Supporting Information

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SI Materials and Methods

Transient Global Cerebral Ischemia. Global cerebral ischemia (GCI) was performed on isoflurane-anesthetized male Sprague–Dawley rats weighing 300 to 350 g (Hilltop Sprague–Dawley) as previously described (1). Blood pressure, blood gases, and blood glucose concentration were monitored and maintained in the normal range throughout the experiments. Rectal temperature was continuously monitored and kept at 37 to 37.5 °C in all animals using a heating pad and a temperature-regulated heating lamp. Brain temperature was monitored in selected animals (the first animal of each surgery day) by a 29-ga thermocouple implanted in the left striatum and kept at 36.4 ± 0.2 °C during ischemia and at 37 to 37.5 °C thereafter. Brain temperature measurement was used to fine tune the animal heating method, and this optimized heating method was applied to all other animals that received rectal but not brain temperature monitoring in the same surgery day. An EEG was 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 anesthesia and surgical exposure procedures except that the arteries were not occluded; these brains were used as non-ischemic controls.

Drug Administration. PACAP38 (His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH2) was obtained from the American Peptide Company and dissolved in artificial cerebrospinal fluid. PACAP38 in artificial cerebrospinal fluid was infused into the rat brain through a pre-implanted 21-ga cannula in the left ventricle (from the bregma: anteroposterior, –0.8 mm; lateral, 1.5 mm; depth, 3.5 mm). For dose-response histology studies, each animal received repetitive ventricular infusions of the indicated doses (0.1, 0.2, 0.5, or 1.0 nmol in 5 μL) at 24, 12, and 6 h before ischemia and 0.5, 6, and 24 h after ischemia unless otherwise indicated (Fig. S2*A*). For time-window of efficacy studies, i.c.v. infusion of PACAP (0.2 nmol) was initiated at 24, 6, or 3 h before ischemia or at 0.5, 1, or 2 h after ischemia and repeated at each time indicated after the first injection (Fig. S2*B*).

Stereological Cell Counting of CA1 Neurons. At 4 or 60 days after GCI, 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 for cresyl violet, and the immediate adjacent section was stained for DNA fragmentation using Klenow-mediated DNA nick-end labeling (2). Briefly, brain sections were incubated in a moist air chamber at 37 °C for 1 h in a labeling mixture containing 10 μM each of dGTP, dCTP, and dTTP; biotinylated dATP; and 40 U/mL *Escherichia coli* Klenow fragment (GibcoBRL) in a 1 × reaction buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl). The reaction was terminated by washing the slides twice in PBS. After washing for 5 min in PBS/BSA (0.5 mg/mL), the slides were incubated with streptavidin/horseradish peroxidase (Vectastain Elite ABC; Vector Laboratories) in PBS/BSA for 1 h at room temperature. The biotin/streptavidin/peroxidase complex was detected by incubating the sections with 0.5 mg/mL diaminobenzidine in PBS (pH 7.4) and 0.05% H_2O_2. For brains obtained at 60 days after GCI, the majority of degenerated CA1 neurons had disappeared, probably because of phagocytosis; thus, DNA fragmentation was not evaluated in those animals.

Viable CA1 neurons (4 and 60 days after GCI) and neurons containing DNA damage (4 d after GCI) were quantified using stereology and the Bioquant Image Analysis program (Bioquant) as described previously (2). The Bioquant software is interfaced with a stage encoder to interpret *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 to 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. Previous study 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 at a constant ratio from section to section. The rostrocaudal length of the CA1 was 2 mm, obtained by multiplying section thickness by the number of sections (50 sections). Every fourth section was 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 $n = \sum$neurons counted × 1/SSF × 1/ASF × 1/TSF). For our study, SSF = 1/4 sections, ASF = 25 × 25 μM/50 × 50 μM, and TSF = 25 μM/40 μM.

In Vitro Model of Neuronal Ischemia. Primary cultures of hippocampal/cortical neurons were prepared from E17 Sprague-Dawley rat embryos and experiments were conducted at 10 to 12 days in vitro when cultures consisted primarily of neurons (>97%), as determined using cell phenotype-specific immunocytochemistry (2). To model ischemia-like conditions in vitro, primary cultures were exposed to transient oxygen and glucose deprivation (OGD) for 60 min as described previously (2, 3). Control glucose-containing cultures were incubated for the same periods of time in humidified 95% air and 5% CO_2.

Cell Death/Viability Assessment in Cultured Neurons. Fluorescence of Alamar blue (Accumed International), an indicator that changes from blue to red and fluoresces when reduced by cellular metabolic activity, was used to measure the viability of cultured neurons at 24 h after OGD (2, 3). One-half of the culture medium was replaced with MEM-Pak containing 10% (vol/vol) Alamar blue, and cultures were incubated for 1.5 h at 37 °C in humidified 95% air and 5% CO_2. Fluorescence was determined in a Millipore CytoFluor 2300 automated plate-reading fluorimeter, with excitation at 530 nm and emission at 590 nm. OGD-induced cell death was confirmed by measuring lactate dehydrogenase (LDH) release from damaged cells into the culture medium. In brief, 10-μl aliquots of medium taken from the cell culture wells were added to 200 μl of LDH reagent (Sigma). Using a spectrophotometer plate reader (Molecular Devices), the emission was measured at 340 nm, which is proportional to the amount of LDH in the medium. The percentage of cell death was calculated as described previously (2). In selective experiments, cell death was evaluated after OGD using Hoechst 33258 nuclear staining. The percentages of cells showing chromatin condensation were quantified by counting at least 3,000 cells under each experimental condition.

Construction of Lentivirus Vectors. To construct lentiviral vectors, each of the cDNAs [hemoagglutinin-tagged, including the human wild-type APE1, the repair-incompetent APE1 mutant, the wild-type cAMP response-element binding (CREB), and the dominant negative CREB mutant] was inserted into the lentiviral transfer vector FSW under the control of neuron-specific Synapsin I promoter. To construct lentiviral vectors expressing shRNA against rat APE1 or ATF-2, the gene-specific targeting sequence (APE1t: 5′-GCAAATCTGCCACACTCAAGA-3′ and 5′- GGATCTCAATGTGGCTCATGA-3′; ATF-2t: 5′-GGAAATACCATTGGCACAAAC-3′ and 5′-GCTATTCCTGCATCAATTACA-3′) or its counterpart scrambled sequence was inserted into the transfer vector FSW under the control of the U6 promoter. The constructed transfer vectors were transformed into S *E. coli*, and then isolated using the EndoFree Plasmid Maxi Kit (Qiagen). For large-scale production of the virus, a plasmid mixture containing 435 μg of pCMV R8.9 (packaging construct), 237 μg of pVSVG (envelope plasmid), and 675 μg of FSW (transfer vector) was suspended in 34.2 mL of $CaCl_2$ (250 mM) and then added volume to volume into 2× BES buffer (pH 6.95). The DNA-$CaCl_2$ precipitate was added into human kidney 293 FT cells (on 15-cm plates at the density of 1.1×10^7/plate) drop by drop (1.125 mL each plate) and allowed to incubate for 12 h before switching to fresh culture medium. The supernatant was collected 72 h after transfection, filtered through the 0.45-μm filter flask and centrifuged at 21,000 rpm for 2 h using the SW28 rotor (Beckman Coulter). Viruses were further purified by sucrose gradient ultracentrifugation. The pellet was suspended in 3 mL of PBS, loaded on top of 2 mL of 20% sucrose solution, and centrifuged at 22,000 rpm for 2 h using the SW50.1 rotor (Beckman Coulter). The resulting pellet was resuspended in 200 μL of DMEM, aliquoted, and stored at −70 °C. The titer of vector stock was determined using ELISA.

For lentivirus vector transfection in primary neurons, the neuronal cultures were transfected for 3 days with the APE1 knockdown shRNA-expressing vectors (APE1t and APE1s) or CREB-expressing vectors (wtCREB and dnCREB) at the range of 10 to 100 TU per cell. Protein expression was assessed by Western blot analysis.

Intracerebral Infusion of Lentivirus Vectors. Lentivirus vectors were infused into the CA1 sector of the dorsal hippocampus (two injection spots; coordinates: anteroposterior, –3.5 mm and lateral, 2.0/3.5 mm from bregma) using convection enhancement technology that was developed previously to enhance the intracerebral delivery of the adeno-associated virus 2 in monkeys and rodents (4). This technique allows a larger distribution volume of viral vector in the targeted brain region after single infusion of the vector (4). Briefly, male Sprague–Dawley rats weighing 250 to 275 g were anesthetized using isoflurane. The vectors were dissolved in physiologic saline (2×10^7 DNase-resistant particles/μL) and infused at the rate of 0.1 μL/min over 50 min via a custom-made convection-enhanced microinfusion system linked to a UMP2 microsyringe pump (World Precision Instruments). The animals were allowed to recover for up to 14 d to enable sufficient gene expression.

APE Promoter Activity Assay. APE1 promoter activity was assessed in neuronal cultures transfected with a luciferase reporter plasmid under the control of rat APE1 promoter. The APE1-LUC reporter was constructed by subcloning the rat APE1 promoter (−1,800 to +170) into pGL3-Basic (Promega). The mutant reporter APE1m-LUC was generated by introducing mutations to the essential CREB/ATF-binding site of the APE1-LUC reporter (wild-type: GCCGTGACGTATGTGCG; mutant: GTAGTGGTGTAGTTGCG) similar to that reported previously by others (5). The mutations were made using the QuikChange XL Site-Directed Mutagenesis kit (Stratagene). The integrity of the constructs and the incorporated mutations were confirmed by sequencing. For APE1-LUC reporter activity assay, hippocampal/cortical neurons cultured in 24-well plates were transfected with 2 μg of reporter DNA together with 100 ng of pRL-TK (Promega) using Lipofectamine 2000 (Invitrogen). Twenty-four hours later, cultures were treated with pituitary adenylate cyclase-activating polypeptide (PACAP) at the indicated concentrations for the indicated periods of time. Luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega) and a Turner TD-20e luminometer according to the manufacturers' instructions. Firefly outputs were normalized to *Renilla* outputs to control for transfection efficiency.

AP Endonuclease Activity Assay. To estimate the ability of nuclear protein extracts to remove apurinic/apyrimidinic (AP) sites in damaged DNA, the oligonucleotide incision assay was performed at 6 and 24 h after GCI, as previously described (6). The repair substrate used in this assay was a 50-mer oligonucleotide with a precisely positioned synthetic tetrahydrofuran (O) AP site at position 26 (5′-TCG GTA CCC GGG GAT CCT CTA GAG TOG ACC TGC AGG CAT GCA AGC TTG GC-3′; O = 8-oxodG or AP site). The oligonucleotide was 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP, and the reaction mixture was passed through a G-25 spin column to remove the free unlabeled [γ-^{32}P]ATP. The labeled oligonucleotide was annealed to the complementary oligonucleotide in 100 mM KCl, 10 mM Tris (pH 7.8), and 1 mM EDTA by heating the samples to 55 °C, and then allowing them to cool down slowly to room temperature. The reaction mixture for the incision assay contained 40 mM hepes-KOH (pH 7.6), 75 mM KCl, 2 mM DTT, 1 mM EDTA, 0.1 mg/mL BSA, 2 mM CaCl, 20 μM zinc acetate, 10% glycerol, 300 fmol of ^{32}P-labeled DNA duplex, and nuclear protein extracts in the indicated amounts. The reaction was incubated at 32 °C for 30 min, and then terminated. The DNA was ethanol-precipitated and then resuspended in formamide dye containing 90% formamide, 0.002% bromophenol blue, and 0.002% xylene cyanol. The samples were heated to 80 °C for 2 min and subjected to electrophoresis on a denaturing 20% polyacrylamide gel containing 7 M urea. The 25-bp incision products were analyzed by autoradiography and densitometry analysis.

Quantitative Measurement of AP Sites in Nuclear DNA. Nuclear DNA was isolated from freshly prepared hippocampal CA1 tissues at 3, 6, 24, and 48 h after GCI or 24 h after sham operation (n = 6–8 per time point). Quantitative measurement of AP sites was performed using the colorimetric assay previously described (6). A biotin-labeled reagent specific for the aldehyde group in the ring-open form of an AP site, designated as Aldehyde Reactive Probe (ARP), was used for the detection of AP sites (Dojindo Molecular Technologies). The purified DNA isolated from brain tissue (ratio of $OD260_{nm}/280_{nm} > 1.8$) was dissolved at a concentration of 100 μg/mL in TE, and 10 μL of DNA solution was incubated with the same volume of ARP solution (5 mM) at 37 °C for 1 h. The ARP-labeled DNA was then ethanol precipitated, and the DNA pellet was suspended in TE and its DNA concentration determined. ARP in the labeled DNA was measured using an ELISA-like assay in a microtiter plate, which was irradiated overnight with a 40W unfiltered lamp before the assay according to the manufacturer's instructions. The ARP-labeled DNA (30 ng in 60 μL) and 90 μL of DNA binding solution (Dojindo Molecular Technologies) were added to each well, and the plate was covered and incubated in the dark at 37 °C overnight. This was followed by incubation in 100 μL of ABC solution (Vector) at 37 °C for 1 h, and then in K-Blue substrate solution (Neogen Corporation) at room temperature for 1 h. The wells were then subjected to OD measurement at 650 nm. All ARP assays were performed in triplicate and the means were calculated. The data, expressed as the number of AP sites per 10^6 nucleotides,

were calculated based on the linear calibration curve generated for each experiment using ARP-DNA standard solutions.

Electrophoretic Mobility Shift Assay. Nuclear proteins were extracted from cultured neurons or hippocampal CA1 tissues using our previously published protocol (6). Protein content in nuclear fractions was measured using the Bradford method (Bio-Rad Bulletin 1177, Bio-Rad). EMSA were performed to determine APE CRE-specific DNA binding activity in nuclear extracts as described previously (7) with modifications. In brief, a 30-bp oligonucleotide was synthesized (GIBCO-BRL) according to the rat APE1 promoter sequence containing the CRE site (5′-TG CCCG GGGCCGTGACGTATGTGCGCCGCG-3′) and then annealed with its antisense sequence in an annealing buffer containing 10 mM Tris (pH 8.0), 100 mM NaCl, and 1 mM EDTA by heating at 90 °C for 5 min and then reducing the temperature to 25 °C over 30 min. The DNA-protein binding reaction was performed in a total volume of 30 μL containing the binding assay buffer (10 mM Tris-HCl, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM $MgCl_2$, 5% glycerol, pH 7.6), 0.0175 pmol of γ-^{32}P-labeled double-stranded probe (>10,000 cpm), 30 μg of nuclear protein, and 1 μg of poly(dI-dC). After incubation for 20 min at room temperature, the reaction mixture was subjected to electrophoresis for 2 h on a nondenaturing 4% polyacrylamide gel in 0.25× TBE (22.5 mM Tris borate and 0.5 mM EDTA). The gel was dried and subjected to autoradiography.

The specificity of APE1-CRE binding activity was determined by performing both competition assays and supershift assays. For the competition assays, a 50-fold molar excess of unlabeled double-stranded oligonucleotide containing the wild-type CRE or mutant CRE (5′-TGCCCGGGATAGTGGTGTGTGTGCGCCGCG-3′) was used as a specific competitor (5). For supershift assays, 5 μg of antibody against CREB (D76D11, rabbit monoclonal antibody, Cell Signaling Technology, Inc.), ATF-2 (20F1, rabbit monoclonal antibody, Cell Signaling Technology, Inc.), or pCREB (87G3, rabbit monoclonal antibody, Cell Signaling Technology, Inc.), was added 30 min before the addition of the oligonucleotide probe and incubated at room temperature.

Chromatin Immunoprecipitation Assay. To determine the effect of PACAP treatment on CREB or ATF2 binding to the APE1 promoter, ChIP was performed using a ChIP assay kit (Millipore) and nuclear extracts from cultured neurons treated with PACAP (0.1 μM) for 2, 4, or 6 h. Nuclear extracts were cross-linked with 1% formaldehyde for 20 min at room temperature. Samples (600 μg of protein) were subjected to immunoprecipitation with anti-CREB (D76D11, rabbit monoclonal antibody, Cell Signaling Technology, Inc.), anti-ATF-1 (C41-5.1, Santa Cruz Biotechnology, Inc.) or anti-ATF-2 (20F1, Cell Signaling Technology, Inc.) antibody. Following precipitation with protein A/G agarose beads, the DNA crosslinked to CREB or ATF protein was released by heating in 0.1 M $NaHCO_3$ for 16 h at 65°C and then purified using a Qiagen PCR clean-up kit. The DNA was subjected to conventional PCR or real time PCR using SYBY green PCR Master Mix (Applied Biosystems). The forward and reverse primers for rat APE1 CRE promoter region were 5′-CAGCAAAGAACCCTTGACAG-3′ and 5′-GAAGGTCCCTTCGCTTGGTTCTGG-3′, respectively.

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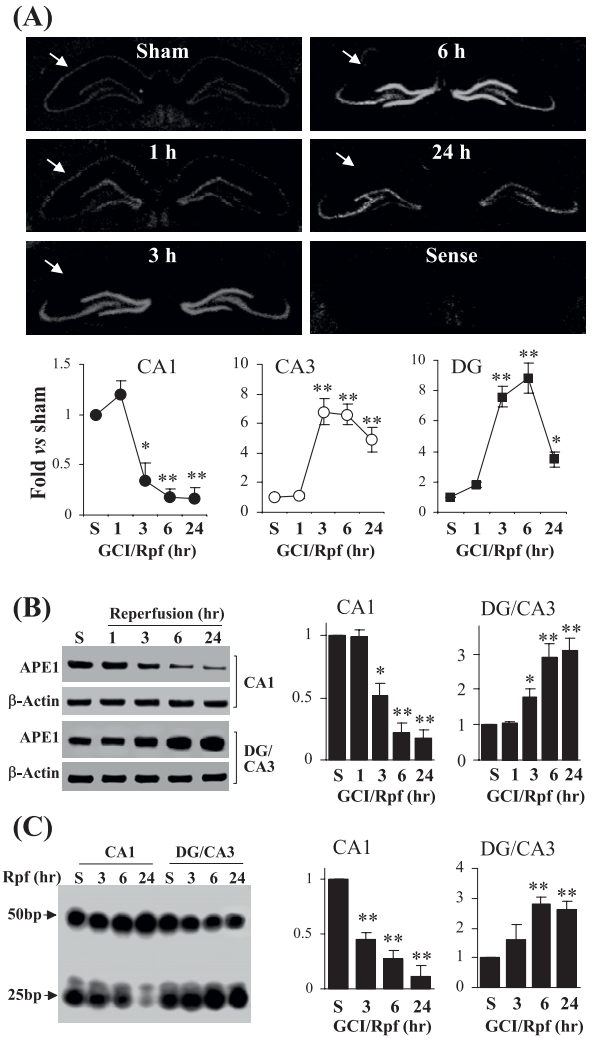

Fig. S1. Global cerebral ischemia (GCI) results in sustained down-regulation of APE1 expression and activity in CA1 neurons (arrows). (*A* and *B*) In situ hybridization (*A*) and Western blot (*B*) show decreased expression of APE1 mRNA and protein in CA1 3 to 24 h after GCI, whereas APE1 expression was markedly up-regulated in hippocampal regions that are resistant to GCI [i.e., CA3 and dentate gyrus (DG)]. Arrows indicate the CA1 subfield of the hippocampus. *, $P < 0.05$; **, $P < 0.01$ vs. sham controls, $n = 4$ per group. (*C*) AP endonuclease activity in hippocampal nuclear extracts (CA1 or DG/CA3) as a function of reperfusion duration, determined by optical density measurements of the 25-bp cleavage products on autoradiographs from three independent experiments. Data were normalized to sham-operated animals. *, $P < 0.05$; **, $P < 0.01$ vs. sham controls.

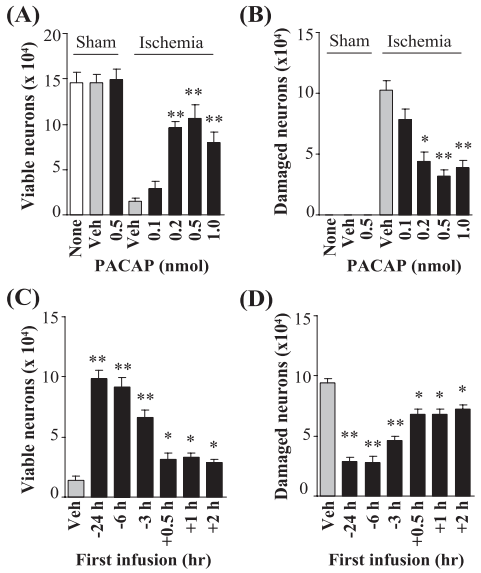

Fig. S2. Pituitary adenylate cyclase-activating polypeptide (PACAP) treatment suppresses DNA fragmentation and cell death in CA1 following GCI. The numbers of viable CA1 neurons (*A* and *C*) and CA1 neurons containing DNA fragmentation as determined using Klenow-mediated DNA nick-end labeling (*B* and *D*) were quantified using stereology 4 d after GCI. (*A* and *B*) Dose-response effects of PACAP treatment against CA1 neuronal death induced by GCI. Rats received repeated injections (at −24, −12, and −6 h) into left ventricle of either vehicle (5 μL) or PACAP at the indicated doses (in 5 μL of volume per injection), and then were subjected to GCI or sham operation. PACAP infusions were repeated at 0.5, 6, and 24 h after GCI. Animals were killed after 4 d. Near maximal protection was obtained with a dose of 0.2 nmol. (*C* and *D*) Therapeutic time window for PACAP protection against GCI. The selected dose of PACAP (0.2 nmol in 5 μL) was injected repetitively into the left ventricle, beginning at the indicated time points before (−24, −6, −3 h) and after (+0.5, +1, +2 h) GCI. For the −24-h treatment group, PACAP infusion was repeated at −12 and −6 h (before GCI) and +0.5, +6, and +24 h (after GCI). For the −6 and −3-h groups, injection was repeated at +0.5, +6, and +24 h (after GCI). For the +0.5, +1, and +2-h treatment groups, injection was repeated at +6 and +24 h (after GCI). *, $P < 0.05$; **, $P < 0.01$ vs. vehicle controls, $n = 8$ per group. Little protection was obtained when the first dose was 0.5 h after GCI.

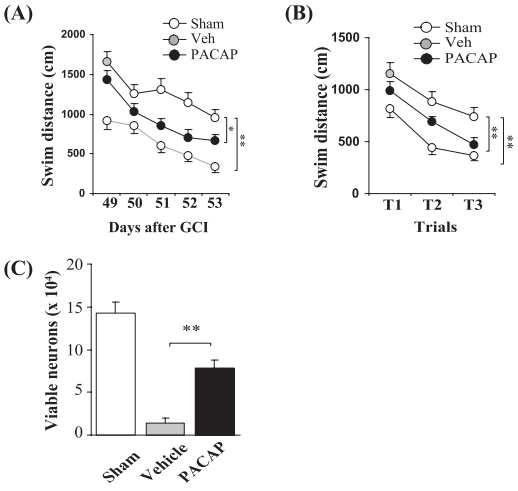

Fig. S3. PACAP treatment improves cognitive performance and long-term CA1 neuronal survival following GCI. (*A*) PACAP treatment (0.2 nmol × 6 times, beginning 24 h before GCI as for Fig. S2 *A* and *B*) significantly improved performance in the Morris water maze compared to vehicle-treated postischemic animals. The Morris water maze is a test for spatial memory [Hartman RE, Lee JM, Zipfel GJ, Wozniak DF (2005) Characterizing learning deficits and hippocampal neuron loss following transient global cerebral ischemia in rats. *Brain Res* 1043:48–56.]. The rats were tested for 5 consecutive days between days 49 and 53 following GCI or sham operation. *, $P < 0.05$; **, $P < 0.01$, $n = 9$ per group. (*B*) PACAP treatment significantly improved performance compared to vehicle-treated postischemic animals on the learning-set water maze, an index of short-term working memory. The rats were tested for the ability to learn a new platform location each day for 5 consecutive days between days 56 and 60 following GCI or sham operation. The rats were given three consecutive trials (T1, T2, and T3) each day, and the data are expressed as the averaged performance on each trial from all 5 days. **, $P < 0.01$, $n = 9$ per group. (C) The number of viable CA1 neurons in postischemic animals was significantly greater in PACAP-treated compared to vehicle-treated animals at 60 d following GCI. **, $P < 0.01$, $n = 9$ per group for vehicle and PACAP treatment, $n = 6$ for the sham control group.

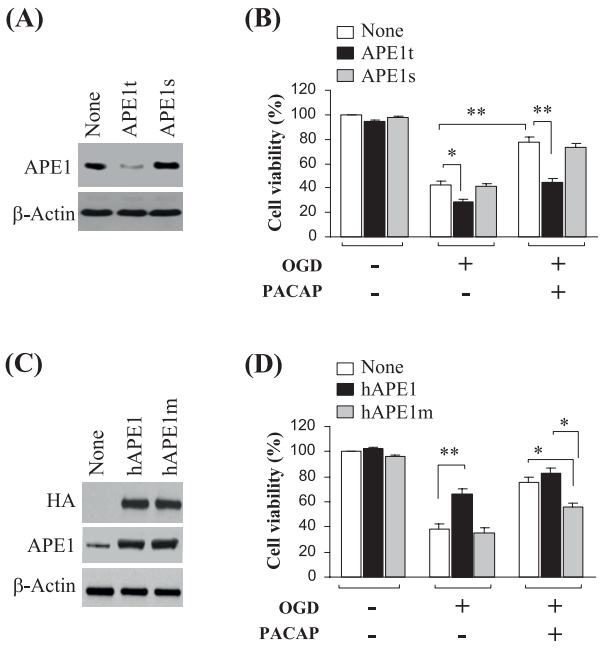

Fig. S4. Targeted knockdown of APE1 or overexpression of a mutant APE1 deficient in DNA-repair activity abrogates PACAP-mediated neuroprotection of cultured neurons against oxygen and glucose deprivation (OGD)-induced death, an in vitro model of neuronal ischemia. (*A*) Lentivirus-mediated transfection for 3 d of APE1-targeting shRNA (APE1t) but not a scrambled control (APE1s) diminished APE1 expression in cultured hippocampal/cortical neurons. (*B*) Targeted knockdown of APE1 expression significantly decreased cell survival 24 h following OGD (1 h) in cultures with or without PACAP treatment. Neurons were infected with lentivirus vectors 3 d before PACAP exposure (0.1 μM); OGD (1 h) was induced 4 h following PACAP treatment. *, $P < 0.05$; **, $P < 0.01$; data were from three independent experiments. (*C*) Lentivirus-mediated transfection for 3 d of human APE1 cDNA (either the wild-type hAPE1 or the repair-incompetent D210A mutant hAPE1m; both were tagged with hemagglutinin, HA) resulted in increased APE1 expression in cultured neurons. Western blots were performed using antibodies against the HA tag and APE1, respectively. Some endogenous expression of APE1 (*First lane*) was detected. (*D*) Overexpression of hAPE1 partially protected against OGD-induced cell death, whereas overexpression of hAPE1m partially blocked neuroprotection afforded by PACAP following OGD. However, hAPE1m did not increase the effect of OGD on untreated cultures. *, $P < 0.05$; **, $P < 0.01$; data were from three independent experiments.

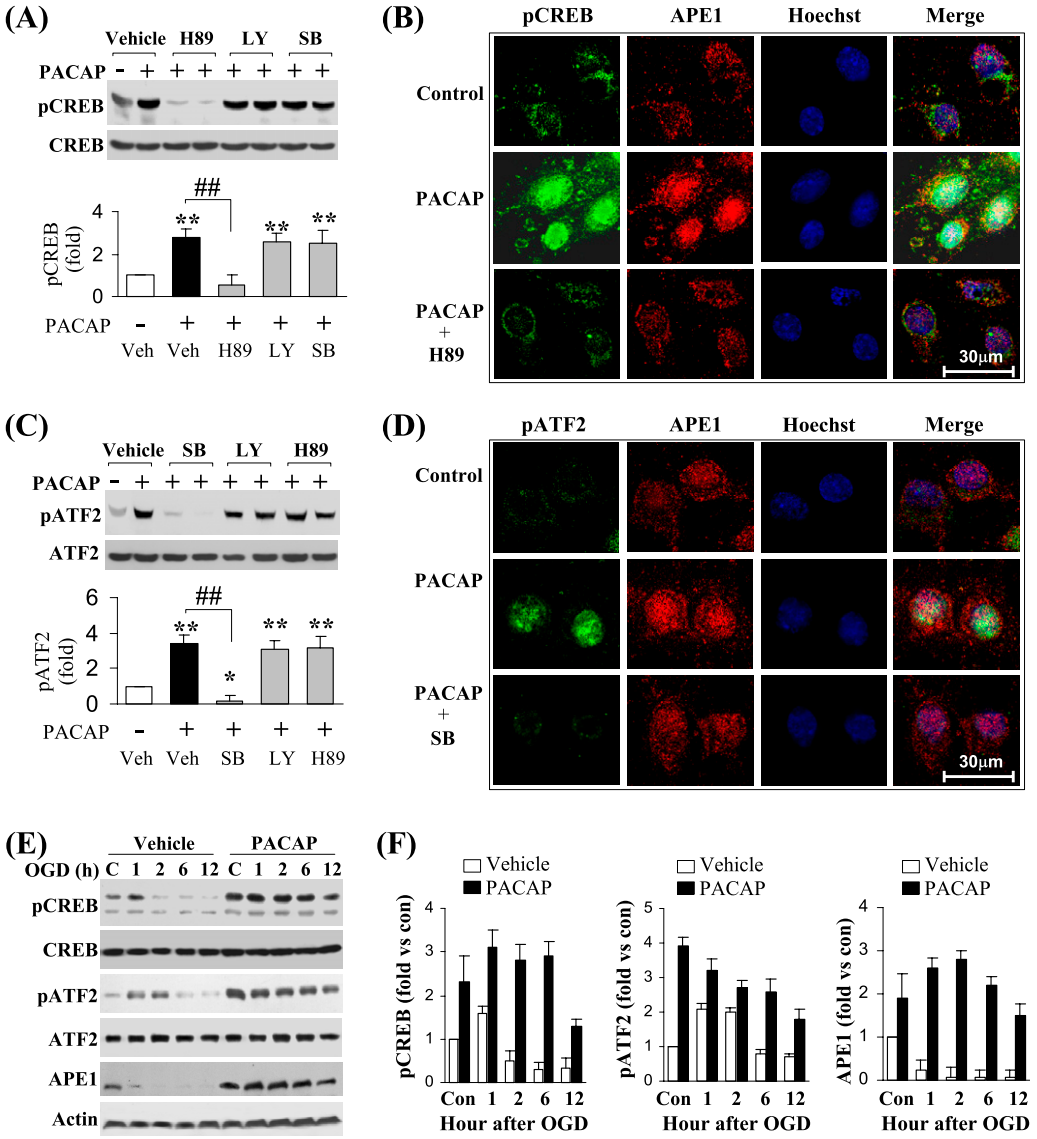

Fig. S5. PACAP induces APE1 expression in cultured neurons via activation of CREB- and ATF2-signaling pathways. (*A*) PACAP treatment (0.1 μM for 4 h) increased the levels of pCREB, and this effect was attenuated by the PKA inhibitor H89 (1 μM), but not by the phosphatidylinositol 3-kinase inhibitor LY294002 (LY, 10 μM) or the p38 inhibitor SB239063 (SB, 10 μM). **, $P < 0.01$ vs. vehicle without PACAP treatment; ##, $P < 0.01$ between the conditions indicated; data were from three experiments. The blots are representative of the data in the bar graph. (*B*) Triple-label fluorescence (pCREB, APE1, and Hoechst) and confocal microscope images show that PACAP-induced increases in pCREB and APE1 expression (*Middle*) were attenuated by H89 (*Bottom*). (*C*) PACAP treatment (0.1 μM for 4 h) increased the levels of pATF2, and this increase was attenuated by the p38 inhibitor SB239063 (SB, 10 μM), but not by LY294002 (10 μM) or H89 (1 μM). *, $P < 0.05$; **, $P < 0.01$ vs. vehicle without PACAP treatment; ##, $P < 0.01$ between the conditions indicated. Data were from three experiments. SB decreased pATF2 to below basal levels, even in the presence of PACAP. The blots are representatives of the data in the bar graph. (*D*) Triple-label fluorescence (pATF2, APE1, and Hoechst) and confocal microscope images show that PACAP-induced increases in pCREB and APE1 expression were attenuated by SB. (*E* and *F*) PACAP treatment (0.1 μM) led to phosphorylation of both CREB and ATF2 and a parallel increase in APE1 following OGD. PACAP was added to cultures 4 h before OGD and maintained throughout the experiments. (*E*) representative blots; (*F*) semiquantitative data were from three experiments.

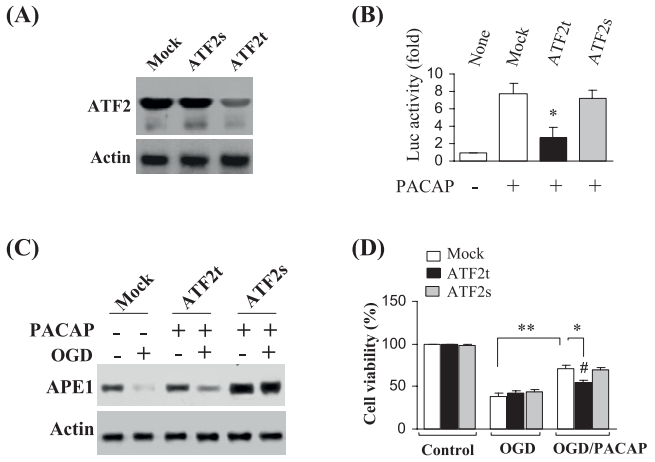

Fig. S6. ATF2 contributes to PACAP-induced APE1 promoter transactivation, protein expression and neuroprotection against OGD in vitro. (*A*) Lentivirus-mediated transfection for 3 d of ATF2-targeted shRNA (ATF2t) but not of a scrambled control (ATF2s) diminished ATF2 protein levels in cultured neurons. (*B*) Targeted knockdown of ATF2 expression decreased PACAP-induced APE1 promoter transactivation, as determined by measuring luciferase activity after PACAP treatment (0.1 μM for 4 h). The APE1-LUC construct was transfected into neurons 24 h before PACAP treatment. *, $P < 0.05$ vs. mock controls, from three experiments. (*C*) Targeted knockdown of ATF2 expression decreased PACAP-induced APE1 protein expression, determined 2 h after OGD. (*D*) Targeted knockdown of ATF2 expression partially but significantly attenuated PACAP-induced neuroprotection against OGD (1 h); cell viability was measured at 24 h after OGD. *, $P < 0.05$; **, $P < 0.01$ between groups indicated in the graph; #, $P < 0.05$ vs. OGD without PACAP; data were from three experiments.

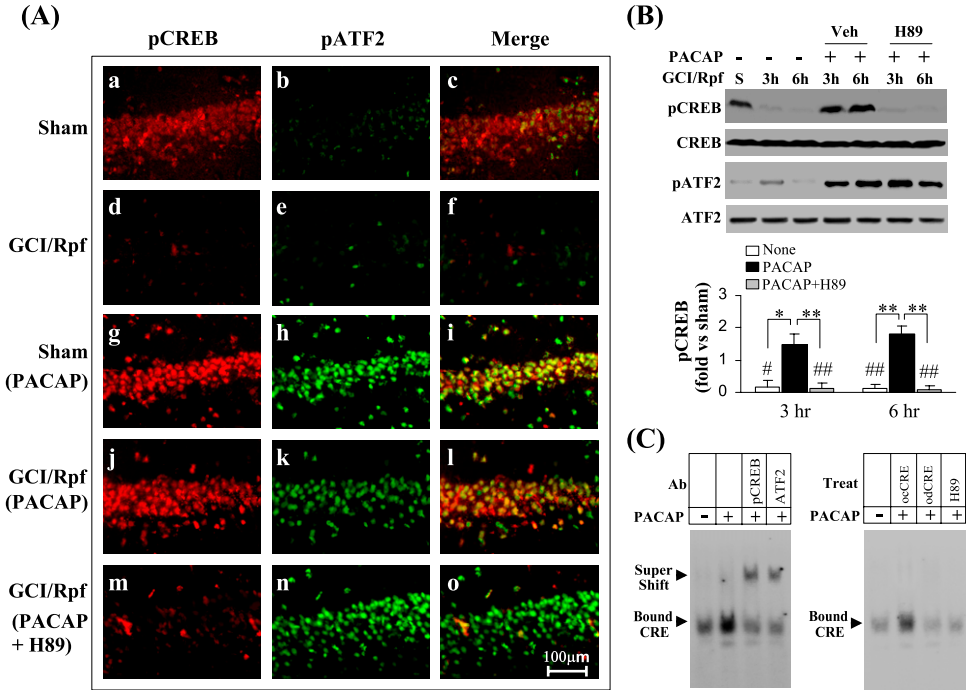

Fig. S7. PACAP activates CREB and ATF2 signaling following GCI. (*A*) Double-label immunofluorescence (pCREB and pATF2) in CA1 at 6 h of reperfusion following GCI (GCI/Rpf) or 6 h after sham operation. PACAP infusions (0.2 nmol at −24, −12, and −6 h) increased pCREB and pATF2 immunoreactivity after either sham operation or GCI. Induction of pCREB but not pATF2 was inhibited by the PKA inhibitor H89, which was administered to animals (10 nmol × 3 times, i.c.v.) 10 min before each PACAP infusion. (*B*) Western blots for pCREB and pATF2 at 3 and 6 h after GCI with or without PACAP infusions. PACAP-induced expression of pCREB, but not of pATF2, after GCI, was inhibited by administration of H89. The quantitative data were from three independent experiments. Data are expressed as normalized to the 6-h sham group (without PACAP treatment). *, $P < 0.05$; **, $P < 0.01$ between the groups indicated in the graph. #, $P < 0.05$; ##, $P < 0.01$ versus the sham group (without PACAP treatment). (*C*) EMSA for APE1-CRE binding was performed using CA1 nuclear extracts after PACAP infusions (0.2 nmol at −24, −12, and −6 h). Supershift assays demonstrate the interaction of pCREB and ATF2 with the APE1-CRE sequence (*Left*). PACAP-induced APE1-CRE binding activity was decreased in brains infused with H89 (10 nmol × 3 times, i.c.v., 10 min before PACAP infusions) or the CRE decoy oligo (odCRE, 5 μL in 10 μmol/L, × 3 times, 10 min before PACAP infusions) but not the control oligo (ocCRE, 5 μL in 10 μmol/L, × 3 times, 10 min before PACAP infusions).