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Knockdown of m-calpain increases survival of primary hippocampal neurons following NMDA excitotoxicity

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Abstract

The calpain family of cysteine proteases has a well-established causal role in neuronal cell death following acute brain injury. However, the relative contribution of calpain isoforms to the various forms of injury has not been determined as available calpain inhibitors are not isoform-specific. In this study, we evaluated the relative role of m-calpain and μ-calpain in a primary hippocampal neuron model of NMDA-mediated excitotoxicity. Baseline mRNA expression for the m-calpain catalytic subunit (calpain 2) was found to be 50-fold higher than for the μ-calpain catalytic subunit (calpain 1) based on quantitative real-time PCR. Adeno-associated viral vectors designed to deliver short hairpin RNAs targeting the catalytic subunits of μ- or m-calpain resulted in 60% and 90% knockdown of message respectively. Knockdown of m-calpain, but not μ-calpain, increased neuronal survival after NMDA exposure at 21 days in vitro. Nuclear translocation of calpain substrates AIF, p35/p25 and CRMP 2-4 was not detected after NMDA exposure in this model. However, nuclear translocation of CRMP-1 was observed and was prevented by m-calpain knockdown. These findings provide insight into potential mechanisms of calpain-mediated neurodegeneration and have important implications for the development of isoform-specific calpain inhibitor therapy.

Keywords

calpain; neuron; excitotoxicity; RNA interference; NMDA; adeno-associated virus

Introduction

Neuronal depolarization leading to release of the excitatory neurotransmitter glutamate and subsequent excitotoxicity contributes to several forms of acute brain injury including stroke, cardiac arrest and traumatic brain injury (for review, see Choi, 1992). A major component of this excitotoxicity is intracellular calcium overload, which leads to activation of the calpain family of calcium-dependent cysteine proteases (EC 3.4.22). Indeed, calpain activity has been observed in models of excitotoxicity (Siman and Noszek, 1988; Siman *et al*, 1989), brain ischemia (Saido *et al*, 1993; Roberts-Lewis *et al*, 1994; Bartus *et al*, 1995; Yamashima *et al*, 1996; Neumar *et al*, 1996; Bartus *et al*, 1998; Neumar *et al*, 2001) and traumatic brain injury (Saatman *et al*, 1996; Saatman *et al*, 2003). Moreover, pharmacologic calpain

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inhibition is neuroprotective in both *in vitro* (Brorson *et al*, 1995; Rami *et al*, 1997) and *in vivo* models of excitotoxic injury (Wu *et al*, 2004), brain ischemia (Lee *et al*, 1991; Rami and Krieglstein, 1993; Hong *et al*, 1994; Bartus *et al*, 1994a; Bartus *et al*, 1994b; Li *et al*, 1998; Markgraf *et al*, 1998; Yokota *et al*, 1999; Kawamura *et al*, 2005) and traumatic brain injury (Buki *et al*, 2003; Ai *et al*, 2007). Similarly, overexpression of calpastatin, the endogenous inhibitor of calpains, is neuroprotective in both *in vivo* excitotoxicity (Takano *et al*, 1999) and global brain ischemia (Cao *et al*, 2007a).

Neither pharmacologic inhibitors nor the endogenous inhibitor, calpastatin, are able to distinguish the roles of different calpain isoforms. The two classical brain calpain isoforms are μ-calpain (EC 3.4.22.52) and m-calpain (EC 3.4.22.53), which exist as heterodimers containing the distinct catalytic subunits calpain 1 and calpain 2, respectively, along with a common regulatory subunit. The importance of determining the contribution of these two isoforms to acute brain injury is highlighted by their differential roles in physiology. These differences are best illustrated by the fact that μ-calpain knockouts are viable with the only reported physiologic defect being in platelet aggregation (Azam *et al*, 2001) while knockout of m-calpain is embryonic lethal (Dutt *et al*, 2006), and calpain 2 RNAi causes aberrant chromosome alignment during mitosis (Honda *et al*, 2004).

The relative role of calpain isoforms may vary based on the injury model, differences in relative expression and subcellular localization. Recently, μ-calpain has been localized to mitochondria (Garcia *et al*, 2005), where it is able to cleave and release apoptosis inducing factor (AIF; Polster *et al*, 2005). Knockdown of μ-calpain in an *in vitro* model of oxygen-glucose deprivation is able to prevent AIF nuclear translocation and increases cell survival (Cao *et al*, 2007a). AIF triggered apoptosis, however, is only one potential mechanism of calpain-mediated cell death. Based on the distribution of alpha-spectrin cleavage in most acute neuronal injury models, calpain activity is extensive outside the mitochondria and numerous extra-mitochondrial calpain substrates have well established causal roles in neuronal death including neurotransmitter receptors such as mGluR1α (Xu *et al*, 2007), calcium regulatory proteins such as the sodium calcium exchanger (Bano *et al*, 2005), and signaling molecules including calcineurin (Wu *et al*, 2004), the p35 activator of cyclin dependent kinase 5 (O'Hare *et al*, 2005; Saito *et al*, 2007; Wang *et al*, 2007) and several members of the collapsin response mediator protein (CRMP) family (Hou *et al*, 2006; Jiang *et al*, 2007; Zhang *et al*, 2007). Numerous other substrates are cleaved by calpain in acute injury models and may also play roles in neurodegeneration (for review, see Bevers and Neumar, 2008).

The major distinction between μ-calpain and m-calpain is the level of calcium required for activation. In *in vitro* assays, m-calpain has been shown to require 400-800 μM Ca^{2+}, while μ-calpain has a lower requirement of 3-50 μM Ca^{2+} (Goll *et al*, 2003). As a result, it is possible that the role of calpain isoforms and mechanism of calpain-mediated injury could depend on the source, severity and duration of intracellular calcium overload as well as the baseline expression of calpain isoforms. In this study we used viral vector-mediated RNA interference to directly compare the ability of μ-calpain or m-calpain knockdown to alter calpain activity and prevent cell death following NMDA-mediated excitotoxicity in rat primary hippocampal neurons at 21 days in vitro.

Materials and Methods

Materials

Rabbit polyclonal antibody to the N-terminal of the μ-calpain catalytic subunit was generated by our laboratory. Rabbit polyclonal antibody to domain III of rat m-calpain was purchased from Triple Point Biologics. Mouse monoclonal antibody to glial fibrillary acidic

protein (GFAP) was purchased from Sigma-Aldrich, USA. Rabbit polyclonal antibody to microtubule associated protein 2 (MAP2) was a gift from Dr. Virginia Lee, University of Pennsylvania. Mouse monoclonal antibody to AIF was purchased from Santa Cruz Biotechnologies. Rabbit polyclonal antibody to the C-terminal of p35/p25 (C-19) was purchased from Santa Cruz Biotechnologies. Rabbit polyclonal antibodies to CRMP-1, -2, -3 and -4 were provided by Dr. Joachim Kappler, Physiologish-chemishes Institut, Rheinische Fredrich-Wilhemls-Universität, Bonn, Germany. Rabbit polyclonal antibody to calpain-cleaved spectrin (Ab38) was a gift from Dr. Robert Siman, University of Pennsylvania. Alexa-568 conjugated secondary antibodies used for immunofluorescence were purchased from Invitrogen. HRP-linked secondary antibodies used for immunoblotting were purchased from Perkin Elmer. Western blots were visualized using enhanced chemiluminescence supplies purchased from Perkin Elmer. Short interfering RNA sequences for the catalytic subunit of rat μ-calpain (capn1) or rat m-calpain (capn2) were obtained from Ambion, USA. Unless otherwise noted, all other chemicals were purchased from Sigma-Aldrich, USA.

Cell culture

Rat2 fibroblasts cells (ATCC No. CRL-1764) were cultured in Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal bovine serum (Gibco) at 37°C and 5% CO_2. Primary hippocampal neurons were cultured as described previously (Cummings *et al*, 1996). Briefly, hippocampi from E19 Sprague-Dawley rat embryos were trypsinized in DMEM (Whittaker Bioproducts) containing 0.027% trypsin at 4°C for 20 min. They were triturated in media consisting of DMEM supplemented with 10% bovine calf serum (Hyclone), 10% Ham's F12 with glutamine (Whittaker Bioproducts), and 50 U/mL penicillin–streptomycin. Dissociated cells were plated on poly-L-lysine coated 35-mm petri dishes with or without glass coverslips and cultured at 37°C in a humidified 5% CO2 incubator. Dissociated cells were plated at a density of 100,000 or 400,000 cells/mL in serum-free Neurobasal medium (Gibco) supplemented by B27 (Gibco). Mitotic inhibitors and antibiotics were not used.

Transfection of short interfering and short hairpin RNA

Rat2 fibroblasts were plated in six-well dishes at a density of 1×10^5 cells per well. Twenty-four hours after plating, cells were transfected with 500 pmol short interfering RNA (siRNA) or 2μg short hairpin RNA (shRNA) plasmid using a lipid-based transfection reagent (Lipofectamine 2000, Invitrogen). Twenty-four hours after transfection, media was replaced with fresh DMEM + 10% FBS. Cells were harvested for Western blot as described below.

Viral vector generation

Recombinant adeno-associated viral (AAV) vectors were generated by the University of Pennsylvania Vector Core by triple transfection of 293 cells and purified by cesium chloride gradient sedimentation as described previously (Fisher *et al*, 1997). AAV 2/1 vectors were designed to express shRNA targeting the catalytic subunit of rat μ-calpain (capn1) or rat m-calpain (capn2). An AAV 2/1 vector expressing shRNA targeting luciferase (luc), which has no homology to any known rat gene, was used as a control. All shRNAs were expressed under the control of the human U6 RNA polymerase III promoter. All vectors also expressed the green fluorescent marker ZsGreen under the control of the CMV promoter.

AAV 2/1 Vector Transduction Efficiency

Cultures of rat primary hippocampal neurons were transduced with 1.5×10^{11} genome copies (GC) of AAV 2/1 vector expressing capn1 or capn2 shRNA at 7 days in vitro (DIV) by adding vector in a vehicle of phosphate-buffered saline (PBS) and 10% glycerol directly to

the culture media. All appropriate biosafety procedures were followed in handling the AAV vectors, which are rated BSL-1 and have never been shown to cause human pathology. Two weeks later (21 DIV) cultures were fixed with paraformaldehyde, immunolabeled for the neuronal marker MAP2, or the glial marker GFAP and counterstained with 4,6-daimidino-2-phenylindole (DAPI) nuclear label. Five random 100× images were taken from each of two 12mm coverslips (10 images per condition). Total transduction efficiency was determined by calculating the percentage of DAPI-labeled cells that expressed ZsGreen. Neuronal transduction efficiency was determined by calculating the percentage of cells that expressed ZsGreen along with MAP2 compared to the total number of ZsGreen-positive cells. A total of four culture dishes were examined for each vector.

Calpain expression assays

Rat2 cells transfected with siRNA were harvested forty-eight hours after transfection, those transfected with plasmid-based shRNA were harvested four days after transfection. Neuronal cultures transduced with capn1 or capn2 shRNA AAV2/1 vectors were harvested two weeks after vector application. All cell types were harvested in homogenization buffer (10mM HEPES, 0.3M sucrose, 0.5mM $MgSO_4$, 2mM EGTA, 0.014% 2-mercaptoethanol) containing protease inhibitors (0.1μM PMSF, 5μg/ml leupeptin, 2μg/ml aprotinin, 2μg/ml pepstatin, 2mM z-VAD-fmk). Protein was quantified using Bradford assay. Proteins were separated using SDS-PAGE on a 7.5% tris-glycine gel, transferred to nitrocellulose membrane, and probed with antibodies specific to μ-calpain and m-calpain, as described under *Materials*. Blots were visualized using enhanced chemiluminescence. All blots used to screen the efficacy of siRNA sequences were repeated in at least two separate Rat2 cultures. AAV-mediated knockdown of μ- and m-calpain in primary hippocampal neurons was tested in three separate cultures. Western blot densitometry was performed with ImageJ software (National Institutes of Health). Band density was corrected against GAPDH loading controls and is reported relative to control (luc shRNA) cultures.

Two weeks after vector transduction, total RNA was isolated from primary rat hippocampal cultures using TRIZOL (Invitrogen) followed by cleanup with an RNEasy Mini Kit (Qiagen). A total of 250ng of RNA was used to generate cDNA using an Oligo-DT primer and Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using SYBR green dye (Applied Biosystems) and primers specific to calpain 1 and 2 catalytic subunits on a Stratagene MX3000P cycler. Expression of calpain 1 and 2 message was normalized against mRNA expression for hypoxanthine phosphoribosyltransferase 1 (HPRT) and was compared between cultures transduced with luc, capn1 and capn2 specific shRNA. Quantification of mRNA was performed in 5 cultures per transduction condition.

NMDA injury characterization

To determine the optimal NMDA concentration for use in injury studies, hippocampal neurons plated on 12mm coverslips were exposed to 10μM, 100μM, 200μM, 400μM or 800μM NMDA in HEPES buffered saline (HBS; 145mM NaCl, 3mM KCl, 10mM HEPES, 8mM glucose, 2mM $CaCl_2$, 1mM $MgCl_2$) or HBS alone for 30 minutes. Culture media was returned to dishes after NMDA exposure. Twenty-four hours after injury, culture media was removed and cells incubated in phosphate buffered saline containing 1μM calcein-AM and 4μg/ml propidium iodide (PI) for 30 minutes. Five random 100× images were taken per coverslip, three coverslips were assessed per condition. Cell survival was determined by calculating the number of calcein-positive, PI-negative cells over the total number of cells. To assess the role of calpain activity in this injury model, separate cultures were pretreated with the calpain inhibitor MDL-28170 at a concentration of 1.0μM for 10 minutes. Cells

were then exposed to NMDA as described above and returned to media containing MDL-28170. Survival was determined by PI labeling 24 hours after injury.

Immunocytochemistry of calpain substrates

To examine the potential role of nuclear translocation of known calpain substrates in this model, separate cultures were exposed to 200μM NMDA or HBS as described above. Thirty minutes or 6 or 24 hours after injury, cells were fixed, nuclei stained with DAPI and immunocytochemistry was performed for AIF, p35/p25, or CRMP-1, 2, 3, or 4. High power images were obtained at each time point to determine if excitotoxicity lead to nuclear translocation of any of the four calpain substrates. At least two independent cultures were immunolabeled for each marker to identify those that translocated to the nucleus following injury.

Effect of calpain isoform knockdown on NMDA-induced calpain activity

Rat primary hippocampal neurons plated on 12mm glass coverslips were transduced with 1.5×10^{11}GC of AAV 2/1 vector expressing luc, capn1 or capn2 shRNA. At 21 DIV, culture media was removed and replaced with HBS with or without 200μM NMDA. Following 30 minutes of NMDA exposure, 3 coverslips were removed from each culture cultures were fixed with 4% paraformaldehyde and immunolabeled for calpain-cleaved spectrin (Ab38). Five random 400× images were taken from each coverslip (15 per condition). A blinded observer counted the images, and cells were scored as Ab38 positive if they displayed any immunolabeling anywhere within the cell. Those cells that displayed Ab38 immunoreactivity were further classified based on the presence of Ab38 labeling in the cell body. Four culture dishes were examined in each condition.

Effect of calpain isoform knockdown on cell survival following NMDA excitotoxicity

Rat primary hippocampal neurons transduced with 1.5×10^{11}GC of AAV 2/1 vector expressing luc, capn1 or capn2 shRNA were injured at 21 DIV. Culture media was removed and replaced with HBS with or without 200μM NMDA. Following 30 minutes of NMDA exposure, the original culture media was restored and cultures were maintained for 24 hours to assess survival.

Twenty-four hours after NMDA exposure, coverslips were stained with PI to label dead cells. Five random 200× images were taken from each coverslip (10 per condition) and the percentage of ZsGreen-expressing, PI-negative cells was determined by a blinded observer. At least four culture dishes were examined in each condition.

Results

Calpain knockdown in Rat2 fibroblasts

To identify target sequences for calpain isoform RNA interference, cultures of rat2 fibroblast cells were transfected with one of three siRNA sequences specific to the catalytic subunit of μ-calpain (capn1), one of three sequences specific to the catalytic subunit of m-calpain (capn2), or a transfection reagent alone (Veh). Sequences of the capn1 and capn2 siRNAs are shown in Table 1. Forty-eight hours after transfection, cells were harvested in the presence of protease inhibitors and expression of μ-calpain and m-calpain was assayed by Western blot. As shown in Figure 1A, all three μ-calpain catalytic subunit (capn1) siRNA sequences reduced expression of μ-calpain without altering expression of m-calpain. Similarly, all three sequences specific to m-calpain catalytic subunit (capn2) reduced expression of that isoform without altering μ-calpain expression (Figure 1B).

A single siRNA sequence for each of the two calpain isoforms was used as the basis for synthesis of a DNA oligonucleotide that codes for a short hairpin RNA (shRNA, target sequences indicated in bold in Table 1). These shRNAs were then inserted into an expression plasmid containing the U6 RNA polymerase III promoter (pSilencer 1.0, Ambion). The U6 plasmids containing shRNA for μ-calpain catalytic subunit (capn1), m-calpain catalytic subunit (capn2) and a scrambled shRNA (scr) were transfected into rat2 fibroblasts. Four days after transfection, cells were harvested in the presence of protease inhibitors and calpain expression was assayed by Western blot. Both the μ-calpain specific (capn1) and m-calpain specific (capn2) shRNAs were able to reduce expression of the targeted isoform without affecting the other calpain isoform (Figure 1C).

Transduction of primary rat hippocampal neurons with adeno-associated viral vectors

To deliver shRNAs targeting the catalytic subunits of μ-calpain (capn1) and m-calpain (capn2) to primary neurons, adeno-associated viral vectors (AAV) of serotype 2/1 were generated. These vectors express shRNA under the control of a U6 promoter along with a green fluorescent marker protein (ZsGreen) under the control of a CMV promoter. A control vector was also generated, containing a luciferase shRNA sequence (luc) with no homology to any rat gene. All three vectors were able to transduce both neurons and astrocytes, as indicated by co-localization of ZsGreen fluorescence with both MAP2 and GFAP markers (Figure 2A, luc images not shown). Overall transduction efficiency, as measured by the percentage of DAPI labeled cells that co-expressed ZsGreen, was 96.3±0.4% for the luc vector, 99.1±0.7% for the capn1 vector and 99.1±1.0% for the capn2 vector (Figure 2B). As expected, the majority of transduced cells were neurons, and the percentage of transduced neuronal cells did not differ significantly between luc, capn1 and capn2 vectors (Figure 2C, luc = 77.8±7.5%, capn1 = 70.2±8.1%, capn2 = 77.6±4.0%; One-way ANOVA, $p > 0.05$).

To evaluate the efficacy of isoform-specific RNA interference, cultures of primary hippocampal neurons were transduced at 7 DIV with AAV2/1 vectors expressing luc, capn1 or capn2 shRNA as described above. At 21 DIV, cells were harvested in the presence of protease inhibitors, and calpain expression was assayed by Western blot (Figure 3A). Transduction with vector expressing capn2 shRNA significantly reduced expression of m-calpain, while transduction with control (luc) or capn1 shRNA did not (Figure 3B). We were unable to consistently detect expression of μ-calpain protein by Western blot in these neuronal cultures, despite being able to detect it in an equivalent amount of Rat2 fibroblast lysate. To address the problem of low μ-calpain protein expression in our neuronal cultures, we performed quantitative real-time PCR for calpain 1 and calpain 2. In control luc shRNA treated cultures, calpain 1 mRNA is expressed at a level approximately 50-fold lower than that for calpain 2, which is consistent with our inability to detect μ-calpain protein (Figure 3C). The effect of transduction with capn2 shRNA replicated the results seen with Western Blot, with a significant reduction in calpain 2 message compared to luc treated controls ($p < 0.05$, One-way ANOVA with Scheffe post-hoc analysis). Both capn1 and capn2 shRNA reduced expression of calpain 1 message (Figure 3D), but given the relatively low baseline expression, the physiologic relevance of this effect is unknown.

Characterization of NMDA injury

To determine the ideal NMDA concentration for excitotoxicity experiments, primary hippocampal neurons were exposed to 10μM, 100μM, 200μM, 400μM or 800μM NMDA or HBS vehicle for 30 minutes at 37˚C. Twenty-four hours after injury, cells were incubated with calcein-AM to label live cells and PI to label dead cells. No cells double-labeled for calcein-AM and PI, confirming the utility of PI as a cell death marker. The percentage of surviving cells decreased as NMDA concentration was increased and concentrations of 200 μM and greater significantly reduced survival compared to HBS-treated cultures (Figure 4A,

One-way ANOVA with Scheffe post-hoc analysis, $p < 0.05$). There was no significant difference in survival between NMDA concentrations of 200μM, 400μM and 800μM. As a result, all subsequent experiments were performed with 200μM NMDA.

To demonstrate that the excitotoxic injury in this model is sensitive to calpain inhibition, separate cultures of primary rat hippocampal neurons were treated with the calpain inhibitor MDL-28170 at a concentration of 1.0μM, and injured by application of 200μM NMDA. Cell survival was assessed at 24 hours following injury by determining the percentage of total cells that did not label with PI (Figure 4B). Treatment with MDL-28170 significantly increased the percentage of surviving cells as compared to vehicle-treated controls (Student's t-test, $p < 0.05$), indicating that cell death in this model is calpain-dependent.

Effect of NMDA exposure on calpain activity in primary hippocampal neurons

To assess the effect of isoform-specific knockdown on NMDA-induced calpain activity, cells were immunolabeled with an antibody that labels calpain-cleaved spectrin (Ab38) immediately following 30 minute NMDA exposure. As shown in Figure 5A, Ab38 immunoreactivity could be identified in ZsGreen-expressing neurons transduced with luc, capn1 and capn2 shRNA. Labeling of calpain-cleaved spectrin was blocked by pre-treatment with the NMDA receptor antagonist MK801 (data not shown). The proportion of transduced cells displaying calpain-cleaved spectrin immunoreactivity did not differ significantly between luc shRNA, capn1 shRNA and capn2 shRNA treated cultures (Figure 5B, one-way ANOVA, $p > 0.05$). However, we did observe two distinct patterns of Ab38 labeling. Some cells diffusely labeled throughout the cell body, while in others, labeling was limited to processes only. To determine if this differential distribution was an effect of isoform-specific knockdown, those cells with spectrin cleavage were further classified based on the presence of immunoreactivity in the cell body versus the processes. Of those injured cells with Ab38 labeling, the percentage of cells with calpain-cleaved spectrin immunoreactivity in the soma was significantly lower in cultures treated with capn2 shRNA than in luc or capn1 shRNA treated cultures (Figure 5C, one-way ANOVA with Scheffe post-hoc analysis, $p < 0.05$). In this injury model, knockdown of m-calpain appears to prevent calpain cleavage of spectrin in the cell body.

Effect of calpain knockdown on neuron survival following NMDA excitotoxicity

To determine the effect of isoform-specific calpain knockdown on neuronal survival, hippocampal neurons were transduced and injured as described above. Twenty-four hours after NMDA exposure, cultures transduced with luc or capn1 shRNA displayed abundant PI labeling as well as morphologic features of degenerating neurons such as swelling and disruption of processes (Figure 6A). In contrast, cultures transduced with capn2 shRNA displayed no such signs of degeneration and appeared qualitatively similar to uninjured controls. Cell survival in HBS-treated controls did not differ between naïve, luc, capn1 and capn2 shRNA treated cultures, indicating that neither vector transduction nor calpain knockdown had an effect on baseline survival. Naïve cultures as well as those transduced with luc or capn1 AAV2/1 vector showed a significant decrease in cell survival after NMDA exposure compared to HBS treated controls. Transduction with capn2 shRNA significantly increased survival compared to NMDA treated luc and capn1 cultures. Furthermore, survival of NMDA-treated cultures transduced with capn2 vector was not different from HBS treated controls (Figure 6B, one-way ANOVA with Scheffe post-hoc analysis, $p < 0.05$) and was equivalent to that in injured cultures pretreated with the calpain inhibitor MDL-28170 (Student's t-test, $p > 0.05$). These results suggest an essential role for m-calpain in mediating NMDA-induced neuronal death.

Nuclear translocation of calpain substrates

In an effort to identify a potential mechanism of m-calpain mediated cell death, we next examined the effect of NMDA toxicity on several known calpain substrates. Given that m-calpain knockdown prevented spectrin cleavage in the cell body immediately surrounding the nucleus, we chose to focus on a number of molecules that undergo nuclear translocation following calpain cleavage. Neuronal cultures were again injured by 30 minute exposure to 200μM NMDA as described above. Thirty minutes, 6 or 24 hours after injury, cells were immunolabeled for AIF, p35, CRMP-1, CRMP-2, CRMP-3 or CRMP-4. Of the six calpain substrates examined, only CRMP-1 displayed differential cellular localization before and after injury. In vehicle treated cells, CRMP-1 immunolabeling was present throughout the cytoplasm and distinctly excluded from the nucleus. As early as 30 minutes following NMDA exposure, CRMP-1 labeling was observed in the nucleus (Figure 7). Nuclear CRMP-1 labeling persisted at 6 hours after injury and was present in PI-positive cells 24 hours after NMDA exposure.

To determine if nuclear CRMP-1 translocation is an isoform specific effect, primary hippocampal neurons were transduced with AAV2/1 vector expressing luc, capn1 or capn2 shRNA at 7 DIV. At 21 DIV, culture media was removed, cells were exposed to 200μM NMDA for 30 minutes, and media was returned to dishes. Twenty-four hours after injury, cells were fixed and immunolabeled for CRMP-1. A significantly smaller percentage of cells transduced with capn2 shRNA displayed nuclear CRMP-1 labeling compared to luc and capn1 shRNA transduced cells (Figure 8, one-way ANOVA with Scheffe post-hoc analysis, $p < 0.05$).

Discussion

While the calpain family of cysteine proteases has a well-established role in brain physiology and pathology, isoform specific roles are incompletely understood. Physiologic calpain activation is required for formation of long-term potentiation (LTP), but μ-calpain (calpain 1) knockout mice do not display any LTP deficiencies (Grammer *et al*, 2005). These findings suggest an isoform specific role in LTP and indirectly implicate m-calpain in normal brain physiology. Similarly, while calpains are known to be key players in neurodegeneration following acute brain injury, isoform specific effects have not been completely examined. Antisense oligonucleotides targeting the calpain 1 catalytic subunit reduced the effects of NMDA excitotoxicity in hippocampal slice culture, however that study did not examine calpain 2 (Bednarski *et al*, 1995). RNA interference for calpain 1, but not calpain 2 has been shown to be protective in neuronal culture injured by oxygen glucose deprivation (OGD) (Cao *et al*, 2007a). By demonstrating that knockdown of calpain 2 improves survival of hippocampal neurons following NMDA excitotoxicity, this study provides the first evidence that m-calpain can play a causal role in neuronal pathology.

We found that baseline expression of calpain 1 mRNA in our cells was approximately 50-fold lower than that for calpain 2, which was consistent with our inability to detect μ-calpain protein by western blot. The relative lack of μ-calpain in our model system may alone explain the importance of m-calpain in the injury studied here. While calpain 2 message has been shown to be more abundant in the adult brain (Li *et al*, 1996), the difference is not as dramatic as we report here in immature neuronal culture. Moving forward, it will be important to consider baseline expression of calpain isoforms when exploring potential mechanisms of calpain-mediated neuronal injury both *in vitro* and *in vivo*.

It is also important to recognize that our capn2 shRNA vector non-specifically reduced expression of calpain 1 mRNA. It is possible, therefore, that μ-calpain protein was present at a level undetectable by western blot, and that knockdown of both calpains was required for

neuroprotection in this injury model. One piece of evidence against a role for μ-calpain in this model is the absence of nuclear translocation of the known μ-calpain substrate AIF. Furthermore, we observed that the capn1 vector produced an equivalent knockdown of calpain 1 message with no effect on calpain 2 and did not produce any neuroprotective effect. Given this evidence, we feel confident in stating that m-calpain played an essential role in mediating NMDA excitotoxicity in our model system.

While we demonstrated increased neuronal survival with knockdown of m-calpain, we surprisingly observed that this increase in survival was not associated with a significant decrease in the number of cells displaying calpain-cleaved spectrin immunoreactivity. Additionally, we are not able to assess any change in the total amount of spectrin cleavage from our immunocytochemical assay. This finding suggests that spectrin cleavage should not be used as an early marker for calpain mediated cell death, as we saw spectrin cleavage even in those cells that did not go on to die. The persistent spectrin cleavage could be explained by either incomplete knockdown of the targeted isoform, or by the presence of other calpain isoforms. In addition to μ- and m-calpain, there is evidence for expression of calpain 3 (Herasse et al, 1999; König et al, 2003; Marcilhac et al, 2006), calpain 5 (Waghray et al, 2004) and calpain 10 (Ma et al, 2001) in neural tissue, any of which may also cleave spectrin following NMDA exposure. We did demonstrate a significant difference in the distribution of calpain activity in the NMDA treated cultures, with knockdown of m-calpain preventing somatic spectrin cleavage. The differential patterns of spectrin cleavage may reflect distinct subcellular localization of each enzyme. This result suggests a potential role for m-calpain in propagation of the excitotoxic signal throughout the cell body leading to eventual cell death.

Somatic calpain activity may be required for cleavage of substrates involved in late stages of cell death signaling, including those that translocate to the nucleus following calpain cleavage. Calpain cleaves AIF, causing it to translocate from mitochondria to the nucleus (Polster *et al*, 2005) in a number of neuronal injury models including *in vitro* excitotoxicity (Yu *et al*, 2002) and oxygen glucose deprivation (Cao *et al*, 2003) and *in vivo* hypoxia (Zhu *et al*, 2003), ischemia (Cao *et al*, 2003) and traumatic brain injury (Zhang *et al*, 2002). Additionally, calpain cleaves the p35 activator protein of cyclin dependent kinase-5 (CDK5) to form the more stable p25 fragment (Kusakawa *et al*, 2000). The p25-CDK5 complex then translocates to the nucleus where it plays an essential role in neuronal cell death following *in vitro* glutamate excitotoxicity (O'Hare *et al*, 2005), endoplasmic reticulum stress (Saito *et al*, 2007) or human immunodeficiency virus neurotoxicity (Wang *et al*, 2007). Calpain also cleaves the collapsin response mediator proteins CRMP-1, -2, and -4 following *in vitro* excitotoxic injury and *in vivo* traumatic brain injury (Zhang *et al*, 2007). Similarly, calpain cleavage of CRMP-1, -3 and -5 has been observed following *in vivo* ischemia (Jiang *et al*, 2007). NMDA injury of cortical neurons causes calpain-mediated cleavage of CRMP-2, which subsequently redistributes from neurites to the cell body (Zhang *et al*, 2007) while calpain-cleaved CRMP-3 translocates to the nucleus after glutamate injury of cerebellar granule neurons or following *in vivo* ischemia (Hou *et al*, 2006). Although each of these calpain substrates has been implicated in acute neuronal injury, they have not previously been examined together in a single study. We examined the cellular distribution of AIF, p35, CRMP-1, -2, -3, and -4 in our injury model, finding that only CRMP-1 translocated to the nucleus following NMDA exposure in our model. Furthermore, knockdown of m-calpain blocked the redistribution of CRMP-1. While m-calpain knockdown also reduced cell death in our model, we cannot, based on the data presented here, causally link CRMP-1 translocation and cell death.

It is also important to note that the results reported here differ from those recently described by Cao *et al* (2007) who demonstrated improved neuronal survival following knockdown of

the μ-calpain catalytic subunit in an injury model associated with AIF cleavage and nuclear translocation. Our results differ from those in Cao *et al* (2007) in that we observed extremely low levels of μ-calpain in our cell culture system and did not observe AIF translocation after injury. One important difference between the Cao study and the results reported here was the injury model used. The Cao study investigated the role of isoform-specific knockdown in oxygen-glucose deprivation (OGD) rather than NMDA excitotoxicity. While excitotoxicity is likely a component of OGD, the two injuries are fundamentally different due to the added component of metabolic interruption in OGD. The lack of oxygen leads to mitochondrial depolarization during OGD, which is followed by substantial mitochondrial calcium overload upon reintroduction of oxygen and glucose. These events could make it more likely for OGD to trigger mitochondrial death pathways such as AIF release.

Although we focused on calpain substrates involved in nuclear signaling, there are numerous other calpain that have known causal roles in neuronal injury pathways including calcium regulatory proteins (Bano *et al*, 2005), neurotransmitter receptors (Xu *et al*, 2007), and a wide range of signaling proteins (Wu *et al*, 2004; O'Hare *et al*, 2005; Hou *et al*, 2006; Jiang *et al*, 2007; Saito *et al*, 2007; Wang *et al*, 2007; Cao *et al*, 2007; Zhang *et al*, 2007; for review, see Bevers and Neumar, 2008). Calpain cleavage of these and other substrates may represent independent injury mechanisms or may be a component of a cell death pathway that includes multiple calpain substrates. Future studies on the isoform-specificity of cleavage of other calpain targets and genetic manipulation of those targets themselves will be necessarily to understand the exact mechanism of calpain-mediated neuronal death.

In this study we demonstrate that knockdown of m-calpain protects mature (21 DIV) primary hippocampal neurons from NMDA excitotoxicity. To the best our knowledge, this is the first study to specifically implicate m-calpain in a model of acute neurodegeneration. We also observed that m-calpain knockdown specifically prevented nuclear translocation of the calpain substrate CRMP-1 after NMDA exposure. Notably absent from our model was nuclear translocation of AIF, which has been reported following oxygen glucose deprivation. These results raise the possibility that the relative contribution of isoform-specific calpain-mediated injury mechanism could vary based on baseline gene expression, injury severity and injury mechanism. Moving forward, it will be particularly important to study isoform-specific calpain-mediated cell death pathways using *in vivo* models examining different mechanisms and severity of injury.

Acknowledgments

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Abbreviations

AAV	adeno-associated virus
AIF	apoptosis inducing factor
capn1	calpain 1 gene name

capn2	calpain 2 gene name
CDK5	cyclin-dependent kinase 5
CRMP	collapsin response mediator protein
DAPI	4,6-daimidino-2-phenylindole
DIV	days in vitro
DMEM	Dulbecco's modified eagle medium
EGFP	enhanced green fluorescent protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
HBS	HEPES buffered saline
HPRT	hypoxanthine phosphoribosyltransferase 1
MAP2	microtubule associated protein 2
PI	propidium iodide
shRNA	short hairpin RNA
siRNA	short interfering RNA

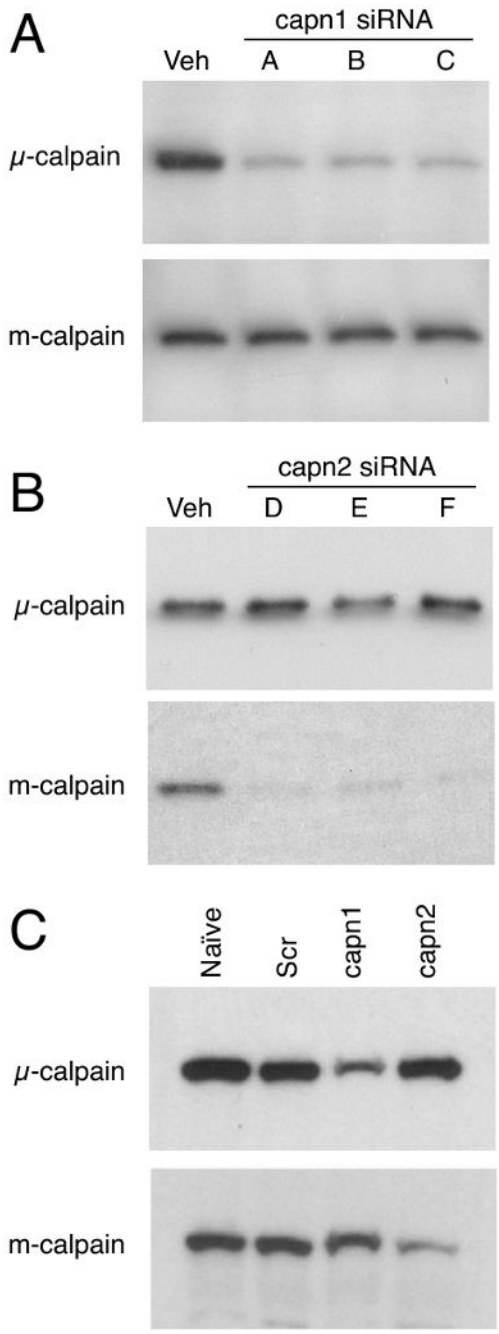

Figure 1.

Calpain knockdown in Rat2 fibroblasts. (A) Rat2 fibroblassts were transfected with three different short interfering RNA (siRNA) sequences targeting the catalytic subunit of μ-calpain (capn1). All three capn1 sequences (lanes A, B and C; see Table 1) reduced expression of μ-calpain compared to controls exposed to transfection reagent alone (Veh), and none of the sequences altered expression of m-calpain. (B) Similarly, three sequences targeting the catalytic subunit of m-calpain (capn2; lanes D, E, and F; see Table 1) decreased expression of that isoform without altering expression of μ-calpain. (C) Sequences B (capn1) and F (capn2) were converted into short hairpin RNA (shRNA) and cloned into a plasmid-based expression system. Transfection of rat2 fibroblasts demonstrated that shRNA

reduced expression of the targeted isoform as compared to naïve cells or those transfected with scrambled shRNA. As in the siRNA experiment, knockdown was isoform-specific.

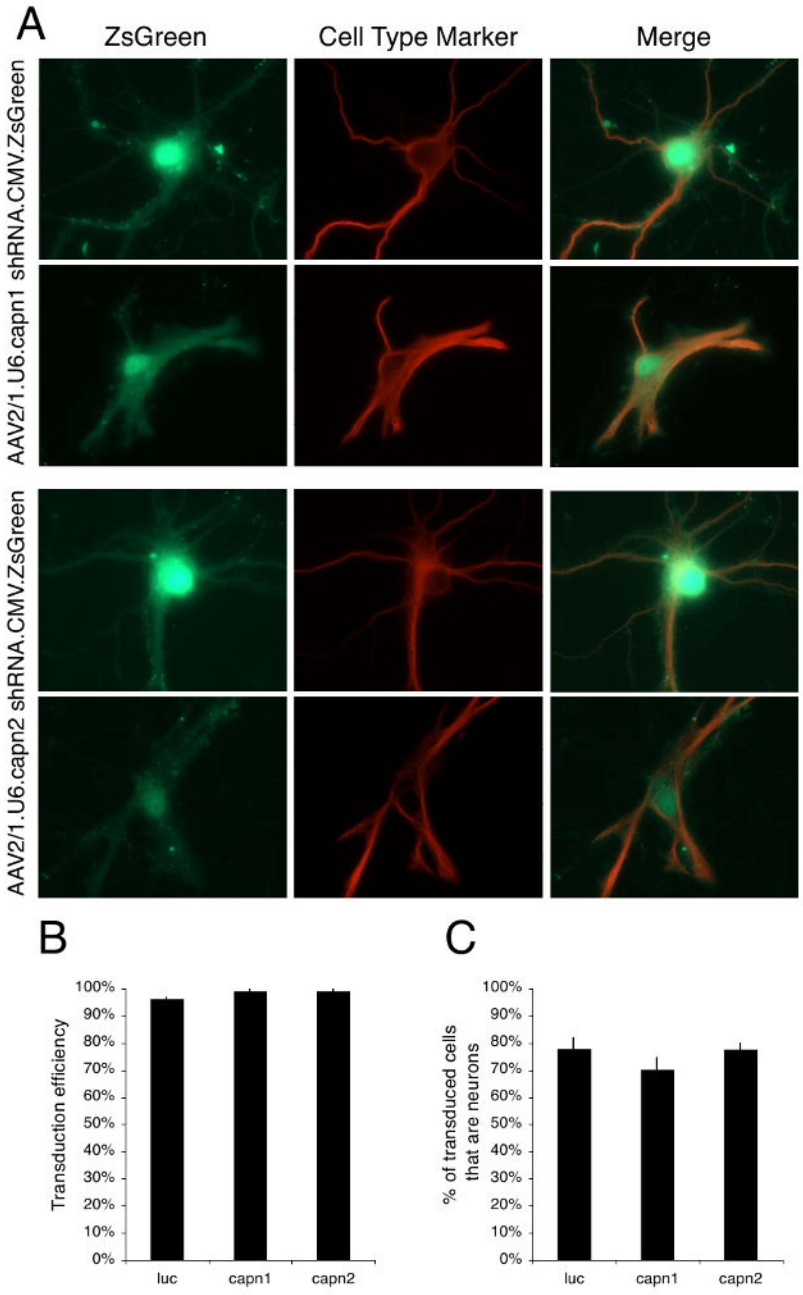

Figure 2.

Transduction of primary hippocampal neuron cultures with adeno-associated viral vectors. (A) AAV2/1 vectors expressing shRNA targeting the catalytic subunit of μ-calpain (capn1) and m-calpain (capn2) were able to transduce both neurons and glia. Transduction was identified by expression of ZsGreen and cell type determined by immunolabeling for MAP2 (neurons) or GFAP (glia). (B) Transduction efficiency was determined by dividing the number of ZsGreen expressing cells over the total number of DAPI-labeled nuclei. Vector expressing luc shRNA transduced 96.3±0.4% of cells, capn1 vector transduced 99.1±0.7% of cells and capn2 vector transduced 99.1±1.0% of cells. (C) The majority of cells in the cultures were neurons, as indicated by the fact that 77.8±7.5% of luc-transduced cells, 70.2

± 8.1% of capn1-transduced cells and 77.6 ± 4.0% of capn2 transduced cells were neurons (mean ± standard deviation). There was no significant difference in the percentage of transduced cells that labeled for the neuronal marker MAP2 between the two conditions (One-way ANOVA, $p > 0.05$; error bars indicate standard error of the mean).

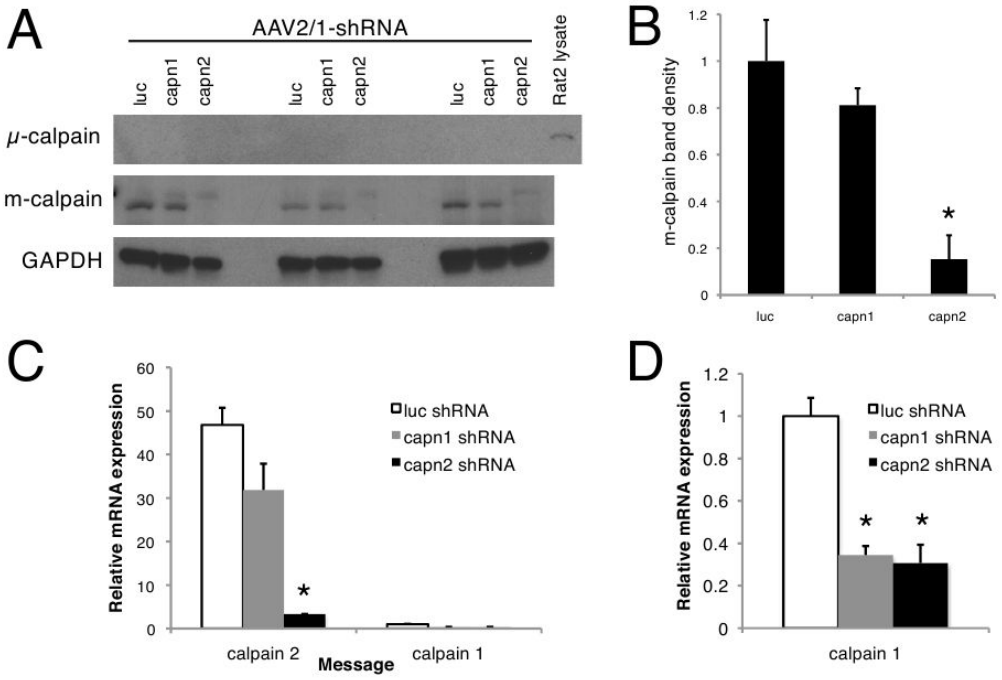

Figure 3.

Calpain isoform knockdown in primary hippocampal neurons. (A) Primary hippocampal neurons were transduced with 1.5×10^{11}GC of vector expressing luc, capn1 or capn2 shRNA. Western blot performed 2 weeks after transduction demonstrates that capn2 shRNA reduces expression of m-calpain, while transduction with luc or capn1 shRNA does not. Expression of μ-calpain could not be detected by Western blot in 20μg of cell lysate from these cultures, even though it can be detected in an equivalent amount of Rat2 fibroblast lysates. (B) Densitometric analysis indicates that capn2 shRNA significantly lowers expression of m-calpain, while transduction with luc and capn1 shRNA has no effect (One-way ANOVA with Scheffe post-hoc analysis, $*p < 0.05$). (C) Quantitative real-time PCR for calpain 1 and 2 message shows that in control luc shRNA-treated cultures, calpain 2 message is approximately 50-fold more abundant than calpain 1 message. Transduction with capn2 shRNA significantly reduces expression of calpain 2 message compared to luc shRNA control ($*p < 0.05$, One-way ANOVA with Scheffe post-hoc analysis). (D) Expanded view of calpain 1 message levels. Both capn1 and capn2 shRNA significantly reduced expression of calpain 1 message ($*p < 0.05$, One-way ANOVA with Scheffe post-hoc analysis), however the physiologic relevance of this knockdown is unknown given the extremely low baseline levels of calpain 1 mRNA.

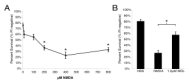

Figure 4.

Characterization of NMDA injury model. (A) Primary hippocampal neurons were exposed to 10μM, 100μM, 200μM, 400μM or 800μM NMDA or HBS vehicle for 30 minutes. Twenty-four hours after injury, live cells were labeled with calcein-AM and dead cells with PI. The percentage of surviving cells was significantly reduced in cultures treated with NMDA concentrations of 200μM and higher (*$p < 0.05$, One-way ANOVA with Scheffe post-hoc analysis). No additional increase in injury was observed at concentrations greater than 200μM, so this concentration was used for all subsequent studies. (B) Primary hippocampal neurons were treated with 1.0μM MDL-28170 and subsequently injured with 200μM NMDA as described. Twenty-four hours after injury, cells were stained with DAPI to label nuclei and stained with propidium iodide to identify dead cells. Treatment with the calpain inhibitor MDL-28170 (1.0μM MDL) significantly increased cell survival compared to injured cultures treated with vehicle (Student's t-test *$p < 0.05$).

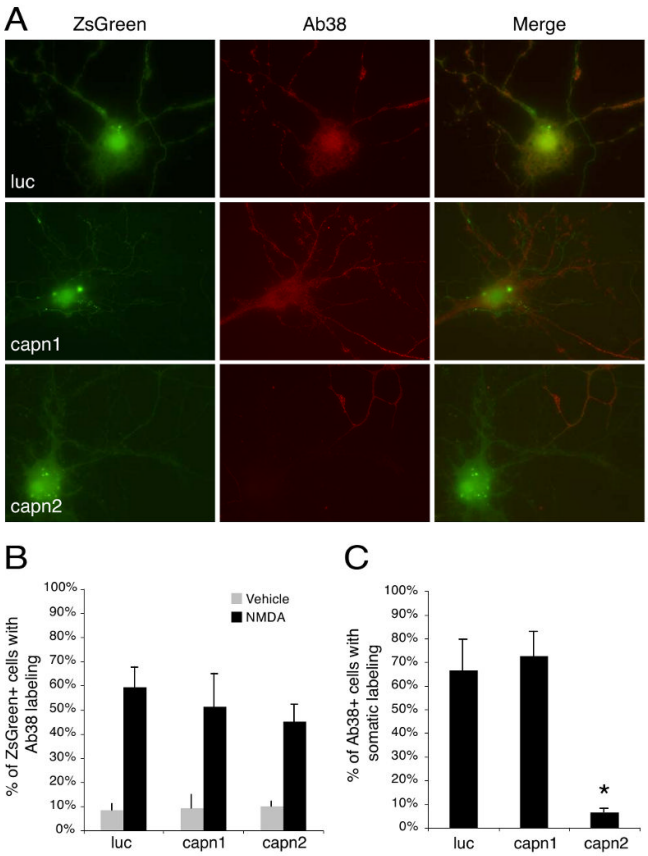

Figure 5.

Calpain activity in neurons transduced with AAV2/1 expressing luc, capn1 or capn2 shRNA. (A) Calpain activity was assessed by immunolabeling cells for calpain-cleaved spectrin (Ab38, red) after 30 minutes of NMDA exposure. Calpain activity was observed in cells transduced with all three shRNA sequences, however the distribution in capn2 shRNA treated cultures was primarily limited to processes, while luc and capn1 shRNA treated cultures displayed labeling both in the soma and processes. (B) Quantification of the percentage of ZsGreen expressing cells that displayed any labeling for calpain-cleaved spectrin. NMDA injury (black bars) increased the percentage of transduced cells with calpain activity in all three transduction conditions as compared to vehicle treated controls (grey bars). No significant difference in the percentage of cells with calpain activity was observed between injured cultures transduced with control (luc), capn1 or capn2 shRNA (One-way ANOVA with Scheffe post-hoc analysis, $p > 0.05$). (C) Quantification of the percentage of Ab38 positive cells with labeling in the cell body. A significantly smaller percentage cells transduced with capn2 shRNA immunolabeled for calpain activity in the cell body (One-way ANOVA with Scheffe post-hoc analysis, $*p < 0.05$).

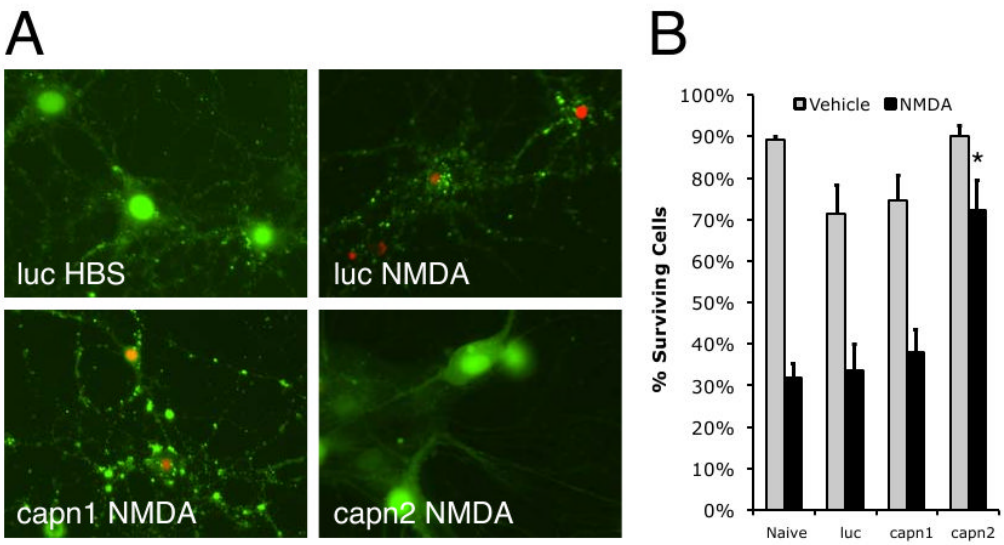

Figure 6.

Cell survival after NMDA injury. (A) Twenty-four hours after NMDA exposure, cultures transduced with AAV2/1 vector expressing luc (luc NMDA) or capn1 shRNA (capn1 NMDA) displayed abundant PI labeling and morphologic signs of cell death, including swelling and breakdown of processes. In contrast, cultures transduced with AAV2/1 vector expressing capn2 shRNA (capn2 NMDA) did not differ qualitatively from control cultures treated with HBS (luc HBS). (B) Quantification of cell survival was performed by calculating the ratio of PI-negative, ZsGreen expressing cells to the total number of ZsGreen expressing cells. Survival in uninjured cultures did not differ between transduction conditions. Cultures transduced with luc and capn1 shRNAs displayed a significant decrease in cell survival following NMDA injury (black bars). The cell death associated with injury in these two transduction conditions did not differ from that observed following NMDA injury in non-transduced controls. In cultures transduced with AAV2/1 vector expressing capn2 shRNA, survival did not differ between vehicle (grey bar) and NMDA treatment (black bar). Furthermore, capn2 knockdown significantly increased survival after NMDA exposure compared to naïve, luc and capn1 shRNA transduced cultures (One-way ANOVA with Scheffe post hoc analysis, * $p < 0.05$ vs. naïve NMDA, luc NDMA and capn1 NMDA).

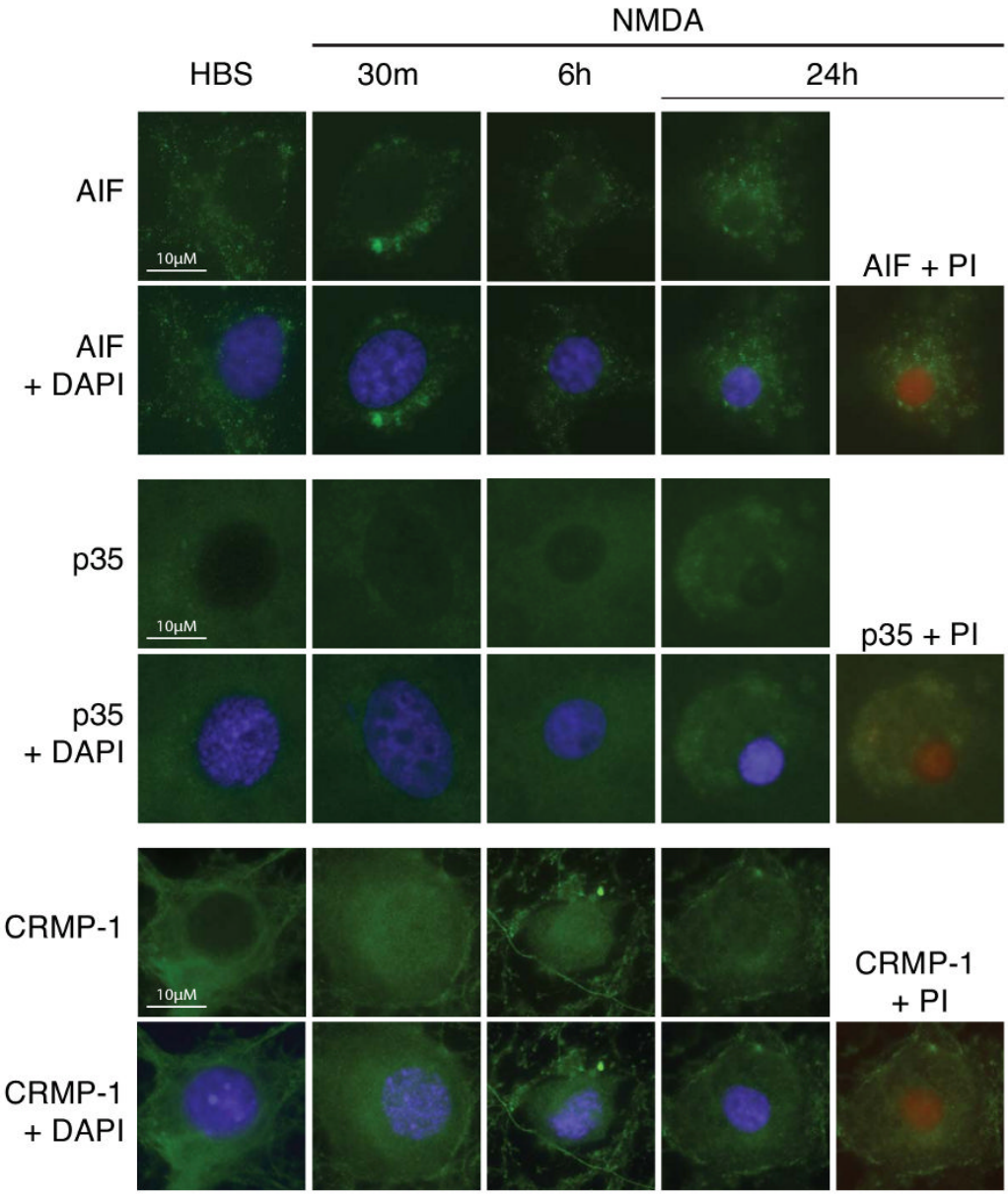

Figure 7.

Translocation of calpain substrates following NMDA exposure. Hippocampal cultures were injured by application of 200μM NMDA for 30 minutes. Immediately following injury or 6 or 24 hours later, cultures were fixed and immunolabeled for the calpain substrates AIF, p35, CRMP-1, CRMP-2, CRMP-3 and CRMP-4. Only CRMP-1 displayed differential cellular localization following injury. Uninjured cultures immunolabeled for CRMP-1 in the cytoplasm, with staining excluded from the nucleus. Following 30 minutes of NMDA exposure, CRMP-1 immunoreactivity was visible throughout both the cell body and nucleus. Nuclear CRMP-1 labeling remained at 6 hours after injury and was visible in dead cells (as identified by PI labeling) 24 hours after NMDA application.

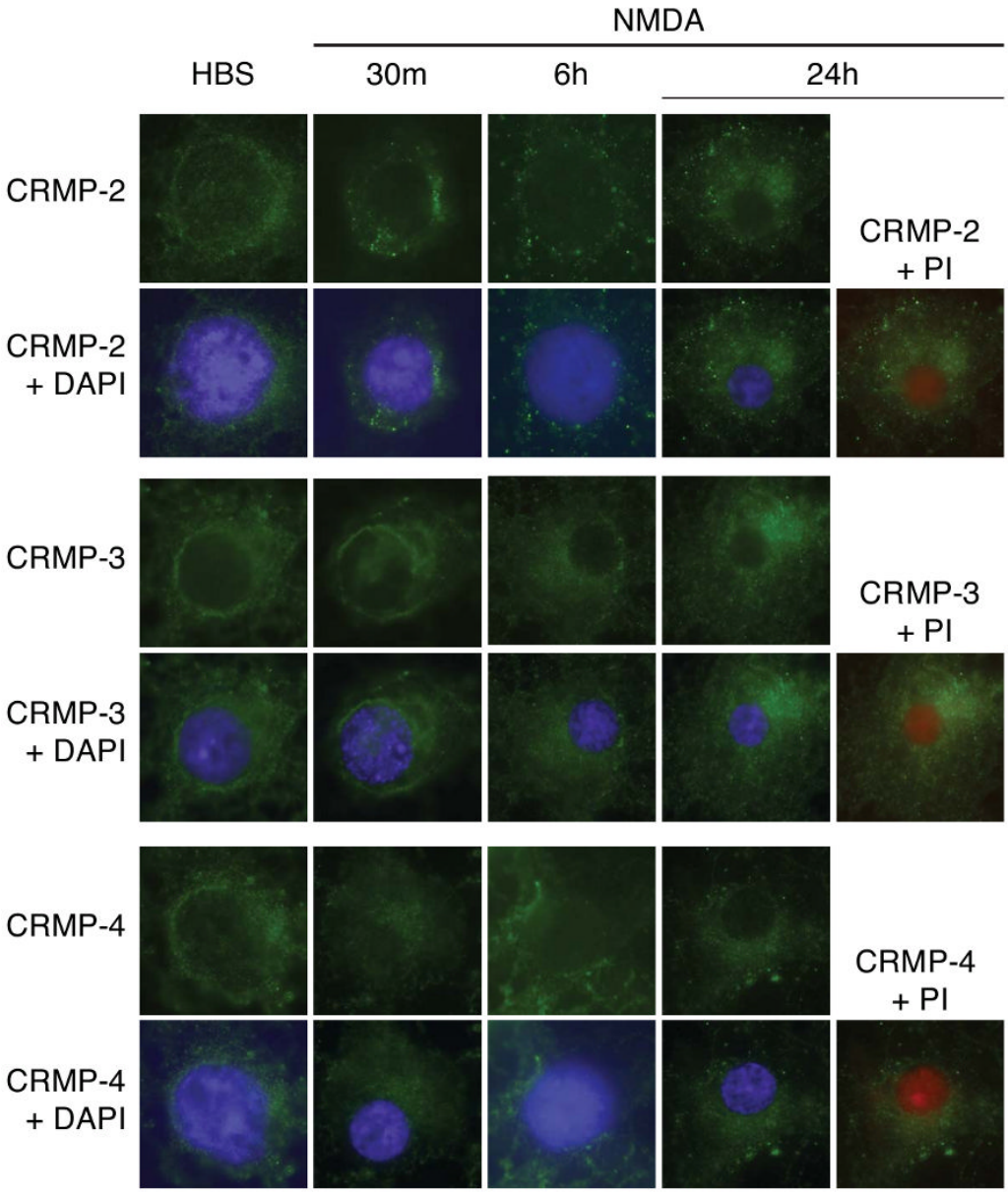

Figure 8.

Effect of m-calpain knockdown on CRMP-1 translocation. Primary hippocampal neurons were transduced with AAV2/1 vector expressing luc, capn1 or capn2 shRNA and injured by application of 200μM NMDA two weeks later. Twenty-four hours after injury cells were fixed and immunolabeled for CRMP-1. A significantly smaller percentage of cells transduced with capn2 shRNA displayed nuclear CRMP-1 labeling compared to luc and capn1 shRNA transduced cultures (One-way ANOVA with Scheffe post-hoc analysis, $*p < 0.05$).

Table 1

Table of siRNA sequences tested in Rat2 fibroblast cells. The sequences selected for conversion to shRNA and used for AAV vector-based RNA interference are shown in bold.

	Gene	Sense siRNA Sequence
A	capn1	GGAUUAUGAAAAUCAACGUtt
B	capn1	**GGAACUGGGUCCCAAUUCCtt**
C	capn1	GGCUAUUACUUUUAAGAACtt
D	capn2	GGAAUUACCCAAAUACCUUtt
E	capn2	GGAAAACUAUGCAGGCAUCtt
F	capn2	**GGCAUCUUCCAUUUCCAGUtt**