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Mechanisms of calpain mediated proteolysis of voltage gated sodium channel α-subunits following *in vitro* dynamic stretch injury

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Abstract

Although enhanced calpain activity is well documented after traumatic brain injury (TBI), the pathways targeting specific substrate proteolysis are less defined. Our past work demonstrated that calpain cleaves voltage gated sodium channel (NaCh) α-subunits in an *in vitro* TBI model. In this study, we investigated the pathways leading to NaCh cleavage utilizing our previously characterized *in vitro* TBI model, and determined the location of calpain activation within neuronal regions following stretch injury to micropatterned cultures. Calpain specific breakdown products of α-spectrin appeared within axonal, dendritic, and somatic regions six hours after injury, concurrent with the appearance of NaCh α-subunit proteolysis in both whole cell or enriched axonal preparations. Direct pharmacological activation of either NMDA receptors (NMDArs) or NaChs resulted in NaCh proteolysis. Likewise, a chronic (6 hour) dual inhibition of NMDArs/NaChs but not L-type voltage gated calcium channels significantly reduced NaCh proteolysis six hours after mechanical injury. Interestingly, an early, transient (30 minutes) inhibition of NMDArs alone significantly reduced NaCh proteolysis. While a chronic inhibition of calpain significantly reduced proteolysis, a transient inhibition of calpain immediately after injury failed to significantly attenuate NaCh proteolysis. These data suggest that both NMDArs and NaChs are key contributors to calpain activation after mechanical injury, and that a larger temporal window of sustained calpain activation needs consideration in developing effective treatments for TBI.

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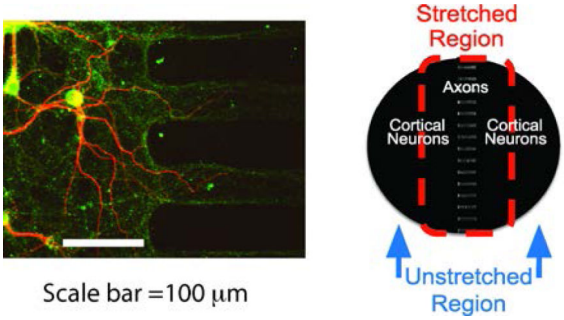
Graphical abstract

Enhanced calpain activity is a hallmark consequence of Traumatic Brain Injury, while the pathways leading to proteolysis of specific substrates are less well defined. Here, we demonstrate how early NMDA receptor activation underlies calpain mediated proteolysis of voltage gated sodium channels following an *in vitro* mechanical insult. Propagating within the first several hours after injury, this signaling pathway may be amenable to new therapeutic approaches.



Keywords

calpain; sodium channel; NMDA receptor; traumatic brain injury; proteolysis; axon

INTRODUCTION

Hallmark pathological consequences of traumatic brain injury (TBI) include activation Ca^{2+} dependent μ and m-calpain (Saatman et al., 2010). Evidence for calpain activation within TBI is plentiful, documented with the presence of calpain specific α-spectrin fragments appearing within somatic, dendritic, and axonal regions particularly vulnerable to degeneration (Roberts-Lewis and Siman, 1993; Kampfl et al., 1996; Saatman et al., 1996; Saatman et al., 2003). It is not known if proteolysis of alternate calpain substrates after TBI follows the subcellular localization of α-spectrin proteolysis. In TBI, calpain activation arises from a chronic loss in intracellular Ca^{2+} regulation (Fineman et al., 1993; Kampfl et al., 1997). Mechanically induced Ca^{2+} dysregulation is studied with *in vitro* stretch injury models, identifying key initiating events in the biochemical cascade (LaPlaca et al., 2007; Spaethling et al., 2007). In particular, activation of NMDA receptors (NMDArs), known modulators of calpain activity, significantly contributes to somatic Ca^{2+} elevation following mechanical injury of dissociated cortical cultures (Siman and Noszek, 1988; Seubert et al., 1989; Roberts-Lewis et al., 1994; Geddes-Klein et al., 2006b).

One key feature of TBI is the remarkable heterogeneity in pathological outcome (Saatman et al., 2008). *In vivo* models, by varying mechanical loading parameters, replicate specific aspects of the heterogeneity presented clinically, ranging from diffuse to focal, white or gray matter injury (O'Connor et al., 2011). *In vitro* TBI models, utilizing neuronal micropatterning techniques, provide evidence that heterogeneity also exists at the subcellular level in terms of the mechanisms underlying Ca^{2+} dysregulation. The mode of calcium influx in neurons exposed to mechanical injury can be primarily mediated through NMDAr activation (Geddes-Klein et al., 2006b) or the appearance of non-specific membrane pores

(Geddes-Klein et al., 2006a; LaPlaca et al., 2007). Mechanical injury restricted to unmyelinated axons increases axoplasmic [Ca^{2+}] through NaCh activation by indirectly opening voltage gated calcium channels (CaChs) and reversing Na^{+}/Ca^{2+} exchangers (Wolf et al., 2001). Although inhibition of both NaChs and CaChs provides substantial neuroprotection *in vivo*, the differential regulation of calpain activation through these two pathways is unknown (Okiyama et al., 1995; Sun and Faden, 1995; Harders et al., 1996). Many channels and exchangers are also calpain substrates (Bi et al., 1998; Bano et al., 2005; von Reyn et al., 2009), highlighting the potential for calpain contributing to further Ca^{2+} dysregulation (Araujo et al., 2010).

Currently, there is no clear linkage between the relative contribution of key initial events leading to early or sustained calpain activation and targeting of specific substrates following stretch injury. Previously, we demonstrated that α-subunits of NaChs are calpain substrates, proteolyzed within an *in vitro* model of TBI (von Reyn et al., 2009). Here, we examine the subcellular location of active calpain in concurrence with NaCh proteolysis, determine the underlying mechanisms leading to calpain activation, and assess how early, transient calpain inhibition influences substrate proteolysis at later timepoints.

METHODS

Cell culture

All reagents were purchased from Sigma-Aldrich (St Louis, MO) unless indicated otherwise. Cortical neurons from embryonic day 18 rats were isolated as described previously (von Reyn et al., 2009), with the following modifications. Embryonic cortices were dissociated and plated in plating media (MEM (Invitrogen, Carlsbad, CA), horse serum (Invitrogen), GlutaMAX (Invitrogen), D-glucose, penicillin/streptomycin (Invitrogen)) on poly-D-lysine (PDL, MW > 300 kDa) coated culture dishes or deformable silicone membranes (Sylgard 184 and 186, Dow Corning, Midland, MI). Cultures were maintained with feeding media (Neurobasal (Invitrogen) , B-27 (Invitrogen), GlutaMAX) including 1 μM Arabinosylcytosine from DIV 5 to DIV 9.

Micropatterning of neuronal cultures

To monitor stretch induced proteolysis within specific subcellular compartments, neuronal cultures containing a 2 mm, integrated axonal compartment were micropatterned using a modified version of the technique described previously (Smith et al., 1999; Tang-Schomer et al., 2010). PDMS (Sygard 184, Dow Corning) negative stamps containing channels of 50 μm height and width were molded from microfabricated masters. Channels traversed through the entire 2 mm stamp width as seen in Figure 1A. Negative stamps were placed on deformable silicone membranes (Specialty Manufacturing) with the channel side down (Figure 1A). Membranes with stamps were treated with 1M NaOH followed by an overnight coating with PDL, restricting substrate absorption on the silicone membrane to areas not in direct contact with the stamp. Cortical cultures, isolated as described above, were plated on the membranes. At DIV 5, stamps were removed, permitting axonal growth restricted to the PDL treated region corresponding to channels on the stamp (Figure 1B). As seen in Figure 1C and 1D, the 2 mm axonal tracts are free of cell bodies, with MAP2 positive dendrites

extending into only the first 200 μm of the tracts. In some experiments, no micropatterning was used and neurons adhered to the substrate to form a randomly interconnected network. We term these “unpatterned” neuronal cultures.

Pharmacological receptor activation

At DIV 18-21, dissociated cortical cultures were rinsed and pre-treated for 15 minutes in saline solution (in mM: 100 NaCl, 5.4 KCl, 2 $MgCl_2$, 1.8mM $CaCl_2$, 26 $NaHCO_3$, 0.9 NaH_2PO_4, 10 HEPES, 25 glucose, pH 7.4) with or without inhibitors of calpain (10 μM MDL28170). Cultures were incubated for one hour at 37°C and 5% CO_2 with the Ca^{2+} ionophore (1 μM ionomycin, Invitrogen), the NMDAr agonist (100 μM NMDA with or without 100 μM glycine), the sodium channel agonist (20 μM veratridine), or the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor agonist (100 μM AMPA) and then lysed in lysis buffer (in mM: 10 Hepes, 200 NaCl, and 30 EDTA with 0.5% Triton-X, pH 7.4) containing freshly added protease (Complete Mini, Roche), tyrosine phosphatase (1mM sodium orthovanadate), kinase (10mM potassium pyrophosphate), and serine/threonine phosphatase (50 mM sodium fluoride) inhibitors.

In vitro stretch injury

An *in vitro* injury model, as described previously (Smith et al., 1999; Lusardi et al., 2004) was used to stretch injure cultures at a controlled strain rate and strain level. Injuries were performed at DIV 15-16 on unpatterned cortical cultures or DIV 15-16 on micropatterned cultures on silicone membranes. The technique is flexible to accommodate three different types of injury: focused injury of only the axonal segment, a more expanded injury of axons and the adjunct cortical networks, or mechanical injury of the entire culture. For all inhibitor studies, cultures were rinsed and preincubated for 5 minutes in a saline solution with or without inhibitors of calpain (10 μM MDL28170, Calbiochem), NMDArs (50 μm APV), L-type voltage gated calcium channels (10 μm nimodipine), or voltage gated sodium channels (1 μM TTX). For BAPTA studies, cultures were loaded with BAPTA-AM (50 μM in 0.2% DMSO, Invitrogen) or DMSO (0.2%) for 30 minutes (37°C, 5% CO_2), rinsed and incubated for 30 min to promote de-esterification (37°C, 5% CO_2), and then stretch injured. Cultures were subjected to a 60-80% strain at a rate of 15-20s^{-1}. For stretch injury of micropatterned cultures, both the patterned axonal and adjacent neuronal (including somas, dendrites, and axons) areas were included in the stretched region, leaving the outer neuronal area unstretched (Figure 1E). Cultures were incubated for six hours (37°C, 5% CO_2), with or without a drug removal step 30 minutes following the injury. Stretch injured and control neuronal culture regions were collected and lysed in lysis buffer. For micropatterned cultures, stretched axonal, stretched neuronal, and unstretched neuronal (uninjured cultures) regions were isolated separately. Since these cultures produce low protein yields, lysates from 8 separate cultures were pooled to obtain protein quantities to run in a single lane on a gel.

Western Blot

Lysates were sonicated and then centrifuged (1000g × 4°C × 10 min) to remove the nuclear fraction and unlysed material. Sample buffer with 2.5% β-mercapto-ethanol was added to supernatants and samples were denatured for 30 minutes at 37 °C. Samples were subjected

to SDS page and western blot. Blots were probed with a pan NaCh antibody (1:1000, Pan, Sigma), stripped, and then reprobed for α-spectrin (1:10,000, MAB1622, Milipore), actin (1:10,000, MAB1501R, Milipore), or the AMPA receptor subunit (GluR1, 1:10,000, Abcam). Blots were quantified using a computer assisted two-dimensional densitometry scanning program (Kodak 1D Image Analysis Software, Eastman Kodak Company, Rochester, NY) and all proteins were normalized to their corresponding actin bands.

Statistics

All data were expressed as means ± SEM. Statistical significance for the data was determined by ANOVA unless otherwise indicated in the results. If data were found to be significant through ANOVA ($p<0.05$), between group tests were conducted using Tukey-Kramer HSD test ($\alpha=0.05$).

Immunocytochemistry

Stretch injured and control cultures were fixed (4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and 3% sucrose in PBS (Invitrogen)) and permeabalized (0.2% Triton-X in PBS). Cultures were blocked with normal goat serum (Invitrogen) and incubated overnight (4°C) with antibodies recognizing βIII-tubulin (1:2500, Covance, Princeton, NJ), the 150 kDa calpain specific N-terminal α-spectrin break down product (1:16,000, Ab38, Roberts-Lewis et al., 1994), Map2 (1:1000, MAB3418, Millipore), and $Na_v1.2$ (1:100, Alomone Labs). Cultures were rinsed and incubated with the corresponding secondary antibodies (goat anti mouse 488 and goat anti rabbit 594, 1:10,000; Invitrogen). Images were acquired at 20X with a CCD camera (C-4742-98, Hamamatsu, Japan), exciting the fluorophores at their respective wavelengths. Images were processed using Metamorph (Molecular Devices, Sunnyvale, CA) and analyzed using a Matlab (MathWorks, Natick, AM) script. Three fields of view were analyzed in each region per culture, evenly spaced within the uninjured region, moving across through the injured region to the central point of the axonal region (1mm away from the edge of the cell body region).

RESULTS

Calpain activation after stretch injury occurs in all subcellular regions, and corresponds with NaCh proteolysis

Given that both NMDARs (localized to dendrites and soma) or NaChs (with different isoforms present on all neuronal regions) might contribute to stretch-induced Ca^{2+} influx leading to calpain activation and NaCh α-subunit proteolysis (Benke et al., 1993; Catterall, 2000; Wolf et al., 2001; Geddes-Klein et al., 2006b), we first investigated the distribution of calpain activation and NaCh proteolysis at 6 hours within subcellular neuronal regions. To isolate subcellular regions for analysis, we micropatterned neuronal cultures separating networks of cortical neurons (containing dendrites, axons, and somas) from unmyelinated axons (Figure 1B-D). We designed our experiment to stretch injure the entire axon region, as well as a portion of the cortical neuron networks bordering each side of the axon region, leaving an outer region of cortical neurons unstretched (Figure 1E). Unlike previous *in vitro* injury models, this model injures all neuronal subcellular compartments yet enables selective analysis of specific compartments, and mimics injury to gray matter populations and their

connecting white matter tracts, as experienced in the brain (Carbonell et al., 1998; Smith et al., 1999; Lusardi et al., 2004). Using an antibody that specifically recognizes the calpain mediated 150 kDa N-terminal α-spectrin breakdown product as an indicator of calpain activation, we identified enhanced immunoreactivity in the cell bodies and processes of stretched cortical neuron regions compared to uninjured cultures (Figure 2A). We also examined unstretched cortical neuron regions in injured cultures, adjacent to the site of mechanical deformation. Although these neurons did not receive a mechanical insult, they displayed enhanced calpain mediated spectrin degradation as compared to control, uninjured cultures, but relatively less immunoreactivity than observed in stretched neurons within the same culture (Figure 2B). Stretch injured cultures at the interface between the neuron and axon regions also displayed enhanced spectrin proteolysis as compared to control cultures (Figure 2C). Finally, stretch injured axon regions displayed enhanced spectrin proteolysis as compared to uninjured, unstretched axons (Figure 3A).

With evidence of pervasive calpain activation following mechanical injury, we evaluated the subcellular distribution of NaCh proteolysis. No antibody currently exists specifically recognizing calpain mediated NaCh α-subunit fragments. Therefore, cortical neuron and axon regions were isolated separately and submitted to Western blot to resolve full length α-subunits from proteolyzed fragments. The AMPA receptor subunit GluR1, with dendritic and somatic expression (Petralia and Wenthold, 1992), was used as a control to evaluate the purity of the axon regions Immunoblots of lysates from patterned cultures displayed increases in proteolysis of NaCh α-subunits and α-spectrin within both the cortical neuron and axon enriched regions as compared to controls (Figure 3B). The relative level of NaCh proteolysis, normalized to actin, in the micropatterned axonal region of injury was significantly elevated over the levels detected in injured cortical neuron region (Figure 3C). However, NaCh cleavage was still detected in the injured cortical neuron population. Therefore, a rapid mechanical deformation encompassing dendritic, somatic, and axonal regions produces diffuse calpain activation, as demonstrated by proteolysis of α-spectrin six hours after injury. At this time, proteolyzed NaCh α-subunits reside in axonal tracts, yet may also exist within somatic and dendritic regions.

With substantial evidence for diffuse calpain activation, all subsequent injury experiments employed whole cell stretch injury on non-patterned cortical cultures. These cultures offer additional benefits of high protein yield (over limited protein availability in the patterned cultures), and permit direct comparisons with past data using non-patterned cultures (von Reyn et al., 2009).

Stretch mediated NaCh proteolysis is Ca^{2+} dependent

Elevated intracellular Ca^{2+} levels presumably contribute to calpain activation following TBI (Kampfl et al., 1997). To determine whether *in vitro* stretch-induced proteolysis of NaChs was dependent on intracellular Ca^{2+} levels, non-patterned cortical cultures were loaded with the cell permeant calcium chelator BAPTA-AM (50 μM) and stretch injured. Six hours following stretch injury, BAPTA treated cultures displayed a significant decrease in both NaCh and α-spectrin proteolysis as compared to untreated controls (Figure 4). Given the significant and nearly complete reduction provided by BAPTA treatment, these data indicate

that intracellular Ca^{2+} elevation underlies the majority of calpain activation after stretch injury.

Direct activation of NaChs and NMDArs leads to NaCh proteolysis

Potential pathways elevating Ca^{2+} following stretch injury include ligand and voltage gated ion channels. Two prominent candidates for channel-mediated influx are NMDArs and NaChs. Both contribute to calcium elevations after stretch injury through direct or indirect mechanisms (Wolf et al., 2001; Geddes-Klein et al., 2006b). Direct activation of NMDArs leads to calpain mediated proteolysis of α-spectrin, (Siman and Noszek, 1988) while inhibition of NaCh activation prevents NaCh proteolysis along stretch injured unmyelinated axons by an unidentified protease (Iwata et al., 2004). Similarly, we achieved significant calpain activation as measured through NaCh α-subunit and α-spectrin proteolysis in our cortical neuron cultures using a one hour application of the respective channel agonist for NMDArs (NMDA) or NaChs (veratridine) (Figure 5). NaCh α-subunit fragments appeared at similar molecular weights to calpain-mediated fragments occurring from ionomycin treatment (von Reyn et al., 2009). Across all conditions, proteolysis was significantly reduced with the calpain inhibitor MDL28170. In comparison, activation of AMPArs with their specific agonist (AMPA) for one hour failed to cause a significant increase in either NaCh or α-spectrin proteolytic fragments. Taken together, these data show that activation of NMDArs or NaChs leads to calpain mediated proteolysis of NaCh α-subunits.

Mechanically induced activation of NMDArs and NaChs contributes to NaCh and spectrin proteolysis after stretch injury to non-patterned cortical cultures

The evidence showing that direct activation of NaChs or NMDArs caused calpain activation and NaCh proteolysis (as demonstrated above), combined with past work showing that stretch activates both of these channels and leads to Ca^{2+} entry (Wolf et al., 2001; Geddes-Klein et al., 2006b), led us to hypothesize that these channels drive calpain activation after stretch injury. To test this hypothesis, we inhibited NaChs and/or NMDArs by pre-treating with the respective antagonist and leaving the antagonist on for the duration of the experiment (Figure 6A, C, D). Inhibition of L-type calcium channels (CaChs) and saline incubation were utilized as comparative controls (Figure 6B, E, F). Dual inhibition of NaChs and NMDArs displayed the maximum effect, significantly decreasing proteolysis of NaChs and α-spectrin (Figure 6C, D). Neither NaCh inhibition nor NMDAr inhibition alone significantly reduced NaCh proteolysis, while NMDAr inhibition produced a significant reduction in α-spectrin proteolysis. In contrast, inhibition of L-type CaChs failed to reduce proteolysis of either substrate (Figure 6E, F). These data suggests NMDArs and NaChs both contribute to calpain activation after stretch injury, and that a long-term dual blockade of both pathways is a highly effective method to reduce NaCh proteolysis.

Early, transient activation of NMDArs and NaChs contribute to NaCh proteolysis after stretch injury

We next considered the timing of inhibitory strategies to reduce or eliminate proteolytic processing of spectrin and NaChs. The peak in elevated intracellular $[Ca^{2+}]$ in the soma and in the axons occurs within the first five to fifteen minutes following stretch injury (Weber et al., 1999; Wolf et al., 2001; Geddes-Klein et al., 2006a; Geddes-Klein et al., 2006b;

Spaethling et al., 2008). We investigated whether blocking this initial rise, through channel inhibition limited to the early stage of injury, would provide the same level of proteolytic protection observed with a chronic inhibition of NMDArs and NaChs. To resolve the early contribution of NaCh or NMDAr activation, cultures were pre-treated with the respective reversible antagonists for 5 minutes and then stretch injured (Bermudez-Rattoni et al., 1991; Golshani et al., 1998). Antagonists were removed 30 minutes after the stretch injury. While this early, transient inhibition of NaChs displayed a trend towards reduction of both spectrin and NaCh proteolysis six hours after the injury, it was not significant (Figure 7). Inhibition of NMDArs, on the other hand, provided significant protection of NaCh proteolysis, while also showing a trend towards a reduction in spectrin fragment accumulation. This suggests that early, transient NMDAr activation provides the predominant pathway for Ca^{2+} entry leading to calpain activation and proteolysis following mechanical injury.

Sustained inhibition of calpain is more effective at reducing NaCh proteolysis than an early, transient inhibition

Since early, transient inhibition of NaChs and NMDArs attenuated proteolysis, we investigated whether a similar inhibition of calpain activation would be effective in attenuating proteolysis observed six hours later. We compared the proteolytic profiles of cultures containing a reversible calpain inhibitor (Bevers and Neumar, 2008) prior to and during the first 30 minutes following stretch injury with those containing the inhibitor the entire six hours following stretch injury (Figure 8). A slight reduction in the 110 kDa NaCh and 145/150 kDa spectrin band intensities occurred for the transient, 30 minute initial treatment. Calpain inhibition over the entire six hours, on the other hand, significantly reduced proteolysis of both NaChs and spectrin to levels not different from uninjured, untreated cultures. In contrast to NMDAr and NaCh blockade, these data show that inhibition of calpain activation beyond 30 minutes following injury is required to significantly influence proteolysis at later time points.

DISCUSSION

Robust calpain activity occurs in numerous neurodegenerative disorders including TBI (Vosler et al., 2008; Saatman et al., 2010). Despite recognition that calpain participates in pathological events following TBI, the mechanisms that cause calpain activation, the subcellular distribution of activated calpain, and the specific targets of this activation are less defined. In this study, we utilize an *in vitro* TBI model to investigate the subcellular localization of calpain activation, identify the pathways causing calpain mediated proteolysis of NaCh α-subunits, and examine the temporal sequence of events influencing this proteolytic process. Employing micropatterned neuronal cultures to isolate axonal compartments from whole cortical neuron (including somas, dendrites, and axons) compartments, we find ubiquitous activation of calpain throughout the neuronal perikarya and processes after mechanical injury, expanding into adjacent, unstretched neuronal populations. Both calpain activation and subsequent calpain-mediated NaCh proteolysis require a mechanically initiated increase in cytoplasmic [Ca^{2+}]. Two pathways previously shown to cause Ca^{2+} influx after stretch injury - activation of NMDArs or NaChs - contribute to calpain mediated proteolysis. An early, transient inhibition of these

mechanoactivated pathways is sufficient to significantly reduce proteolysis six hours after injury. An early, transient inhibition of calpain, however, does not significantly reduce either α-spectrin or NaCh proteolysis six hours after injury. Instead, a sustained calpain inhibition for the entire six hours is required. Together, these data show the primary pathways for calpain activation and subsequent NaCh proteolysis following mechanical injury *in vitro*, and highlight important regulating steps in calpain activation within the first several hours after injury that may be amenable to new therapeutic approaches.

Our micropatterned *in vitro* stretch injury model permits investigation of the subcellular distribution of NaCh α-subunit proteolysis, overcoming immunocytochemical requirements for antibodies discriminating between proteolyzed and non-proteolyzed substrates. NaCh α-subunit proteolysis is enhanced along axons, consistent with past studies showing that mechanical injury to unmyelinated axons causes proteolysis of the $Na_v1.2$ α-subunit (Iwata et al., 2004). The sodium channel isoform $Na_v1.2$ is expressed *in vivo* within unmyelinated axons, initial segments, and within the early formation of the Nodes of Ranvier (Westenbroek et al., 1989; Boiko et al., 2001). Although the pan NaCh antibody utilized in our studies is able to recognize other NaCh α-subunits that can be expressed in the soma and dendritic compartments (Rasband et al., 1999), the NaCh fragments selected for analysis are those previously identified as $Na_v1.2$ (von Reyn et al., 2009). Therefore, these data support proteolysis of $Na_v1.2$ as an axonal pathology, in agreement with previous data (Iwata et al., 2004; Jette et al., 2006), and an excellent biomarker for axonal injury.

Ample evidence suggests that activation of NMDArs, a major source of Ca^{2+} entry under both synaptic communication and excitotoxicity, can directly activate calpain (Siman and Noszek, 1988; DeRidder et al., 2006; Xu et al., 2009). Moreover, NMDArs show an enhanced calcium influx in response to NMDA agonist after mechanical injury, owing to the partial loss of the magnesium block caused by injury (Zhang et al., 1996). Elevation of extracellular $[Mg^{2+}]$, significantly reduces calpain mediated proteolysis of α-spectrin after TBI (Saatman et al., 2001). Therefore, it is not surprising that somatic and dendritically localized NMDArs contribute to NaCh proteolysis, even with substantial NaCh proteolysis along axons in our injury model (Benke et al., 1993). A continual NMDAr stimulation, elevates Ca^{2+} in axons (Cochilla and Alford, 1999), and activation of NaChs is not required for axoplasmic Ca^{2+} elevation (Christie and Jahr, 2008). Therefore, part of the NaCh cleavage process can be considered a more distal effect from activation of NMDArs on dendrites and soma. Our data also emphasize the early role NMDArs play on cleavage, where early, transient inhibition of the receptor is enough to significantly reduce proteolysis. This is consistent with our past work showing activation of NMDARs following mechanical injury contributing to an overwhelming majority of the calcium influx in this injury model (Geddes-Klein et al., 2006b).

NaCh proteolysis occurs in cortical neurons following application of a NaCh agonist. Likewise, blocking NaChs reduces proteolytic events after mechanical injury, linking NaCh activation to calpain activation. When mechanical injury is applied only to unmyelinated axons, NaCh activation is the dominant driving force for indirect Ca^{2+} entry and NaCh proteolysis by an unspecified protease (Wolf et al., 2001; Iwata et al., 2004). In our data, although NMDAr activation appears to be a dominant mechanism for calpain activation and

NaCh cleavage, a dual inhibition of NMDArs and NaChs is the most effective treatment to significantly reduce cleavage of NaChs and spectrin. Enhanced protection with NaCh inhibition may be due to a reduction in glutamate release in the postacute injury phase (Prakriya and Mennerick, 2000). If correct, this would highlight the importance of non-NMDA glutamate receptors in calpain activation after mechanical injury. To this end, recent reports indicate the increased appearance of calcium permeable AMPA receptors after mechanical injury (Spaethling et al., 2008; Goforth et al., 2011), and separate reports show that these AMPA receptors are linked to calpain activation (Araujo et al., 2004), an additional path to explore in future studies to identify emerging therapeutic targets that could affect the timing and extent of channel cleavage. Mild (2-10% strain) *in vitro* injury to isolated axon bundles results in cytoskeletal disruption and delayed axotomy similar to that seen following depletion of intracellular calcium stores with thapsigargin (Staal et al., 2010). As thapsigargin inhibition of the sarcoplasmic-endoplasmic recticulum calcium-ATPase leads to an increase in calpain activation (Nguyen et al., 1999), it would be interesting to compare calpain activation through extracellular calcium entry with calcium release from intracellular stores, particularly in the context of injury severity. Finally, although our data indicate a prominent role for voltage and ligand-gated channels in the ensuing NaCh cleavage, we do not exclude the possibility that a similar process will occur if non-specific increases in membrane permeability arise after injury (Geddes et al., 2003). Calpain activation can be attenuated upon treatment with Poloxamer 188, targeting non-specific membrane pores, following fluid shear stress injury to neuronal cultures (Kilinc et al., 2009).

Our data suggests that activation of both NMDArs and NaChs after mechanical injury is the predominant mechanisms leading to calpain activation and proteolysis of NaCh α-subunits and that a prolonged inhibition of activated calpain is key for blunting NaCh proteolysis. Past studies demonstrate how NMDAr and NaCh inhibition within *in vivo* models of TBI leads to tissue sparing and significant improvements in neurological outcomes (Faden et al., 1989; Okiyama et al., 1995; Sun and Faden, 1995; Harders et al., 1996; Smith et al., 1997), and highlight the efficacy of several classes of compounds on reducing the extent of calpain activation following TBI (Saatman et al., 2010), yet the corresponding effect on NaCh proteolysis is not known. Our data suggest that the targeting and timing of treatments are important, as seen through the failure of L-type CaCh inhibition to attenuate proteolysis and the need to deliver a sustained calpain inhibition to reduce proteolysis six hours after injury. Moreover, balancing the potential benefit of specific antagonist approaches (e.g., NMDAR antagonists) with the pro-survival signaling roles played by NMDArs (Hardingham, 2009) and, more broadly, neural activity after injury (Soriano et al., 2006) suggests a coordinated approach will be necessary to achieve optimal protection. One potentially important regulation point worth future study is the mitochondria, which are key in buffering calcium influx through NMDArs (Peng and Greenamyre, 1998), participate in calcium homeostasis (Babcock et al., 1997), and contain calpain (Garcia et al., 2005). One possible reason for the need to provide sustained calpain inhibition to prevent NaCh cleavage is that mechanically injured neurons will exhibit a tonic, enhanced uptake of cytosolic calcium into the mitochondria that will cause a later opening of the mitochondrial permeability transition pore. Either the release of mitochondrial calcium or activated calpain from the mitochondria could lead to a secondary phase of activated calpain that would be blunted with a sustained

calpain inhibitor strategy. Finally, the effectiveness of NaCh and NMDAr antagonists may occur from separate NMDAr and NaCh activation pathways, and the mechanical circumstances of the injury may be the important factor dictating the relative contribution of each pathway. For example, mechanically initiated damage to axons in circumstances with predominant white matter injury may increase the importance of NaCh activation as a therapeutic target and lessen the need to target the NMDAr, thereby avoiding challenges raised recently with NMDAr mediated neuroprotection strategies (Papadia and Hardingham, 2007). However, circumstances causing more widespread mechanical damage to the grey and white matter would enhance the synergistic contribution of both pathways, and likely warrant a more multifaceted treatment approach.

Future therapies will also require an understanding of the functional significance of calpain mediated proteolysis of NaChs. While evidence suggests that proteolysis of the inactivation loop of NaChs may contribute to loss of ionic homeostasis after injury (Iwata et al., 2004), the pathological outcome of calpain specific proteolysis of NaChs has yet to be determined. As a subset of NaChs remain in the plasma membrane following calpain proteolysis, NaChs are poised to contribute to the loss of ionic homeostasis if proteolysis decreases inactivation (von Reyn et al., 2009). Alternatively, proteolysis may suppress activity (Yuen et al., 2007), in this case engaging a protective mechanism to curtail ionic influx. With the potential for either neuroprotective or neurodegenerative outcomes, and evidence that calpain contributes to physiological signaling (Vanderklish et al., 1996), the most successful therapeutic strategies may emerge from targeting proteolysis of specific substrates rather than a broad calpain inhibition.

In conclusion, we have shown that calpain activation and subsequent proteolysis of NaCh α-subunits are mediated by NMDArs and NaChs following stretch injury. Calpain activation occurs throughout the injured neuron, while NaCh proteolysis has a substantial presence within axonal tracts. The axonal distribution of proteolyzed NaChs suggests a necessary focus on targeting calpain activation and its downstream effects within clinically relevant focal and diffuse axonal injury as prime neuroprotective strategies. Furthering our understanding of how these pathologies occur, as investigated here, will aid in developing these strategies.

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Abbreviations

TBI	Traumatic Brain Injury
NMDAr	NMDA receptor
NaCh	voltage gated sodium channel
TTX	tetrodotoxin
Nimo	Nimodipine

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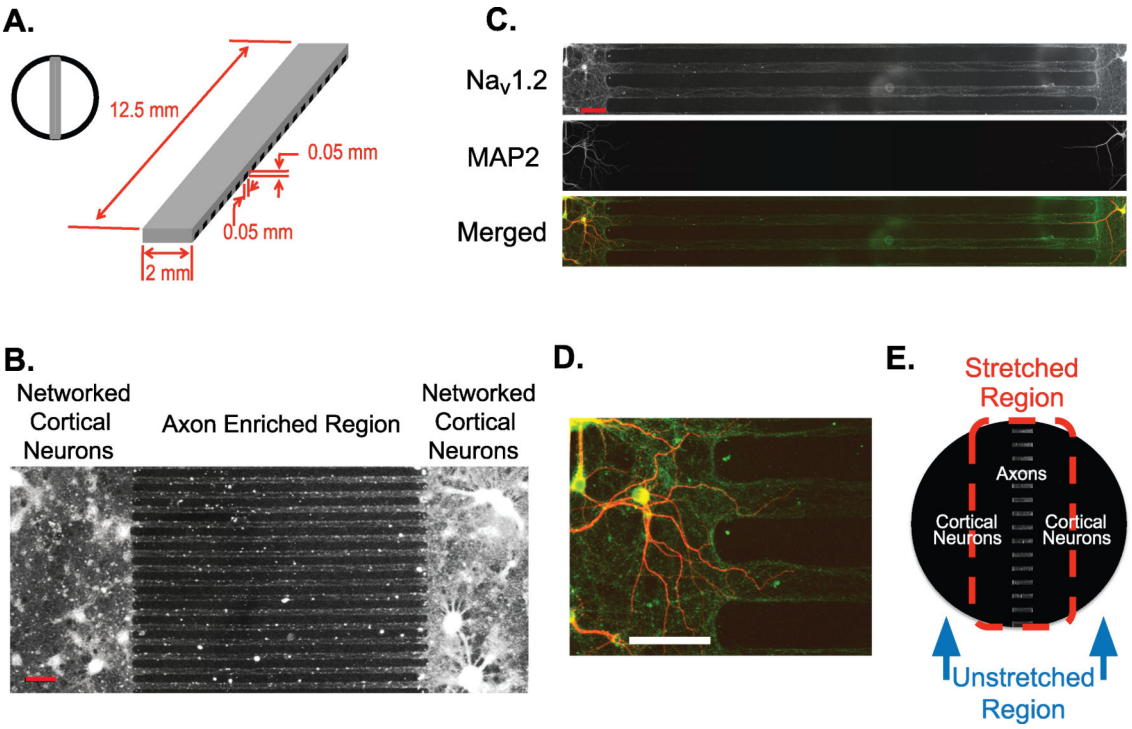

Figure 1.

Fabrication and characterization of micropatterned cultures for the *in vitro* stretch injury model ***A.*** Schematic of silicone insert containing gaps to permit axon growth. Inset displays how micropatterning is used to develop oriented axons that bridge two separate populations of networked cortical neurons. ***B.*** Image of a micropatterned culture at 10x. Scale bar = 200 μm. ***C.*** Antibody labeling of $Na_V1.2$ and MAP2 displays the isolation of the axonal and dendritic (MAP2 positive) regions. Scale bar = 100 μm. ***D.*** Magnification of the boundaries between the cortical neuronal region and the axonal region shown in *C.* Scale bar = 100 μm. ***E.*** Diagram labeling area of stretch injury targeting both the axonal and medial cortical culture region, leaving the lateral cortical neurons uninjured.

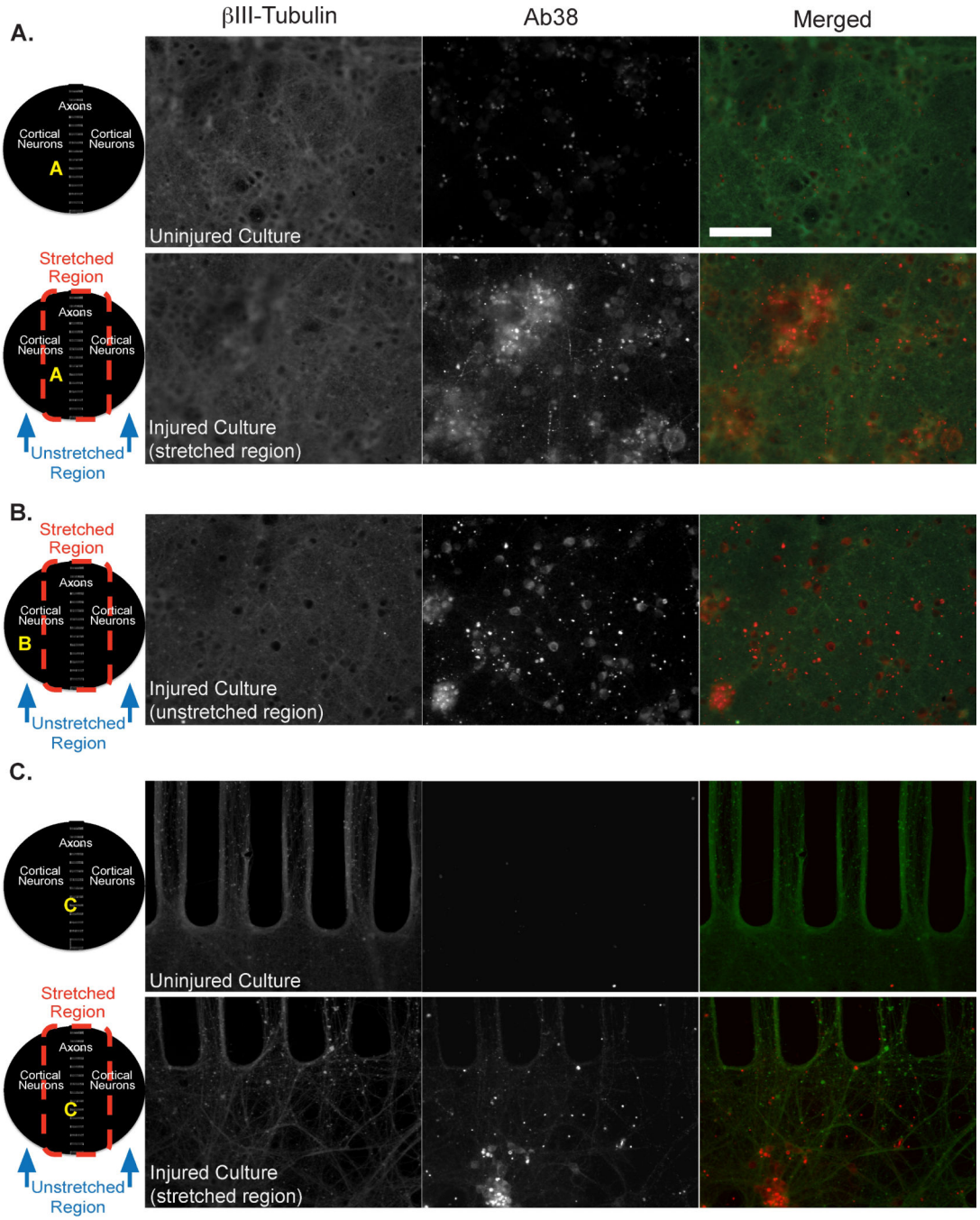

Figure 2.

Widespread distribution of calpain mediated α-spectrin proteolysis within subcellular regions after injury. Region of interest is indicated by a yellow letter in the leftmost pane. ***A.*** Representative images of Ab38 (calpain specific α-spectrin breakdown product, red) and βIII-tubulin (green) immunoreactivity in control cortical neuron regions or stretch injured cortical neuron regions 6 hours after injury (scale bar, upper right =100 μM) ***B.*** Representative images of Ab38 (calpain specific α-spectrin breakdown product) and βIII-tubulin immunoreactivity in injured but unstretched cortical neuron regions 6 hours after

injury. ***B.*** Representative images of Ab38 (calpain specific α-spectrin breakdown product) and βIII-tubulin immunoreactivity in the interface between cortical neuron regions and axon regions in control or stretch injured cultures 6 hours after injury. (n = 3 cultures per condition).

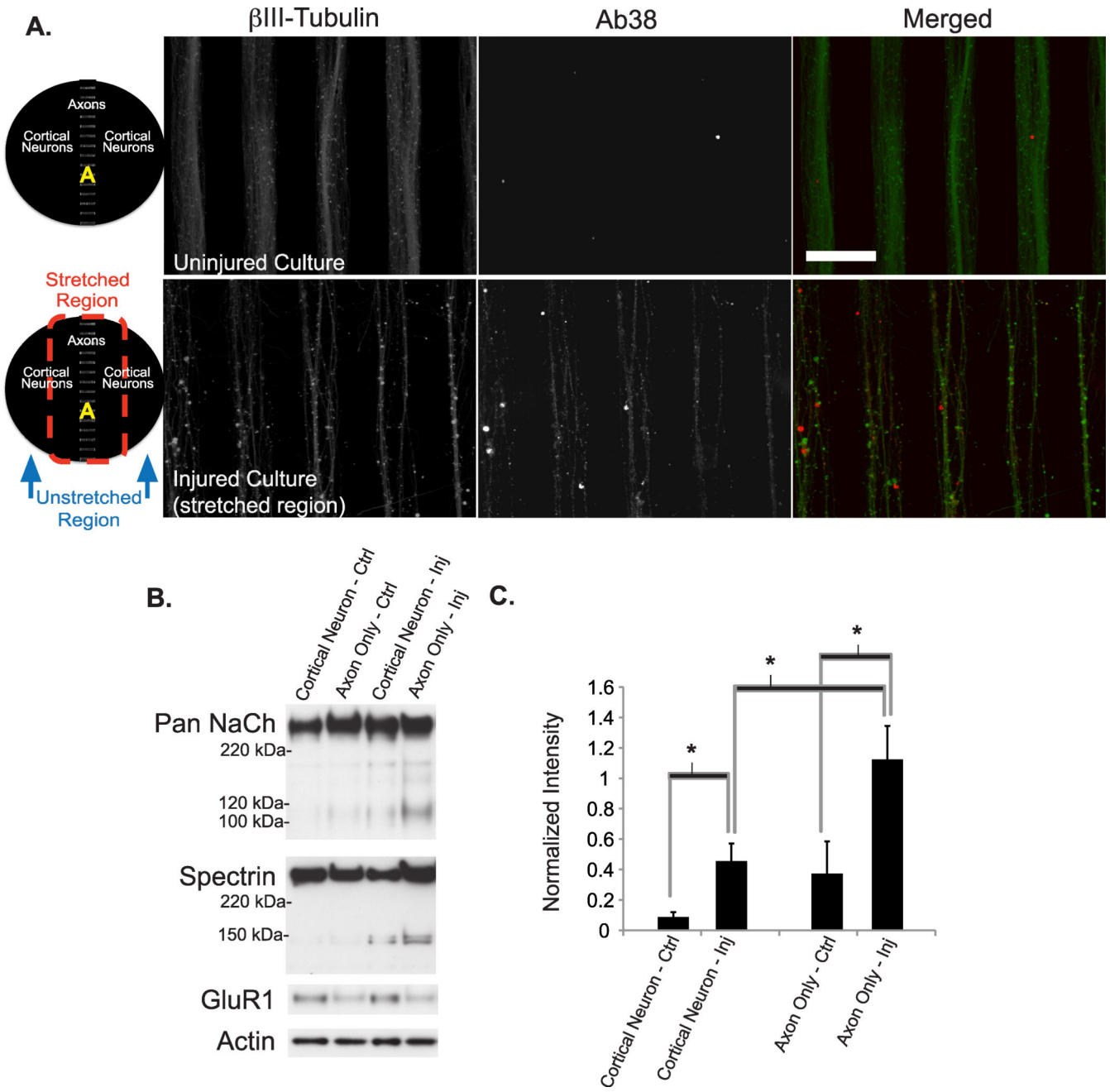

Figure 3.

NaCh proteolysis is found within the cortical neuronal networks and axon only regions and corresponds with the appearance of calpain specific spectrin breakdown in both areas. ***A.*** Representative images of βIII-tubulin (green) and Ab38 (red) immunoreactivity in control or stretch injured axon regions 6 hours after injury. Region of interest is indicated by a yellow letter in the leftmost pane. (scale bar, upper right = 100 μm; n = 3 cultures per condition). ***B.*** Representative western blot displaying NaCh proteolysis distributed within both the cortical neuron (whole cell) and axon region. The dendritic and somatically localized AMPA receptor subunit GluR1 is used to indicate the purity of the axon cultures. ***C.*** Quantification

of the levels of the 110 kDa NaCh fragment recognized by the pan NaCh antibody and normalized over actin, as seen in *A*. (*=$p<0.05$)

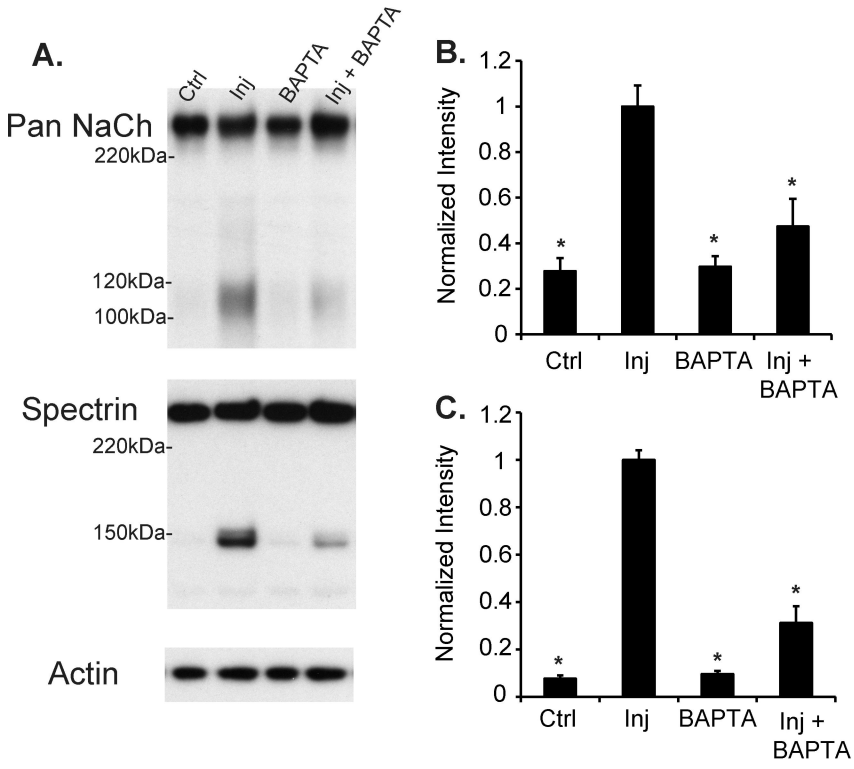

Figure 4.

Stretch injury induced NaCh and α-spectrin proteolysis is dependent upon intracellular [Ca^{2+}]. ***A.*** Representative western blots illustrating a significant decrease in fragment accumulation after stretch injury upon intracellular loading with the Ca^{2+} chelator BAPTA-AM. Both the injured and uninjured control cultures were treated with 0.2% DMSO. ***B.*** Quantification of the pan NaCh 110 kDa band shown in *A*. ***C.*** Quantification of the 145/150 kDa spectrin bands shown in *A*. (n = 4-5 per condition, *=$p<0.05$ from untreated stretch injured cultures)

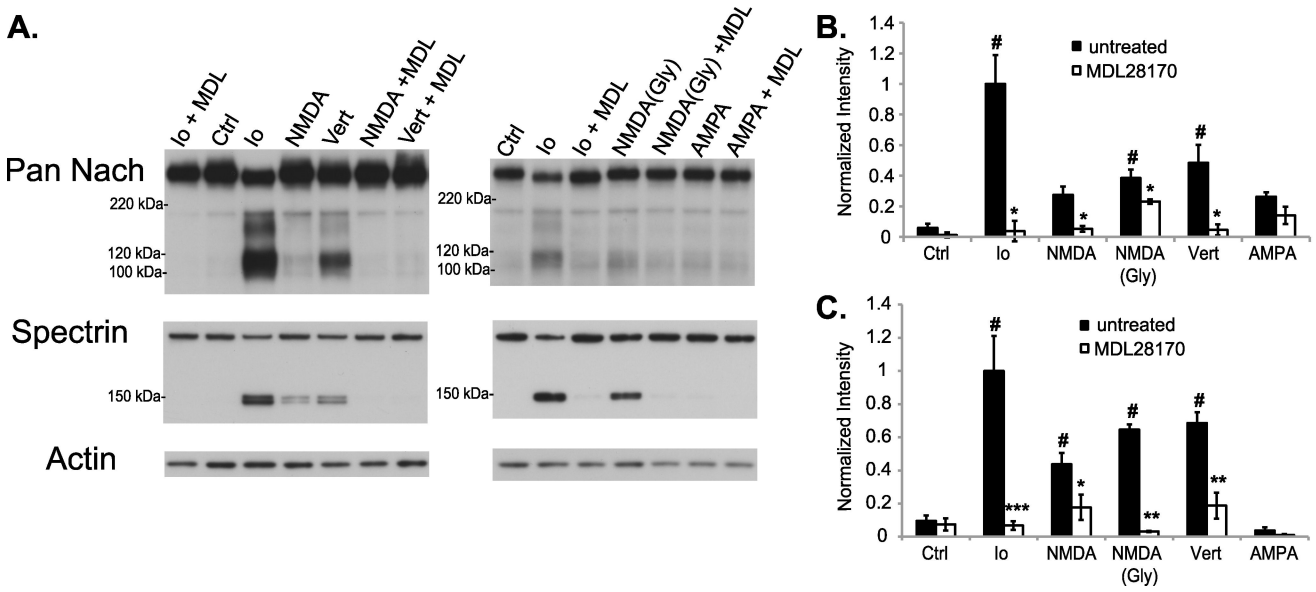

Figure 5.

Activation of both NMDArs and NaChs leads to calpain mediated NaCh and spectrin proteolysis. *A.* Representative western blots illustrating significantly enhanced NaCh and α-spectrin fragment formation upon treatment with the NMDAr (100 μM NMDA with or without 100 μM glycine) or NaCh (20 μM veratridine) agonist but not the AMPAr (100 μM AMPA) agonist. Fragments are significantly reduced in the presence of the calpain inhibitor MDL28170 (10 μM). Ionomycin (Io, 1 μM) and saline treated control cultures are shown for comparison. ***B.*** Quantification of the pan NaCh 110 kDa band shown in *A.* ***C.*** Quantification of the 145/150 kDa spectrin bands shown in *A.* (n = 3-7, #=p<0.05 from agonist free control, ANOVA and Tukey-Kramer HSD test, *=p<0.05, **=p<0.005, ***=p<0.001 from respective uninhibited calpain treatment, Student's t-test)

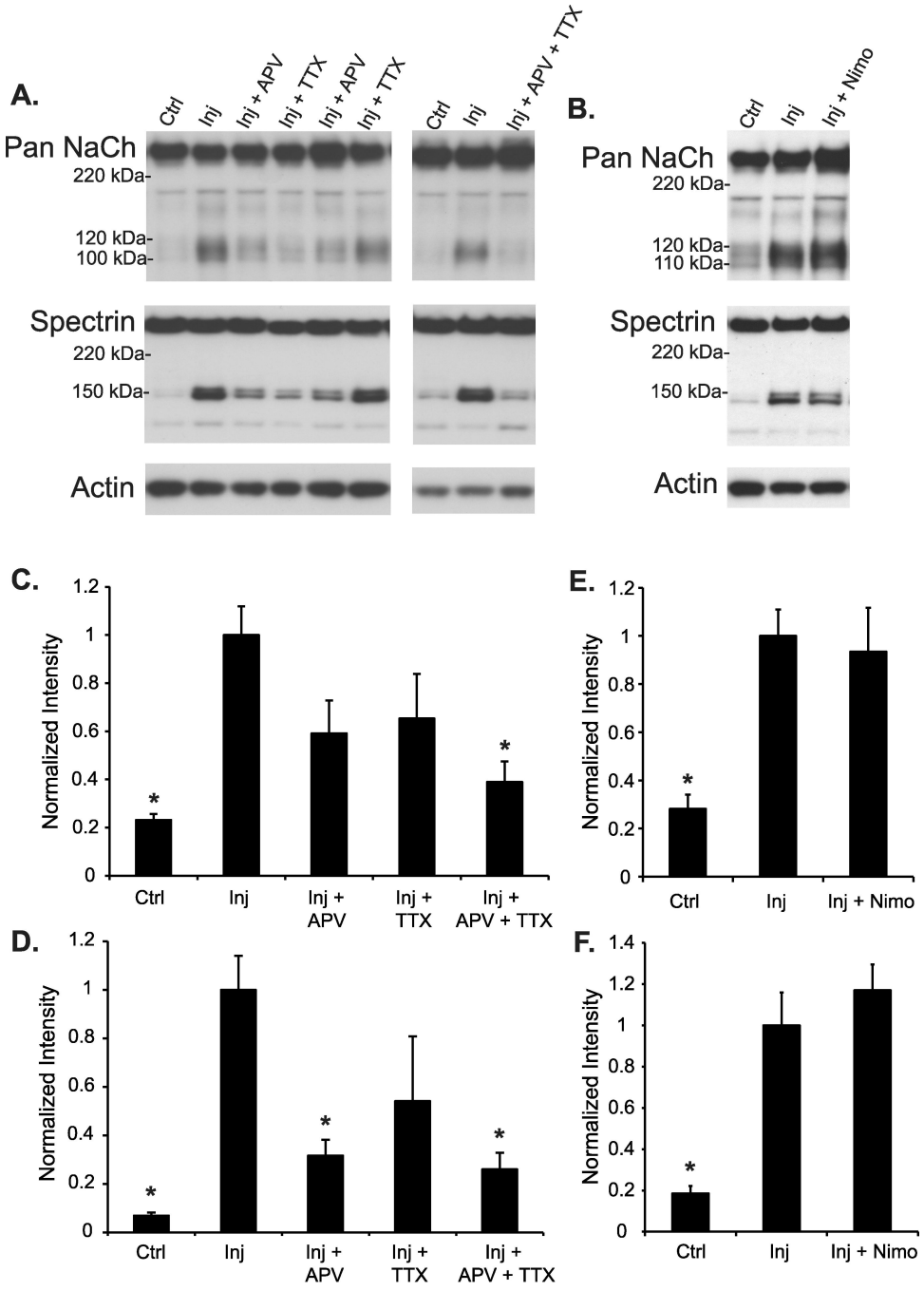

Figure 6.

Chronic inhibition of NMDArs and NaChs, but not L-type CaChs, significantly inhibits NaCh and spectrin proteolysis following whole cell stretch injury of non-patterned cortical cultures. ***A.*** Representative western blots showing a significant decrease in NaCh and spectrin proteolysis at 6 hours when the NMDAr antagonist APV (50 μM) and NaCh antagonist TTX (1 μM) are applied for the duration of the experiment. Both antagonists applied alone show a trend in reducing NaCh proteolysis. APV alone significantly reduces spectrin proteolysis. ***B.*** Representative western blots showing no decrease in NaCh or

spectrin proteolysis upon blocking L-type CaChs with nimodipine (Nimo, 10 μm). Uninjured, saline treated cultures were used as a control. ***C.*** Quantification of the pan NaCh 110 kDa band shown in *A.* ***D.*** Quantification of the 145/150 kDa spectrin bands shown in *A.* (n = 4-5, *=p<0.05 from untreated control) ***E.*** Quantification of the pan NaCh 110 kDa band shown in *B.* ***F.*** Quantification of the 145/150 kDa spectrin bands shown in *B.* (n = 4-5, *=p<0.05 from injured untreated control)

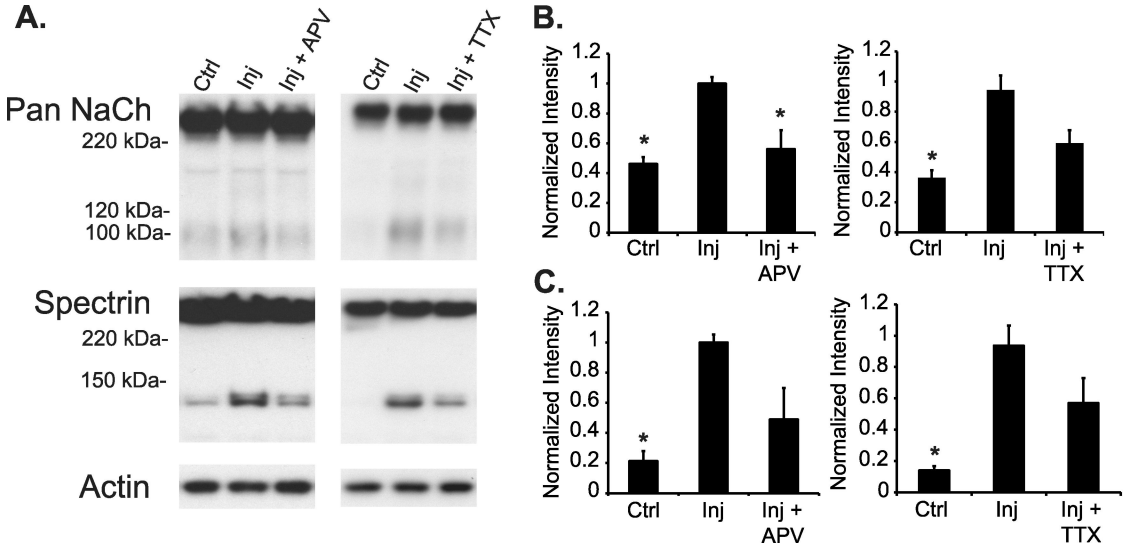

Figure 7.

Inhibition of NMDArs prior to and 30 minutes following whole cell stretch injury of non-patterned cortical cultures attenuates NaCh proteolysis at 6 h. ***A.*** Representative western blots illustrating that the 30 minute APV (50 μM) treatment significantly attenuates NaCh proteolysis and reduces spectrin proteolysis at six hours. Inhibition of the NaCh for 30 minutes with TTX (1 μM) shows a trend towards reduction in both spectrin and NaCh proteolysis at 6 hours. Uninjured, saline treated cultures were used as a control. ***B.*** Quantification of the pan NaCh 110 kDa band shown in *A.* ***C.*** Quantification of the 145/150 kDa spectrin bands shown in *A.* (n = 5-6 per condition, *=$p<0.05$ from injured untreated control)

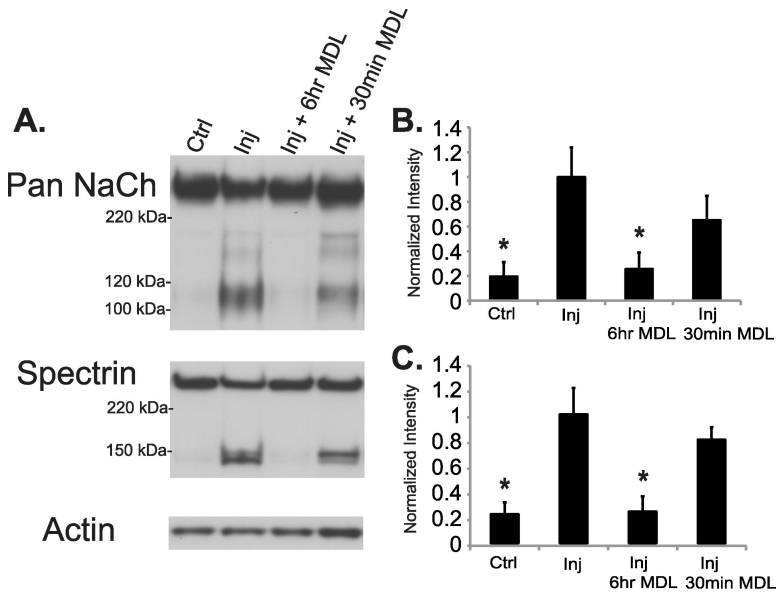

Figure 8.

Chronic inhibition of calpain following whole cell stretch injury of non-patterned cortical cultures is required to significantly attenuate NaCh and spectrin proteolysis. ***A.*** Representative western blots displaying the failure of a 30 minute MDL28170 (10 μM) application to significantly reduce NaCh and spectrin proteolysis compared to application of the drug throughout the entire six hours following stretch injury. Uninjured, saline treated cultures were used as controls. ***B.*** Quantification of 110 kDa pan NaCh band displayed in *A.* ***C.*** Quantification of 145/150 kDa spectrin bands displayed in *A.* (n = 3 per condition, *=p<0.05 from untreated injury)