Supporting Information

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SI Materials and Methods

Primary Neuronal Culture and Oxygen–Glucose Deprivation. Cortical neurons were dissociated, suspended in Neurobasal medium supplemented with B27, and plated in six-well dishes at 2×10^5 cells/cm^2 for immunoblotting and activity assays, 1.6×10^5 cells/cm^2 for cell death assays and protein synthesis assays, and 8×10^4 cells/cm^2 for immunocytochemistry. Experiments were performed on day in vitro (DIV) 11–12 to ensure development of a mature neuronal phenotype with expression of glutamate receptors (1) and where cultures consisted of >97% neurons determined by neuron- and glial-specific immunocytochemistry (2).

Oxygen–glucose deprivation (OGD) was used as an in vitro model of ischemia. Briefly, maintenance medium was replaced with medium lacking glucose and other components known to be substrates for glycolysis; culture dishes were placed in an airtight chamber (Billups-Rothenberg) and flushed with 100% argon gas for 3 min; and neurons were incubated at 37 °C for 0.5, 1.0, or 1.5 h. Following OGD, medium was replaced and incubated (reperfused) for the times indicated (medium was not replaced for the "0" time point). Control cultures had their medium changed with maintenance medium the same number of times as OGD-treated cultures and were incubated at 37 °C in humidified 95% air and 5% CO_2 for equivalent periods.

Alamar Blue, Lactate Dehydrogenase (LDH), and Hoechst Staining. Neuronal viability was assessed with Alamar Blue (TREK Diagnostic Systems). Alamar Blue is a blue nonfluorescent dye that fluoresces red when reduced by cellular metabolic activity. The amount of fluorescence is proportional to the number of viable cells (3). Medium was replaced with medium containing Alamar Blue reagent diluted at 1:30 in Neurobasal medium without B27 supplement. Neurons were incubated at 37 °C in humidified 95% air and 5% CO_2 for 30 min. Fluorescence was read with a Millipore CytoFluor 2300 automated plate-reading fluorometer set at excitation of 530 nm and emission of 590 nm.

LDH release is an indicator of cell membrane integrity and correlates with the number of dead cells induced by excitotoxic stimuli (4). Aliquots of 100 μL were taken from culture wells and were added to 150 μL of LDH reagent (Sigma-Aldrich). Absorbance of the reaction was measured at 340 nm over a period of 5 min. The slope of the absorbance versus time correlates with the amount of cell death. Data are presented as a percentage of the level of LDH release in control neurons.

Nuclear staining with Hoechst 33258 was used to confirm the results of the Alamar Blue and LDH assays. Neurons were fixed with 4% paraformaldehyde for 10 min, washed six times with PBS, stained with Hoechst for 15 min, and washed three more times in PBS (5). Cells with normal and with condensed and fragmented nuclei were counted and considered viable and nonviable, respectively. At least 3,000 cells were counted under each condition (three fields per condition, four to six wells per condition, and three independent experiments). Results are presented as the percentage of viable cells (set as 100%) relative to experimental control.

Protein Synthesis Measurement in Vitro. Culture medium was replaced with methionine-free medium (Invitrogen) supplemented with 10 μCi [^{35}S]methionine (MP Biomedicals), and cultures were incubated at 37 °C for 15 min. This incubation time ensured assessment of protein synthesis initiation without appreciable protein degradation. Pulse-labeling was stopped by washing in ice-cold PBS three times. Neurons were lysed in 250 μL of 1 M NaOH/2% H_2O_2 and incubated at 37 °C for 10 min. Aliquots were taken from lysed samples to measure protein content. Proteins in lysates were precipitated with 750 μL of 20% TCA, and samples were incubated on ice for 30 min. Pellets were then collected by vacuum filtration on Whatman GF/A glass fiber filters, washed three times with 1 mL 5% TCA and once with 1 mL acetone, and allowed to dry. Incorporation of [^{35}S]methionine was measured by scintillation counting. Incorporation was measured as cpm per microgram protein, and data were expressed as mean percentage of control ± SD.

Western Blot Analysis. Western blot was performed by gel electrophoresis of cell lysates on SDS/PAGE gels, and proteins were detected using enhanced chemiluminescence reagents (GE Healthcare). The following antibodies were used: goat polyclonal antibodies against eIF4G1 N terminus (N-20) and C terminus (D-20) (1:400) (both antibodies recognized both rat and human forms), eIF4A (1:400), and β-actin (1:10,000), purchased from Santa Cruz Biotechnology; rabbit polyclonal antibodies against PABP (1:1,000), p97 (1:1,000), eIF4B (1:1,000), p70$^{S6\ kinase}$ (1:1,000), eIF2α (1:1,000), S6 (1:1,000), 4E (1:1,000), and 4EBP (1:1,000), and rabbit monoclonal antibodies for P-p70$^{S6\ kinase}$ (1:1,000) and P-eIF2α (1:1,000) and a mouse monoclonal antibody against HA (1:1,000), purchased from Cell Signaling; a mouse monoclonal antibody against α-spectrin (1:1,000) purchased from BIOMOL that recognized full length (280 kDa), the calpain cleavage product (145 kDa), caspase cleavage product (120 kDa), and the common 150-kDa cleavage product; a rabbit polyclonal antibody for eIF3 (p110) with a 1:15,000 dilution purchased from Bethyl Laboratories; and a rabbit polyclonal antibody for eIF4G2 (1:400) produced as described previously (6). The secondary antibodies recognizing the goat, rabbit, and mouse were diluted 1:2,000 and were all purchased from Santa Cruz Biotechnology. Immunoreactivity was semiquantified using the MCID gel densitometric-scanning program.

Immunocytochemistry. Triple-label staining was performed to examine colocalization of HA tag with the neuronal marker MAP2, along with Hoechst stain in transfected primary neurons. Twenty-four hours after OGD, neuron viability was assessed by Alamar Blue fluorescence and LDH in the medium. The cultures were then fixed with 4% paraformaldehyde for 10 min, washed six times with PBS, and then blocked for 1 h at 4 °C in 2% BSA with 0.5% Triton X-100 for cell permeabilization. The cells were incubated overnight at 4 °C with rabbit anti-MAP2 antibody (1:1,000) (Santa Cruz Biotechnology) and mouse anti-HA (1:1,000) (Cell Signaling) antibody diluted in blocking solution. After incubation, cells were washed six times and incubated for 1 h at 4 °C with Alexa 488-conjugated goat anti-rabbit antibody and Cy3-conjugated rabbit anti-mouse antibody (Jackson ImmunoResearch) diluted 1: 2,000. After another three consecutive washes with PBS, cells were stained with Hoechst 33258 to visualize changes in nuclear morphology. Finally, cells were observed and imaged with an inverted fluorescent microscope and a CCD camera. Digital images were captured with the Metavue program (MDS Analytical Technologies).

Caspase and Calpain Activity Assays. Caspase and Calpain activity assays were performed as previously described (7, 8). Briefly, neurons were collected from six-well plates, centrifuged, and lysed by adding 0.15 mL lysis buffer (Cell Signaling). The samples were sonicated and then centrifuged at 16,000 × *g* for 10 min at 4 °C. After centrifugation, protein concentrations of the su-

pernatants were determined using the Bio-Rad Protein Assay Kit. For caspase activity measurement samples containing 100 μg protein were mixed with 20 μM of the fluorogenic substrate Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethyl-coumarin (Chemicon International), followed by a 2-h incubation at 37 °C. The changes in fluorescence were quantified every 20 min, using a luminescence spectrometer (Winlab; Perkin-Elmer) (excitation, 400 nm; emission, 505 nm). Results are presented as percentage fluorescence of experimental groups relative to control read at 2 h incubation.

Calpain activity assay was performed as previously described (7), and neurons were collected as described in the caspase activity assay. In brief, 100 μg of protein was incubated with calpain reaction buffer [20 mM Hepes, pH7.6, 1 mM EDTA, 50 mM NaCl, and 0.1% (vol/vol) 2-mercaptoethanol] containing 10 μM calpain I fluorescent substrate H-E(EDANS)PLF_AERK(DABCYL)-OH (Calbiochem). The reaction was initiated by addition of $CaCl_2$ to a final concentration of 5 μM, and the mixtures were incubated at 37 °C for 30 min. The activity of calpain was measured by detecting the increase in fluorescence using excitation/emission wavelengths of 335/500 nm. Calpain activity as measured by relative fluorescence was presented as percentage fluorescence of experimental groups relative to control.

Caspase Inhibitor Application. The pan-caspase inhibitor Z-Val-Ala-Asp(OCH3)-fluoromethylketone (ZVAD-fmk) and the caspase-3/7 inhibitor *N*-benzyloxycarbonyl-Asp(Ome)-Glu(Ome)-Val-Asp-(Ome)-fluoro-methylketone (*z*-DEVD-fmk) (Calbiochem) were dissolved in DMSO to a stock concentration of 10 mM. The stock solutions were further diluted in maintenance and OGD culture medium to a final concentration of 100 μM as indicated in Fig. 2 *E* and *F*. Control cultures received equally diluted DMSO in PBS added to the culture medium. The medium with the drugs or the control DMSO medium was added 1 h before OGD, for the entire 1-h duration of OGD, and for 24 h after OGD.

Truncated eIF4G1 Recombinant Protein Preparation. A construct of pGex-eIF4G1(197-674)-His and pGex-eIF4G1(675-1129)-His eIF4G1 fragments containing a C-terminal His tag were generated by PCR using pcDNA3-HA-eIF4G1(1-1599) (9). GST-eIF4G1 (197-674)-His and GST-eIF4G1(675-1129)-His were expressed in *Escherichia coli* BL21(DE3) and purified by Ni-nitrilotriacetic acid agarose (Qiagen) followed by purification using glutathione Sepharose 4B (GE Healthcare) chromatography. The GST tag was excised by PreScission protease (GE Healthcare).

Cell-Free Proteolytic Cleavage of eIF4G1 by Calpain. The cell-free calpain cleavage assay was conducted as previously described (10, 11). Briefly, recombinant μ-calpain (Calbiochem) was incubated with either full-length or truncated forms of eIF4G1 in a calpain assay buffer (25 mM Tris·HCl, pH 7.3 and 150 mM NaCl). Calcium at a concentration of 1 mM was added as indicated in the experiments.

Incubation of calpain with eIF4G1 occurred either in the absence or in the presence of equal molar concentrations of calpastatin (Calbiochem) or 10 μM of the peptide calpain inhibitor ALLN (Calbiochem). Reactions were incubated for the given times at 25 °C and were stopped by addition of 10 mM EDTA. The calpain-treated samples were resolved on SDS/PAGE. The gels were stained with Coomassie blue, and bands were excised for MALDI-TOF mass spectrometry. In other experiments, the gels were transferred to PVDF membranes and probed for the indicated antibodies.

Lentiviral Vector Construction and Production. Lentiviral vectors overexpressing human full-length HA-eIF4G were constructed by inserting the HA-eIF4G into the FUW transfer vector, which is under control of the ubiquitin promoter, using the restriction endonucleases HindIII and XhoI. A control vector with the EGFP ORF was also constructed and inserted into the FUW transfer vector as previously described (12). The constructed transfer vectors were transformed into JM109 cells (Promega) and isolated using the EndoFree Plasmid Maxi Kit (Qiagen). Large-scale production of virus was performed as previously described (13) with minor modifications. A mixture of plasmids containing 675 μg FUW transfer vector, 435 μg pCMVΔR8.9 (packaging construct), and 237 μg pVSVG (envelope plasmid) was added to 24 mL Opti-MEM (Invitrogen). Lipofectamine 2000 (Invitrogen) solution (900 μL in 24 mL Opti-MEM) was also prepared, and the plasmid mixture and Lipofectamine 2000 solution were added and incubated at room temperature for 20 min. Six microliters of plasmid–Lipofectamine solution was added to 15-cm plates with a 70% confluent monolayer of HEK293T cells. Cells were incubated for 4 h, and the medium was replaced with fresh culture medium. The supernatant was collected 72 h after transfection, filtered through a 0.45-μm filter flask, and centrifuged at 21,000 rpm for 1.5 h using the SW28 rotor (Beckman Coulter). Further purification of viruses was performed using sucrose gradient ultracentrifugation. The pellet was suspended in 3 mL Opti-MEM, loaded on top of 3 mL 20% sucrose solution, and centrifuged at 22,000 rpm for 1.5 h using the SW50.1 rotor (Beckman Coulter). The pellet was then resuspended in 200 μL Opti-MEM, aliquoted, and stored at −70 °C.

Transfection Protocol for Calpastatin and Lentiviral Vectors. The calpastatin plasmids or GFP control plasmids (Amaxa) were transfected immediately before plating. Following the rat neuron Nucleofector kit protocol, 4×10^6 cells were transfected with each reaction. Neurons were then plated at the densities described above.

Biological titer was determined for each batch of lentivirus made. A range of virus volume (1–20 μL) was added to individual wells on 24-well plates and incubated for 72 h. Transfection efficiency was assessed by immunolabeling the HA tag. Following immunocytochemical detection, the volume of virus suspension was scaled proportionally to infect the cells of a six-well plate, and transfection was assessed by Western blot. For eIF4G1 overexpression experiments, neurons at DIV 8 were infected with lentivirus. Experiments were then performed on DIV 11.

Lentiviral-shRNA Infection. Lentiviral shRNA directed at rat eIF4G1 (sc-155983-V) and a lentivirus containing a proprietary control (nontargeting) sequence of shRNA (sc-108080) was procured from Santa Cruz Biotechnology. Virus was stored in aliquots at 5×10^3 infectious units of virus/μL. Biological titer was performed to determine efficient knockdown in primary neurons as assessed by Western blot at indicated incubation times. For neuronal viability and protein synthesis measurements, neurons were transfected with 1.5 μL virus per well in six-well plates 72 h for before being subjected to OGD.

HeLa Cell Extract and Calpain Incubation. HeLa cells were grown to confluence in 15-cm plates in DMEM supplemented with 10% bovine serum (Invitrogen). The cells were then collected, lysis buffer (Cell Signaling) was added, and the pellet was sonicated. HeLa cell supernatent (100 μg protein), deionized H_2O, 0.2 μM calpain 1 (Calbiochem), and 2.5 mM $CaCl_2$ were added to a total volume of 80 μL. Calpain was the last component added and the timer was then started. Extracts were incubated at 37 °C, and loading buffer containing 10 mM EDTA was added at 5 s, 10 s, 30 s, 1 min, 2 min, 5 min, 10 min, and 30 min to stop the reaction. Western blot analysis was performed and membranes were probed for eIF4G1, eIF4G2, and p97.

Luciferase Vectors, in Vitro Transcription, mRNA Transfection, and Luciferase Assays. The β-globin and encephalomyocarditis virus (EMCV) reporter plasmids were prepared by cloning their 5′-UTRs into pSVN luciferase vector as previously described (14, 15). The cloning cassettes consisted of the T7 promoter, the various experimental 5′-UTRs, the *Renilla* or *Firefly* luciferace ORF, and a 50-mer poly(A) tail.

For in vitro transcription, the β-globin reporter plasmid was linearized with BamHI and the EMCV reporter was linearized with ClaI and transcribed with T7 RNA polymerase (Ambion) to produce mRNAs. Reactions were performed in the presence of either the 7-methyl-guanosine cap (7mG) (β-globin) or the cap analog G(ppp)A (EMCV) to create capped and uncapped mRNA. Enzymatic cleanup was performed using an RNeasy kit (Qiagen), mRNA concentration was determined, and each mRNA was inspected on an agarose gel to determine correct size and screen for possible degradation.

Transfection of primary neurons was performed using 20 μL DMRIE-C reagent (Invitrogen) per well on 24-well plates. A blank control with no transfection reagent and a well with DMRIE-C reagent only (no mRNA) were used for each experimental condition. Immediately before transfection, DMRIE-C reagent was mixed with culture medium and 1 μg mRNA. Culture medium was aspirated from neurons, and medium containing transfection mix was added. All conditions were transfected with the experimental *Renilla*-containing ORF and an EMCV *Firefly* luciferase-containing mRNA to serve as an internal control. Neurons were exposed to 1 h OGD, and mRNA was transfected into neurons 1 h before the indicated reperfusion time and neurons were lysed 2 h later (total of 2 h presence of mRNA); i.e., for the 2-h reperfusion data point, β-globin mRNA was transfected at 1 h reperfusion and cells were lysed at 3 h reperfusion.

Following incubation, neurons were processed and luciferase assays were performed using the Dual Luciferase Reporter Assay system (Promega). *Renilla* and *Firefly* luciferase activities were measured according to the manufacturer's protocol (Promega) on an LMaxII luminometer (Molecular Devices). A minimum of three independent experiments consisting of three to six wells each were carried out for each mRNA. Standardized luminescence was determined for each experimental time point by dividing relative light units (RLU) obtained from the *Renilla* luciferase-containing mRNA by the RLU of the EMCV *Firefly* luciferase control. Data are presented as β-globin *Renilla* luciferase/EMCV *Firefly* luciferase.

In Vivo Ischemia Model. Global cerebral ischemia (GCI) was performed as previously described (16), using isoflurane-anesthetized male Sprague–Dawley rats weighing 300–350 g (Hilltop Sprague–Dawley). Physiologic parameters including blood pressure, blood gases, and glucose concentration were monitored and maintained within normal limits throughout the experiments. Rectal temperature was continuously monitored and kept at 37–37.5 °C in all animals, using a heating pad and a temperature-regulated heating lamp. Select animals had their brain temperature monitored (the first animal of each surgery day) using a 29-ga thermocouple implanted in the left striatum and kept at 36.4 ± 0.2 °C during ischemia and at 37–37.5 °C thereafter. This measurement was used to optimize the animal heating method, which was applied to all other animals that received rectal but not brain temperature monitoring during the same surgery day. Electroencephalograms were monitored in all animals to ensure isoelectricity within 10 s after carotid artery occlusion. A sham operation was performed in additional animals, using the same anesthesic and surgical exposure procedures except that the arteries were not occluded; these brains were used as nonischemic controls.

Determination of [^{14}C]Leucine Incorporation Rate in Rat Brain. The measurement of [^{14}C]leucine incorporation rate was based on a comprehensive kinetic model (17) and performed using the quantitative autoradiographic method as described previously (18, 19). Briefly, L-[^{14}C]leucine (specific activity >300 mCi/mmol; PerkinElmer) was infused i.v. (15 μCi/animal in 500 μL of normal saline) with an automatic infusion pump over a 32-min period before animals were killed at 24 h and 8 wk after GCI or sham. Timed arterial samples were taken throughout the period of tracer infusion. The blood samples were centrifuged immediately. The plasma was deproteinized by the addition of 10% trichloroacetic acid (TCA) for further processing. At the end of the tracer infusion, the animals were killed and transcardially perfused, and the brains were rapidly removed and frozen for cryostat sectioning. Tissue samples were taken from various brain regions and sonicated in 10% TCA for biochemical analysis. The plasma and brain tissue samples were then subjected to HPLC and fluorometric detection, as previously described (20), to determine the specific radioactivity of [^{14}C]leucine in plasma and brain tissue and the concentration of free leucine in plasma.

For quantitative autoradiography, the cryostat sections were processed according to a previously described protocol (21) and exposed to film for 14 d. Autoradiographic images were analyzed densitometrically. The local tissue concentrations of ^{14}C-labeled protein were then determined depending on the tissue radioactivity, which was quantified using the image optical density/radioactivity relationship obtained with ^{14}C polymer standards. Rates of leucine incorporation into protein were calculated from local tissue concentrations of ^{14}C-labeled protein, the radioactivity of [^{14}C] leucine, the concentration of free leucine in plasma, and λ, the ratio of labeled to unlabeled precursor pool distribution space in tissue, using the operational equation of Smith et al. (18). The value of λ used in this paper was estimated from the ratio of tissue to plasma [^{14}C]leucine specific activity as described previously (19). Data are presented in Table S1.

MDL 28170 Administration in Vivo. The calpain inhibitor MDL 28170 (Enzo Life Sciences) was dissolved in DMSO/polyethylene glycol 300 (1:8). For the short-term [^{14}C]leucine incorporation study (24 h after GCI), a 30 mg/kg bolus of MDL 28170 was administered via the tail vein at the onset of post-GCI reperfusion, followed by a 6-h continuous infusion at 5.0 $mg{\cdot}kg^{-1}{\cdot}h^{-1}$ delivered by an external pump in unanesthetized rats (22). For histology studies (4 d or 8 wk after GCI) or long-term [^{14}C] leucine incorporation study (4 d or 8 wk after GCI), i.p. injections of MDL 28170 (30 mg/kg) were given to rats at 24, 48, and 72 h, and at 4 and 8 wk after GCI.

Cresyl Violet Staining and Hippocampal Cell Counts. At 4 d or 8 wk after GCI, animals were killed and transcardially perfused, and coronal sections (40 μm thick) were cut throughout the dorsal hippocampal formation at the coronal levels between –2.5 and –4.5 mm from bregma. Every fourth section was stained with cresyl violet. Viable CA1 neurons were quantified using stereology and the Bioquant Image Analysis program as described previously (12). The Bioquant software is interfaced with a stage encoder to determine *x*-, *y*-, and *z*-axis movement of the microscope stage. The entire CA1 subfield from a given section was captured with a color CCD camera, and grid squares of 50 × 50 μm were generated over the region of interest. The optical dissector method requires that 100–150 cells be counted in a given structure to estimate the total number. The number of points needed to be assessed within the outlined area was empirically determined. A previous study (23) has shown that a 25 × 25-μm counting frame and a focus depth of 25 μm in a 40-μm section with 7.5-μm guard regions above and below should be used. The 25 × 25 × 25-μm box in which the neurons were counted was defined as a dissector. An average of nine dissectors per section

yields the desired number of counted neurons throughout the CA1 at the levels of the dorsal hippocampus. The number of dissectors in the CA1 of a given section was maintained from section to section. The rostrocaudal length of the CA1 volume measured was 2 mm, obtained by multiplying section thickness by the number of sections (50 sections). Every fourth section was stained and counted. The total number of neurons was calculated using the optical dissector, equal to the quotient of the total number of neurons counted and the product of the fractions for sampling section frequency (SSF) (fraction of sections counted), area section frequency (ASF) (sampling area/area between dissectors), and thickness section frequency (TSF) (dissector depth/section thickness), or $n = \Sigma$neurons counted $\times 1/\text{SSF} \times 1/\text{ASF} \times 1/\text{TSF}$. For our study, SSF = 1/4 sections, ASF = 25×25 μm/50×50 μm, and TSF = 25 μm/40 μm.

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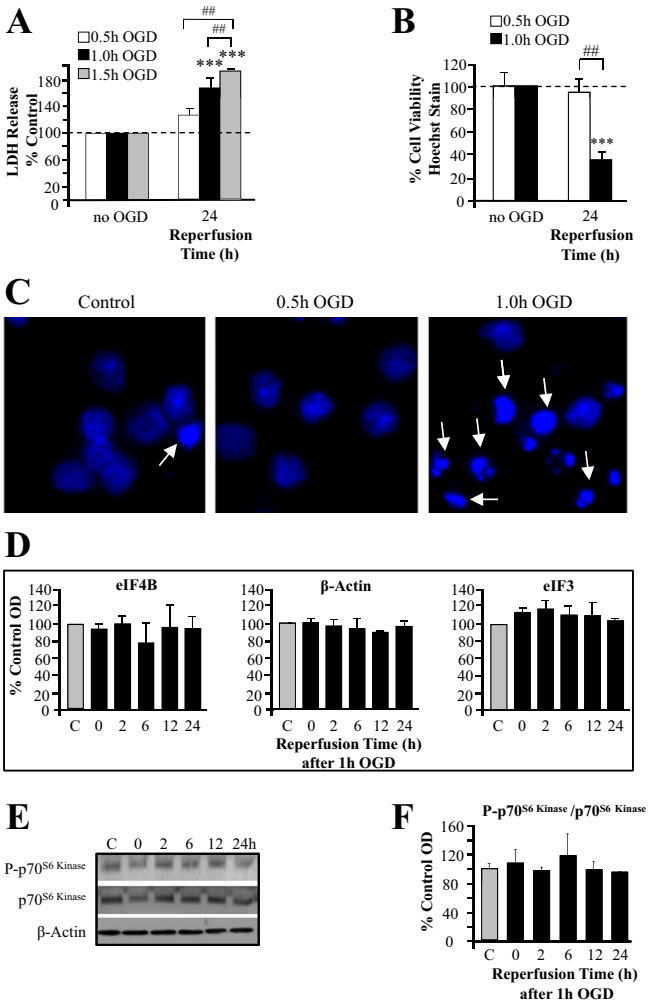

Fig. S1. Cell viability and translation initiation factor expression following OGD. (*A*–*C*) Cell death was measured following OGD by assay of LDH release (*A*) and of nuclear fragmentation using Hoechst stain (*B* and *C*). (*C*) Representative images of control neurons and neurons subjected to 0.5 and 1 h of OGD and stained with Hoechst 33258 after 24 h reperfusion. After 0.5 h OGD, the cells were little affected, but after 1 h OGD many nuclei were condensed or fragmented (indicated by arrows). (*D*) In Western blots optical densities of initiation factors eIF4B and eIF3 normalized to β-actin loading controls were little changed relative to control from immediately after 1 h OGD to 24 h later. (*E*) Western blot comparing total $p70^{S6\ Kinase}$ and $P\text{-}p70^{S6\ Kinase}$ following 1 h OGD and up to 24 h reperfusion. (*F*) Quantitation of *E*. There was little change in $P\text{-}p70^{S6\ Kinase}$ or total $p70^{S6\ Kinase}$ at any tested time after OGD. Bars represent mean ± SD values of n = 3–4 independent experiments. ANOVA with Fisher's PLSD was conducted to determine statistical significance: $^{***}P < 0.001$, OGD vs. control; $^{\#\#}P < 01$, intra-OGD comparison. Abbreviations: LDH, lactate dehydrogenase; OD, optical density; eIF4B, eukaryotic (translation) initiation factor 4B; eIF3, eukaryotic (translation) initiation factor 3.

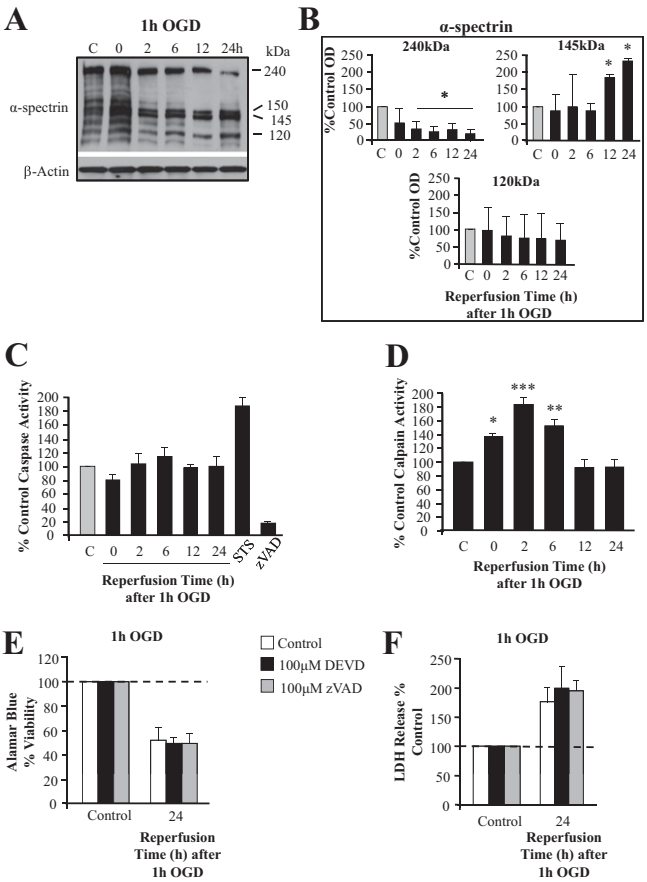

Fig. S2. Evaluation of caspase and calpain activation following OGD. (*A* and *B*) Primary neurons were subjected to 1 h OGD, lysates were taken at the indicated reperfusion times, and Western analysis was performed using an antibody directed against α-spectrin with β-actin loading controls. Immunoblots show decrease in full-length α-spectrin (240 kDa), increase in the calpain-generated145-kDa α-spectrin fragments, but no consistent change in the 120-kDa caspase-generated fragments. (*B*) Quantitation of *A*: optical density relative to control of α-spectrin fragments following OGD and reperfusion. (*C*) Caspase activity, assayed as described in *Materials and Methods*, remained near the control level from 0 to 24 h after 1 h OGD. Neurons were treated for 1 h with 200 nM staurosporine (STS) as a positive control or 200 nM STS and 100 μM zVAD, a caspase inhibitor, as a negative control. (*D*) Calpain activity was increased immediately after OGD and until at least 6 h reperfusion. (*E* and *F*) Primary neurons were treated with the indicated concentrations of the pan-caspase inhibitor zVAD and the caspase-3–specific inhibitor DEVD for 1 h before OGD and throughout 1 h OGD and 24 h reperfusion. Cell viability assessed by Alamar Blue fluorescence (*E*) and LDH release (*F*) were assessed after 1 h OGD and 24 h reperfusion was not affected by the caspase inhibitiors. Inhibitor concentration used was the highest that did not cause cell death. Bars represent mean ± SD of $n = 3–4$ independent experiments. ANOVA with Fisher's PLSD determined statistical significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Abbreviations: OD, optical density.

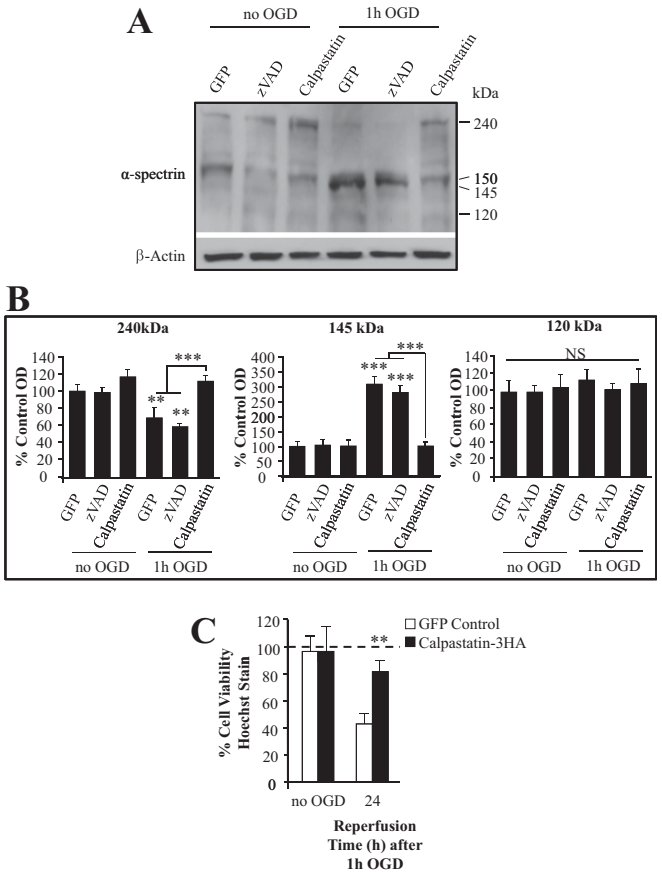

Fig. S3. Calpastatin transfection reduces α-spectrin cleavage and increases neuronal viability following OGD. (*A*) Calpastatin overexpression (by viral transduction 11 d earlier) reduced α-spectrin cleavage to the 145-kDa fragment induced after 1 h OGD and 24 h reperfusion. Neurons were transfected with either calpastatin or GFP plasmids. GFP-transfected cells were used for control or were treated with 100 μM zVAD, a caspase inhibitor, for 1 h before, during, and for 24 h after 1 h OGD. Calpastatin increased full-length α-spectrin and decreased formation of the 145-kDA calpain-mediated cleavage product after OGD compared with GFP-transfected control. There was no change in the level of the 120-kDa fragment. (*B*) Quantitation of *A*. (*C*) HA-tagged calpastatin transfection increased cell viability assayed by Hoechst staining after 1 h OGD and 24 h reperfusion. Bars represent mean ± SD of $n = 3–4$ independent experiments. ANOVA with Fisher's PLSD determined statistical significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Abbreviations: MAP2, microtubule-associated protein; OD, optical density.

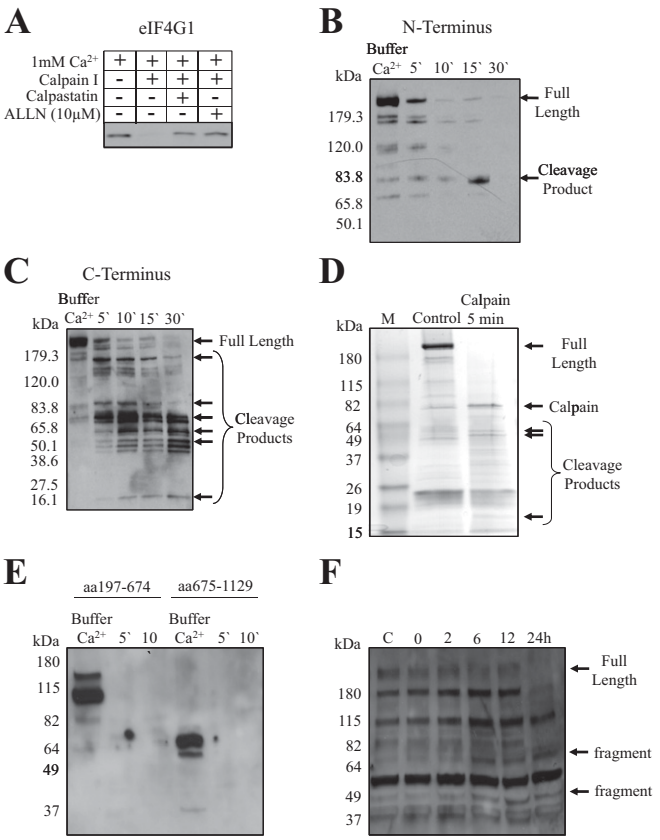

Fig. S4. Calpain cleaves eIF4G1 in a cell-free assay. (*A*) Recombinant eIF4G1 (400 ng) was incubated with 10 nM calpain I (50:1 molar ratio of eIF4G1:calpain) and activated by 1 mM Ca^{2+} at 25 °C for 1 h in a calpain reaction buffer. The reaction was stopped by the addition of 10 mM EDTA in an SDS loading buffer. The protein was then resolved on 4–15% gradient gels. Calpain greatly decreased the density of the eIF4G1 band (lane 2). Calpain cleavage of eIF4G1 was inhibited by either recombinant calpastatin or the pharmacological calpain inhibitor ALLN (lanes 3 and 4). Gels were probed with an N-terminal eIF4G1 antibody. (*B* and *C*) Reaction mixtures incubated for the indicated times (5–30 min) with the same amounts of recombinant eIF4G1 and calpain as in *A* were probed for eIF4G1 using antibodies directed against the N terminus (*B*) or the C terminus (*C*). *B* and *C* show bands of putative cleavage products of eIF4G1 produced by calpain I. (*D*) Recombinant eIF4G1 (10 μg) was incubated with Ca^{2+} (control) or Ca^{2+} and calpain for 5 min. Coomassie-blue–stained eIF4G1 cleavage by calpain I was analyzed by mass spectrometry. Three bands were positively identified as cleavage products of eIF4G1 with molecular weights of 60, 50, and 16 kDa (bracketed arrows). The large subunit of calpain is visualized at 80 kDa. (*E*) Recombinant truncated eIF4G1 proteins (100 ng) were assessed for susceptibility to calpain cleavage as above. Molecular weights of eIF4G1 amino acids 197–674 and amino acids 675–1129 are 105 kDa and 55 kDa, respectively. The immunoblot was probed for a C-terminal His tag to visualize recombinant proteins. Calpain activity generated no discernable cleavage products produced from these truncated proteins. (*F*) Lysates of primary neuronal cultures at various reperfusion times following 1 h OGD were probed with a C-terminal eIF4G1 antibody. The fragments found in neuronal culture at ~66 and 50 kDa correspond to the fragments observed in the cell-free assays and following focal ischemia in vivo (24). Blots are representative of n = 3–4 independent experiments. Abbreviations: M, molecular weight marker.

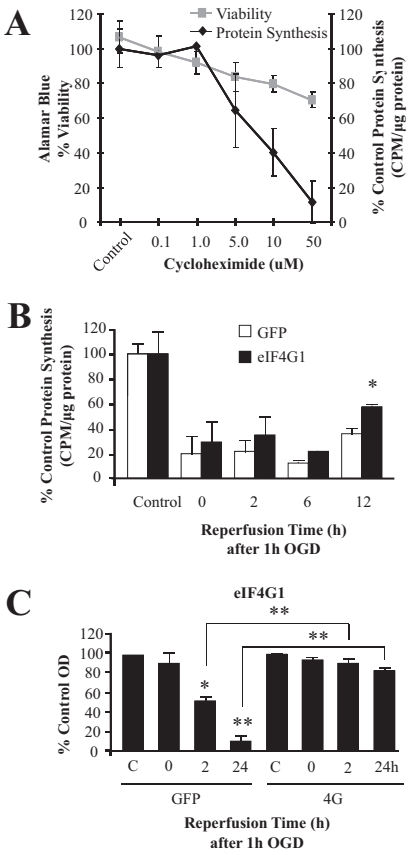

Fig. S5. Cycloheximide-induced PSI and cell death: protein synthesis time course and eIF4G1 levels following OGD. (*A*) Concentration effect on neuronal viability, measured by Alamar Blue fluorescence, and rate of protein synthesis by neurons exposed to different concentrations of cycloheximide (CHX) for 24 h. Viability and protein synthesis decreased with increasing concentrations of CHX. (*B*) Overexpression of eIF4G1 had little effect on protein synthesis relative to the GFP-transfected control at early reperfusion times after 1 h OGD, but moderately increased synthesis at 12 h. (*C*) Quantitation of Western blots for eIF4G1 as in Fig. 3*E*. In control, GFP transfected cells, the level of eIF4G1 after 1 h OGD decreased to 50% at 2 h reperfusion and to ~10% at 24 h reperfusion. Overexpression of eIF4G1 largely maintained eIF4G1 levels up to 24 h reperfusion. Bars in *A* represent mean ± SD of $n = 3–4$ independent experiments. ANOVA with Fisher's PLSD determined statistical significance: $*P < 0.05$ and $**P < 0.01$ versus control and where indicated.

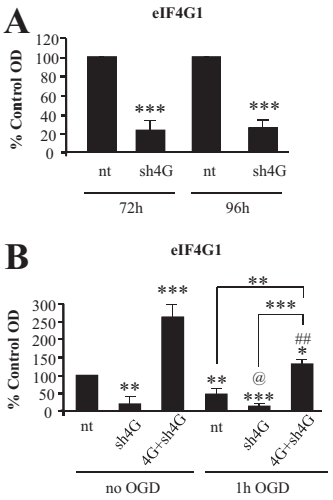

Fig. S6. shRNA-mediated knockdown of eIF4G1 and recovery of eIF4G1 levels with concomitant human eIF4G1 overexpression. (*A*) Infection with a lentivirus containing a shRNA sequence targeting rat eIF4G1 (sh4G) results in an ~80% decrease in expression of eIF4G1 compared with neurons infected with a lentivirus containing a control, nontargeting shRNA sequence (nt) at both 72 and 96 h after infection. (*B*) Knockdown of rat eIF4G1 resulted in decreased expression of the protein (sh4G) as seen in *A* whereas concomitant overexpression of human HA-eIF4G1 (4G+sh4G) resulted in an ~2.5-fold increase in expression compared with neurons infected with the lentivirus containing the nontargeting sequence (nt). Expression of eIF4G1 decreased to ~50% in neurons transfected with the nontargeting sequence (nt) following 1 h OGD and 24 h reperfusion (compare with Fig. 1*D*). Knockdown of eIF4G1 using shRNA resulted in further, but not significant decreases in eIF4G1 from 23% to 11% of control levels of eIF4G1 after 1 h OGD, whereas neurons transfected with eIF4G1 and the shRNA (4G+sh4G) maintained eIF4G1 levels above control values after 1 h OGD and 24 h reperfusion. Bars represent mean ± SD of $n = 3–4$ independent experiments. ANOVA with Fisher's PLSD determined statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control or as indicated. $^{@}P < 0.05$ versus nt and 1 h OGD. $^{\#\#}P < 0.01$ versus 4G+sh4G and no OGD.

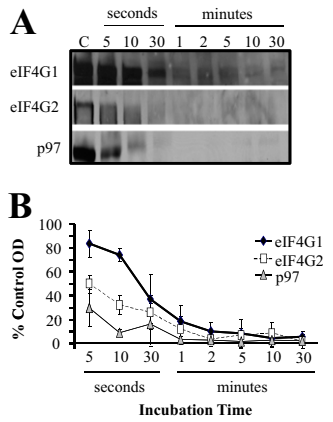

Fig. S7. Other eIF4G1 family members are susceptible to calpain. HeLa cell extracts were incubated with Ca^{2+} and 0.2 μM calpain for the times indicated. (*A*) Western blots probing for eIF4G1, eIF4G2, and p97 show rapid degradation of all three proteins. (*B*) Quantitation of data like those in *A*. Calpain cleavage of these proteins was virtually complete in <1 min . Blots are representative of $n = 3–4$ independent experiments.

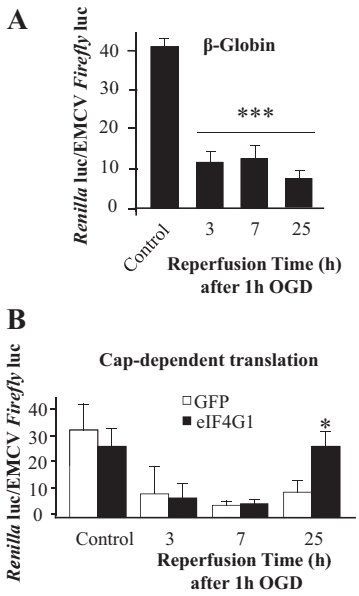

Fig. S8. Cap- and IRES-dependent translation following in vitro ischemia. (*A*) Plasmid DNA carrying β-globin and endomyocarditis virus (EMCV) 5′-UTRs upstream of the *Renilla* luciferase reporter ORF were linearized, and mRNA transcription was performed in the presence of the 7-methyl guanosine cap or the G(ppp)A cap analog to produce capped and uncapped mRNA corresponding to their in situ transcription (i.e., β-globin was capped and the EMCV 5′-UTR was incubated with the cap analog and was considered an uncapped transcript). In all conditions neurons were transfected with the β-globin–*Renilla* mRNA and an EMCV *Firefly* luciferase-containing mRNA to serve as an internal control. Neurons were subjected to 1 h OGD; transfected with mRNA after 1, 5, and 23 h reperfusion; and lysed 2 h later at 3, 7, and 25 h reperfusion, respectively. Luminescence was determined for each experimental time point as the ratio of relative light units (RLU) from the *Renilla* luciferase to the RLU from the EMCV *Firefly* luciferase. This ratio, a measure of cap-dependent translation, was greatly decreased at 3, 7, and 25 h reperfusion. (*B*) Neurons were transfected with GFP lentivirus or HA-eIF4G1 (4G) lentivirus for 72 h, subjected to 1 h OGD and transfected with β-globin mRNA during reperfusion as in *A*. Overexpression of eIF4G1 increased cap-dependent translation at 25 h reperfusion to initial levels, whereas translation in GFP-transfected controls remained low. Bars represent mean ± SD of $n = 3$–4 independent experiments. ANOVA with Fisher's PLSD determined statistical significance: $^{*}P < 0.05$; $^{***}P < 0.001$. Abbreviations: EMCV, encephalomyocarditis virus.

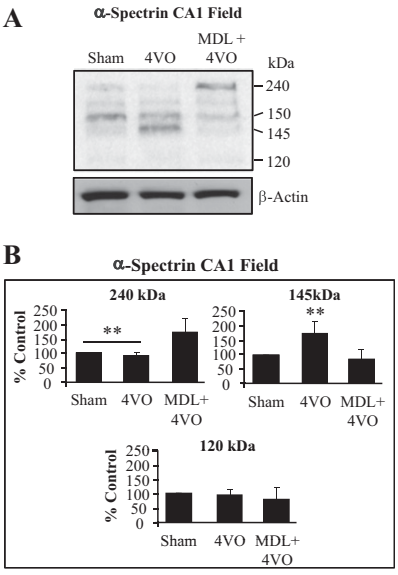

Fig. S9. Degradation of eIF4G1 induced by OGD in culture is induced by global ischemia in vivo. (*A*) α-Spectrin Western blot measuring calpain activity in CA1 neurons from rats 4 d after sham operation (left lane), after 12-min four-vessel occlusion (4VO, center lane), or after 4VO and treatment with the calpain inhibitor MDL 28170 (right lane; see *SI Materials and Methods* for details on drug administration). The calpain-specific 145-kDa band was present only in the 4VO lane and not in the sham or MDL + 4VO lanes. There was no 120-kDa band typical of caspase cleavage of α-spectrin. (*B*) Quantitation of data like those in *A*. Relative optical density of full-length, 240-kDa spectrin was greater in MDL+ 4VO-treated rats and density of the calpain cleavage product, a 145-kDa band, was greater in 4VO-only rats. There was no 120-kDa band typical of caspase cleavage of α-spectrin. Bars represent mean ± SD of $n = 6$–8 rats. ANOVA with Fisher's PLSD determined statistical significance: $^{**}P < 0.01$. Abbreviations: CA1/3, hippocampal cornu ammonis fields 1 and 3; OD, optical density.

Table S1. [^{14}C]Leucine incorporation rate after transient global cerebral ischemia with and without calpain inhibition

Brain ROI		Sham 1	24 h	Sham 2	8 wk
CA1	Vehicle	23.1 ± 1.8	2.4 ± 0.7**	18.4 ± 1.5	2.8 ± 1.9**
	MDL	22.5 ± 1.5	19.3 ± 2.3$^{\#\#}$	20.7 ± 1.8	22.0 ± 2.1$^{\#\#}$
CA3	Vehicle	21.8 ± 2.4	22.7 ± 2.5	17.1 ± 2.2	19.8 ± 2.8
	MDL	22.3 ± 1.3	22.9 ± 2.0	17.6 ± 2.3	18.3 ± 2.4
Cortex	Vehicle	25.5 ± 2.5	19.4 ± 2.4	22.3 ± 2.2	18.7 ± 2.6
	MDL	24.8 ± 2.2	20.6 ± 2.4	22.8 ± 2.0	20.2 ± 2.1

[^{14} C]Leucine incorporation rates in various brain regions of interest (ROI) are presented as nmol·g^{-1}·min^{-1} in sham-treated rats and in rats that received global ischemia induced by 12-min vessel occlusion followed by reperfusion for either 24 h or 8 wk. Sham 1 and Sham 2 are the sham-operated group compared with the groups that received global ischemia at 24 h and 8 wk, respectively. Vehicle was an equal volume and concentration of DMSO/polyethylene glycol compared with the MDL 28170 treated rats. Global ischemia induced pronounced depression of protein synthesis in CA1 at 24 h and 8 wk reperfusion. MDL, a calpain inhibitor, protected protein synthesis at both times. Cortex and CA3 did not show significant depression of protein synthesis. Data are mean ± SEM (n = 6–8/group). **$P < 0.01$ vs. sham group; $^{\#\#}P < 0.01$ vs. vehicle treatment group. Statistical analysis was performed using ANOVA and post hoc Student–Newman–Keul tests. Abbreviations: CA1 and -3, cornu ammonis 1 and 3; MDL, MDL 28170.