Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2012 | 123 | 147–158 doi: 10.1111/j.1471-4159.2012.07859.x

ORIGINAL ARTICLE

Calpain-cleaved type 1 inositol 1,4,5-trisphosphate receptor impairs ER Ca^{2+} buffering and causes neurodegeneration in primary cortical neurons

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Abstract

Disruption of neuronal Ca^{2+} homeostasis plays a well-established role in cell death in a number of neurodegenerative disorders. Recent evidence suggests that proteolysis of the type 1 inositol 1,4,5-trisphosphate receptor ($InsP_3R1$), a Ca^{2+} release channel on the endoplasmic reticulum, generates a dysregulated channel, which may contribute to aberrant Ca^{2+} signaling and neurodegeneration in disease states. However, the specific effects of $InsP_3R1$ proteolysis on neuronal Ca^{2+} homeostasis are unknown, as are the functional contributions of this pathway to neuronal death. This study evaluates the consequences of calpain-mediated $InsP_3R1$ proteolysis on neuronal Ca^{2+} signaling and survival using adeno-associated viruses to express a recombinant cleaved form of the channel (capn-$InsP_3R1$) in rat primary cortical neurons. Here, we demonstrate that expression of capn-$InsP_3R1$ in cortical cultures reduced cellular viability. This effect was associated with increased resting cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), increased $[Ca^{2+}]_i$ response to glutamate, and enhanced sensitivity to excitotoxic stimuli. Together, our results demonstrate that $InsP_3R1$ proteolysis disrupts neuronal Ca^{2+} homeostasis, and potentially acts as a feed-forward pathway to initiate or execute neuronal death.

Keywords: calcium, calpain, endoplasmic reticulum, excitotoxicity, inositol 1,4,5-trisphosphate receptor, neuron.

J. Neurochem. (2012) **123**, 147–158.

Spatial and temporal regulation of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) plays a vital role in neuronal signaling, and dysregulation of $[Ca^{2+}]_i$ can trigger cell death (Berridge *et al.* 2000, 2003; Orrenius *et al.* 2003; Mattson 2007). Under physiologic conditions, changes in neuronal $[Ca^{2+}]_i$ are tightly controlled by Ca^{2+} pumps, Ca^{2+}-binding proteins, and intracellular organelles. The endoplasmic reticulum (ER), the primary Ca^{2+} storage organelle in neurons, is capable of both Ca^{2+} sequestration and release. Proper ER Ca^{2+} regulatory function is essential to neuronal $[Ca^{2+}]_i$ buffering, signaling, and survival (Mattson *et al.* 2000; Bardo *et al.* 2006; Stutzmann and Mattson 2011).

A ubiquitous mechanism for Ca^{2+} signaling in neurons is Ca^{2+} release from the ER lumen through the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) (Foskett *et al.* 2007). Of the three identified $InsP_3R$ genes, type 1 $InsP_3R$ ($InsP_3R1$) is the most abundant isoform expressed in brain (Sharp *et al.* 1993). Physiologic $InsP_3R1$-mediated Ca^{2+} signaling in neurons is important for gene expression, membrane excitability, and synaptic plasticity (Bardo *et al.*

Received April 27, 2012; revised manuscript received June 21, 2012; accepted June 26, 2012.

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Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; AAV, adeno-associated virus; capn-$InsP_3R1$, calpain-cleaved $InsP_3R1$; DIV, days *in vitro*; ER, endoplasmic reticulum; $InsP_3R1$, type 1 $InsP_3$ receptor; MAP2, microtubule-associated protein 2; PI, propidium iodide; Tg, thapsigargin.

2006; Stutzmann and Mattson 2011). $InsP_3R1$ dysfunction has been implicated in a number of neurodegenerative disease states as a mechanism for impaired ER Ca^{2+} buffering, disruption of Ca^{2+} homeostasis, and neuronal death (Verkhratsky and Toescu 2003; Foskett 2010). In most of these cases, $InsP_3R1$ dysfunction has been associated with allosteric modulation of the channel (Foskett *et al.* 2007). However, increasing evidence suggests that proteolytic modification of $InsP_3R1$ can also dramatically disrupt channel physiology (Assefa *et al.* 2004; Nakayama *et al.* 2004; Verbert *et al.* 2008; Kopil *et al.* 2011), and may potentially contribute to neurodegeneration.

$InsP_3R1$ is a target for caspase-3 and calpain-mediated proteolysis, which generate similarly sized ~95 kDa carboxyl-terminal fragments (Hirota *et al.* 1999; Kopil *et al.* 2011). Both caspase and calpain-mediated proteolysis of $InsP_3R1$ remove the amino-terminal ligand-binding domain and a large portion of the coupling domain, leaving the pore-forming transmembrane domains intact. Studies examining the functional properties of the caspase-derived carboxyl-terminal $InsP_3R1$ fragment demonstrated increased ER Ca^{2+} leak, and corresponding decreased ER $[Ca^{2+}]$ ($[Ca^{2+}]_{ER}$), and buffering capacity in cell lines expressing the recombinant channel (Assefa *et al.* 2004; Nakayama *et al.* 2004; Verbert *et al.* 2008). These effects were mediated by $InsP_3$-independent Ca^{2+} release through caspase-cleaved $InsP_3R1$, and expression of the truncated channel increased cellular sensitivity to apoptotic stimuli (Assefa *et al.* 2004). Our laboratory recently identified the calpain-cleavage site for $InsP_3R1$ and determined that the calpain-derived carboxyl-terminal channel fragment displayed $InsP_3$-independent gating and constitutive channel activity (Kopil *et al.* 2011). When expressed in cell lines, calpain-cleaved $InsP_3R1$ decreased $[Ca^{2+}]_{ER}$ via increased ER Ca^{2+} leak (Kopil *et al.* 2011). Although no study to date has investigated the effects of caspase- or calpain-cleaved $InsP_3R1$ on neuronal Ca^{2+} homeostasis, these observations suggest that proteolysis of $InsP_3R1$ may transform spatial and temporal regulation of neuronal $[Ca^{2+}]_i$ and potentially signal cell death. Consistent with this hypothesis, we previously identified calpain-cleaved $InsP_3R1$ in selectively vulnerable cerebellar Purkinje cells at both early and late times following ischemic brain injury (Kopil *et al.* 2011).

To define the role of $InsP_3R1$ proteolysis in neuronal Ca^{2+} homeostasis and neurodegeneration, we examined responses of primary cortical neurons to expression of recombinant calpain-cleaved $InsP_3R1$. Expression of the cleaved channel reduces viability of cortical cultures at baseline. Using single-cell Ca^{2+} imaging, we determined that calpain-cleaved $InsP_3R1$ increases resting neuronal $[Ca^{2+}]_i$ and leads to greater rises in $[Ca^{2+}]_i$ in response to physiologic stimuli. Calpain-cleaved $InsP_3R1$ also sensitized cultures to excitotoxic injury. Together, these data suggest a specific role for proteolyzed $InsP_3R1$-mediated Ca^{2+} signaling in the molecular injury cascade in neurons, which has important therapeutic implications for neurodegenerative disorders associated with Ca^{2+} dysregulation.

Materials and methods

Materials

Unless otherwise noted, all chemicals were purchased from (Sigma-Aldrich, St Louis, MO USA).

Antibodies

Rabbit polyclonal antibody targeted against the 20 carboxyl-terminal residues of rat $InsP_3R1$ was generated using previously described methods (Joseph and Samanta 1993; Kopil *et al.* 2011) (Covance ImmunoTechnologies, Denver, PA, USA). Rabbit polyclonal antibody to microtubule-associated protein 2 (MAP2) was a gift from Dr. Virginia Lee (University of Pennsylvania). Mouse monoclonal antibody to BiP/GRP78 was purchased from BD Biosciences. Mouse monoclonal antibody to α-spectrin (Ab1622) was purchased from Chemicon, Temecula, CA, USA. Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies used for immunofluorescence were purchased from Invitrogen, Carlsbad, CA, USA. Horseradish peroxidase-linked secondary antibodies used for immunoblotting were purchased from Perkin Elmer, Waltham, MA, USA.

Rat primary mixed cortical cultures

Primary dissociated cultures were prepared from embryonic rat cortices as described previously (Cummings *et al.* 1996). Briefly, E19 embryos from pregnant female Sprague–Dawley rats (Charles River Laboratories Inc., Wilmington, MA, USA) were removed, the brains extracted, and cortices dissected. Cortices were trypsinized in Dulbecco's minimum essential medium (Whittaker Bioproducts, Walkersville, MD, USA) at 4°C for 20 min. Cells were triturated in media consisting of Dulbecco's minimum essential medium supplemented with 10% bovine calf serum (HyClone, Logan, UT, USA), 10% Ham's F12 with glutamine (Whittaker Bioproducts), and 50 U/mL penicillin–streptomycin. Dissociated cortical cultures were plated on poly-L-lysine-coated 35-mm dishes (4.8×10^5 cells/well; Western blot), 12 mm glass coverslips in 24-well plates (8×10^4 cells/well; immunofluorescence), or 20×50-mm glass coverslips in four-well plates (3.2×10^6 cells/well; single-cell Ca^{2+} imaging). Dissociated cells were grown in serum-free Neurobasal medium (Gibco, Rockville, MD, USA) supplemented by B27 (Gibco) and cultured at 37°C in a humidified 5% CO_2 incubator. Mitotic inhibitors and antibiotics were not used. This study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Constructs and site-directed mutagenesis

The transgene for rat calpain-cleaved $InsP_3R1$ (capn-$InsP_3R1$; Fig. 1a) was previously generated (Kopil *et al.* 2011). To construct the pore-inactivating D2550A mutation (capn-$InsP_3R1$ D2550A), site-directed mutagenesis was performed with QuikChange (Stratagene) using primers (5′–GGCGGAGTAGGAGCT GTGCTCAGGAAG–3′ and 5′–CTTCCTGAGCACAGCTCCTAC TCCGCC–3′ (codon change is shown in boldface type and underlined)) and capn-$InsP_3R1$ as a template. The mutation was confirmed by DNA sequencing. For transduction of primary

neurons, both capn-$InsP_3R1$ and capn-$InsP_3R1$ D2550A were subcloned into a plasmid containing the adeno-associated viral (AAV) inverted terminal repeats and polyA sequence for viral vector generation.

Viral vector generation

Recombinant AAV vectors were generated by the University of Pennsylvania Vector core as previously described (Fisher *et al.* 1997). Briefly, AAV vectors were prepared by triple transfection of HEK293 cells and purified by cesium chloride gradient sedimentation. AAV 2/1 vectors were designed to express capn-$InsP_3R1$ (lots V2049 and V2451), capn-$InsP_3R1$ D2550A (lots V2521 and V2601), or lacZ (lot V0861). All transgenes were expressed under the control of the cytomegalovirus promoter.

AAV 2/1 Vector transduction of primary neurons

Rat primary cortical cultures were transduced at 7 days *in vitro* (DIV) by adding AAV 2/1 vector in a vehicle of phosphate-buffered saline and 10% glycerol directly to the culture media. Cultures were transduced with 7.5×10^{10} genome copies (GC) per mL of media. All cell viability, biochemical, and functional assays were performed 1 week later (14 DIV).

Cell viability assays

Transduced cultures were fixed with 4% paraformaldehyde, immunolabeled for the neuronal marker MAP2 with a goat anti-rabbit secondary antibody (Alexa Fluor 568), and counter-stained with 4,6-diamidino-2-phenylindole nuclear label (Vector Laboratories, Burlingame, CA, USA). Five random 100× images were taken from each 12-mm coverslip. The number of surviving MAP2-labeled neurons per coverslip was estimated by blinded counting of all captured fields. For each transduction condition, 8–12 coverslips were counted from four independent experiments. To control for variability in culture health between experiments, the percentage of viable cells was determined by normalizing to the average cell count from lacZ-transduced cultures for each independent experiment and the average of the normalized experiments is shown. Reported SEM and error bars reflect propagated error through normalization.

Cell viability was independently measured by staining cultures with calcein-AM and propidium iodide (PI; Invitrogen). Live cultures were incubated with 3 μM calcein-AM and 5 μM PI in Dulbecco's phosphate-buffered saline (DPBS; Gibco) for 30 min at 37°C and 5% CO_2. Cultures were rinsed with DPBS and immediately imaged. Five random 100× images were taken from each 12-mm coverslip. The number of surviving, calcein-AM-positive, PI-negative cells per coverslip was estimated by blinded counting of all captured fields. For each transduction condition, 6–14 coverslips were counted from three independent experiments. The number of viable cells in each transduction condition was normalized to lacZ controls as described above.

Western blotting of primary neuron cultures

Media was removed from transduced cultures and replaced with DPBS at 4°C. Cells were harvested by scraping, and resuspended in homogenization buffer (50 mM Tris, 150 mM NaCl, 2 mM EGTA) with a protease inhibitor cocktail and sonicated. Lysates were treated with sodium dodecyl sulfate loading buffer, boiled, and analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. Western blots were visualized using enhanced chemiluminescence supplies purchased from Perkin Elmer. Protein and polypeptide densities were quantified using computer densitometry (ImageJ, US National Institutes of Health, Bethesda, MD, USA) and normalized to lacZ controls. Error bars reflect propagated error through normalization.

Single-cell Ca^{2+} imaging

Cortical cultures were plated onto glass coverslips (Warner Instruments, Hamden, CT, USA) and transduced with AAV 2/1 at 7 DIV. One week following transduction, cells on coverslips were secured in a perfusion chamber, and mounted on the stage of an inverted microscope (Nikon Eclipse TE2000; Nikon Inc., Melville, NY, USA). Cells were loaded with Fura-2-AM (2.5 μM; Molecular Probes, Eugene, OR, USA) for 45 min at 37°C and 5% CO_2 in Ca^{2+}-containing extracellular solution (in mM: 120 NaCl, 4 KCl, 20 HEPES, 15 Glucose, 2 $CaCl_2$, 1 $MgCl_2$, pH 7.3). Fura-2 was alternately illuminated at 340/380 nm, and fluorescence intensity filtered at 510 nm. Data were collected and recorded as described previously (White *et al.* 2005; Cheung *et al.* 2008). Cells were perfused with 2 mM Ca^{2+} extracellular solution to establish baseline $[Ca^{2+}]_i$ before (S)-3,5-dihydroxyphenylglycine (DHPG; 10 μM), glutamate (10 μM), thapsigargin (Tg; 1 μM), or KCl (50 mM) was applied in the extracellular solution. At the end of the experiment, Mn^{2+} was used to quench Fura-2 fluorescence (Ca^{2+}-containing extracellular solution supplemented with 10 mM $MnCl_2$ and 10 μM ionomycin). The remaining background fluorescence following Mn^{2+} quench was subtracted during analysis. Glial cells were identified by their morphology and ability accumulate high levels of Fura-2 compared with neurons (Ikegaya *et al.* 2005), and were excluded from analysis. Experiments using DHPG, glutamate alone, glutamate plus thapsigargin, and KCl were performed on different weeks, in cultures from different platings. Within each experiment using DHPG, glutamate alone, glutamate plus thapsigargin, or KCl, however, the studied lacZ-, capn-$InsP_3R1$ D2550A-, and capn-$InsP_3R1$-transduced cultures were derived from the same plating. To compare between experiments using glutamate alone and glutamate plus thapsigargin, the percent of maximum $[Ca^{2+}]_i$ was determined by normalizing to the average peak $[Ca^{2+}]_i$ of capn-$InsP_3$ R1-transduced cultures from each plating. Reported SEM and error bars reflect propagated error through normalization.

Changes in $[Ca^{2+}]_i$ are presented as changes in fluorescence ratio. Dye calibration was achieved by applying experimentally determined constants to the equation: $[Ca^{2+}] = K_d\ \beta(R-R_{min})/(R_{max}-R)$. Macros used for analysis were custom macros written for IGOR Pro (WaveMetrics, Portland, OR, USA).

Glutamate injury characterization

HEPES-buffered saline (HBS; in mM: 145 NaCl, 3 KCl, 10 HEPES, 8 Glucose, 2 $CaCl_2$, 1 $MgCl_2$, pH 7.4) with or without L-glutamic acid was added to the media of transduced cortical cultures at 14 DIV. Twenty-four hours after HBS or glutamate application, cultures were fixed with 4% paraformaldehyde, immunolabeled for MAP2, and counter-stained with 4,6-diamidino-2-phenylindole. Five random 100× images were taken from each 12-mm coverslip. The number of surviving MAP2-labeled neurons per coverslip was estimated by blinded counting of all captured fields. For each transduction condition, six to eight coverslips were counted from three independent experiments. To control for variability in culture health between experiments and differences in baseline toxicity, the

percent of viable cells at each glutamate dose was determined by normalizing within transduction conditions to the average cell count from HBS-treated cultures. Data were normalized within independent experiments and the average of the normalized experiments is shown. Reported SEM and error bars reflect propagated error through normalization. Dose-response curves were calculated by fitting plotted mean data points using the Hill equation and holding the base and max at 0 and 100, respectively. Reported error for LD_{50} values represents error of the fit.

Data analysis and statistics

Data are presented as the mean ± SEM, and statistical significance of differences between the means was assessed using either unpaired *t*-tests or ANOVA for repeated measures using Barlett's test for equal variances and a Bonferroni correction. Differences between means were accepted as statistically significant at the 95% level ($p < 0.05$).

Results

Expression of recombinant $InsP_3R1$ constructs in primary cortical neurons

Previously, we showed that expression of a recombinant form of rat $InsP_3R1$ corresponding to the stable carboxyl-terminal fragment generated by calpain proteolysis (capn-$InsP_3R1$; Δ1-1917; Fig. 1a) altered $[Ca^{2+}]_i$ homeostasis in immortalized cell lines. In this study, we examined the functional consequences of capn-$InsP_3R1$ expression in primary neurons. To achieve high transduction efficiency of primary cortical cultures, we utilized AAV 2/1-mediated gene delivery of capn-$InsP_3R1$, which transduces over 90% of cells in primary neuron cultures (Royo *et al.* 2008; Bevers *et al.* 2009). As a control for $InsP_3$-independent Ca^{2+} release through the truncated channel, we generated AAV 2/1 vector expressing capn-$InsP_3R1$ with a D2550A point mutation in the pore-forming region of the channel (capn-$InsP_3R1$ D2550A; Fig. 1a). This point mutation eliminates Ca^{2+} permeability of wild-type (wt) $InsP_3R1$ (Boehning and Joseph 2000; Boehning *et al.* 2001; Cardenas *et al.* 2010). Because of the genome size of AAV, viral packaging capacity of transgenes is limited (Dong *et al.* 1996), and we were unable to generate vectors expressing full-length $InsP_3R1$ with or without the D2550A mutation. We used AAV 2/1 expressing lacZ to control for effects of viral transduction.

To examine expression of $InsP_3R1$ constructs in neurons, we stably transduced cortical cultures with AAV 2/1 expressing capn-$InsP_3R1$, capn-$InsP_3R1$ D2550A, or lacZ at 7 DIV and harvested cells 1 week later (14 DIV). Western blotting of whole-cell lysates using a carboxyl-terminal $InsP_3R1$ antibody demonstrated expression of endogenous wt-$InsP_3R1$ in all samples (Fig. 1b). In lysates from cultures

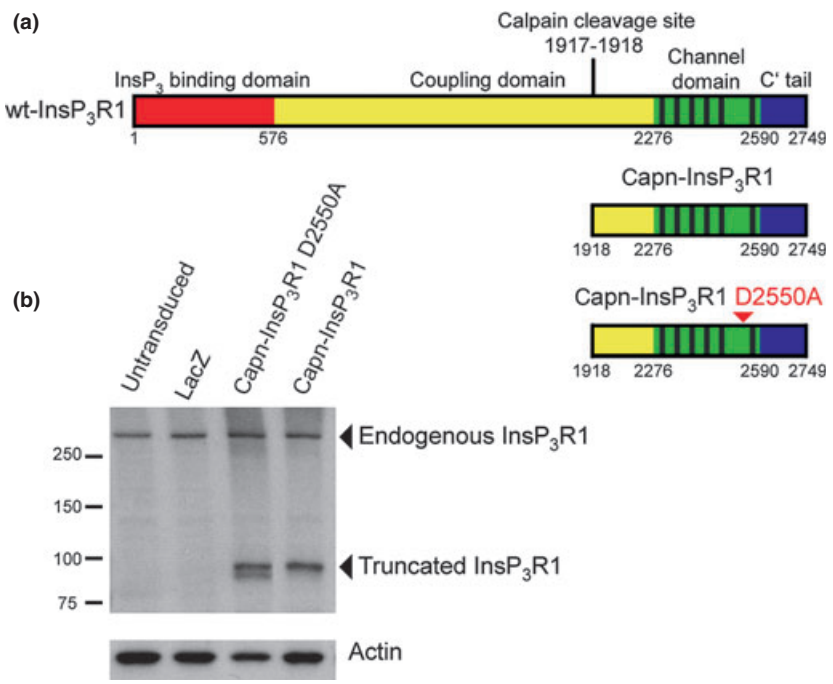

Fig. 1 Schematic representation and expression of recombinant $InsP_3R1$ constructs in primary neurons. (a) Protein domain structure of wt-$InsP_3R1$ (top), calpain-cleaved $InsP_3R1$ recombinant (middle), and calpain-cleaved $InsP_3R1$ with the D2550A point mutation (bottom; arrowhead). Residues numbered according to rat type 1 SI+, SII+, SIII- sequence (protein accession NP_001007236.1). (b) Western blot analysis of whole-cell lysates from untransduced rat primary cortical cultures and primary cortical cultures transduced with AAV 2/1 expressing lacZ, capn-$InsP_3R1$ D2550A, or capn-$InsP_3R1$ at 1 week post-transduction (14 DIV). Carboxyl-terminal $InsP_3R1$ antibody was used to detect endogenous and recombinant truncated rat $InsP_3R1$. Antibody against actin was used as a loading control.

transduced with capn-$InsP_3R1$ and capn-$InsP_3R1$ D2550A, we also detected the 95 kDa form of the channels, confirming expression of recombinant $InsP_3R1$ constructs.

Capn-$InsP_3R1$ reduces neuronal viability

To determine the effects of capn-$InsP_3R1$ on neuronal viability, we transduced 7 DIV cultures with capn-$InsP_3R1$, capn-$InsP_3R1$ D2550A, or lacZ control vectors. One week post-transduction (14 DIV), we immunostained cultures for MAP2 (Fig. 2a, left) and quantified the number of surviving neurons (Fig. 2b). Expression of capn-$InsP_3R1$ resulted in significant decrease in the percentage of viable neurons compared with expression of capn-$InsP_3R1$ D2550A or lacZ (capn-$InsP_3R1$ = 65.3 ± 5.5%, capn-$InsP_3R1$ D2550A = 112.7 ± 5.8%, lacZ = 100.0 ± 3.8%; $p < 0.001$). To confirm the capn-$InsP_3R1$ toxicity phenotype, we incubated transduced cultures with calcein-AM and PI to identify living cells and dead cells, respectively (Fig. 2a, right). A significant decrease in percentage of surviving cells was again observed in cultures transduced with capn-$InsP_3R1$ compared with controls (capn-$InsP_3R1$ = 76.7 ± 4.5%, capn-$InsP_3R1$ D2550A = 112.5 ± 6.5%, lacZ = 100.0 ± 4.0%; $p < 0.001$; Fig. 2c). Interestingly, the percentage of viable cells in capn-$InsP_3R1$-transduced cultures determined by calcein-AM and PI staining was significantly higher than survival quantified by counts of MAP2-labeled cells ($p < 0.05$). This likely reflects the mixed constituents of the cortical cultures, which contain ~10% glia (data not shown) that are identified using calcein-AM but not MAP2, suggesting that glial viability is not altered by capn-$InsP_3R1$. Together, these results suggest that expression of capn-$InsP_3R1$ specifically reduces neuronal viability.

We hypothesized that expression of capn-$InsP_3R1$ reduced neuronal viability by disrupting intracellular Ca^{2+} homeostasis. The proteolyzed channel, which has $InsP_3$-independent gating and increases the ER Ca^{2+} leak (Kopil *et al.* 2011), could potentially trigger neurodegeneration through either ER Ca^{2+} depletion and induction of ER stress, or by increased $[Ca^{2+}]_i$ and feed-forward activation of proteases. To examine the mechanism responsible for capn-$InsP_3R1$ reductions in neuronal viability, we performed Western blotting of whole-cell lysates from transduced cultures. Using an antibody against the stress-inducible ER chaperone protein BiP (Figure S1a), we did not detect any difference in activation of the ER stress pathway between cultures (Figure S1b). Nor did we detect differences in calpain or caspase-3 protease activation using an antibody against a shared substrate, α-spectrin (Figure S1c and d). Absence of Western blot evidence of increased ER stress or protease activation with expression of capn-$InsP_3R1$ does not conclusively imply that neither pathologic cascade plays a causal role in capn-$InsP_3R1$-induced neuronal death. However, if either pathway is responsible for executing cell death in capn-$InsP_3R1$-transduced cultures, Western blotting of neuronal lysates is not sensitive enough to detect it at the times examined here.

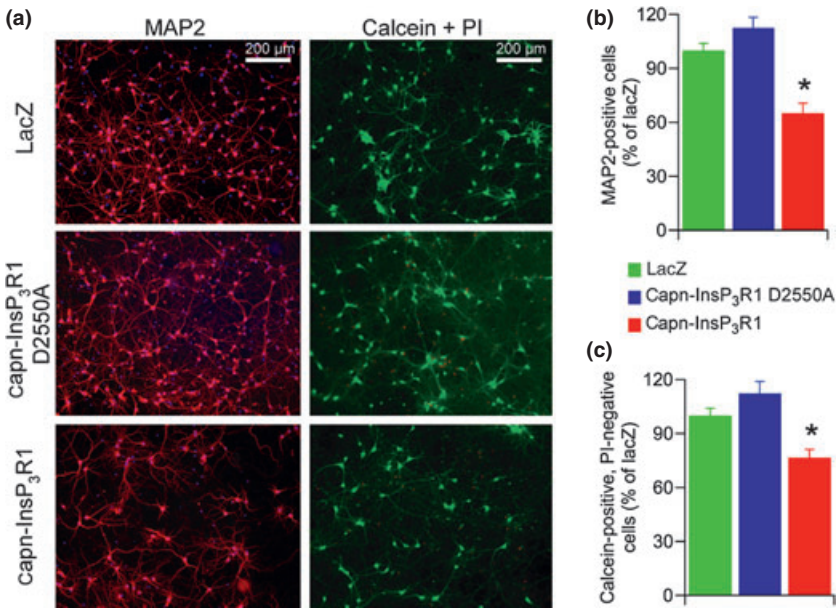

Fig. 2 Primary cortical neuron viability is reduced by expression of capn-$InsP_3R1$. Rat primary cortical cultures were transduced with adeno-associated virus 2/1 expressing lacZ, capn-$InsP_3R1$ D2550A, or capn-$InsP_3R1$ (7 DIV). (a) One week following transduction (14 DIV), cultures were stained for microtubule-associated protein 2 (MAP2) (red) and nuclei (blue; left panel), or incubated with calcein-AM (green) and propidium iodide (PI) (red; right panel). Representative 100× epifluorescence images captured for counting are shown. (b) Percent neuronal survival as determined by counts of MAP2-reactive cells (one-way ANOVA; $^*p < 0.001$). (c) Percent cell survival as determined by counts of calcein-AM positive, PI-negative cells (unpaired *t*-tests with unequal variance; $^*p < 0.001$). Expression of capn-$InsP_3R1$ resulted in a significant decrease in percent viable neurons compared with lacZ and capn-$InsP_3R1$ D2550A as determined by both assays.

Capn-InsP$_3$R1 does not deplete intracellular Ca^{2+} stores

Previous data from our laboratory indicated that capn-InsP$_3$R1 functions as a Ca^{2+} leak channel in the ER (Kopil *et al.* 2011). To examine whether this leak was sufficient to deplete intracellular Ca^{2+} stores in neurons, we utilized single-cell Ca^{2+} imaging to measure InsP$_3$-induced changes in [Ca^{2+}]$_i$. We monitored somatic [Ca^{2+}]$_i$ using Fura-2 in primary cortical cultures transduced with capn-InsP$_3$R1, capn-InsP$_3$R1 D2550A, or lacZ (Fig. 3a). To stimulate Ca^{2+} release from InsP$_3$-sensitive stores, we applied saturating concentrations of the Group I metabotropic glutamate receptor agonist DHPG (10 μM). Expression of capn-InsP$_3$R1 significantly increased resting [Ca^{2+}]$_i$ in neurons (90 ± 3.3 nM) compared with capn-InsP$_3$R1 D2550A (70 ± 1.8 nM) and lacZ controls (70 ± 1.6 nM; $p < 0.001$; Fig. 3b), supporting previous evidence that capn-InsP$_3$R1 leaks Ca^{2+} from the ER. Application of DHPG elicited responses in a majority of neurons from all transduced cultures (Fig. 3c). Despite the apparent basal Ca^{2+} leak through the cleaved channel, expression of capn-InsP$_3$R1 did not affect the DHPG-induced peak [Ca^{2+}]$_i$ in neurons (Fig. 3d) or the maximum change in [Ca^{2+}]$_i$ from baseline to peak (Fig. 3e). These data demonstrate that expression of the leaky channel, while sufficient to increase resting [Ca^{2+}]$_i$, does not deplete [Ca^{2+}]$_{ER}$.

Decreased ER Ca^{2+} buffering in neurons expressing capn-insP$_3$R1

To examine the effects of calpain-cleaved InsP$_3$R1 on neuronal Ca^{2+} homeostasis more broadly, we measured changes in [Ca^{2+}]$_i$ in response to acute glutamate (10 μM) application (Fig. 4a), which activates both ionotropic and metabotropic glutamate receptors (Michaelis 1998). Consistent with results from Ca^{2+} imaging experiments above, resting [Ca^{2+}]$_i$ was significantly increased in neurons expressing capn-InsP$_3$R1 compared with capn-InsP$_3$R1 D2550A- and lacZ-expressing cells ($p < 0.001$; Fig. 4b). In addition, capn-InsP$_3$R1 significantly increased the glutamate-induced peak [Ca^{2+}]$_i$ (1,520 ± 43 nM) compared with capn-InsP$_3$R1 D2550A (1,030 ± 36 nM) and lacZ controls (940 ± 25 nM; $p < 0.001$; Fig. 4c). Stimulation of an alternate pathway for Ca^{2+} influx also resulted in a significantly increased peak [Ca^{2+}]$_i$ in cultures transduced with capn-InsP$_3$R1 (50 mM KCl; Figure S2). This result suggests that the increased glutamate-induced [Ca^{2+}]$_i$ in capn-InsP$_3$R1-transduced neurons is not dependent on increased glutamate receptor expression. Instead, the increased [Ca^{2+}]$_i$ rise is likely because of an impaired ability of the ER to buffer Ca^{2+} influx from the extracellular space. To test this hypothesis, we measured changes in [Ca^{2+}]$_i$ in response to acute glutamate (10 μM) application following a 3-min

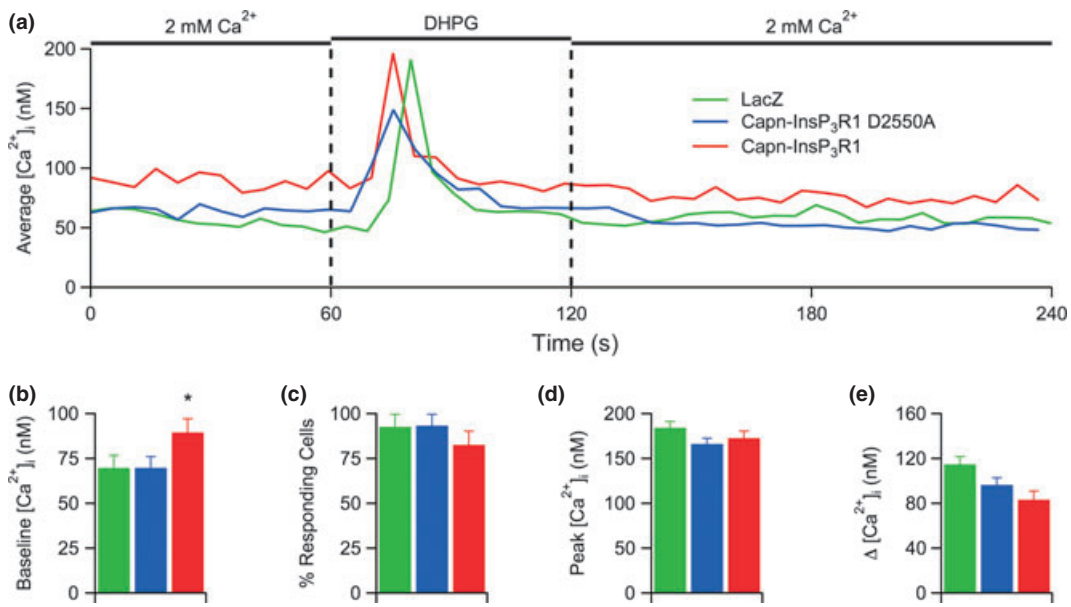

Fig. 3 Capn-InsP$_3$R1 does not deplete InsP$_3$-sensitive stores. (a) Representative single cell [Ca^{2+}]$_i$ responses to (S)-3,5-dihydroxyphenylglycine (DHPG) in Fura-2-loaded primary cortical neurons (14 DIV) transduced with lacZ, capn-InsP$_3$R1 D2550A, or capn-InsP$_3$R1. Total number of single-cell Ca^{2+} responses analyzed in these experiments was 219, 246, and 172 for lacZ-, capn-InsP$_3$R1 D2550A-, and capn-InsP$_3$R1-transduced cultures, respectively. (b) Summary of average resting [Ca^{2+}]$_i$ in neurons from cultures used for DHPG Ca^{2+} imaging experiments. Cultures expressing capn-InsP$_3$R1 demonstrate increased baseline [Ca^{2+}]$_i$ (unpaired *t*-tests with unequal variance; *$p < 0.001$). (c) Percentage of cells in transduced cortical cultures that exhibited > 10% increase in [Ca^{2+}]$_i$ in response to DHPG. (d) Summary of average peak [Ca^{2+}]$_i$ responses elicited by DHPG. (e) Summary of average change in [Ca^{2+}]$_i$ from resting to peak [Ca^{2+}]$_i$. Despite significant differences in resting [Ca^{2+}]$_i$ in these experiments, no statistical differences were observed in average change in [Ca^{2+}]$_i$.

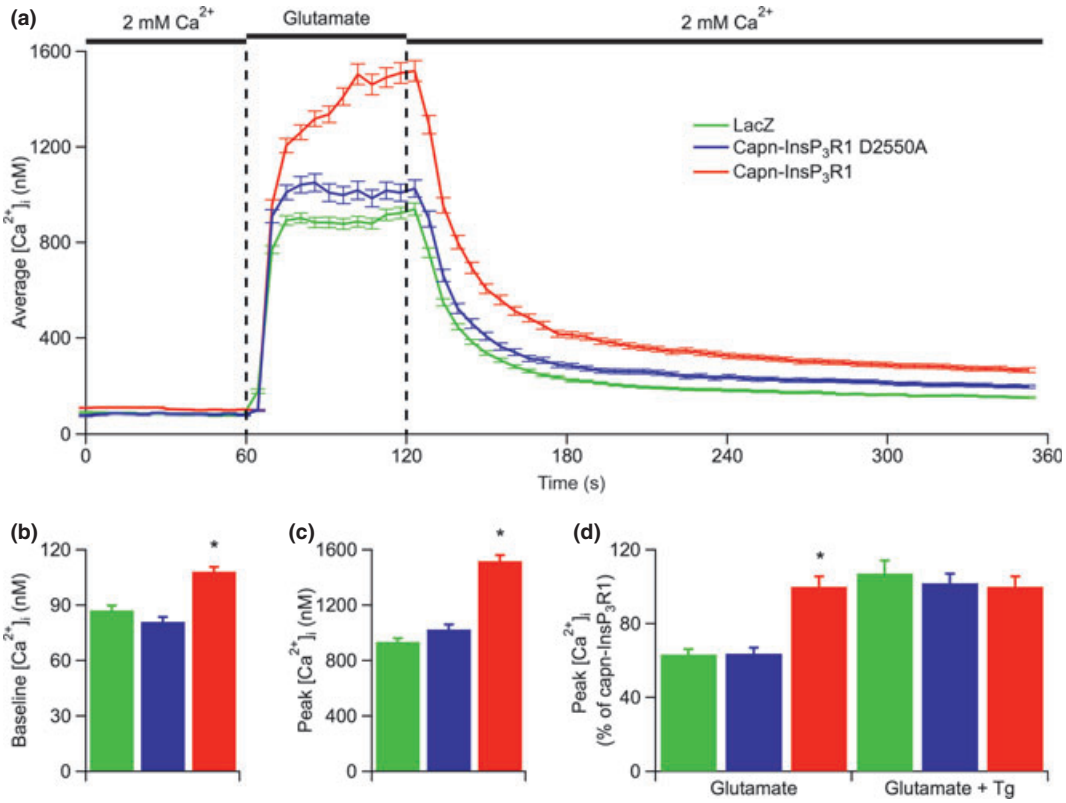

Fig. 4 Neurons expressing capn-InsP$_3$R1 have increased glutamate-induced rises in $[Ca^{2+}]_i$. (a) Averaged single-cell $[Ca^{2+}]_i$ responses to glutamate in Fura-2-loaded primary cortical neurons (14 DIV) transduced with lacZ, capn-InsP$_3$R1 D2550A, or capn-InsP$_3$R1. Total number of single-cell Ca^{2+} responses analyzed in these experiments was 491, 271, and 396 for lacZ-, capn-InsP$_3$R1 D2550A-, and capn-InsP$_3$R1-transduced cultures, respectively. (b) Summary of average resting $[Ca^{2+}]_i$ in neurons from cultures used for glutamate Ca^{2+} imaging experiments. Expression of capn-InsP$_3$R1 significantly increased resting $[Ca^{2+}]_i$ in neurons (108.2 ± 2.6 nM) compared with capn-InsP$_3$R1 D2550A (81.0 ± 2.5 nM) and lacZ controls (87.3 ± 2.6 nM; unpaired *t*-tests with unequal variance; $^*p < 0.001$). (c) Summary of average peak $[Ca^{2+}]_i$ responses elicited by glutamate shows an increased maximum $[Ca^{2+}]_i$ achieved in capn-InsP$_3$R1-expressing cells (unpaired *t*-tests with unequal variance; $^*p < 0.001$). (d) Summary of average peak $[Ca^{2+}]_i$ elicited by glutamate in the absence or presence of Tg (normalized data for glutamate alone (left) are the same as absolute data shown Fig. 4c). Total number of single-cell Ca^{2+} responses analyzed in experiments with Tg was 182, 209, and 146 for lacZ-, capn-InsP$_3$R1 D2550A-, and capn-InsP$_3$R1-transduced cultures, respectively. Tg eliminated the capn-InsP$_3$R1-associated glutamate-induced enhanced peak $[Ca^{2+}]_i$ (lacZ, 1,357 ± 66 nM; capn-InsP$_3$R1 D2550a, 1,276 ± 44 nM; capn-InsP$_3$R1, 1,288 ± 54 nM).

pre-incubation with Tg (1 μM) to inhibit the sarco-endoplasmic reticulum Ca^{2+}-ATPase. Blocking ER Ca^{2+} uptake eliminated the difference in the glutamate-induced peak $[Ca^{2+}]_i$ between neurons expressing capn-InsP$_3$R1 and control cultures expressing lacZ or capn-InsP$_3$R1 D2550A (Fig. 4d). Together, these data indicate that capn-InsP$_3$R1 disrupts normal intracellular Ca^{2+} homeostasis and impairs the ability of the ER to buffer physiologic rises in $[Ca^{2+}]_i$.

Capn-InsP$_3$R1 increases sensitivity to glutamate-induced neurotoxicity

To determine whether the observed capn-InsP$_3$R1-induced impairment of ER Ca^{2+} buffering increased neuronal susceptibility to excitotoxic injury, we exposed transduced 14 DIV primary cortical cultures to 1 μM glutamate or HBS vehicle. We selected this dose from initial studies performed in untransduced cultures, where treatment with 1 μM glutamate resulted in negligible injury (Figure S3). After incubation in glutamate for 24 h, the cultures were immunostained for MAP2 to quantify the number of surviving neurons (Fig. 5a). To account for baseline differences in viability between capn-InsP$_3$R1- and control-transduced cultures, the percentage of MAP2-positive neurons in glutamate-injured cultures was normalized to vehicle-treated cultures transduced with the same vector. Exposure to 1 μM glutamate resulted in significant loss of neurons in all cultures ($p < 0.05$). Of note, cultures transduced with capn-InsP$_3$R1 were particularly susceptible (survival: capn-InsP$_3$R1 = 36.9 ± 10.6%, capn-InsP$_3$R1 D2550A = 77.2 ± 10.1%, lacZ = 79.5 ± 9.2%; $p < 0.001$). This result indicates that capn-InsP$_3$R1 increases neuronal susceptibility to excitotoxic injury.

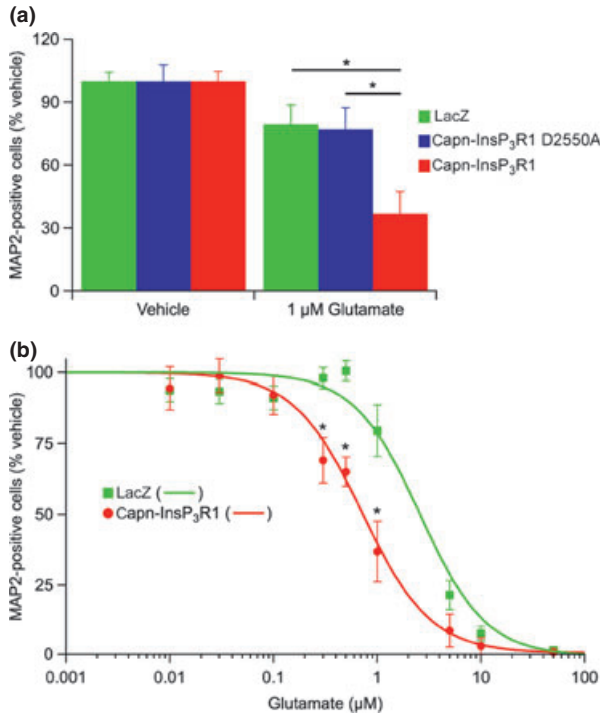

Fig. 5 Expression of capn-$InsP_3R1$ increases neuronal susceptibility to glutamate-mediated injury. (a) Transduced primary cortical cultures (14 DIV) were exposed to 1 μM glutamate or HEPES-buffered saline vehicle. Twenty-four hours later, cultures were stained for microtubule-associated protein 2 (MAP2) and percent neuronal survival quantified relative to vehicle-treated cultures. Expression of capn-$InsP_3R1$ resulted in a significant decrease in percentage of MAP2-positive cells following glutamate injury compared with lacZ and capn-$InsP_3R1$ D2550A (one-way ANOVA; $^*p < 0.01$). (b) Glutamate dose-response curves for lacZ and capn-$InsP_3R1$-transduced cultures (data at 1 μM glutamate are the same as shown in Fig. 5a). Expression of capn-$InsP_3R1$ significantly decreased percent MAP2-positive cells following treatment with 0.3, 0.5, and 1 μM glutamate compared with lacZ (unpaired *t* tests; $^*p < 0.01$) and resulted in a shift in the glutamate dose-response curve. LD_{50} for glutamate in neurons expressing capn-$InsP_3R1$ (0.75 ± 0.19 μM) is reduced compared with neurons expressing lacZ (2.61 ± 0.78 μM).

To further examine the enhanced vulnerability to glutamate-mediated injury in capn-$InsP_3R1$-transduced cultures, we treated primary cortical neurons with a wide range of glutamate doses (0.01–50 μM). As we did not observe differences between neuronal survival in lacZ- and capn-$InsP_3R1$ D2550A-transduced cultures at baseline or following glutamate injury, we chose a single control (lacZ) for these experiments. Transduced primary cortical neurons were incubated with glutamate or HBS vehicle and the number of surviving neurons was quantified 24 h later by MAP2 staining (Fig. 5b). Glutamate concentrations ≥ 0.3 μM induced significant loss of neurons in cultures transduced with capn-$InsP_3R1$ ($p < 0.01$), whereas lacZ-transduced cultures were only sensitive at concentrations ≥ 1 μM. To facilitate comparison across doses, we fit data using a Hill equation to produce normalized dose-response curves. Capn-$InsP_3R1$ expression resulted in a leftward shift of the glutamate dose-response curve compared with lacZ control. These results suggest that the proteolyzed channel increases neuronal sensitivity to glutamate-mediated injury and decreases the threshold for excitotoxicity.

Discussion

While several studies have examined the effects of $InsP_3R1$ proteolysis on cellular Ca^{2+} homeostasis (Assefa *et al.* 2004; Nakayama *et al.* 2004; Verbert *et al.* 2008; Kopil *et al.* 2011), the current study presents the first functional investigation of calpain-cleaved $InsP_3R1$ in neurons. We have demonstrated that capn-$InsP_3R1$ disrupts $[Ca^{2+}]_i$ homeostasis and decreases neuronal viability. Impaired ER Ca^{2+} buffering caused by the leaky channel likely accounts for the increased sensitivity to excitotoxic injury observed in primary cortical neurons expressing capn-$InsP_3R1$. Together, these results suggest that $InsP_3R1$ proteolysis is a novel, possible feed-forward pathway for executing neuronal cell death.

Capn-$InsP_3R1$ is sufficient to signal neuronal death in culture

Previous studies examining caspase- and calpain-cleaved $InsP_3R1$ in cell lines did not report effects of the dysregulated channel on baseline cell viability (Assefa *et al.* 2004; Nakayama *et al.* 2004; Verbert *et al.* 2008; Kopil *et al.* 2011). This is likely because of the relatively low open probability (P_o) of the cleaved channel at resting $[Ca^{2+}]_i$ (Kopil *et al.* 2011). Unlike cell lines, however, primary neurons in culture form complex networks with spontaneous activity and neurotransmission, leading to transient increases in $[Ca^{2+}]_i$ (Murphy *et al.* 1992; Maeda *et al.* 1995). Thus, we hypothesized that expression of capn-$InsP_3R1$ in neurons would be sufficient to signal cell death through disruption of neuronal Ca^{2+} homeostasis. To examine the effect of capn-$InsP_3R1$ on neuronal viability, we employed AAV-mediated gene delivery to achieve high transduction efficiency in primary cortical cultures. We determined that expression of capn-$InsP_3R1$ indeed significantly decreased neuronal viability at baseline (Fig. 2). However, capn-$InsP_3R1$ expression did not lead to complete cell loss, as the majority (~65%) of capn-$InsP_3R1$-transduced neurons were viable, at least at the time studied. This likely explains why we were unable to determine the mechanism(s) for capn-$InsP_3R1$-induced cell death using Western blotting (Figure S1). The moderate effect of capn-$InsP_3R1$ on baseline neuron viability may suggest gene expression heterogeneity within the population of neurons in primary cortical cultures. Identifying gene expression differences, and understanding their potential interactions with capn-$InsP_3R1$ that modulate neuronal death requires additional investigation.

Studying capn-$InsP_3R1$ in neurons using AAV-mediated gene delivery has some limitations. Foremost, the limited packaging capacity of AAV prohibits viral vector expression of full-length $InsP_3R1$ (Dong *et al.* 1996). As an alternative control, we expressed capn-$InsP_3R1$ with a point mutation in the putative pore selectivity filter between transmembrane domains 5 and 6. In wt-$InsP_3R1$, replacing the aspartate at residue 2550 with alanine (D2550A) results in a channel that is still able to bind $InsP_3$ and gates normally, but lacks Ca^{2+} permeability and does not have Ca^{2+}-release activity (Boehning and Joseph 2000; Boehning *et al.* 2001; Cardenas *et al.* 2010). The capn-$InsP_3R1$ D2550A mutant channel is expected to have reduced Ca^{2+}-release activity similar to the mutant full-length channel, but with $InsP_3$-independent gating, similar to capn-$InsP_3R1$. Thus, the structure and function of capn-$InsP_3R1$ D2550A serve as a specific control for the effects of $InsP_3$-independent Ca^{2+} release through capn-$InsP_3R1$.

Studying the Ca^{2+}-permeable and impermeable capn-$InsP_3R1$ in neurons may be complicated by heteroligomerization of the recombinant truncated channels with endogenous $InsP_3R1$. At 14 DIV, we observed expression of each 95 kDa $InsP_3R1$ construct at an approximately 1 : 1 ratio with endogenous 260 kDa $InsP_3R1$ (Fig. 1b; densitometry analysis not shown). Additional studies are required to fully elucidate the stochiometry and functional properties of the different heteroligomeric channels. The single-cell Ca^{2+} imaging results may provide some initial insights into the function of heteroligomeric channels. Specifically, we did not observe a reduction in the percentage of cells with $InsP_3$-induced Ca^{2+} release in capn-$InsP_3R1$ D2550A-transduced cultures (Fig. 3c). Nor did we observe a reduction in Ca^{2+} released from $InsP_3$-sensitive stores (Fig 3d and e). Assuming that recombinant $InsP_3R1$ subunits oligomerize with endogenous $InsP_3R$ in transduced neurons, we conclude that expression of the D2550A mutant under the conditions of our experiments did not exert a dominant negative effect on Ca^{2+} permeability. This is consistent with theoretical models of a similar mutation in ryanodine receptors, where mutating a negatively charged residue in the selectivity filter of an individual subunit resulted in only a partial decrease in Ca^{2+} conductance (Gao *et al.* 2000; Wang *et al.* 2005). Similarly, there is no expectation of a strong dominant negative effect of capn-$InsP_3R1$ on the $InsP_3$ requirement for channel gating. This is based on evidence that binding of four $InsP_3$ molecules is not required for channel gating. Instead, binding of each $InsP_3$ molecule increases the likelihood that the channel will open (Mak *et al.* 2003; Foskett *et al.* 2007; Mak *et al.* 2007), although it is unknown if the absence of some $InsP_3$ binding sites in a heteroligomeric channel of truncated and full-length $InsP_3R$ subunits affects the sensitivity to activation by $InsP_3$. By studying the effects of stable capn-$InsP_3R1$ expression in neurons with endogenous $InsP_3R$, we are perhaps more closely modeling a physiologically relevant pathologic process of limited channel proteolysis.

Neuronal Ca^{2+} homeostasis is disrupted by capn-$InsP_3R1$

How does $InsP_3$-independent Ca^{2+} release through capn-$InsP_3R1$ affect neuronal Ca^{2+} homeostasis? Using single-cell imaging, we observed normal $InsP_3$-induced Ca^{2+} release in capn-$InsP_3R1$-transduced cortical neurons through activation of mGluR1 and mGluR5 receptors (Fig. 3b, d and e) (Conn and Pin 1997; Fagni *et al.* 2000). This result is consistent with previous reports showing that expression of caspase- or calpain-cleaved $InsP_3R1$ does not deplete $[Ca^{2+}]_{ER}$, even though it increases the rate of ER Ca^{2+} leak (Assefa *et al.* 2004; Verbert *et al.* 2008; Kopil *et al.* 2011). This increased ER Ca^{2+} leak in neurons expressing capn-$InsP_3R1$ was manifested as elevated resting $[Ca^{2+}]_i$ (Figs 3b and 4b, Figure S2b). While the resting $[Ca^{2+}]_i$ was variable across experiments performed on cortical cultures from different embryos and platings, the ~20 nM increase in $[Ca^{2+}]_i$ in capn-$InsP_3R1$-transduced neurons versus controls remained consistent. This elevated resting $[Ca^{2+}]_i$ suggests that capn-$InsP_3R1$ generates an ER Ca^{2+} leak that cannot be fully compensated for by other Ca^{2+} transport mechanisms. This is in contrast to previous data from Neuro-2A cells, where we did not detect an effect of capn-$InsP_3R1$ on resting $[Ca^{2+}]_i$ (Kopil *et al.* 2011). The elevated basal $[Ca^{2+}]_i$ observed here could be a cell-type specific effect, or a consequence of chronic capn-$InsP_3R1$ expression, as our previous study examined changes in $[Ca^{2+}]_i$ at 6 h post-transfection. In either case, sustained elevations in resting $[Ca^{2+}]_i$ may explain the decreased neuronal viability in primary cortical cultures expressing capn-$InsP_3R1$.

The apparent increased ER Ca^{2+} leak through capn-$InsP_3R1$ resulted in more pronounced changes in neuronal $[Ca^{2+}]_i$ in response to Ca^{2+} influx. Expression of capn-$InsP_3R1$ significantly increased the peak $[Ca^{2+}]_i$ response to glutamate (Fig. 4c). This likely reflects a decreased capacity of normal ER Ca^{2+} buffering in neurons expressing capn-$InsP_3R1$, as the significant difference in peak $[Ca^{2+}]_i$ between capn-$InsP_3R1$ and controls was eliminated by inhibiting ER Ca^{2+} uptake with Tg (Fig. 4d). The kinetics of the glutamate Ca^{2+} responses were also altered in capn-$InsP_3R1$-transduced cultures. $[Ca^{2+}]_i$ in control neurons rapidly reached a plateau after the initial rise, whereas $[Ca^{2+}]_i$ in capn-$InsP_3R1$-transduced neurons continued to rise until glutamate was removed from the bath (Fig. 4a). Interestingly, average peak $[Ca^{2+}]_i$ in capn-$InsP_3R1$-expressing neurons approached 2 μM, which is optimal $[Ca^{2+}]_i$ for wt-$InsP_3R1$ and capn-$InsP_3R1$ activation (Foskett *et al.* 2007; Kopil *et al.* 2011). We expect that increased channel P_o of capn-$InsP_3R1$ at this higher $[Ca^{2+}]_i$ compromises ER Ca^{2+} buffering, and provides a potential mechanism for increased sensitivity to glutamate injury (Figure 5) through irreversible disruption of neuronal Ca^{2+} homeostasis.

It is important to note that we measured somatic $[Ca^{2+}]_i$. As neuronal dendritic and synaptic structure is complex, with heterogeneous distributions of ER and $InsP_3R$ (Bardo *et al.* 2006; Stutzmann and Mattson 2011), capn-$InsP_3R1$ may exert varying influences in these cellular compartments. Understanding the spatial complexity and functional consequences of capn-$InsP_3R1$-mediated Ca^{2+} signaling in dendrites and synapses warrants future investigation.

Role of calpain-cleavage of $InsP_3R1$ in neurodegeneration

Sustained pathologic rises in $[Ca^{2+}]_i$ are a key component of both apoptotic and necrotic cell death (Berridge *et al.* 2000; Orrenius *et al.* 2003; Mattson 2007). Therefore, we hypothesized that the dramatic glutamate-induced rise in $[Ca^{2+}]_i$ in capn-$InsP_3R1$-expressing primary cortical neurons would increase their sensitivity to excitotoxic injury. We tested this by exposing transduced cultures to glutamate rather than NMDA, as expression of pathogenic NMDA receptor subunits is developmentally regulated in primary neurons (O'Donnell *et al.* 2006), and glutamate produced a more consistent, dose-dependent injury in untransduced 14 DIV cultures (data not shown). As predicted, expression of capn-$InsP_3R1$ significantly decreased the percentage of surviving neurons over a range of glutamate concentrations (Fig. 5). Enhanced glutamate-induced cell death is likely precipitated by Ca^{2+}-induced Ca^{2+} release through capn-$InsP_3R1$ in addition to decreased basal ER Ca^{2+} buffering in these cells. Together, these impairments of neuronal Ca^{2+} homeostasis increase sensitivity to excitatory stimuli, leading to $[Ca^{2+}]_i$ overload. The downstream effects of this $[Ca^{2+}]_i$ overload may signal cell death through either necrotic or apoptotic pathways. Sustained elevations of $[Ca^{2+}]_i$ could directly lead to pathologic activation of calpains and calpain-mediated cell death (Goll *et al.*, 2003). Alternatively, $[Ca^{2+}]_i$ overload could enhance mitochondrial Ca^{2+} uptake, triggering mitochondrial permeability transition and caspase-mediated apoptosis (Orrenius *et al.* 2003; Taylor *et al.* 2008). Future mechanistic studies are required to clarify the precise pathologic pathway(s) activated by capn-$InsP_3R1$-mediated Ca^{2+} signaling. Current available data suggest that calpain proteolysis of $InsP_3R1$ in neurons acts to activate and accelerate cell death pathways after injury through $[Ca^{2+}]_i$ overload. Interestingly, chronic activation and Ca^{2+} release through $InsP_3R1$ can signal ubiquitination and subsequent channel degradation by the proteasome (Alzayady and Wojcikiewicz 2005; Bhanumathy *et al.* 2006). However, calpain proteolysis of the channel removes all of the potential polyubiquitination sites within the $InsP_3R1$-coupling domain (Bhanumathy *et al.* 2006; Sliter *et al.* 2008), which are required for proteasomal degradation. Thus, calpain cleavage of $InsP_3R1$ may be critical to executing cell death by removing activity-dependent regulation of the channel, thereby causing Ca^{2+} overload.

Conclusions

In summary, our results indicate that expression of calpain-cleaved $InsP_3R1$ impairs neuronal ER Ca^{2+} buffering, leading to increased sensitivity to excitotoxic stimuli and neurodegeneration in primary neuron cultures. As an early part of the molecular injury cascade (Kopil *et al.* 2011), calpain proteolysis of $InsP_3R1$ could contribute a feed-forward pathway to accelerate neuronal death through $[Ca^{2+}]_i$ overload. Thus, inhibiting Ca^{2+} release through calpain-cleaved $InsP_3R1$ is a potentially important therapeutic strategy for intervention in disorders associated with calpain activity and disruption of Ca^{2+} homeostasis.

Acknowledgements

We thank Margaret Maronski for the preparation of cortical cultures. We thank Dr. Virginia Lee for supplying antibody. We thank Dr. Shu-Jen Chen, Dr. Arbansjit Sandhu, and the University of Pennsylvania Vector Core for technical expertise in cloning and viral vector generation. We also thank Dr. Don-On Daniel Mak for IGOR macros and helpful discussions, and Dr. Horia Vais for comments on the manuscript. This work was supported by National Institutes of Health grants NS039481 (RWN), NS069951 (RWN), NS071828 (CMK), MH059937 (JKF), and GM065830 (JKF).

Conflict of interest

The authors declare no conflict of interest.

Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Activation of ER stress markers and proteases in transduced primary cortical neurons.

Figure S2. Neurons expressing capn-$InsP_3R1$ have increased rises in$[Ca^{2+}]$i in response to depolarization.

Figure S3. Characterization of glutamate injury model.

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