and caspase-9 and is considered a key executioner of apoptosis (Eldadah and Faden, 2000). Similar to the current study, caspase-3 mRNA peaked 24 h after injury in the ipsilateral cortex and hippocampus (Yakovlev et al., 1997). Activation of constitutively expressed caspase-3 may be involved in the acute period, while *de novo* synthesis of caspase-3 may be required for delayed apoptotic cell death.

### *Caspase-9*

Activation of the intrinsic caspase-9-mediated pathway following TBI (Nathaniel et al., 2000; Knoblach et al., 2002; Yakovlev et al., 2001) has been suggested to be due to loss of mitochondrial integrity (Verweij et al., 1997; Xiong et al., 1997; Sullivan et al., 1998; Verweij et al., 2000) and release of cytochrome-c (Buki et al., 2000; Thompson et al., 2000). A small but significant increase in caspase-9 mRNA was seen in the ipsilateral cortex across time after 1.6 mm (severe) injury magnitude, while no increases were detected in the ipsilateral hippocampus, a finding similar to that observed in a focal model of ischemia (Harrison et al., 2001). The increased caspase-9 protein activity observed after lateral fluid percussion injury (Knoblach et al., 2002; Yakovlev et al., 2001) and CCI (Nathaniel et al., 2000) may be the result of differences in constitutive versus *de novo* expression of caspase-9 protein.

### *Caspase-8*

FAS (Beer et al., 2000; Lenzlinger et al., 2002; Matsushita et al., 2000) and TNF (Shohami et al., 1994; Fan et al., 1996) both increase after TBI and likely activate the extrinsic pathway employing caspase-8 and BID. Caspase-8 mRNA upregulation supports recent work by Beer et al. (2001), who reported an increase in caspase-8 mRNA from 1 to 72 h in the ipsilateral cortex after CCI. In the present study, increased injury magnitude plays a key role in the temporal peak of caspase-8 mRNA.

### *BID*

This is the first paper to report an increase in both cortical and hippocampal BID message after TBI. Increased levels of caspase-8 and BID message after TBI further suggest that the extrinsic pathway may be a vital part of cell death after TBI. Caspase-8 may directly activate caspase-3 or indirectly through BID release mitochondrial factors that activate caspase-9 and caspase-3 (Budihardjo et al., 1999). Recent work has shown that BID protein was increased from 6 h to 7 days after controlled cortical impact (Franz et al., 2002). BID appears to have a significant contribution to ischemic neuronal cell death (Benchoua et al., 2001; Plesnila et al., 2001), and the continued increase in BID mRNA likely has an equally significant role in delayed or secondary neuronal cell death after TBI.

### *Calpain-1 and -2*

Calpain, a cysteine protease, is activated in necrotic and apoptotic models of cell death (Wang et al., 2000; Chan and Mattson, 1999).

The relative gene expression of calpain-2 markedly increased in the hippocampus and peaked 24 h after all three injury magnitudes studied. A role for calpain-2 in the activation of caspase-3 has been suggested *in vitro* and *in vivo* in a neonatal hypoxia-ischemia model (Blomgren et al., 2001). Importantly, caspase-3 and calpain-2 had similar temporal peaks in response to injury magnitude in the ipsilateral cortex and their relationship warrants further attention.

Rapid activation of calpain after TBI most likely stems from activation of constitutively expressed calpains. However, *de novo* synthesis of calpains may be required for delayed apoptotic cell death. In addition, calpains may play a role in membrane repair and resealing as well as in dendritic remodeling after injury (Faddis et al., 1997). Thus, increased mRNA levels of calpain may be indicative of both a pathological as well as a normal physiological role. Additional studies are required to clarify calpain's complex role in TBI pathology.

*Calpastatin*

Calpastatin is an endogenous inhibitor of calpain. Previous studies by our group showed that calpastatin protein increased as calpain activity decreased in a CCI model of TBI (Newcomb et al., 1999b); however, a reciprocal relationship was not detected at the message level between calpastatin and calpain-1 and -2. Rather, calpastatin mRNA had a similar pattern of increase to calpain-1 mRNA across all three injury magnitudes in the ipsilateral cortex and hippocampus. Increases in mRNA levels for both calpains and calpastatins likely reflect the tight regulatory control of calpastatin and calpains at the cellular level.

**CONCLUSION**

In conclusion, we have presented the first studies to examine concurrent temporal and regional changes in mRNA levels of calpains and caspases after three levels of injury magnitude. Importantly, the magnitude of injury affected the regional distribution and temporal pattern of mRNA expression. The significant and concurrent increases in caspase-8, caspase-3, and BID implicate the extrinsic pathway for a promising target of therapeutic intervention. It is hoped that this information will further our ability to understand the pathophysiological response to different magnitudes of injury and optimize the therapeutic window. Future studies are required to address the important implications raised by this study—that injury severity causes differential expression of numerous effectors of cellular pathology and that a targeted therapeutic approach must take issues of injury severity and time post injury into account.

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