SUPPLEMENTARY INFORMATION

Supplemental Figures and Figure Legends

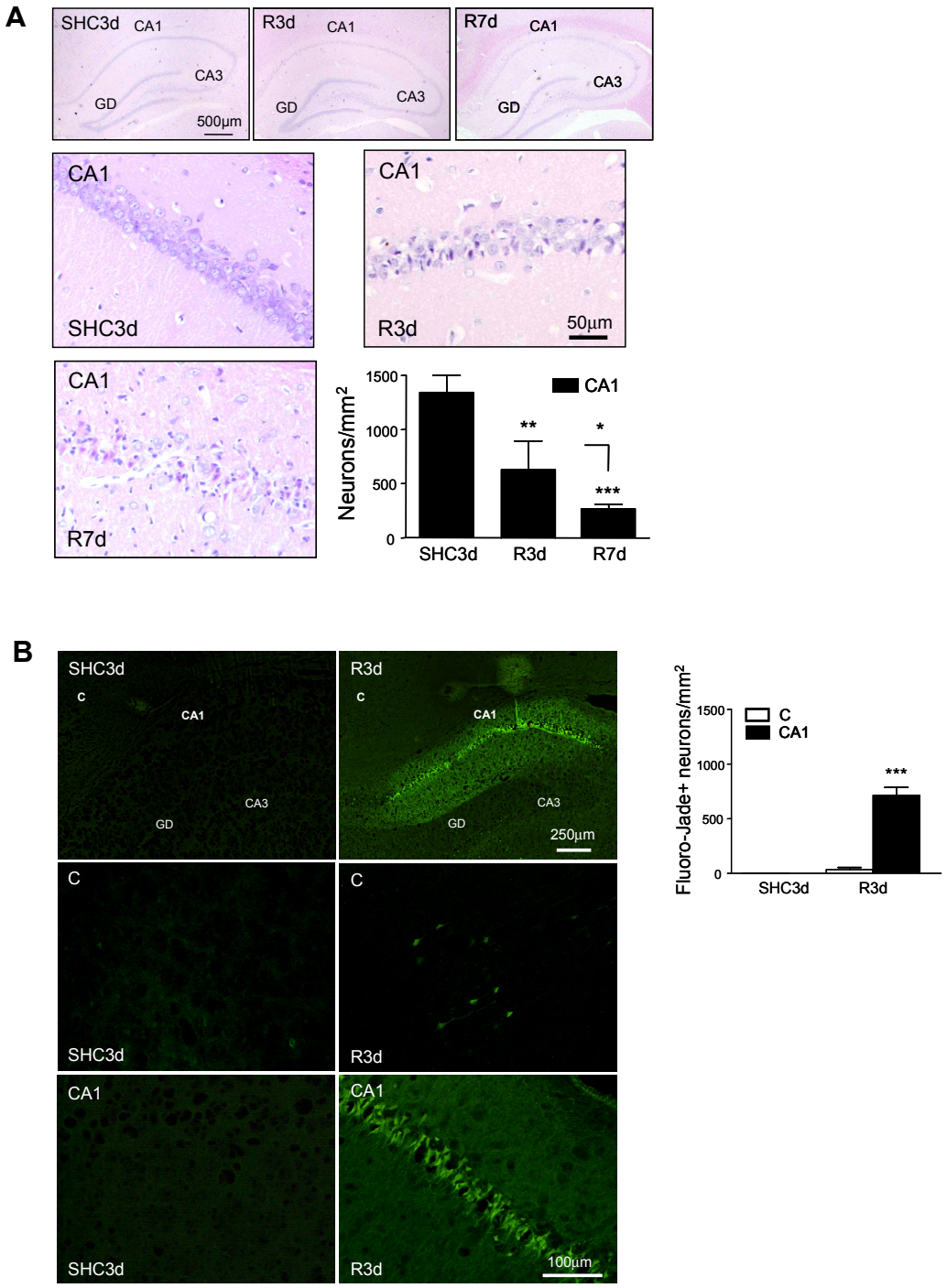

Fig. S1. (related to Figure 1) Analysis of neuronal viability after ischemia shows the delayed neuronal death induced during reperfusion in the hippocampal CA1 region. *A*, Brain paraffin sections of the hippocampal region from control (SHC3d) and ischemic animals with reperfusion for 3 or 7 days (R3d and R7d, respectively) were used after fixation to visualize neuronal viability by hematoxylin-eosin staining [1]. The small images show representative photomicrographs of neuronal viability in the hippocampal region (CA1 and

CA3, hippocampal *cornu ammonis* 1 and 3 region, respectively; GD, *gyrus dentatus*), while the large images are from the middle linear part of CA1. Bar graph: Stained neurons were counted in hippocampal CA1 fields as described in Supplemental Methods. Data are from three different animals run in duplicate. Error bars indicate SD. **$p < 0.01$ and ***$p < 0.001$, compared to SHC3d control; *$p < 0.05$, R3d compared to R7d, by Newman-Keuls' post test after analysis of variance ($p < 0.001$). *B*, Brain cryosections from control (SHC3d) and ischemic animals that underwent reperfusion for 3 days (R3d) were used after fixation to detect neuronal death by Fluoro Jade B staining [2] and visualized by fluorescence microscopy (in green). Upper images are representative photomicrographs of neuronal death in the hippocampal region; middle images are from the cerebral cortex (C); and lower images are from the middle linear part of the hippocampal CA1 region (CA1). Bar graph: Fluoro Jade-positive neurons were counted in C or CA1 fields as described in Supplemental Methods. Data are from four different animals run in duplicate. Error bars indicate SD. $p < 0.001$, compared to CA1 R3d by Newman-Keuls' post test after analysis of variance ($p < 0.0001$).

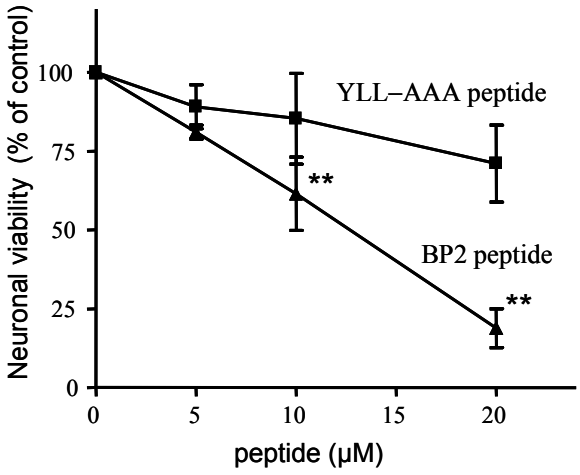

Fig. S2. (related to Figure 4) Induction of cell damage by 4E-BP2 peptide in neuronal cultures. Primary neuronal cultures (7 days *in vitro*) were prepared as described previously [3, 4] and exposed for 1 h to varying concentrations of peptides. The cells were then incubated for 1.5 h in 0.2 mg/ml MTT. Cells were lysed with 10 mM HCl and 10% SDS and quantified by absorbance (test 595 nm, reference 690 nm). Decreased MTT activity denotes impairment of mitochondrial function and is considered to be an index of cell damage. The neuronal viability corresponding to that of control cells (1.06 ± 0.10 AU) was considered to be 100%. The values represent the average of three independent experiments; error bars indicate SD. Squares, YLL-AAA peptide; triangles, BP2 peptide; **$p < 0.01$ compared to control by Newman-Keuls' post test after analysis of variance ($p < 0.001$). BP2 peptide induced a dose-dependent neuronal death.

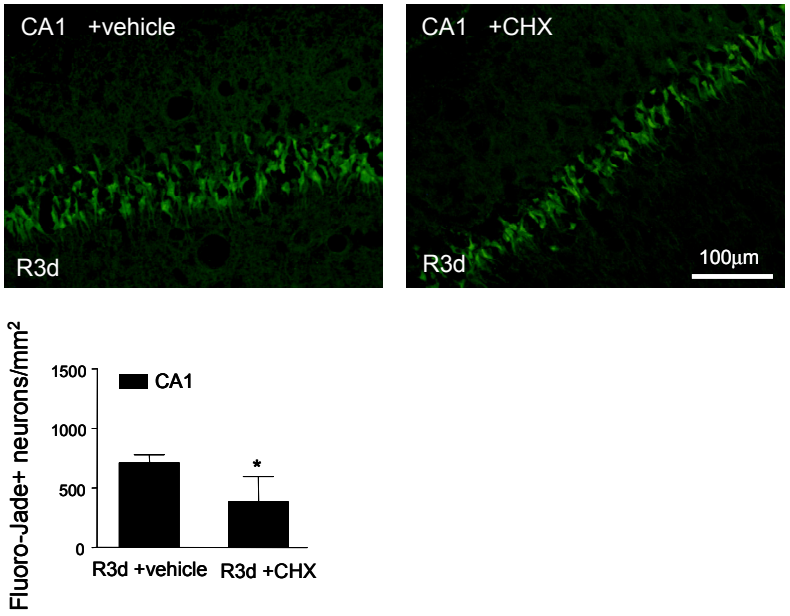

Fig. S3. (related to Figure 5) Cycloheximide prevents the ischemia-reperfusion-induced neuronal death in the hippocampal CA1 region. Brain cryosections of the hippocampal CA1 region (CA1) from untreated (vehicle) or treated animals [1 mg/kg cycloheximide (CHX) in vehicle] that underwent ischemia and 3 day-reperfusion (R3d +vehicle and R3d +CHX, respectively) were used to detect neuronal death by Fluoro Jade B staining [2] and visualized by fluorescence microscopy (in green). Figures shown are representative results. Bar graph: Fluoro Jade-positive neurons were counted in hippocampal CA1 fields as described in Supplemental Methods. Data are from four to five different animals run in duplicate. Error bars indicate SD. *$p < 0.05$, compared to R3d +vehicle (control).

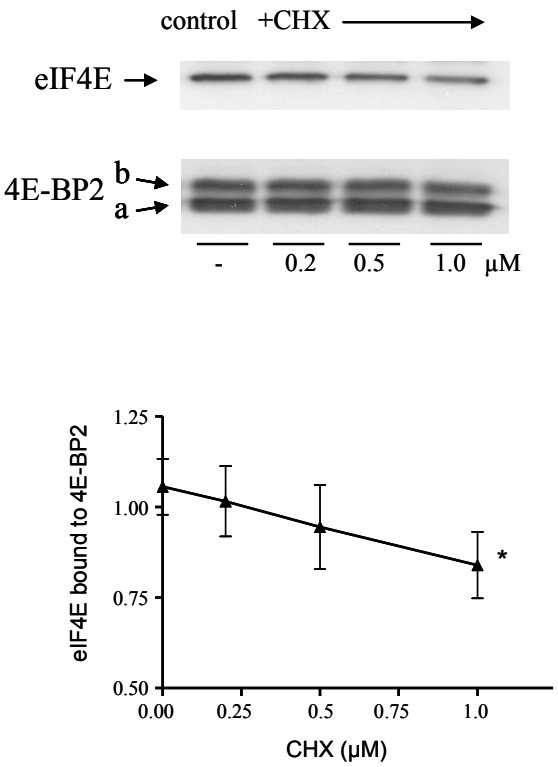

Fig. S4. (related to Figure 6) Cycloheximide interferes the association of 4E-BP2 with eIF4E *in vitro*. PMS samples (200 µg) from control animals were incubated for 10 min at 30 ºC in the presence of varying concentrations of CHX. Samples were then placed on ice and immunoprecipitated with anti-4E-BP2 antibody as described in [3], washed and centrifuged for 5 min at 2500 × *g* 3 times in buffer A. Immunoprecipitates were then analyzed by SDS-PAGE and western blotting for anti-eIF4E and anti-4E-BP2 antibodies. Arrows show the relative position for eIF4E and the *a* and *b* forms of 4E-BP2. Plot graph shows the quantification of eIF4E bound to 4E-BP2 immunoprecipitates in the presence of different CHX concentrations. Data are the quantification of eIF4E with respect to 4E-BP2 (*a* + *b* forms) levels (ratios). The values represent the average of three independent experiments; error bars indicate SD. *$p < 0.05$ compared to control by Newman-Keuls' post test after analysis of variance ($p < 0.05$). CHX induced dissociation between eIF4E and 4E-BP2 in a dose-dependent fashion.

Supplemental Methods

Hippocampal slices

Hippocampal slices were prepared from 20- to 23-day-old Sprague-Dawley rats. Animals were sacrificed by decapitation and the brain was rapidly removed and placed in cool and fresh Krebs-Henseleit-20 mM HEPES, pH 7.4 solution (KHH) containing 120 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 10 mM glucose (300-310 mOs/kg). Brain coronal sections, 1 mm or 200 μm thick, were prepared in KHH at the level of interaural +5.5 ± 0.5 mm by tissue chopper sectioning or vibratome, respectively, and the hippocampus was dissected under a magnifying glass. Sections containing the hippocampus were kept in KHH for 3 h at 37 °C in a 6.5% CO_2 atmosphere, prior to incubation with or without additives.

Western blot analysis

Samples of PMS (35 μg) or m^7GTP-Sepharose of each different experimental condition were analyzed by SDS-PAGE (7.5% or 12% acrylamide, 3% crosslinking), transferred onto PVDF membranes (GE Healthcare), incubated with the primary antibody against the specific protein, and after the blots were incubated with peroxidase-conjugated anti-mouse, -rabbit (both from GE Healthcare) or -goat (Santa Cruz Biotechnology) IgG, and developed with ECL reagent (GE Healthcare). The western blots were quantified using Quantity-One software (Bio-Rad). Internal standards (tubulin) were included to normalize the different immunoblots.

TUNEL assay and Hoechst nuclear staining

Apoptotic cells within brain sections were detected using the Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling (TUNEL) assay (Promega). Coronal cryotome brain sections containing the dorsal hippocampal formation were cut at 5 μm as described for the immunohistochemistry procedure (see above). For hippocampal slices, cryotome sections were cut at 10 μm thick by the anterior face. Sections were postfixed with 4% formaldehyde in PBS for 20 min and permeabilized with 20 μg/ml proteinase K in PBS for 10 min at room temperature, washed in PBS and a terminal deoxynucleotidyl transferase (TdT) incubation was carried out for 1.5 h at 37 °C with fluorescein-12-dUTP as described by the supplier. The reaction was terminated by extensive washing in PBS and deionized water at room temperature. A positive control was performed by nicking the nuclear DNA with DNase I as specified by the supplier. A negative control was achieved by excluding the TdT enzyme

from the reaction. The sections were then mounted with coverslips in anti-fade solution with glycerol-buffer containing p-phenylenediamine and 30 μM bisbenzimide (Hoechst 33342) for nuclear staining.

The hippocampal CA1 subfield from a given section was analyzed with fluorescence microscopy (40x objective) to count the number of apoptotic nuclei (green). Grid rectangles of 286 x 215 μm were drawn on the region of interest and digitized with a color CCD camera (1280 x 960 pixel resolution). The number of TUNEL-positive cells per image was counted by two independent observers with a total area of 1.236 mm^2 per section analyzed from the CA1 region. Four sections per brain sample were averaged per experiment and treatment information was kept concealed throughout the study.

Immunohistochemistry and confocal fluorescence microscopy

The animals were killed by transcardiac perfusion performed under deep anesthesia. Perfusion via the left ventricle was started with a washout of 200 ml of 0.9% NaCl and the brains, following perfusion and fixing with 4% (w/v) paraformaldehyde solution in PBS, were removed and postfixed in the same solution overnight at 4 °C. Brains were washed sequentially with 10, 20 and 30% (w/v) sucrose in PBS, embedded in Tissue-Tek O.C.T. (Sakura Finetek) and frozen at -80 °C prior to cryostat sectioning. Brain coronal sections (10 μm thick) were prepared at the level of interaural +5.7 ± 0.2 mm on Real Capillary Gap microscope slides (Dako). Those brain sections containing the hippocampus were postfixed with 4% paraformaldehyde in PBS for 5 min, washed in PBS and incubated in 10 mM sodium citrate (pH 6.0) at 95 °C for 3 min, cooled for 20 min and washed in PBS three times. Sections were incubated in a blocking solution (5% heat-inactivated goat serum, 0.1% Triton X-100 in PBS) for 1 h at room temperature and were subsequently incubated with primary antibodies overnight at 4 °C followed by specific fluorochrome-conjugated secondary antibodies for 1 h at room temperature added sequentially. Preparations were mounted with coverslips in anti-fade solution and examined using an MRC-1024 confocal laser scanning microscope (Bio-Rad) equipped with an IX70 inverted microscope and argon-krypton laser. The secondary antibodies used were Alexa 488-conjugated goat anti-rabbit IgG antibody and Alexa 568-conjugated goat anti-mouse IgG antibody (Molecular Probes). Samples were scanned under both 488 and 568 nm wavelength excitation controlled by Bio-Rad LaserSharp software (Bio-Rad) with all acquisition settings kept constant for all images. Data acquisition was performed sequentially in order to avoid cross-talk between the emission spectra of the

fluorochromes. Unrelated rabbit and mouse antibodies were used to test background staining. Image acquisition was performed more than five times and the most representative results are shown. The images were obtained using a 100x objective and colocalization was analyzed by thresholding the background using the LaserSharp software (Bio-Rad). The LaserSharp software provides quantitative analysis of the degree of colocalization of two fluorophores on a pixel-by-pixel basis. The degree of colocalization can be evaluated by the percentage of green objects co-localizing with red objects in the area of interest.

Supplemental References

1. Burda J, Hrehorovsk M, Bonilla LG, Danielisova V, Cizkov D, Burda R *et al.* Role of protein synthesis in the ischemic tolerance acquisition induced by transient forebrain ischemia in the rat. *Neurochem. Res.* 2003; 28**:** 1213-1219.
2. Burda J, Matiasov M, Gottlieb M, Danielisov V, Nemethov M, Garcia L *et al.* Evidence for a role of second pathophysiological stress in prevention of delayed neuronal death in the hippocampal CA1 region. *Neurochem. Res.* 2005; 30**:** 1397-1405.
3. Quevedo C, Salinas M, Alcazar A. Initiation factor 2B activity is regulatad by protein phosphatase 1, which is activated by the mitogen-activated protein kinase-dependent pathway in insulin-like growth factor 1-stimulated neuronal cells. *J. Biol. Chem.* 2003; 278**:** 16579-16586.
4. Ayuso MI, Hernandez-Jimenez M, Martin ME, Salinas M, Alcazar A. New hierarchical phosphorylation pathway of the translational represor eIF4E-binding protein 1 (4E-BP1) in ischemia-reperfusion stress. *J. Biol. Chem.* 2010; 285**:** 34355-34363.