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Traumatic mechanical injury to the hippocampus in vitro causes regional caspase-3 and calpain activation that is influenced by NMDA receptor subunit composition

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Apoptotic or necrotic cell death in the hippocampus is a major factor underlying the cognitive impairments following traumatic brain injury. In this study, we examined if traumatic mechanical injury would produce regional activation of calpain and caspase-3 in the in vitro hippocampus and studied how the mechanically induced activation of NR2A and NR2B containing *N*-methyl-D-aspartate receptors (NMDARs) affects the activation of these proteases following mechanical injury. Following a 75% stretch, significant levels of activated caspase-3 and calpain-mediated spectrin breakdown products were evident only in cells within the dentate gyrus, and little co-localization of the markers was identified within individual cells. After 100% stretch, only calpain activation was observed, localized to the CA3 subregion 24 h after stretch. At moderate injury levels, both caspase-3 and calpain activation was attenuated by blocking NR2B containing NMDARs prior to stretch or by blocking all NMDARs prior to stretch injury. Treatment with an NR2A selective NMDAR antagonist had little effect on either activated caspase-3 or Ab38 immunoreactivity following moderate injury but resulted in the appearance of activated caspase-3 in the dentate gyrus following severe mechanical stretch. Together, these studies suggest that the injury induced activation of NR2A containing NMDARs functions as a pro-survival signal, while the activation of NR2B containing NMDARs is a competing, anti-survival, signal following mechanical injury to the hippocampus.

Keywords: Traumatic brain injury; Organotypic; Hippocampus; Caspase-3; Calpain; *N*-methyl-D-aspartate receptor

Introduction

The common neuropathological changes in the hippocampus in human closed head injury and experimental models of traumatic brain injury (TBI) lead many to suggest that the hippocampus is selectively vulnerable after TBI (Kotapka et al., 1994; Smith et al., 1991). The potential selective vulnerability plays a prominent role when developing treatment strategies for TBI, since cell death in the hippocampus is thought to be one underlying component for the cognitive impairments that occur in human TBI (Enriquez and Bullock, 2004; Maas, 2001). Both apoptotic and necrotic cell death mechanisms are common features in the traumatically injured brain, although the distribution of these pathways among the different cell types and regions of the brain is still being fully described (reviewed in Raghupathi, 2004; Yakovlev and Faden, 2001).

Past studies examining cell death following TBI have concentrated mainly on the activation of two distinct proteases-caspase-3 and calpain-as precursors to cell death. Morphologic evidence of apoptosis following TBI is supported by observations of neuronal and glial activation of caspase-3 in the cortex (Clark et al., 2000; Beer et al., 2000) and in the hippocampus (Keane et al., 2001). In comparison, the role of calpains is often suggested as a primary mediator of necrotic cell death following CNS injury (Wang, 2000), supported by biochemical and immunohistochemical evidence of calpain activation in injured brain regions exhibiting necrosis (Saatman et al., 1996; Kampfl et al., 1996; Newcomb et al., 1999). In some experimental models, areas demonstrating apoptosis occur in regions that contain necrotic cells (Hicks et al., 1996; Dietrich et al., 1994; Conti et al., 1998; Pike et al., 1998, 2000). In other experimental models, the high levels of mechanical stress in the cortex during injury (Ueno et al., 1995) produce

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predominantly necrotic cell death (Sutton et al., 1993; Newcomb et al., 1999).

Across all models, the activation of the glutamatergic receptors and, in particular, the *N*-methyl-D-aspartate receptors (NMDARs) subtype is implicated in mediating traumatic neuronal death (see Arundine and Tymianski, 2004 for recent review). In several laboratories, the treatment of brain-injured animals with NMDAR antagonists results in a decreased cell loss in both the hippocampus and the cortex (Bernert and Turski, 1996; Faden et al., 1989; Hicks et al., 1993; Hayes et al., 1988; Okiyama et al., 1998; Shapira et al., 1990; Toulmond et al., 1993). Recently, though, the role of NMDARs in playing only a detrimental role on cell fate has been questioned. New evidence suggests a more complex role for the NMDAR, where a stimulation of the receptor as early as 24 h following TBI can result in a significant improvement in impairments following TBI (Biegon et al., 2004).

One potential reason for the complex role of the NMDAR in cell death after TBI is that the activation of different NMDAR populations can stimulate either pro- or anti-survival downstream signaling pathways. In the hippocampus, the NMDAR is composed of 2 NR1 subunits and 2–3 additional subunits that are exclusively NR2B, NR2A, or a mix of NR2A and NR2B (see Cull-Candy et al., 2001 for review). Recent data show that the preferential activation of NR2A containing receptors located synaptically can promote pro-survival signaling in neurons and provide protection against apoptosis induced by trophic factor withdrawal (Papadia et al., 2005). In contrast, the stimulation of NR2B containing NMDARs located extrasynaptically provides a counterbalancing role on cell fate by promoting a preferred route for extracellular calcium to the mitochondria and causing a breakdown of mitochondria membrane potential (Sattler et al., 1998; Hardingham et al., 2002). To date, though, there is no information on whether NMDAR can preferentially mediate either necrotic or apoptotic cell death after traumatic mechanical injury nor is it known if NR2A or NR2B containing receptors play different roles in either necrotic or apoptotic cell death cascades.

In this study, we assessed the role of NMDAR on mediating the activation of both calpain and caspase-3 following mechanical injury and the relative role of these two subunits in the hippocampus (NR2A, NR2B) on the activation of these two proteases following injury. Our hypotheses were that (a) a uniform stretch injury to the organotypic hippocampus would produce activation of either caspase-3 or calpain in only selected subfields of the hippocampus, (b) the activation of both proteases would be dependent upon NMDA receptor activation, and (c) the activation of NR2A containing receptors was key in minimizing the extent of caspase-3 activation in the hippocampus, while the selective inhibition of NR2B containing NMDAR would be effective in reducing the extent of both calpain and caspase-3 activation after mechanical injury. To examine this hypothesis, an organotypic culture of the hippocampus was subjected to a single, transient stretch and evaluated for regional activation of calpains and caspase-3 using immunohistochemical methods. The results demonstrate that (a) both calpain and caspase-3 activation are dependent on the severity of the injury and are activated to different levels in select subfields of the hippocampus, (b) calpains and caspase-3 are activated in separate cell populations, and (c) stretch-induced activation of NMDARs leads to activation of calpain and caspase-3. Importantly, blockade of the NR2B subunit results in reduced calpain and caspase-3 activation, while blockade of the synaptically localized NR2A subunit increased caspase-3 and calpain activation. Collectively, these data demonstrate that severity of mechanical injury regulates the regional extent of traumatic protease activation and identifies the delicate balance between synaptic and extrasynaptic NMDARs in the activation of both apoptosis- and necrosis-related pathways following TBI.

Materials and methods

Organotypic culture isolation

All procedures involving animal use were approved by the Institutional Animal Use and Care Committee at the University of Pennsylvania. The methods for isolating organotypic cultures are similar to those described previously, with some modifications (Morrison et al., 2000). Briefly, brains were aseptically removed from 6-day-old CD®IGS rat pups (Charles River Laboratories, Inc., Wilmington, MA), transferred to ice-cold Gey's salt solution (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 6.5% glucose, and sectioned coronally (350-μm sections) on a McIlwain Tissue Chopper (Brinkman Instruments, Westbury, NY). The slices were carefully separated, and the hippocampus was dissected out of each section. Hippocampal slices were plated onto a flexible silicone membrane (Dow Corning, Midland, MI) stretched across the bottom of a stainless steel well and treated with poly-L-lysine (20 μg/ml, Sigma) and laminin (50 μg/ml, BD Biosciences, Bedford, MA). Neurobasal-A media (Invitrogen Corp., Carlsbad, CA) supplemented with 6.5% glucose, B-27 (Invitrogen), and 1 μg/ml L-glutamine (Sigma) was added to the culture well. Cultures were kept in a humidified incubator at 37°C, 5% CO_2 on a rocker (Elmeco Engineering, Rockville, MD) for 10 days in vitro (DIV) with media changes occurring every 2–3 days.

In vitro model of TBI

To controllably stretch the organotypic cultures, we used a custom-designed injury device described previously (Lusardi et al., 2004). Cultures were placed on top of a steel plate and positioned over a 6 mm × 18-mm rectangular cutout section on the plate (Fig. 1A). Pressurizing the top surface of the membrane caused only the section over the rectangular cutout region to deform. The slit was positioned to deliver a uniform, uniaxial stretch the hippocampal slice in each culture. Each slice culture was aligned to match the direction of stretch to the medial–lateral axis of the slice culture. A transient pressure pulse was delivered to mimic the rapid deformations that occur in TBI (Meaney et al., 1995), and the pressure was adjusted to deliver a precise amount of stretch to the isolated hippocampal slice cultures (Fig. 1B). For these experiments, we used pressure to achieve stretch levels of 50, 75, or 100 ± 2%. We verified these stretch levels by tracking the position of beads on the surface of the membrane during the pressurization, measuring the bead motion to calculate strain applied to the slice cultures (Lusardi et al., 2004). At these levels of mechanical injury, we observed no signs of detachment from the membrane, and there was no obvious tearing of the tissue. Therefore, these levels of mechanical injury approximate clinical injuries that cause impairment without immediate tearing or laceration in the hippocampal region. Two control groups were also included—a naïve sham and a 'pressure' sham, where pressure was

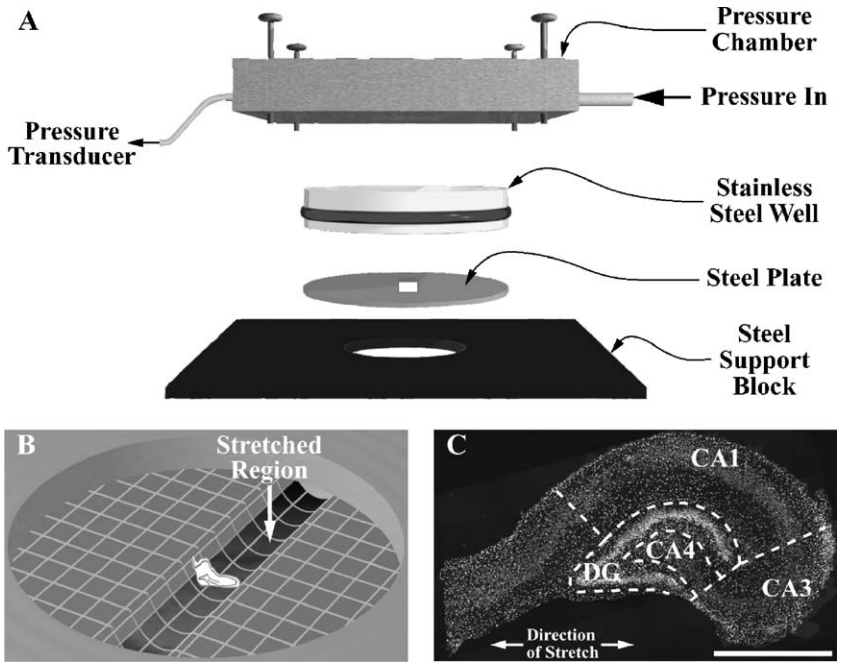

Fig. 1. (A) Overview of the stretch injury device, consisting of a stainless steel well that is placed between a stainless steel chamber and a steel base plate. (B) Hippocampal cultures on a flexible elastic membrane were exposed to a transient pressure. The culture was aligned along the axis of stretch and deformed along with the substrate. (C) The injured tissue was sectioned, stained, and the CA1, CA3, dentate gyrus (DG), and CA4 regions were analyzed for apoptotic and necrotic cell death. Scale bar = 1 mm. Overview (A) and representative culture on membrane (B) not drawn to scale.

applied to the culture, but the underlying membrane was not allowed to deflect.

In a selected subset of experiments, we preincubated cultures with compounds to inhibit either all NMDARs (D,L-2-amino-5-phosphonovaleric acid (APV), 25 μM, Sigma), NR2A containing NMDARs (500 nM NVP-AMM077) that are localized synaptically in hippocampal neurons (Tovar and Westbrook, 1999), or NR2B containing receptors located extrasynaptically (3 μM ifenprodil (Tovar and Westbrook, 1999)). All compounds were added to media 5 min prior to injury and remained on the cultures until fixation at 24 h.

At 2, 6, and 24 h post-stretch, cultures were fixed for 30 min with 4% paraformaldehyde (Sigma) at room temperature and stored for 48–72 h in 50% sucrose at 4°C. The tissue was sectioned on a frozen sliding microtome into 20-μm thick sections. The sections were then stored in cryoprotectant at −20°C until the time of staining.

Double-label immunofluorescence

To identify calpain and caspase-3 activation in hippocampal tissue, tissue was double-labeled for markers of the two pathways. Cells showing calpain activation were identified using an antibody to detect calpain-mediated spectrin breakdown products (Ab38, R. Siman). Caspase-3 activation was identified using a commercial antibody (Promega Corp., Madison, WI). Because both of these antibodies were generated in rabbit hosts, direct fluorescent labeling via tyramide signal amplification (TSA) (Perkin-Elmer Life Sciences, Boston, MA) was used with the activated caspase-3 antibody. Ab38 was visualized with a secondary antibody conjugated to Alexa 594 (Molecular Probes, Inc., Eugene, OR). Control experiments were done to ensure that the concentration of activated caspase-3 antibody was high enough to generate a signal using TSA, but low enough so as to not generate a signal when the cross-reacting secondary antibody to Ab38 was applied (Teramoto et al., 1998; Wang et al., 1999). Moreover, camptothecin (10 μM) was used to elicit a maximum caspase-3 activation, while maitotoxin (1 nM) was used as a positive control for AB38(+) immunoreactivity.

Sections were rinsed three times in 0.1 M Tris-buffered saline + 0.1% Triton X-100 (TBST) and endogenous peroxidases were quenched using a 30-min incubation in 3% H_2O_2 in 50/50 ddH_2O/MeOH. Sections were then rinsed three times in TBST and blocked for 20 min in TSA blocking buffer. Next, sections were washed twice in 0.1 M Tris and incubated overnight at 4°C in 1:6000 activated caspase-3 primary antibody diluted in 5% normal goat serum (NGS) in TBST. The following day, the sections were rinsed three times in TBST and incubated for 1 h in 1:1000 biotinylated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted in TSA blocking buffer, followed by incubation for 1 h in 1:200 dilution of streptavidin–horseradish peroxidase in TSA blocking buffer. Following this step, the sections were rinsed three times and incubated in a 1:300 dilution of the fluorescein–tyramide reagent in 1:5 amplification diluent and 0.1 M Tris for 10 min. Finally, the tissue was rinsed three times with TBST and blocked in 5% NGS in TBST for 1 h. After blocking, the sections were incubated overnight at 4°C in Ab38 primary antibody (1:6000) using 5% NGS in TBST. The next day, sections were rinsed three times with TBST and incubated for 1 h in 1:500 goat anti-rabbit Alexa-594 secondary antibody diluted in 5% NGS in TBST. After washing the sections three times, they were counterstained with Hoechst 33342 (2.5 μg/ml in TBST, Molecular Probes). A total of three non-adjacent sections were stained from each organotypic culture.

To determine the cell type of activated caspase-3 positive cells, double-label immunohistochemistry with TSA was employed.

Procedures identical to those described previously were used, with cell-specific antibodies replacing Ab38 in the TSA procedure. Antibodies specific to neurons (1:200 mouse anti-NeuN, Chemicon International, Inc., Temecula, CA), astrocytes (1:1000 rabbit anti-GFAP, Chemicon), and oligodendrocytes (1:500 mouse anti-CNPase, Sigma) were used. Although NeuN and CNPase are monoclonal antibodies, all procedures were done using TSA for consistency. Cell-specific antibodies were identified using 1:500 goat anti-rabbit Alexa-594 secondary antibody or 1:1000 goat anti-mouse Alexa 594 secondary antibody (Molecular Probes).

Data analysis

Composite montage images of the stained sections were collected using a Leica microscope (Leica Microsystems, Wetzlar, Germany) and Image Pro software (Phase 3 Imaging, Inc., Glen Mills, PA). Separate images were collected for each of the different labels used in the tissue. Tissue was then quantified using Metamorph (Universal Imaging, Inc., Downingtown, PA) using a novel algorithm that corrected for variations in the area across hippocampal regions. For quantification of tissue labeled for activated caspase-3 and Ab38, image stacks were created of the three different labels in a single section of tissue, and a custom program was used to identify random areas (100 pixels × 100 pixels) inside a designated region (CA1, CA3, dentate gyrus, or CA4) of interest for quantification. In each region, 10.5 ± 0.5% of the area was selected for quantification (Fig. 1C) to avoid area biasing across regions. Positively labeled cells were counted in each of these random areas and the total summed. Density was calculated by summing the number of positive cells across sections in a region of interest from a culture and dividing by the quantified area of the region. Density was then converted from cells/pixel^2 to cells/mm^2 by multiplying the area values by (0.6417 μm/pixel)^2, as provided by the Image Pro software.

Quantification of the cell phenotype showing caspase-3 activation was done in a similar manner. After collecting montage images of the different labels, Metamorph was again used to create stacks of images. The number of caspase-3 positive cells in each region of the tissue (CA1, CA3, dentate gyrus, CA4) was counted, and the number of these cells that were also labeled with a cell-specific marker was noted. The percentage of caspase-3 positive cells which were also labeled with a specific cell marker was then calculated.

For all results, data are presented as mean ± standard error of the mean (SEM). Hippocampal cultures were taken from a total of 67 rats. Between four and six hippocampal cultures were injured under each condition, and cultures from a minimum of two isolations were used for each condition. Statistical significance was determined using one-way ANOVA with Tukey post hoc comparisons, except where noted (Statistica, StatSoft, Inc, Tulsa, OK), and P values of less than 0.05 were considered significant.

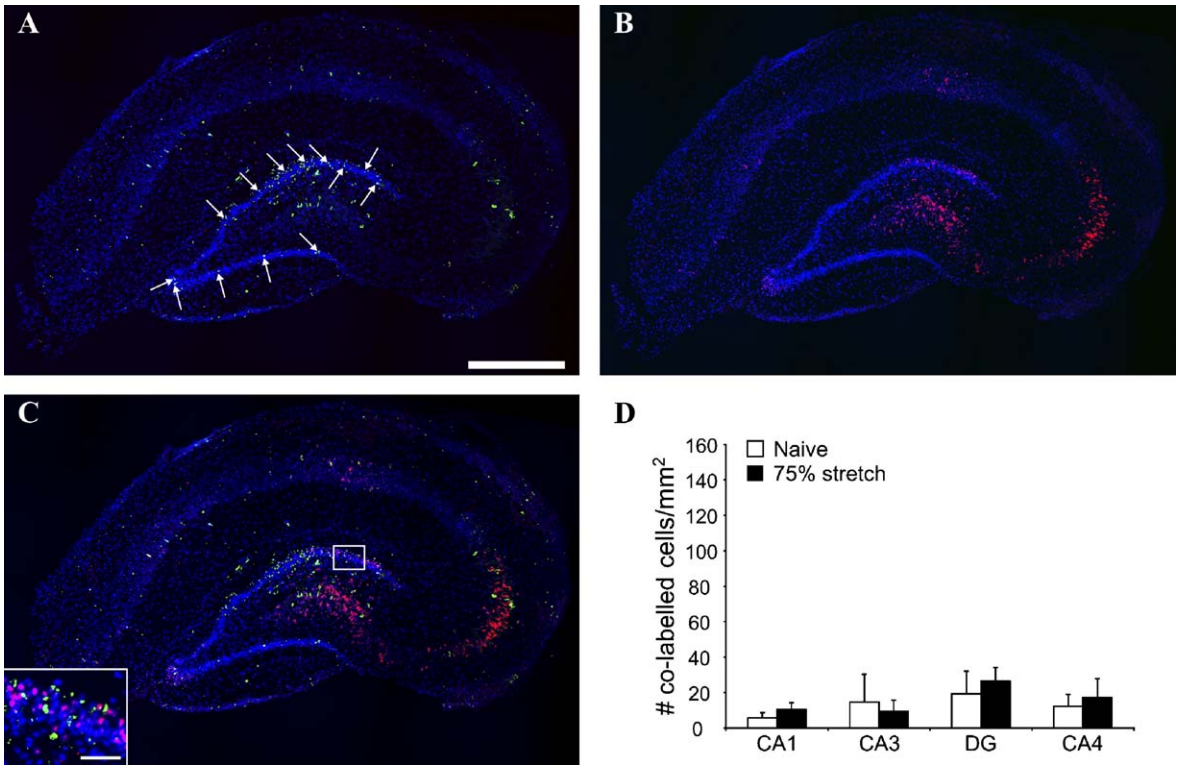

Fig. 2. Activation of caspase-3 and calpain occurred in separate cells and in distinct regions of the hippocampus. (A) Caspase-3 activity (green) was concentrated in the granule cell layer of the dentate gyrus. Activation was also found in other regions but tended to be scattered among the different strata of the hippocampus. (B) Labeling for the calpain-mediated fragment of spectrin (red) was localized to the CA3 and CA4 subregions, with some additional activity in the dentate gyrus. (C) A combined image of the individual stains revealed that few cells displayed activation of both caspase-3 and calpain (inset figure; scale bar = 50 μm) indicating that the two death pathways occurred in distinct cell populations. (D) The presence of co-localization was not significantly higher in stretch injured cultures at levels where both significant caspase-3 and Ab38 immunoreactivity were observed (75% stretch; 24 h following injury). Blue stain = Hoechst 33342; scale bar = 500 μm.

Results

Stretch injury results in activation of caspase-3 and calpains in separate cell populations

Activation of both caspase-3 and calpains in the hippocampus is often reported following brain trauma in vivo (Raghupathi, 2004). We first examined if a similar activation occurs in vitro following mechanical injury and if the mechanical injury levels required to cause calpain activation are different from levels causing caspase-3 activation across the entire hippocampal slice culture. At 50% stretch, protease activation was not observed in any region of the hippocampal culture (not shown). At 75% stretch, activation of both caspase-3 and calpains was observed (Fig. 2), while after 100% stretch, only calpain was activated (not shown). However, both caspase-3 and calpain were activated in distinct cell populations of the injured hippocampal culture. Following 75% stretch injury, activated caspase-3 immunoreactivity was primarily observed in the granule cell layer of the dentate gyrus (Fig. 2A). By contrast, Ab38(+) cells were observed almost exclusively in the pyramidal cell layer of areas CA3 and CA4 and also the granule cell layer of the dentate gyrus (Fig. 2B). Importantly, superimposition of images revealed a distinct lack of co-localization of activated caspase-3 and Ab38 immunoreactivities (Fig. 2C). Quantification of the co-localization showed that, across all regions, co-localization in stretch injured cultures was not significantly different than unstretched controls (Fig. 2D).

Quantification of activated caspase-3(+) cells revealed that 75% stretch injury resulted in a significant, 3-fold increase in caspase-3 activity across the entire hippocampal slice culture compared to control tissue ($P < 0.01$, Fig. 3A). A similar threshold level of stretch was required for calpain activation (Fig. 3B). The number of cells exhibiting Ab38 immunoreactivity was similar in sham cultures and those stretched at 50% but was significantly greater following 75% stretch ($P < 0.01$, Fig. 3B). In contrast, 100% stretch resulted in significant numbers of Ab38(+) cells ($P < 0.05$, Fig. 3B) but not caspase-3 activation.

Regional activation of caspase-3 and calpain is dependent on the level of applied stretch and time after stretch

We next examined if specific regions of the hippocampal culture showed significant calpain or caspase-3 activation following stretch. Activation of caspase-3, which occurred only after 75% stretch, was predominant in the dentate gyrus and was observed to a much lower extent in the CA1 (Fig. 3C). The number of active caspase-3(+) cells in the dentate gyrus ($P < 0.01$) and in the CA1 ($P < 0.01$) was significantly greater compared to sham tissue (Fig. 3C). In comparison, the regional profile of calpain activation after either 75% or 100% stretch changed with the level of injury (Fig. 3D). Following 75%

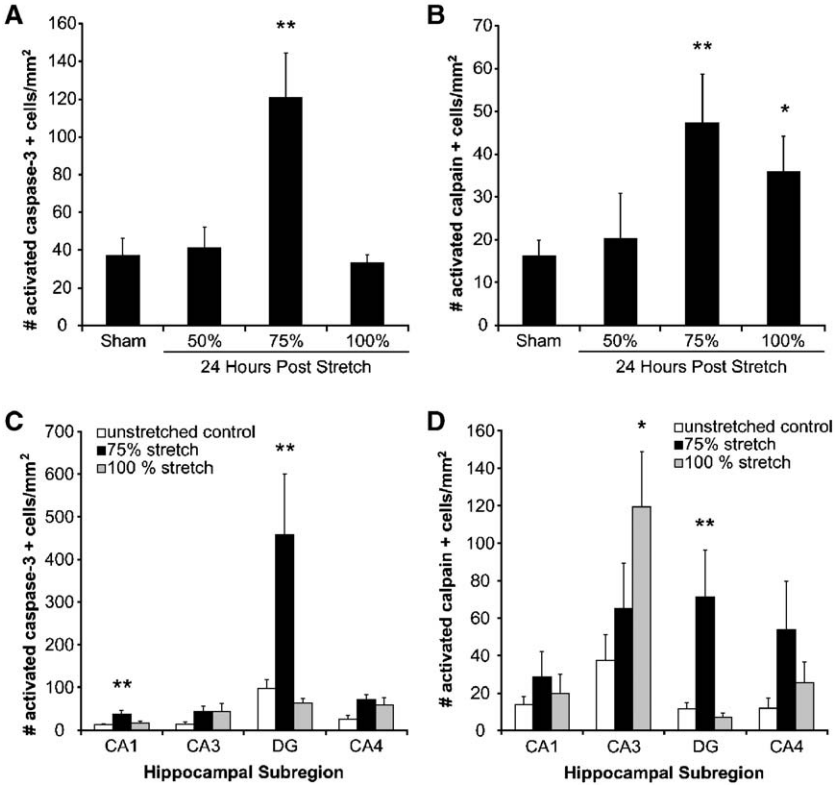

Fig. 3. Changes in level of stretch insult resulted in differential activation of death pathways. (A) Caspase-3 activity was significantly elevated 24 h after a 75% stretch but remained near the baseline activity level at a higher level of stretch. (B) Ab38 immunoreactivity significantly increased 24 h after a 75% stretch and was also significantly elevated following a 100% stretch. (C) Following a 75% stretch, a significant increase in the number of activated caspase-3 positive cells was found in both the dentate gyrus and CA1 subregion. (D) A significant number of cells in the dentate gyrus displayed Ab38 immunoreactivity following a 75% stretch. Other regions were not significantly elevated over controls. Following a 100% stretch, a significant increase in calpain-mediated spectrin breakdown fragment was found in the CA3 subfield. Stretch levels less than 75% failed to cause significant activation of either caspase-3 or calpain. Data presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

stretch, the number of Ab38(+) cells was significantly greater in the dentate gyrus ($P < 0.01$) (Fig. 3D). At 100% stretch, Ab38(+) cells were restricted to the CA3 region and were nearly 3-fold higher in number than sham control tissue ($P < 0.05$, Fig. 3D).

Activation of caspase-3 and calpains in the dentate gyrus after 75% stretch increased with time after stretch (Fig. 4). In sham tissue (Fig. 4A) and at 2 h post-stretch (Fig. 4B), activated caspase-3(+) cells were occasionally present. At 6 h (Fig. 4C) and 24 h (Fig. 4D), the extent of active caspase-3(+) cells was greater than that in sham tissue; immunoreactivity tended to be localized in the cell body. Quantification revealed that the number of active caspase-3(+) cells was significantly greater at 6 h ($P < 0.05$) and 24 h ($P < 0.001$) compared sham sections (Fig. 4E).

In contrast to caspase-3 activation, calpain activation was present in the dentate gyrus after 75% stretch and in the CA3 after 100% stretch. The number of Ab38(+) cells in the dentate gyrus after 75% stretch was significantly greater than that in sham sections observed as early as 2 h, with significant numbers of Ab38(+) between 2 and 24 h ($P < 0.05$ compared to sham, Fig. 4F). In area CA3, Ab38(+) cells in sham tissue sections were confined to the pyramidal cell layer (Fig. 5A). Between 2 and 24 h following a 100% stretch, the pyramidal cell layer in the CA3 subfield contained numerous Ab38(+) cells (Figs. 5B–D). The presence of Ab38 immunoreactivity was observed primarily in the cell bodies and, to a lesser extent, in cell processes at all times post-stretch. The number of Ab38(+) cells was significantly different from that in sham tissue sections only at 24 h post-stretch ($P < 0.05$); at 2 h, there was a small but non-significant increase in labeling ($P = 0.092$), while at 6 h, the cell number was not statistically different than in sham (Fig. 5E).

Stretch-induced caspase-3 activation occurs in both neurons and astrocytes

Following 75% stretch, the presence of activated caspase-3 in neurons, astrocytes, and oligodendrocytes was quantified. Cells containing activated caspase-3 were present in all layers of the hippocampus and co-localized with both GFAP(+) cells (Fig. 6A) and NeuN(+) cells (Fig. 6B). Active caspase-3(+) was present both in neurons that did not exhibit overt chromatin condensation (Fig. 6B) and those that contained apoptotic bodies (not shown). Few, if any, oligodendrocytes were observed to contain activated caspase-3 (Fig. 6C). A subset of the activated caspase-3(+) cells failed to label for any of the 3 cell-specific markers used in the present study. Quantification of the cell types revealed that the majority of the activated caspase-3(+) astrocytes were present in the CA1/CA3 regions, while the dentate gyrus contained a large number of activated caspase-3(+) neurons (Fig. 6D).

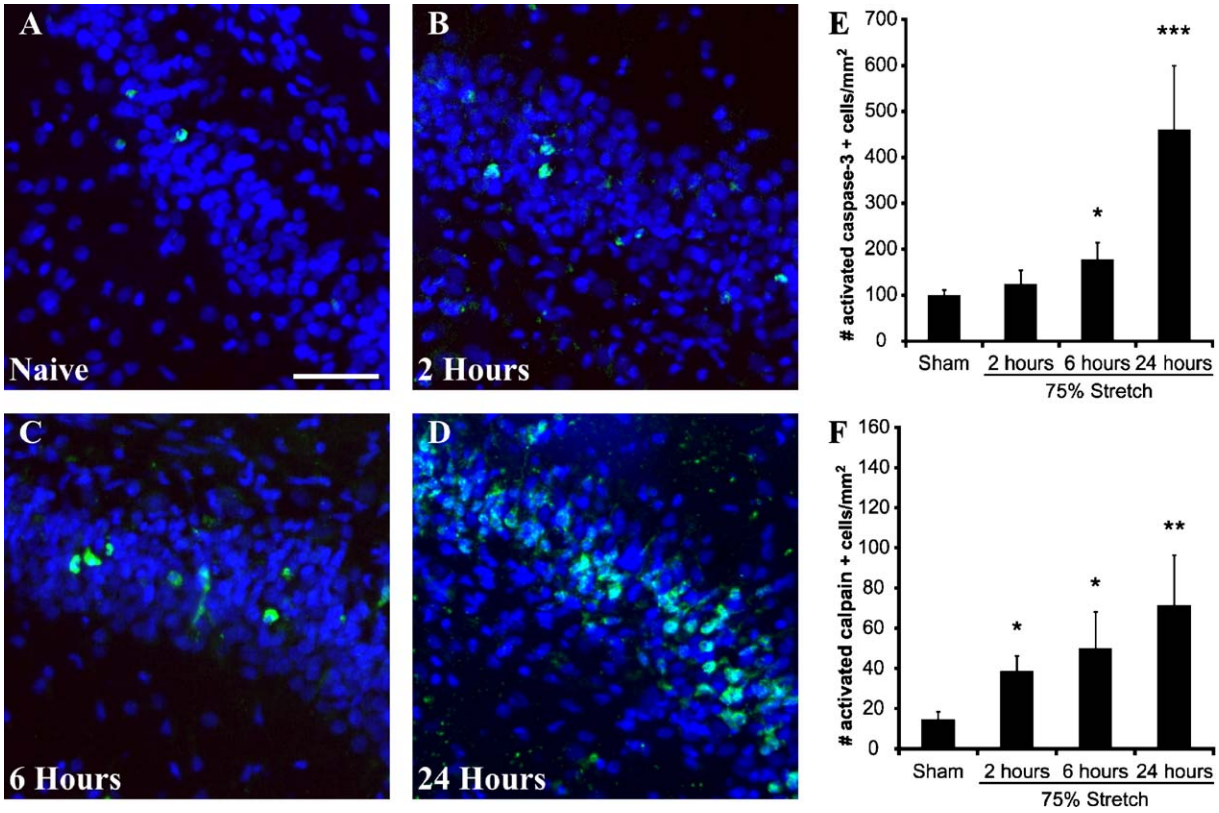

Fig. 4. Temporal activation of caspase-3 in the dentate gyrus (DG) following 75% stretch. (A) Few activated caspase-3 positive cells (green stain) were found in control tissue. (B) At 2 h post-stretch, there was no noticeable increase in the number of positive cells. (C) Activated caspase-3 immunoreactivity increased in the granule cell layer 6 h after stretch. (D) By 24 h post-stretch activation was robust. (E) Quantification of the number of activated caspase-3 positive cells revealed that activity was significant by 6 h post-stretch and increased at 24 h after stretch. (F) Quantification of Ab38 immunoreactivity in the DG revealed that significant activity was detectable within 2 h of stretch, indicating that calpain activation occurred prior to caspase-3 activation in this model. Blue stain = Hoechst 33342. Data presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; scale bar = 50 μm.

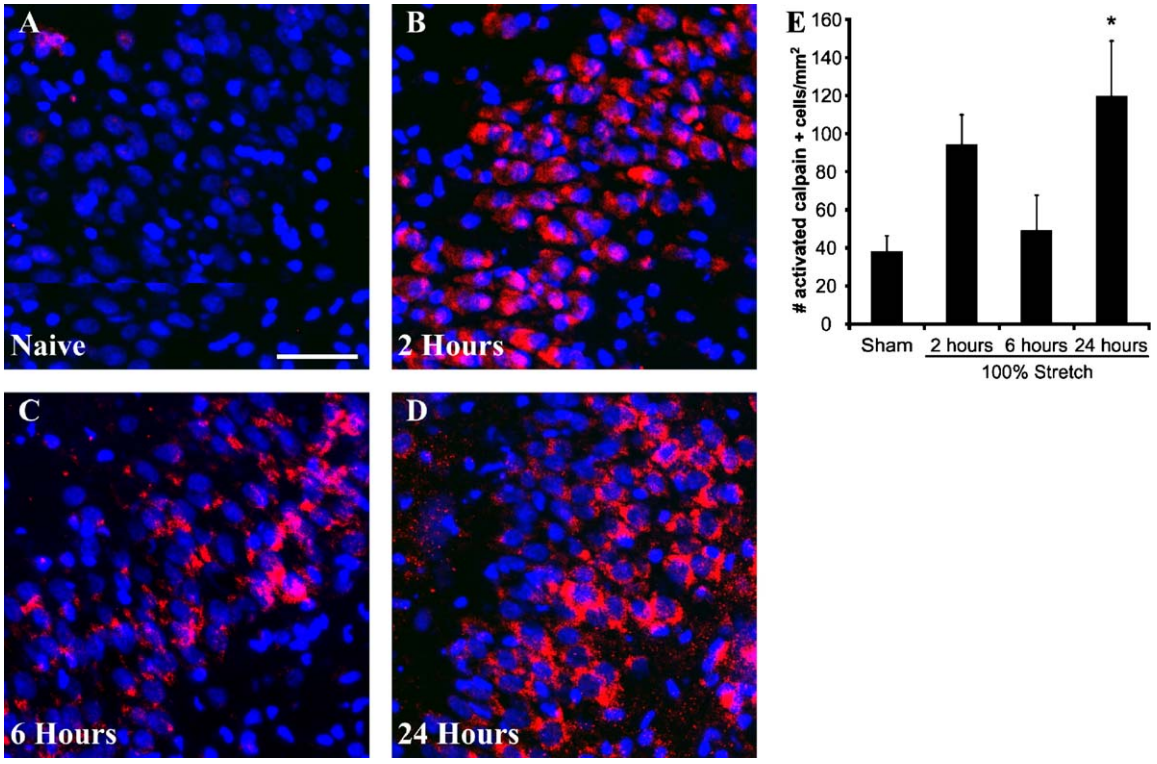

Fig. 5. Temporal detection of calpain-mediated spectrin breakdown product in CA3 following 100% stretch. (A) Few Ab38(+) cells (red stain) was detected in control cultures. (B) At 2 h post-stretch, robust Ab38 immunoreactivity was observed throughout the pyramidal cell layer of the CA3. Cell bodies appeared to be mostly intact and nuclei of typical size. (C) Ab38 immunoreactivity was reduced but still present 6 h after stretch. (D) High levels of immunoreactivity in the pyramidal cell layer were observed 24 h after stretch. Cell bodies appeared to be losing integrity at this time point. (E) Quantification of Ab38(+) cells in the CA3 region suggested biphasic activation. However, only activity 24 h after stretch was significant versus control cultures. Blue stain = Hoechst 33342. Data presented as mean ± SEM. *$P < 0.05$; scale bar = 50 μm.

Inhibiting NMDAR activation attenuates both calpain and caspase-3 activation

Based on past evidence indicating the immediate activation of the NMDAR in neurons following traumatic mechanical injury (Zhang et al., 1996), we evaluated the effect of blocking the NMDAR on the subsequent activation of calpains and caspase-3. The pretreatment of cultures with APV, a broad-spectrum NMDA antagonist, significantly reduced the number of active caspase-3(+) cells in the dentate gyrus at 24 h following a 75% stretch ($P < 0.05$) compared to untreated, injured cultures (Fig. 8A). Calpain activation in the dentate gyrus following 75% stretch (Fig. 8B) and in the CA3 region following 100% stretch (Fig. 8D) was also reduced significantly by APV pretreatment.

Extrasynaptic NMDARs played a key role in the activation of both caspase-3 and calpain following stretch. Using a dosage of ifenprodil (3 μM) to preferentially block extrasynaptic NMDARs prior to stretch (Williams, 1993), we found that caspase-3 activation was completely attenuated following 75% injury ($P < 0.05$, Fig. 7B). Using the same pretreatment, we observed that calpain activation in the dentate gyrus following 75% stretch was reduced to nearly sham levels ($P = 0.054$, Fig. 7E), and the activation of calpain following 100% stretch was significantly attenuated ($P < 0.05$, Fig. 7K).

In comparison, inhibition of the synaptic NMDARs showed that this receptor population played a role different from extrasynaptic NMDARs in calpain and caspase-3 activation. Representative images of the level of activated caspase-3(+) and Ab38(+) demonstrate the effects of pretreatment at different levels of stretch (Fig. 7). Pretreatment with an antagonist directed towards NR2A subunit containing NMDARs (Auberson et al., 2002), which are primarily located at synapses (Tovar and Westbrook, 1999), did not significantly change caspase-3 activation in the dentate gyrus following 75% stretch (Figs. 7C; 8A), unlike the protection offered by extrasynaptic blockade. Similarly, blocking synaptic NMDARs did not reduce the calpain activation in the dentate gyrus following 75% injury (Figs. 7F; 8B) and caused a modest increase in calpain activation following 100% injury (Figs. 7L; 8D). Importantly, blocking synaptic NMDARs following 100% stretch would significantly increase the caspase-3 activation in the CA3 region (Fig. 8C).

Discussion

Mechanical injury to the organotypic hippocampus produced regionally distinct patterns of calpain and caspase-3 activation in the acute period following injury. Although the presence of Ab38(+) cells was localized to cells in the pyramidal cell layer, caspase-3 positive cells were observed throughout layers of the CA1 and CA3 subregions and, in these regions, a roughly equal number of cells staining for activated caspase-3 also labeled for astrocytic and neuronal cell markers. The pattern of labeling was different in the dentate gyrus, where caspase-3 positive cells localized to the granule cell layer, and a majority of the cells were found to be neurons. Interestingly, activation of caspase-3 occurred

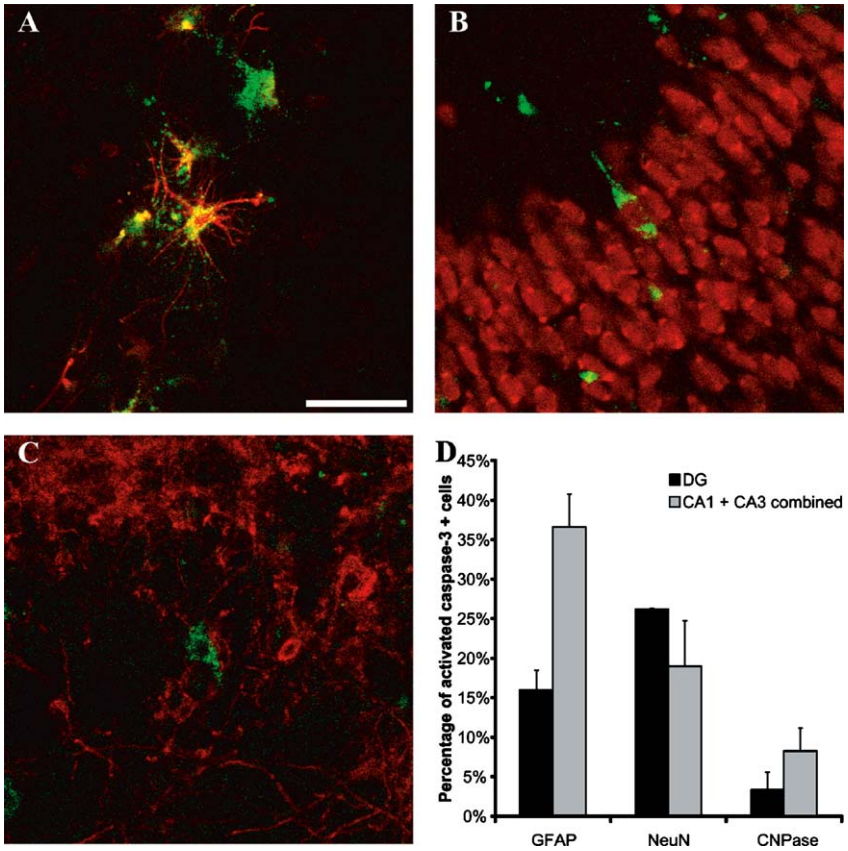

Fig. 6. The majority of caspase-3 positive cells were astrocytes or neurons. (A) Cells labeled with activated caspase-3 (green stain) were likely to co-label with the astrocyte marker GFAP (red stain) and indicated an activated form in both the CA1 and CA3. (B) Caspase-3 positive cells in the granule cell layer of the dentate gyrus were highly likely to co-label with NeuN (red stain), but this co-labeling was uncommon in other regions of the hippocampus, such as the CA3. (C) Cells in all regions were also likely to be surrounded by CNPase positive oligodendrocytes (red stain) rather than co-labeled with them. (D) Quantification of the number of caspase-3 positive cells that co-labeled with a cell-specific marker across all regions indicated that the percentage of astrocytes and neurons undergoing apoptosis were not significantly different. The data also indicated that fewer oligodendrocytes show positive caspase-3 labeling, and that a subpopulation of cells failed to co-label with any of the three cell-specific indicators. Scale bar = 50 μm.

only after a 75% stretch. In comparison, calpain activity appeared at this level of injury but was more pronounced and highly localized in the CA3 region at 100% stretch. Both calpain and caspase-3 activation were reduced significantly by blocking NMDARs prior to mechanical injury. The NR2B containing NMDARs appear key in controlling the activation of these two separate processes, as inhibiting this receptor subpopulation significantly reduced the activation of both calpain and caspase-3 in all hippocampal regions following mechanical injury. In contrast, inhibiting NR2A containing synaptic NMDARs increased the amount of caspase-3 activation in the dentate gyrus at the most severe levels of mechanical injury.

The results from this study highlight the potentially complex progression of caspase-3 and calpain activation in the hippocampus following TBI. Although both apoptosis and necrosis occur following TBI, much less is known on how the severity of injury will preferentially activate either pathway in the acute or intermediate period after injury. Our data are consistent with an in vitro investigation of apoptotic and necrotic cell death following stretch injury, showing that an increase in a caspase-3-specific breakdown product of spectrin peaks and then decreases with increasing stretch, while calpain activation is more broadly distributed across injury severity conditions and precedes caspase-3 activity in time (Pike et al., 2000). Although animal models of TBI show a progression in caspase activation over time in the cortex and the hippocampus (Colicos and Dash, 1996; Yakovlev et al., 1997; Conti et al., 1998; Clark et al., 2000; Keane et al., 2001; Sullivan et al., 2002), calpain activation in parallel with caspase activation is not well described within a single study. Our data show little co-activation of calpain and caspase-3 in the same cell, consistent with a recent report showing that mild and moderate levels of controlled cortical impact injury (CCI) will produce a more extensive pattern of both calpain-specific spectrin breakdown and caspase-3 activation (Clouse et al., 2003). These limited data on the co-activation of apoptotic and necrotic cell death within the same cell differ from evidence reported from in vivo ischemia studies (Snider et al., 1999; Zhang et al., 2002; Neumar et al., 2003), where the co-activation does appear in a subset of cells within the hippocampus. Taken together, our data suggest that there may be a continued and unique enhancement of calpain activation in trauma at higher levels of injury in vivo, with a corresponding decrease in the relative amount of caspase-3 activation, and there is less co-activation of both proteases within the same cell when compared to ischemic brain injury.

The progressive change in the pattern of caspase-3 and calpain activation observed in mechanically injured hippocampal cultures

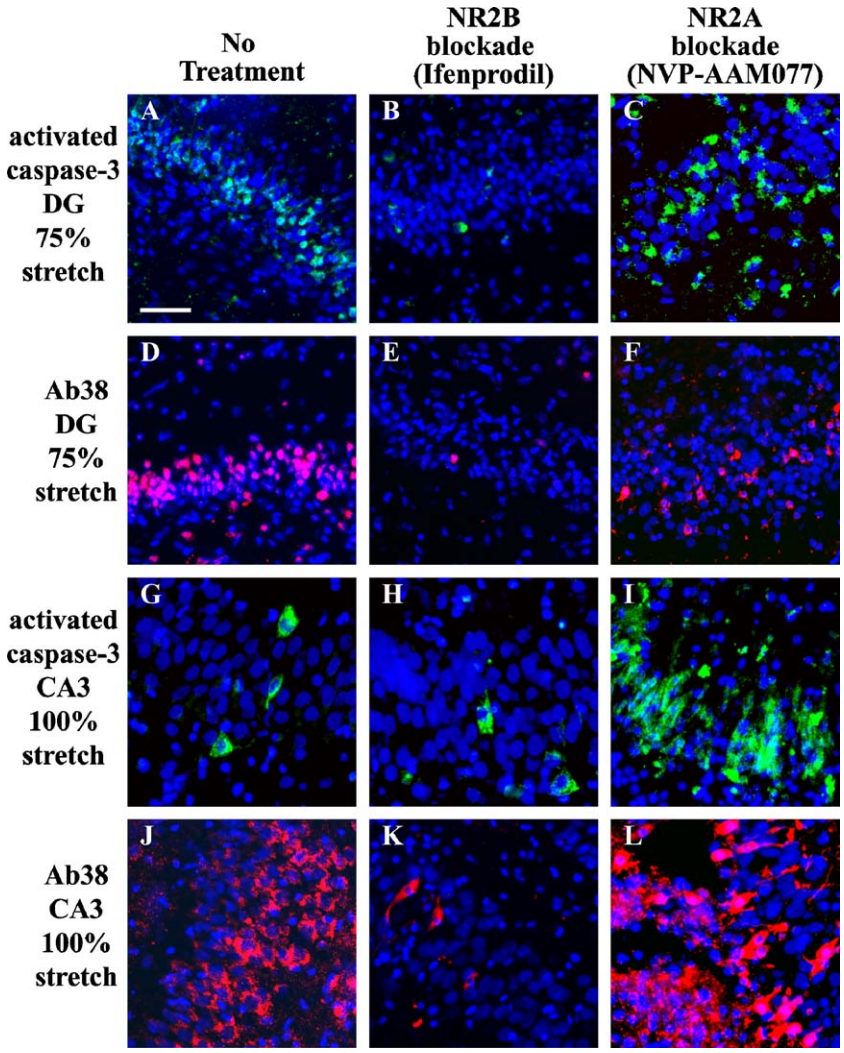

Fig. 7. Blockade of NR2A containing receptors resulted in an increase in activated caspase-3 and Ab38. Following a 75% stretch, levels of both activated caspase-3 and Ab38 immunoreactivity increased in the DG (A, D) and treatment with an extrasynaptic dose of an NR2B-specific antagonist (ifenprodil) resulted in a significant attenuation of this activity (B, E). In comparison, an NR2A-specific antagonist block of synaptically localized NMDA receptors (NVP-AAM077) caused little reduction in activated caspase-3 and Ab38 (C, F). In contrast, very little activated caspase-3 is observed in the CA3 following a 100% stretch (G) and blocking NR2B receptors had no effect (H). Blocking NR2A receptors, however, resulted in robust caspase-3 activity in this region (I). There was a robust level of Ab38 immunoreactivity observed following a 100% stretch (J), which was reduced by the blocking of NR2B receptors (K). The amount of Ab38 immunoreactivity was not reduced by the blocking of NR2A receptors (L). Scale bar = 50 μm.

also shows an intriguing shift across hippocampal regions. Although past studies using in vivo models show activated caspase-3 labeling of neurons and astrocytes in the dentate gyrus and, to a much lesser extent, throughout the CA1 and CA3 hippocampal subfields (Clark et al., 2000; Beer et al., 2000), we believe that this is the first evidence of a shift in the type and location of protease activation after traumatic mechanically injury. Intriguingly, the absence of caspase-3 activation in the dentate gyrus was not replaced with an increase in calpain activation in the same region at the most severe injury level. Instead, calpain activity unexpectedly shifted from the dentate gyrus (75% stretch injury) to the CA3 pyramidal cell layer (100% stretch injury), consistent with the proposed vulnerability of the CA3 subfield at more severe levels of injury in vivo (Baldwin et al., 1997; Smith et al., 1997; Grady et al., 2003). However, we must consider the possibility that the absence of calpain labeling in the dentate gyrus at the highest level of injury is because calpain activation occurred quickly and was soon followed with a later stage of cell death not detected by either antibody. Clarifying this process in the future will identify whether there is a consistent shift in vulnerability across different hippocampal subregions, a shift in the timing of these cell death processes, or both at higher levels of stretch.

After establishing the regional patterns of calpain and caspase-3 activation in the in vitro hippocampus after mechanical injury, we examined the role of NMDARs on these immunoreactive changes. To our knowledge, no study has directly examined how NMDAR activation following traumatic mechanical injury can lead directly to calpain activation. With the evidence from past studies showing that the NMDAR loses its characteristic Mg^{+2} block following mechanical injury (Zhang et al., 1996), the mechanically initiated change in the NMDAR would appear a central mechanism for the calpain activation. In support of this mechanism, we observed the calpain activation appearing in the CA3 pyramidal cell layer, and the granule cell layer of the dentate gyrus was significantly attenuated if we

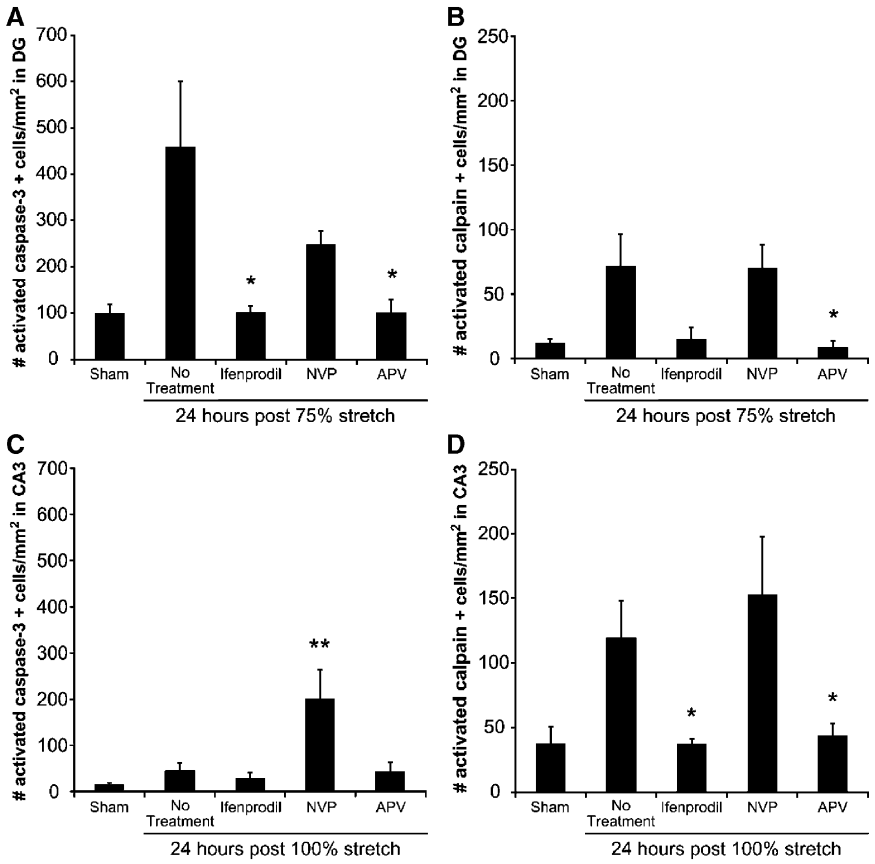

Fig. 8. Role of NMDA receptors on caspase-3 activation and the presence of Ab38 immunoreactivity after mechanical injury. (A) Blockade of NR2B receptors with ifenprodil resulted in the attenuation of activated caspase-3 in the dentate gyrus (DG) 24 h after a 75% stretch while blocking NR2A receptors with NVP did not result in a similar reduction. However, blocking all NMDA receptors with APV also significantly reduced the level of activated caspase-3. (B) Treatment with ifenprodil also resulted in decreased levels of Ab38 in the DG ($P = 0.054$). No reduction in Ab38 immunoreactivity resulted from treatment with NVP. A significant reduction was achieved with exposure to APV. (C) Following a 100% stretch, no increase in the level of activated caspase-3 was found in the CA3 subregion. Blocking NR2A receptors resulted in a significant increase in the number of activated caspase-3 positive cells. (D) Treatment with either ifenprodil or APV resulted in a significant decrease in Ab38(+) cells in the CA3 subregion 24 h after a 100% stretch. In contrast, NVP treatment resulted in a slight increase in the density of Ab38(+) cells. Data presented as mean ± SEM. $^*P < 0.05$.

blocked NMDAR activation prior to stretch. Further, our data show that NR2B containing NMDARs appear key in this calpain activation process, since the selective targeting of these NMDARs also proved an effective method to attenuate spectrin breakdown. These data point out the importance of the NR2B containing NMDAR subpopulation, which comprises a large majority of extrasynaptic NMDARs, in calpain activation following TBI. These data may be of particular significance for developing new approaches for NMDAR antagonist therapies, as the complete blockade of NMDARs is difficult to implement clinically, and a more directed targeting of the extrasynaptic NMDARs may also extend the therapeutic window for NMDAR antagonists. Defining the timing of a delayed blocking strategy would be useful for determining if the NMDAR represents a potential target for treatment or if one needs to consider targeting secondary signaling cascades.

The modulation of caspase-3 activation by NMDARs appears more complex. First, the ability of NMDAR blockade to more completely inhibit apoptosis across non-neuronal cell types was unexpected. Although recent reports show that NMDARs are present in astrocytes (Gottlieb and Matute, 1997; Schipke et al., 2001; Krebs et al., 2003), the role of NMDARs in astrocytic caspase-3 activation is not known. We cannot rule out the possibility that NMDA receptors in astrocytes are activated following mechanical injury, causing the subsequent caspase-3 activation. The data from this study suggest that this potential link should be explored in more detail, since it may be important for understanding apoptosis in glial cells following mechanical injury. The reduction in caspase-3 activation in neurons could be explained partly by mechanically activated NMDARs following 75% stretch injury. Indeed, several reports show that transient NMDA stimulation leads to apoptotic cell death within 24 h (Bonfoco et al., 1995; van Lookeren Campagne et al., 1995; Lesort et al., 1997; Tenneti et al., 1998; Yu et al., 1999). Previously, it was shown that NMDA-mediated apoptosis is controlled preferentially through the extrasynaptic NMDARs (Sattler et al., 1998), similar to our finding that blocking NR2B containing NMDARs would also significantly attenuate caspase-3 activation. Interestingly, the activation of synaptically localized NR2A containing NMDARs following the highest level of stretch injury appears to play a pro-survival role in the dentate gyrus, as specifically inhibiting these NMDARs enhanced caspase-3 activation 24 h following injury. When the initial level of mechanical injury was reduced to 75% stretch, the inability to block caspase-3 activation in the dentate gyrus with the inhibition of synaptic NMDARs suggests these receptors may also play a similar role at

this less severe level of injury. Alternatively, it is possible that synaptic NMDARs may not be activated at the 75% injury level, and therefore, the inhibition of these receptors would play no role in either caspase-3 or calpain activation. The balance between NR2A and NR2B containing NMDAR activation in traumatic injury is consistent with recent data showing that the coordinated activation of synaptic and extrasynaptic NMDARs can, through the mitogen activated protein kinase cascades, result in the downstream activation of either pro-survival or pro-apoptotic pathways (Paul et al., 2003; Papadia et al., 2005). A critical key that remains to be determined is whether each of these NMDAR populations are either differentially or equally activated at different mechanical injury severities, clarifying if there is a change in the balance of NMDA-mediated signaling across different levels of injury.

In summary, we show that the activation of both caspase-3 and calpain in the hippocampus occurs within the first 24 h following mechanical injury, and that co-activation of these proteases does not occur within the same cell. The pattern of activation is influenced by the severity of mechanical injury and is controlled centrally by the extrasynaptic NMDARs. A more precise examination of the staging between these two cell death processes represent an important next step in understanding new treatment strategies for progressive patterns of apoptosis and necrosis that appear in the traumatically injured brain.

Acknowledgments

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