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Calpain-cleaved type 1 inositol 1,4,5-trisphosphate receptor impairs ER Ca²⁺ buffering and causes neurodegeneration in primary cortical neurons

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Abstract

Disruption of neuronal Ca²⁺ 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 (InsP₃R1), a Ca²⁺ release channel on the endoplasmic reticulum, generates a dysregulated channel, which may contribute to aberrant Ca²⁺ signaling and neurodegeneration in disease states. However, the specific effects of InsP₃R1 proteolysis on neuronal Ca²⁺ homeostasis are unknown, as are the functional contributions of this pathway to neuronal death. This study evaluates the consequences of calpain-mediated InsP₃R1 proteolysis on neuronal Ca²⁺ signaling and survival using adeno-associated viruses to express a recombinant cleaved form of the channel (capn-InsP₃R1) in rat primary cortical neurons. Here, we demonstrate that expression of capn-InsP₃R1 in cortical cultures reduced cellular viability. This effect was associated with increased resting cytoplasmic Ca²⁺ concentration ([Ca²⁺]_i), increased [Ca²⁺]_i response to glutamate, and enhanced sensitivity to excitotoxic stimuli. Together, our results demonstrate that InsP₃R1 proteolysis disrupts neuronal Ca²⁺ homeostasis, and potentially acts as a feed-forward pathway to initiate or execute neuronal death.

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Conflict of interest

The authors declare no conflict of interest.

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Keywords

calcium; calpain; endoplasmic reticulum; excitotoxicity; inositol 1,4,5-trisphosphate receptor; neuron

Spatial and temporal regulation of cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) plays a vital role in neuronal signaling, and dysregulation of $[\text{Ca}^{2+}]_i$ can trigger cell death (Berridge *et al.* 2000, 2003; Orrenius *et al.* 2003; Mattson 2007). Under physiologic conditions, changes in neuronal $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 (InsP₃R) (Foskett *et al.* 2007). Of the three identified InsP₃R genes, type 1 InsP₃R (InsP₃R1) is the most abundant isoform expressed in brain (Sharp *et al.* 1993). Physiologic InsP₃R1-mediated Ca^{2+} signaling in neurons is important for gene expression, membrane excitability, and synaptic plasticity (Bardo *et al.* 2006; Stutzmann and Mattson 2011). InsP₃R1 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 (Verkhatsky and Toescu 2003; Foskett 2010). In most of these cases, InsP₃R1 dysfunction has been associated with allosteric modulation of the channel (Foskett *et al.* 2007). However, increasing evidence suggests that proteolytic modification of InsP₃R1 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.

InsP₃R1 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 InsP₃R1 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 InsP₃R1 fragment demonstrated increased ER Ca^{2+} leak, and corresponding decreased ER $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_{\text{ER}}$), 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 InsP₃-independent Ca^{2+} release through caspase-cleaved InsP₃R1, 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 InsP₃R1 and determined that the calpain-derived carboxyl-terminal channel fragment displayed InsP₃-independent gating and constitutive channel activity (Kopil *et al.* 2011). When expressed in cell lines, calpain-cleaved InsP₃R1 decreased $[\text{Ca}^{2+}]_{\text{ER}}$ via increased ER Ca^{2+} leak (Kopil *et al.* 2011). Although no study to date has investigated the effects of caspase- or calpain-cleaved InsP₃R1 on neuronal Ca^{2+} homeostasis, these observations suggest that proteolysis of InsP₃R1 may transform spatial and temporal regulation of neuronal $[\text{Ca}^{2+}]_i$ and potentially signal cell death. Consistent with this hypothesis, we

previously identified calpain-cleaved InsP₃R1 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 InsP₃R1 proteolysis in neuronal Ca²⁺ homeostasis and neurodegeneration, we examined responses of primary cortical neurons to expression of recombinant calpain-cleaved InsP₃R1. Expression of the cleaved channel reduces viability of cortical cultures at baseline. Using single-cell Ca²⁺ imaging, we determined that calpain-cleaved InsP₃R1 increases resting neuronal [Ca²⁺]_i and leads to greater rises in [Ca²⁺]_i in response to physiologic stimuli. Calpain-cleaved InsP₃R1 also sensitized cultures to excitotoxic injury. Together, these data suggest a specific role for proteolyzed InsP₃R1-mediated Ca²⁺ signaling in the molecular injury cascade in neurons, which has important therapeutic implications for neurodegenerative disorders associated with Ca²⁺ 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 InsP₃R1 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 \times 50-mm glass coverslips in four-well plates (3.2×10^6 cells/well; single-cell Ca²⁺ 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% CO₂ 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 InsP₃R1 (capn-InsP₃R1; Fig. 1a) was previously generated (Kopil *et al.* 2011). To construct the pore-inactivating D2550A mutation (capn-InsP₃R1D2550A), site-directed mutagenesis was performed with Quik-Change (Stratagene) using primers (5′-GGCGGAGTAGGAGCT GTGCTCAGGAAG-3′ and 5′-CTTCCTGAGCACAGCTCCTAC TCCGCC-3′ (codon change is shown in boldface type and underlined)) and capn-InsP₃R1 as a template. The mutation was confirmed by DNA sequencing. For transduction of primary neurons, both capn-InsP₃R1 and capn-InsP₃R1 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-InsP₃R1 (lots V2049 and V2451), capn-InsP₃R1 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% CO₂. 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 Ca²⁺ 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% CO₂ in Ca²⁺-containing extracellular solution (in mM: 120 NaCl, 4 KCl, 20 HEPES, 15 Glucose, 2 CaCl₂, 1 MgCl₂, 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 Ca²⁺ extracellular solution to establish baseline [Ca²⁺]_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, Mn²⁺ was used to quench Fura-2 fluorescence (Ca²⁺-containing extracellular solution supplemented with 10 mM MnCl₂ and 10 μM ionomycin). The remaining background fluorescence following Mn²⁺ quench was subtracted during analysis. Glial cells were identified by their morphology and ability to 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-InsP₃R1 D2550A-, and capn-InsP₃R1-transduced cultures were derived from the same plating. To compare between experiments using glutamate alone and glutamate plus thapsigargin, the percent of maximum [Ca²⁺]_i was determined by normalizing to the average peak [Ca²⁺]_i of capn-InsP₃ 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 CaCl₂, 1 MgCl₂, 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 LD₅₀ 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 Bartlett'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 InsP₃R1 constructs in primary cortical neurons

Previously, we showed that expression of a recombinant form of rat InsP₃R1 corresponding to the stable carboxyl-terminal fragment generated by calpain proteolysis (capn-InsP₃R1; 1–1917; Fig. 1a) altered $[Ca^{2+}]_i$ homeostasis in immortalized cell lines. In this study, we examined the functional consequences of capn-InsP₃R1 expression in primary neurons. To achieve high transduction efficiency of primary cortical cultures, we utilized AAV 2/1-mediated gene delivery of capn-InsP₃R1, which transduces over 90% of cells in primary neuron cultures (Royo *et al.* 2008; Bevers *et al.* 2009). As a control for InsP₃-independent Ca²⁺ release through the truncated channel, we generated AAV 2/1 vector expressing capn-InsP₃R1 with a D2550A point mutation in the pore-forming region of the channel (capn-InsP₃R1 D2550A; Fig. 1a). This point mutation eliminates Ca²⁺ permeability of wild-type (wt) InsP₃R1 (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 InsP₃R1 with or

without the D2550A mutation. We used AAV 2/1 expressing lacZ to control for effects of viral transduction.

To examine expression of InsP₃R1 constructs in neurons, we stably transduced cortical cultures with AAV 2/1 expressing capn-InsP₃R1, capn-InsP₃R1 D2550A, or lacZ at 7 DIV and harvested cells 1 week later (14 DIV). Western blotting of whole-cell lysates using a carboxyl-terminal InsP₃R1 antibody demonstrated expression of endogenous wt-InsP₃R1 in all samples (Fig. 1b). In lysates from cultures transduced with capn-InsP₃R1 and capn-InsP₃R1 D2550A, we also detected the 95 kDa form of the channels, confirming expression of recombinant InsP₃R1 constructs.

Capn-InsP₃R1 reduces neuronal viability

To determine the effects of capn-InsP₃R1 on neuronal viability, we transduced 7 DIV cultures with capn-InsP₃R1, capn-InsP₃R1 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-InsP₃R1 resulted in significant decrease in the percentage of viable neurons compared with expression of capn-InsP₃R1 D2550A or lacZ (capn-InsP₃R1 = 65.3 ± 5.5%, capn-InsP₃R1 D2550A = 112.7 ± 5.8%, lacZ = 100.0 ± 3.8%; $p < 0.001$). To confirm the capn-InsP₃R1 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-InsP₃R1 compared with controls (capn-InsP₃R1 = 76.7 ± 4.5%, capn-InsP₃R1 D2550A = 112.5 ± 6.5%, lacZ = 100.0 ± 4.0%; $p < 0.001$; Fig. 2c). Interestingly, the percentage of viable cells in capn-InsP₃R1-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-InsP₃R1. Together, these results suggest that expression of capn-InsP₃R1 specifically reduces neuronal viability.

We hypothesized that expression of capn-InsP₃R1 reduced neuronal viability by disrupting intracellular Ca²⁺ homeostasis. The proteolyzed channel, which has InsP₃-independent gating and increases the ER Ca²⁺ leak (Kopil *et al.* 2011), could potentially trigger neurodegeneration through either ER Ca²⁺ depletion and induction of ER stress, or by increased [Ca²⁺]_i and feed-forward activation of proteases. To examine the mechanism responsible for capn-InsP₃R1 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-InsP₃R1 does not conclusively imply that neither pathologic cascade plays a causal role in capn-InsP₃R1-induced neuronal death. However, if either pathway is responsible for executing cell death in capn-InsP₃R1-

transduced cultures, Western blotting of neuronal lysates is not sensitive enough to detect it at the times examined here.

Capn-InsP₃R1 does not deplete intracellular Ca²⁺ stores

Previous data from our laboratory indicated that capn-InsP₃R1 functions as a Ca²⁺ leak channel in the ER (Kopil et al. 2011). To examine whether this leak was sufficient to deplete intracellular Ca²⁺ stores in neurons, we utilized single-cell Ca²⁺ imaging to measure InsP₃-induced changes in [Ca²⁺]_i. We monitored somatic [Ca²⁺]_i using Fura-2 in primary cortical cultures transduced with capn-InsP₃R1, capn-InsP₃R1 D2550A, or lacZ (Fig. 3a). To stimulate Ca²⁺ release from InsP₃-sensitive stores, we applied saturating concentrations of the Group I metabotropic glutamate receptor agonist DHPG (10 μM). Expression of capn-InsP₃R1 significantly increased resting [Ca²⁺]_i in neurons (90 ± 3.3 nM) compared with capn-InsP₃R1 D2550A (70 ± 1.8 nM) and lacZ controls (70 ± 1.6 nM; *p* < 0.001; Fig. 3b), supporting previous evidence that capn-InsP₃R1 leaks Ca²⁺ from the ER. Application of DHPG elicited responses in a majority of neurons from all transduced cultures (Fig. 3c). Despite the apparent basal Ca²⁺ leak through the cleaved channel, expression of capn-InsP₃R1 did not affect the DHPG-induced peak [Ca²⁺]_i in neurons (Fig. 3d) or the maximum change in [Ca²⁺]_i from baseline to peak (Fig. 3e). These data demonstrate that expression of the leaky channel, while sufficient to increase resting [Ca²⁺]_i, does not deplete [Ca²⁺]_{ER}.

Decreased ER Ca²⁺ buffering in neurons expressing capn-insP₃R1

To examine the effects of calpain-cleaved InsP₃R1 on neuronal Ca²⁺ homeostasis more broadly, we measured changes in [Ca²⁺]_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 Ca²⁺ imaging experiments above, resting [Ca²⁺]_i was significantly increased in neurons expressing capn-InsP₃R1 compared with capn-InsP₃R1 D2550A- and lacZ-expressing cells (*p* < 0.001; Fig. 4b). In addition, capn-InsP₃R1 significantly increased the glutamate-induced peak [Ca²⁺]_i (1,520 ± 43 nM) compared with capn-InsP₃R1 D2550A (1,030 ± 36 nM) and lacZ controls (940 ± 25 nM; *p* < 0.001; Fig. 4c). Stimulation of an alternate pathway for Ca²⁺ influx also resulted in a significantly increased peak [Ca²⁺]_i in cultures transduced with capn-InsP₃R1 (50 mM KCl; Figure S2). This result suggests that the increased glutamate-induced [Ca²⁺]_i in capn-InsP₃R1-transduced neurons is not dependent on increased glutamate receptor expression. Instead, the increased [Ca²⁺]_i rise is likely because of an impaired ability of the ER to buffer Ca²⁺ influx from the extracellular space. To test this hypothesis, we measured changes in [Ca²⁺]_i in response to acute glutamate (10 μM) application following a 3-min pre-incubation with Tg (1 μM) to inhibit the sarco-endoplasmic reticulum Ca²⁺-ATPase. Blocking ER Ca²⁺ uptake eliminated the difference in the glutamate-induced peak [Ca²⁺]_i between neurons expressing capn-InsP₃R1 and control cultures expressing lacZ or capn-InsP₃R1 D2550A (Fig. 4d). Together, these data indicate that capn-InsP₃R1 disrupts normal intracellular Ca²⁺ homeostasis and impairs the ability of the ER to buffer physiologic rises in [Ca²⁺]_i.

Capn-InsP₃R1 increases sensitivity to glutamate-induced neurotoxicity

To determine whether the observed capn-InsP₃R1-induced impairment of ER Ca²⁺ 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-InsP₃R1- 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-InsP₃R1 were particularly susceptible (survival: capn-InsP₃R1 = $36.9 \pm 10.6\%$, capn-InsP₃R1 D2550A = $77.2 \pm 10.1\%$, lacZ = $79.5 \pm 9.2\%$; $p < 0.001$). This result indicates that capn-InsP₃R1 increases neuronal susceptibility to excitotoxic injury.

To further examine the enhanced vulnerability to glutamate-mediated injury in capn-InsP₃R1-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-InsP₃R1 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-InsP₃R1 ($p < 0.01$), 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-InsP₃R1 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 InsP₃R1 proteolysis on cellular Ca²⁺ 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 InsP₃R1 in neurons. We have demonstrated that capn-InsP₃R1 disrupts [Ca²⁺]_i homeostasis and decreases neuronal viability. Impaired ER Ca²⁺ buffering caused by the leaky channel likely accounts for the increased sensitivity to excitotoxic injury observed in primary cortical neurons expressing capn-InsP₃R1. Together, these results suggest that InsP₃R1 proteolysis is a novel, possible feed-forward pathway for executing neuronal cell death.

Capn-InsP₃R1 is sufficient to signal neuronal death in culture

Previous studies examining caspase- and calpain-cleaved InsP₃R1 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 [Ca²⁺]_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 [Ca²⁺]_i (Murphy *et al.* 1992; Maeda

et al. 1995). Thus, we hypothesized that expression of capn-InsP₃R1 in neurons would be sufficient to signal cell death through disruption of neuronal Ca²⁺ homeostasis. To examine the effect of capn-InsP₃R1 on neuronal viability, we employed AAV-mediated gene delivery to achieve high transduction efficiency in primary cortical cultures. We determined that expression of capn-InsP₃R1 indeed significantly decreased neuronal viability at baseline (Fig. 2). However, capn-InsP₃R1 expression did not lead to complete cell loss, as the majority (~65%) of capn-InsP₃R1-transduced neurons were viable, at least at the time studied. This likely explains why we were unable to determine the mechanism(s) for capn-InsP₃R1-induced cell death using Western blotting (Figure S1). The moderate effect of capn-InsP₃R1 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-InsP₃R1 that modulate neuronal death requires additional investigation.

Studying capn-InsP₃R1 in neurons using AAV-mediated gene delivery has some limitations. Foremost, the limited packaging capacity of AAV prohibits viral vector expression of full-length InsP₃R1 (Dong *et al.* 1996). As an alternative control, we expressed capn-InsP₃R1 with a point mutation in the putative pore selectivity filter between transmembrane domains 5 and 6. In wt-InsP₃R1, replacing the aspartate at residue 2550 with alanine (D2550A) results in a channel that is still able to bind InsP₃ and gates normally, but lacks Ca²⁺ permeability and does not have Ca²⁺-release activity (Boehning and Joseph 2000; Boehning *et al.* 2001; Cardenas *et al.* 2010). The capn-InsP₃R1 D2550A mutant channel is expected to have reduced Ca²⁺-release activity similar to the mutant full-length channel, but with InsP₃-independent gating, similar to capn-InsP₃R1. Thus, the structure and function of capn-InsP₃R1 D2550A serve as a specific control for the effects of InsP₃-independent Ca²⁺ release through capn-InsP₃R1.

Studying the Ca²⁺-permeable and impermeable capn-InsP₃R1 in neurons may be complicated by heterooligomerization of the recombinant truncated channels with endogenous InsP₃R1. At 14 DIV, we observed expression of each 95 kDa InsP₃R1 construct at an approximately 1 : 1 ratio with endogenous 260 kDa InsP₃R1 (Fig. 1b; densitometry analysis not shown). Additional studies are required to fully elucidate the stoichiometry and functional properties of the different heterooligomeric channels. The single-cell Ca²⁺ imaging results may provide some initial insights into the function of heterooligomeric channels. Specifically, we did not observe a reduction in the percentage of cells with InsP₃-induced Ca²⁺ release in capn-InsP₃R1 D2550A-transduced cultures (Fig. 3c). Nor did we observe a reduction in Ca²⁺ released from InsP₃-sensitive stores (Fig 3d and e). Assuming that recombinant InsP₃R1 subunits oligomerize with endogenous InsP₃R1 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 Ca²⁺ 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 Ca²⁺ conductance (Gao *et al.* 2000; Wang *et al.* 2005). Similarly, there is no expectation of a strong dominant negative effect of capn-InsP₃R1 on the InsP₃ requirement for channel gating. This is based on evidence that binding of four InsP₃ molecules is not required for channel gating. Instead, binding of each InsP₃ 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 InsP₃ binding sites in a heteroligomeric channel of truncated and full-length InsP₃R subunits affects the sensitivity to activation by InsP₃. By studying the effects of stable capn-InsP₃R1 expression in neurons with endogenous InsP₃R, we are perhaps more closely modeling a physiologically relevant pathologic process of limited channel proteolysis.

Neuronal Ca²⁺ homeostasis is disrupted by capn-InsP₃R1

How does InsP₃-independent Ca²⁺ release through capn-InsP₃R1 affect neuronal Ca²⁺ homeostasis? Using single-cell imaging, we observed normal InsP₃-induced Ca²⁺ release in capn-InsP₃R1-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 InsP₃R1 does not deplete [Ca²⁺]_{ER}, even though it increases the rate of ER Ca²⁺ leak (Assefa *et al.* 2004; Verbert *et al.* 2008; Kopil *et al.* 2011). This increased ER Ca²⁺ leak in neurons expressing capn-InsP₃R1 was manifested as elevated resting [Ca²⁺]_i (Figs 3b and 4b, Figure S2b). While the resting [Ca²⁺]_i was variable across experiments performed on cortical cultures from different embryos and platings, the ~20 nM increase in [Ca²⁺]_i in capn-InsP₃R1-transduced neurons versus controls remained consistent. This elevated resting [Ca²⁺]_i suggests that capn-InsP₃R1 generates an ER Ca²⁺ leak that cannot be fully compensated for by other Ca²⁺ transport mechanisms. This is in contrast to previous data from Neuro-2A cells, where we did not detect an effect of capn-InsP₃R1 on resting [Ca²⁺]_i (Kopil *et al.* 2011). The elevated basal [Ca²⁺]_i observed here could be a cell-type specific effect, or a consequence of chronic capn-InsP₃R1 expression, as our previous study examined changes in [Ca²⁺]_i at 6 h post-transfection. In either case, sustained elevations in resting [Ca²⁺]_i may explain the decreased neuronal viability in primary cortical cultures expressing capn-InsP₃R1.

The apparent increased ER Ca²⁺ leak through capn-InsP₃R1 resulted in more pronounced changes in neuronal [Ca²⁺]_i in response to Ca²⁺ influx. Expression of capn-InsP₃R1 significantly increased the peak [Ca²⁺]_i response to glutamate (Fig. 4c). This likely reflects a decreased capacity of normal ER Ca²⁺ buffering in neurons expressing capn-InsP₃R1, as the significant difference in peak [Ca²⁺]_i between capn-InsP₃R1 and controls was eliminated by inhibiting ER Ca²⁺ uptake with Tg (Fig. 4d). The kinetics of the glutamate Ca²⁺ responses were also altered in capn-InsP₃R1-transduced cultures. [Ca²⁺]_i in control neurons rapidly reached a plateau after the initial rise, whereas [Ca²⁺]_i in capn-InsP₃R1-transduced neurons continued to rise until glutamate was removed from the bath (Fig. 4a). Interestingly, average peak [Ca²⁺]_i in capn-InsP₃R1-expressing neurons approached 2 IM, which is optimal [Ca²⁺]_i for wt-InsP₃R1 and capn-InsP₃R1 activation (Foskett *et al.* 2007; Kopil *et al.* 2011). We expect that increased channel P_o of capn-InsP₃R1 at this higher [Ca²⁺]_i compromises ER Ca²⁺ buffering, and provides a potential mechanism for increased sensitivity to glutamate injury (Figure 5) through irreversible disruption of neuronal Ca²⁺ homeostasis.

It is important to note that we measured somatic [Ca²⁺]_i. As neuronal dendritic and synaptic structure is complex, with heterogeneous distributions of ER and InsP₃R (Bardo *et al.* 2006;

Stutzmann and Mattson 2011), capn-InsP₃R1 may exert varying influences in these cellular compartments. Understanding the spatial complexity and functional consequences of capn-InsP₃R1-mediated Ca²⁺ signaling in dendrites and synapses warrants future investigation.

Role of calpain-cleavage of InsP₃R1 in neurodegeneration

Sustained pathologic rises in [Ca²⁺]_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 [Ca²⁺]_i in capn-InsP₃R1-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-InsP₃R1 significantly decreased the percentage of surviving neurons over a range of glutamate concentrations (Fig. 5). Enhanced glutamate-induced cell death is likely precipitated by Ca²⁺-induced Ca²⁺ release through capn-InsP₃R1 in addition to decreased basal ER Ca²⁺ buffering in these cells. Together, these impairments of neuronal Ca²⁺ homeostasis increase sensitivity to excitatory stimuli, leading to [Ca²⁺]_i overload. The downstream effects of this [Ca²⁺]_i overload may signal cell death through either necrotic or apoptotic pathways. Sustained elevations of [Ca²⁺]_i could directly lead to pathologic activation of calpains and calpain-mediated cell death (Goll *et al.*, 2003). Alternatively, [Ca²⁺]_i overload could enhance mitochondrial Ca²⁺ 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-InsP₃R1-mediated Ca²⁺ signaling. Current available data suggest that calpain proteolysis of InsP₃R1 in neurons acts to activate and accelerate cell death pathways after injury through [Ca²⁺]_i overload. Interestingly, chronic activation and Ca²⁺ release through InsP₃R1 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 InsP₃R1-coupling domain (Bhanumathy *et al.* 2006; Sliter *et al.* 2008), which are required for proteasomal degradation. Thus, calpain cleavage of InsP₃R1 may be critical to executing cell death by removing activity-dependent regulation of the channel, thereby causing Ca²⁺ overload.

Conclusions

In summary, our results indicate that expression of calpain-cleaved InsP₃R1 impairs neuronal ER Ca²⁺ 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 InsP₃R1 could contribute a feed-forward pathway to accelerate neuronal death through [Ca²⁺]_i overload. Thus, inhibiting Ca²⁺ release through calpain-cleaved InsP₃R1 is a potentially important therapeutic strategy for intervention in disorders associated with calpain activity and disruption of Ca²⁺ homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used:

[Ca ²⁺] _i	cytoplasmic free Ca ²⁺ concentration
AAV	adeno-associated virus
capn-InsP ₃ R1,	calpain-cleaved InsP ₃ R1
DIV	days in <i>vitro</i>
ER	endoplasmic reticulum
InsP ₃ R1	type 1 InsP ₃ receptor
MAP2	microtubule-associated protein 2
PI	propidium iodide
Tg	thapsigargin

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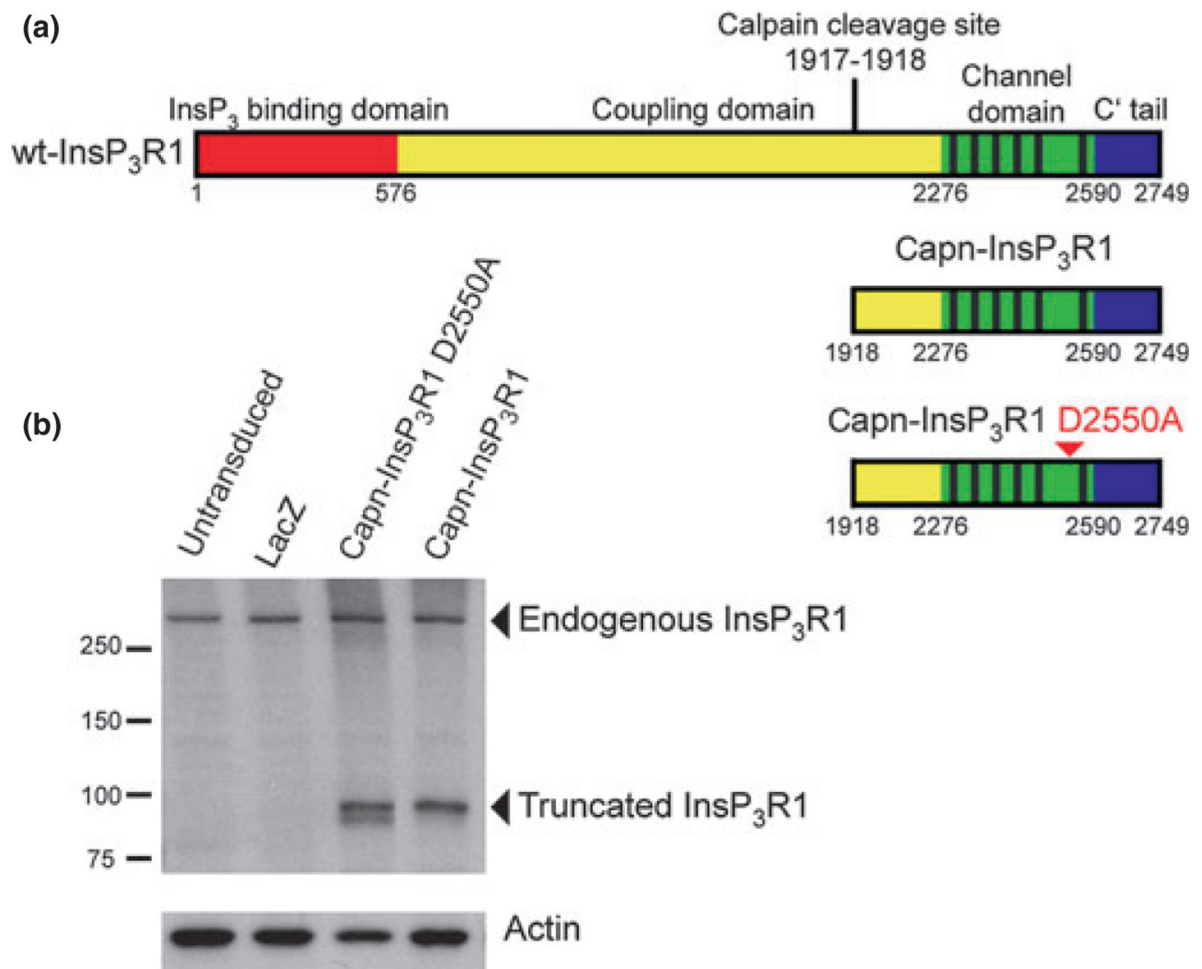


Fig. 1. Schematic representation and expression of recombinant InsP₃R1 constructs in primary neurons. (a) Protein domain structure of wt-InsP₃R1 (top), calpain-cleaved InsP₃R1 recombinant (middle), and calpain-cleaved InsP₃R1 with the D2550A point mutation (bottom; arrowhead). Residues numbered according 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-InsP₃R1 D2550A, or capn-InsP₃R1 at 1 week post-transduction (14 DIV). Carboxyl-terminal InsP₃R1 antibody was used to detect endogenous and recombinant truncated rat InsP₃R1. Antibody against actin was used as a loading control.

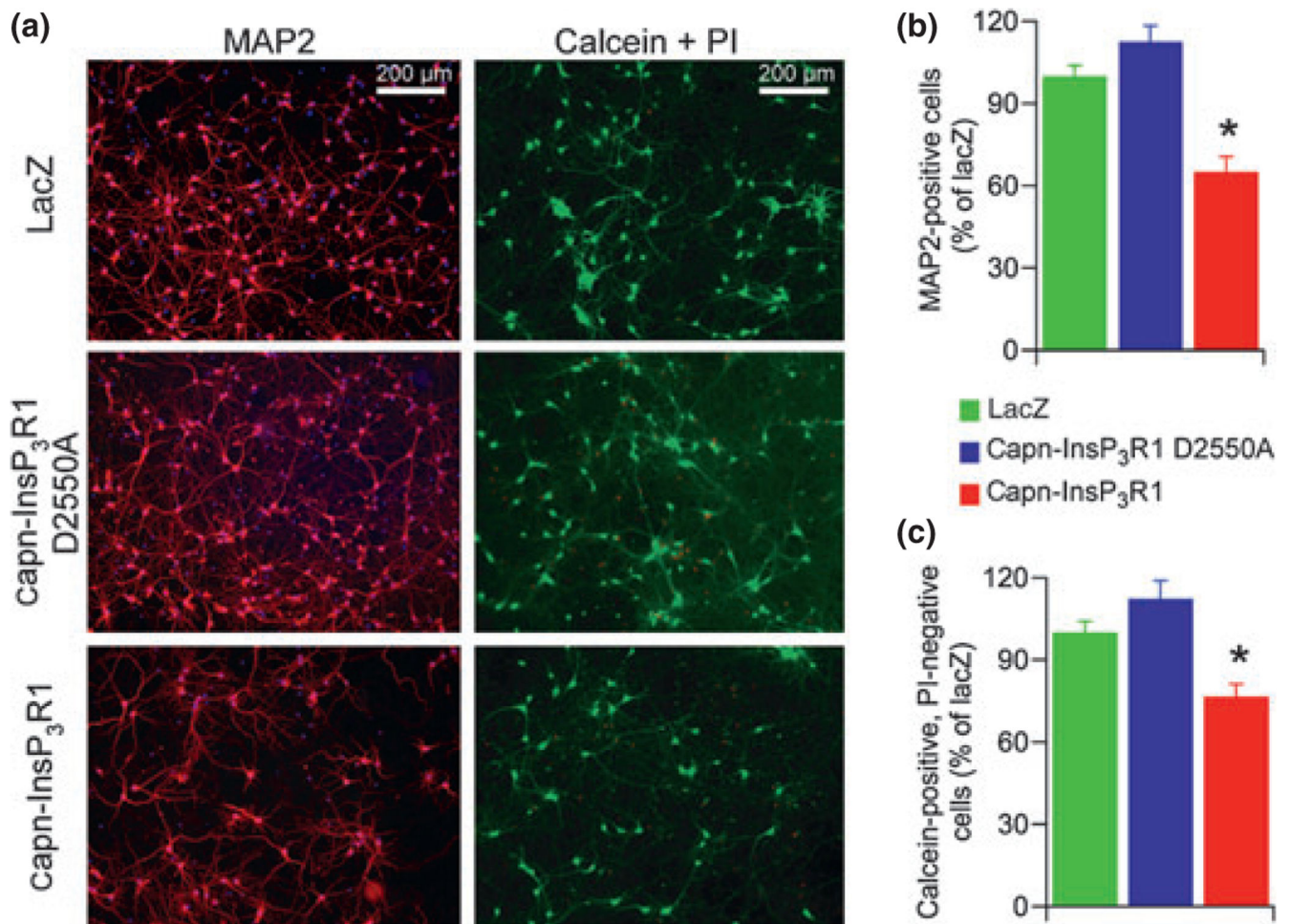
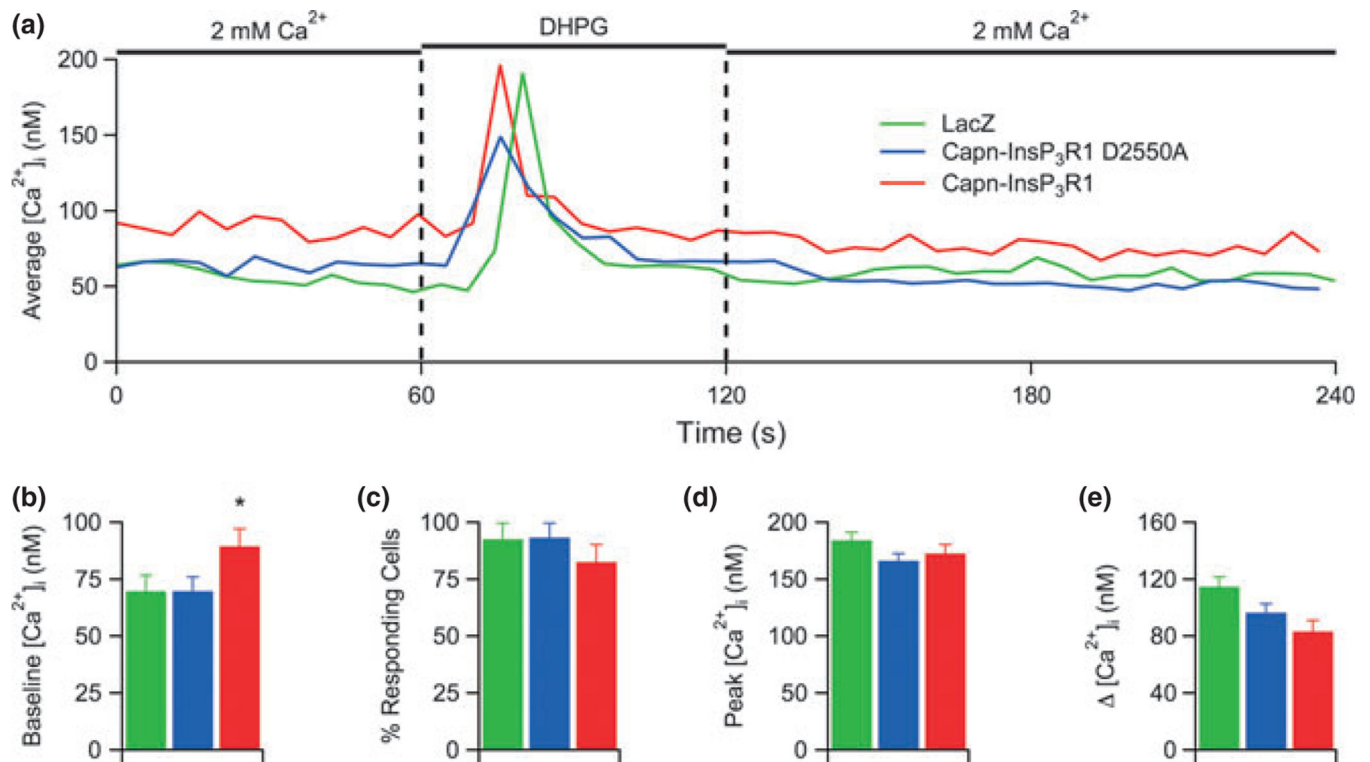


Fig. 2.

Primary cortical neuron viability is reduced by expression of capn-InsP₃R1. Rat primary cortical cultures were transduced with adeno-associated virus 2/1 expressing lacZ, capn-InsP₃R1 D2550A, or capn-InsP₃R1 (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 \times 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-InsP₃R1 resulted in a significant decrease in percent viable neurons compared with lacZ and capn-InsP₃R1 D2550A as determined by both assays.

**Fig. 3.**

Capn-InsP₃R1 does not deplete InsP₃-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-InsP₃R1 D2550A, or capn-InsP₃R1. Total number of single-cell Ca^{2+} responses analyzed in these experiments was 219, 246, and 172 for lacZ-, capn-InsP₃R1 D2550A-, and capn-InsP₃R1-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-InsP₃R1 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 $[Ca^{2+}]_i$.

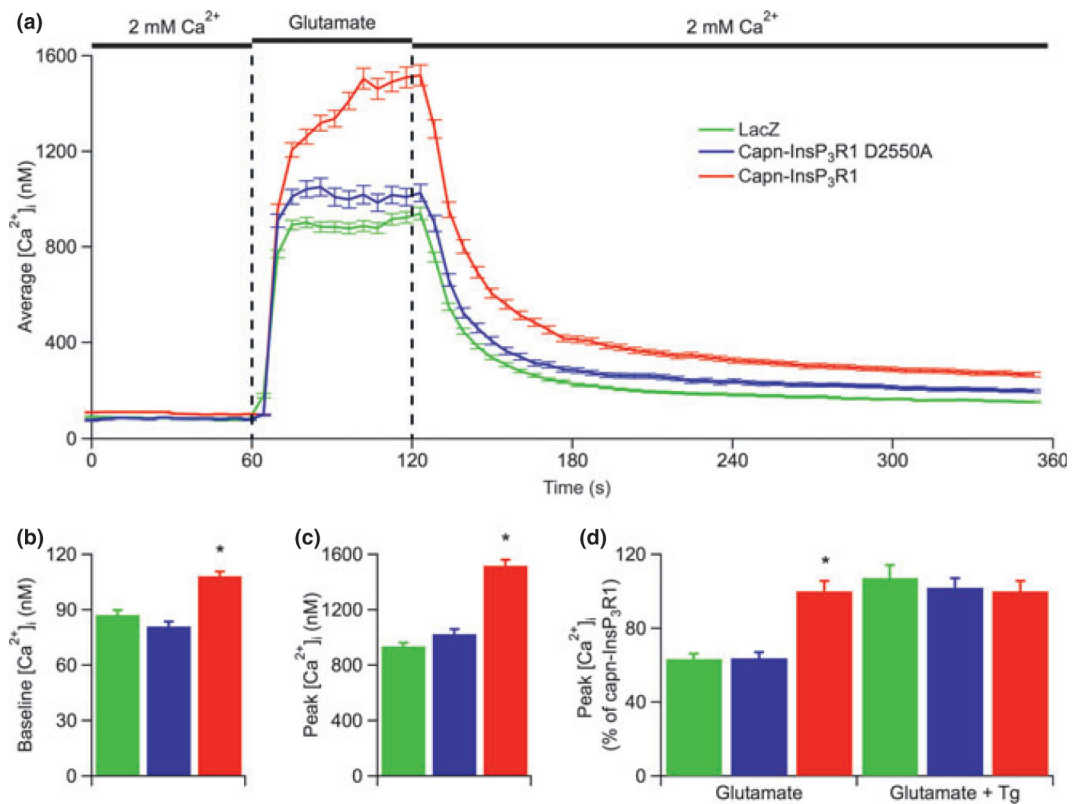


Fig. 4.

Neurons expressing capn-InsP₃R1 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-InsP₃R1 D2550A, or capn-InsP₃R1. Total number of single-cell Ca^{2+} responses analyzed in these experiments was 491, 271, and 396 for lacZ-, capn-InsP₃R1 D2550A-, and capn-InsP₃R1-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-InsP₃R1 significantly increased resting $[Ca^{2+}]_i$ in neurons (108.2 ± 2.6 nM) compared with capn-InsP₃R1 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-InsP₃R1-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-InsP₃R1 D2550A-, and capn-InsP₃R1-transduced cultures, respectively. Tg eliminated the capn-InsP₃R1-associated glutamate-induced enhanced peak $[Ca^{2+}]_i$ (lacZ, $1,357 \pm 66$ nM; capn-InsP₃R1 D2550a, $1,276 \pm 44$ nM; capn-InsP₃R1, $1,288 \pm 54$ nM).

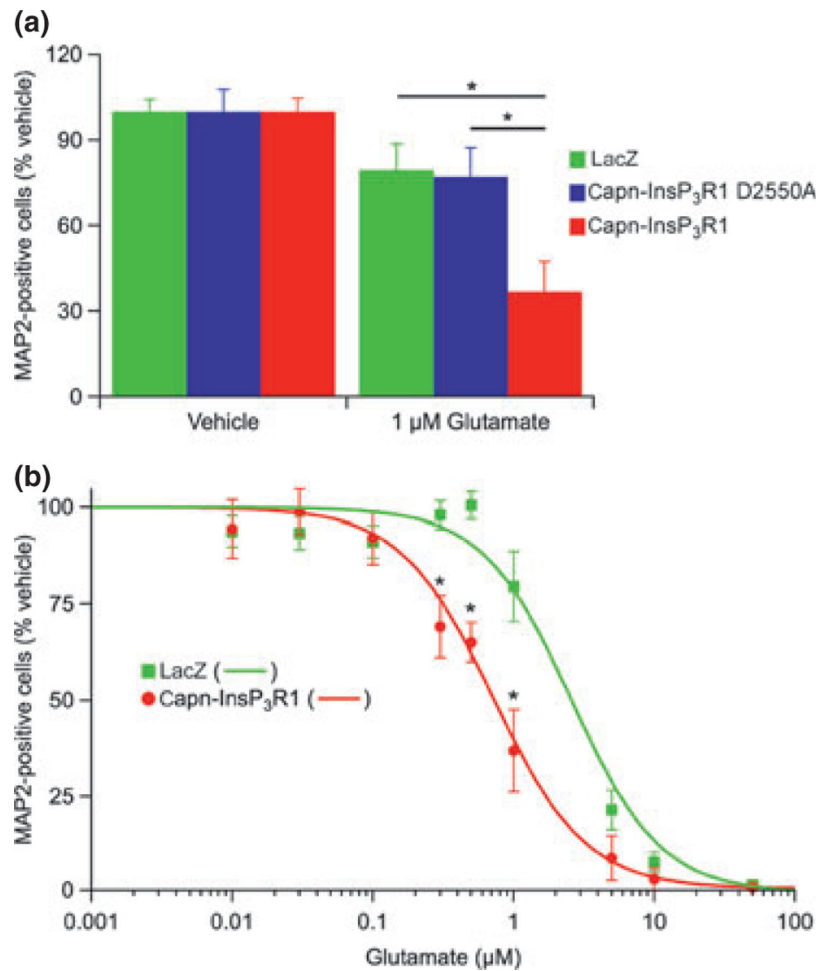


Fig. 5. Expression of capn-InsP₃R1 increases neuronal susceptibility to glutamate-mediated injury. (a) Transduced primary cortical cultures (14 DIV) were exposed to 1 μM glutamate or HEPES-buffered saline vehicle. Twenty-four hours later, cultures were stained for microtubule-associated protein 2 (MAP2) and percent neuronal survival quantified relative to vehicle-treated cultures. Expression of capn-InsP₃R1 resulted in a significant decrease in percentage of MAP2-positive cells following glutamate injury compared with lacZ and capn-InsP₃R1 D2550A (one-way ANOVA; **p* < 0.01). (b) Glutamate dose-response curves for lacZ and capn-InsP₃R1-transduced cultures (data at 1 μM glutamate are the same as shown in Fig. 5a). Expression of capn-InsP₃R1 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. LD₅₀ for glutamate in neurons expressing capn-InsP₃R1 ($0.75 \pm 0.19 \mu\text{M}$) is reduced compared with neurons expressing lacZ ($2.61 \pm 0.78 \mu\text{M}$).