The endogenous inhibitor of Akt, CTMP, is critical to ischemia-induced neuronal death

Supplementary Fig. 1 Ischemia induces and preconditioning blocks selective, delayed death of CA1 neurons. Adult male Sprague-Dawley rats (3-4 weeks of age) were subjected to sham operation, preconditioning (PC), global ischemia (ischemia) or preconditioning followed 48 h later by ischemia (PC+ischemia), and sacrificed 7 days after the last surgery for histology. (a) Representative toluidine blue-stained coronal brain sections at the level of the dorsal hippocampus from sham (a’,b’), preconditioning (c’,d’), ischemia (e’,f’) or preconditioning+ischemia (g’,h’) animals. Left, representative low magnification photomicrographs of hippocampus at 7 d. Right, representative high magnification images showing surviving neurons in the hippocampal CA1 pyramidal cell layer. (b) Quantification of neuronal counts in the CA1 pyramidal layer from photomicrographs like those shown in a. Neuronal counts/250 μm of length were as follows: sham, 92 ± 2, n = 5; preconditioning, 89 ± 1, n = 5; ischemia, 5 ± 2, n = 5, P < 0.01; preconditioning+ischemia, 86 ± 2, n = 10; P < 0.01 for ischemia vs. control and ischemia vs. preconditioning+ischemia). **P < 0.01.
Supplementary Fig. 2  Protein standard curves for Westerns. Analysis of standards containing varying protein concentrations indicated that optical densities of bands are linearly related to protein concentration. Standard curve of (○) p-Akt ($r = 0.993$, $P < 0.01$), (●) Akt ($r = 0.999$, $P = 0.02$), (▲) CTMP ($r = 0.999$, $P < 0.01$), (■) p-FOXO3A ($r = 0.999$, $P < 0.01$) and (□) p-PTEN ($r = 0.99$, $P < 0.01$). $n = 4$ animals per group.
Supplementary Fig. 3  CTMP localizes to the cytosol in control and ischemic CA1. **Upper,** Global ischemia promotes marked increase in CTMP levels in the cytosol of hippocampal CA1. Little or no CTMP was localized to the nuclear or mitochondrial fractions in either control or ischemic CA1 tissue. Animals were subjected to global ischemia or sham surgery and killed at 3 h after reperfusion. The CA1 was rapidly dissected and processed for cytosol, nuclear or mitochondrial-enriched fractions. Similar results were observed in samples from 4 control and 4 ischemic animals. **Lower,** N2A cells expressing CTMP-RFP. Under physiological conditions, CTMP does not localize to nuclei marked by DAPI. A typical coverslip contained 100-150 CTMP-RFP-positive cells per field of 300 N2A cells. Similar data were observed in 6-8 independent experiments.
Supplementary Fig. 4  CTMP overexpression suppresses Akt kinase activity. N2A cells were transfected with hCTMP-IRES-GFP or GFP alone and 1 day later, processed for Akt kinase assays. (a) Akt activity in the absence or presence of CTMP plotted as relative fluorescence units vs. time. (b) Summary of data in (a). CTMP overexpression reduces Akt kinase activity (to 78% ± 4 of control; n = 4 per group; P < 0.01 for CTMP vs. mock-transfected cells).
Supplementary Fig. 5  hCTMP rescues RNAi-mediated CTMP knockdown in N2A cells. (a) CTMP-miRNA-1 (directed to bp’s 144-164 of mCTMP) and CTMP-miRNA-2 (directed to bps 453-473 of mCTMP) are not complementary to the hCTMP sequence. hCTMP differs in sequence from mCTMP in the region targeted by CTMP miRNA and is therefore RNAi resistant (rescue construct). (b) N2A cells were transfected with nontargeting (NT) or CTMP miRNA-1/2 by means of lipofectamine in the absence (endogenous) or presence (rescue) of hCTMP. Cell lysates were subjected to Westerns and probed for CTMP, p-Ser473-Akt, Akt and β-actin. (c) Summary of CTMP data in (b). (d) Summary of p-Ser473-Akt/Akt data in (b). Endogenous, nontransfected N2A cells (lane 1) express constitutive levels of CTMP, pAkt and Akt. Transfection with NT miRNA (lane 2) does not significantly alter CTMP or Akt abundance or Akt phosphorylation status. In contrast, CTMP miRNA (lane 3) markedly attenuates CTMP expression (to 59% ± 6 control (nontransfected); n = 4-5; P < 0.01 vs. CTMP miRNA alone) and enhances Akt phosphorylation with little or no change in total Akt abundance (pAkt/Akt = 188% ± 5 control; n = 4-5; P < 0.01 vs. CTMP miRNA alone). Rescue, N2A cells expressing hCTMP (lane 4) exhibit high CTMP and markedly reduced pAkt; total Akt abundance does not significantly differ from that of nontransfected cells (compare lanes 1 and 4). In cells co-expressing hCTMP with NT (lane 5) or CTMP (lane 6) miRNA, levels of CTMP protein remain high.
Supplementary Fig. 6  Activation of the miRNA silencing machinery by GFP miRNA does not alter CTMP expression. N2A cells were transfected with GFP in the absence or presence of GFP-miRNA or mock-transfected and collected 2 days after transfection. Protein samples were subjected to Western blot analysis and probed for CTMP and GFP. (a) Western blots showing CTMP and GFP expression. (b) Summary of data in (a). GFP miRNA reduces GFP (lane 3), but not CTMP (lanes 2,3), expression. CTMP and GFP band densities were normalized to β-actin (loading control). n = 3-4 samples per group.
Supplementary Fig. 7 CTMP miRNA attenuates CTMP expression and rescues Akt kinase activity in insulted CA1. NT or CTMP miRNA was transduced unilaterally into the right CA1 of intact rats by means of the lentivirus expression system. Fourteen days after viral injection, rats were subjected to either global ischemia (10 min, 4 VO) or sham surgery. Animals were sacrificed 3 h after reperfusion, the CA1 was microdissected and lysates processed for Western blot analysis (a,b) or kinase assays (b,c). (a) Western blots showing CTMP expression in the CA1 of animals expressing NT or CTMP miRNA and subjected to sham surgery or global ischemia. (b) Summary of data in (a). In sham animals expressing NT miRNA, CTMP expression is relatively low in CA1. Ischemia markedly increases CTMP expression (to 182% ± 13 of control; *P < 0.01 vs. sham+NT miRNA; compare lane 3 with 1). In sham animals, CTMP miRNA, modestly (but not significantly) reduces CTMP expression relative to that expressed after transduction of NT miRNA (to 74% ± 7 of control; n.s. vs. NT miRNA; compare lane 2 with 1). In ischemic animals, CTMP miRNA significantly reduces CTMP expression (from 182% ± 13 to 122% ± 10 of control; n = 4-6 animals per group; **P < 0.01 vs. NT miRNA; compare lane 4 with 3). (c) Akt kinase activity in fluorescence units vs. time. (d) Mean Akt kinase activity in CA1, expressed as a ratio of the kinase activity in ipsilateral to contralateral CA1. Ratios greater than 100% indicate an increase in kinase activity. Ischemia markedly enhances Akt phosphorylation (Fig. 1a), but does not significantly alter Akt kinase activity, relative that of control CA1, presumably due to CTMP inhibition. CTMP miRNA significantly reduces CTMP expression (a,b) and significantly increases Akt kinase activity (c,d) in postischemic CA1 (to 119% ± 8 of control; n = 3-4 animals per group; # P < 0.05 vs. NT miRNA). Error bars represent means ± SEM. Significance of experimental vs. control (sham) animals is denoted as (*); significance of CTMP miRNA1/2 vs. NT miRNA is denoted as (# ). * / # P < 0.05, ** / ###P < 0.01.
Supplementary Fig. 8  CTMP over-expression in hippocampal neurons does not induce TUNEL positivity or neuronal death. Upper, Hippocampal neurons (DIV 14-18) transfected with GFP (top row) or CTMP-IRES-GFP (second row) were assayed for loss of viability by either TUNEL (terminal deoxynucleotidyl-transferase mediated dUTP nick-end labeling) staining (red) or by visualization of nuclear condensation and/or fragmentation in DAPI-stained cultures (blue) at 48 h after transfection. Nuclear alteration is a late, but definitive sign of cell death1. Lower, histograms showing the percentage of CTMP-transfected cells that are TUNEL-positive, TUNEL-negative and surviving cells. Left, TUNEL-positive cells (GFP cells, 7 ± 0.57%; n = 29 cells, 4 coverslips; CTMP cells, 4 ± 0.22%; n = 30 cells, 4 coverslips; n.s. vs. GFP cells). Center, TUNEL-negative cells (GFP cells, 93 ± 2.5%; n = 29 cells, 4 coverslips; CTMP cells, 97 ± 4.2%; n = 30 cells, 4 coverslips; n.s. vs. GFP cells). Right, surviving cells (GFP cells: 93 ± 2.5%; n = 29 cells, 4 coverslips; CTMP cells, 97 ± 4.2%; n = 30 cells, 4 coverslips; n.s. vs. GFP-transfected). A typical coverslip contained 8-13 transfected neurons in a field of 100 neurons. The data are the mean of 3 independent experiments. Each experiment included 2 cover slips.