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

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Abstract

Dysregulation of Akt signaling is a critical player in a broad range of diseases including cancer, diabetes and heart disease. The role of Akt signaling in brain disorders is less clear. Here we show that global ischemia in intact rats triggers expression and activation of the Akt inhibitor CTMP (Carboxyl-Terminal Modulator Protein) in vulnerable hippocampal neurons, that CTMP binds and extinguishes Akt activity and that CTMP is essential to ischemia-induced neuronal death. Whereas ischemia induces a dramatic phosphorylation and nuclear translocation of Akt, p-Akt in postischemic neurons is not active, as assessed by kinase assays and phosphorylation of downstream targets GSK-3β and FOXO3A. RNA-interference-mediated depletion of CTMP in a clinically relevant model of stroke restores Akt activity and rescues hippocampal neurons. These findings document a critical role for CTMP in the neurodegeneration associated with stroke and identify CTMP as a novel therapeutic target for amelioration of hippocampal injury and cognitive deficits.

Introduction

Transient global or forebrain ischemia arising as a consequence of cardiac arrest or open heart surgery elicits selective, delayed death of hippocampal CA1 neurons and cognitive deficits1–4. The relative contributions of apoptosis and necrosis remain controversial.

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AUTHOR CONTRIBUTIONS

T.M. and D.O. designed and conducted the experiments, prepared all of the figures and participated in writing the manuscript. K.-M.N. designed experiments and (with A.F.) provided guidance in the cloning of miRNA and cDNA constructs into the lentiviral vector. A.L.-B. performed Westerns and immunoprecipitation experiments. B.A.H. provided the CTMP constructs. A.F. provided the lentiviral vector and wrote the lentiviral methods. R.S.Z. designed experiments, supervised the study and wrote the manuscript.

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Postischemic neurons exhibit many of the biochemical hallmarks of apoptosis including mitochondrial release of cytochrome *c*, cleavage of procaspase-9 to generate the "initiator" caspase-9 and generation and activation of the "terminator" caspase-3 prior to the onset of neuronal death5–8, but show morphologic hallmarks of neuronal necrosis including dilated organelles and intranuclear vacuoles concurrent with cell death9. Ischemic tolerance is a well-established phenomenon in which brief ischemic insults (or preconditioning) acts downstream of caspase-3 to suppress apoptosis and protect CA1 neurons against a subsequent severe ischemic challenge4,10–13. Neuroprotective strategies such as ischemic preconditioning promote phosphorylation of Akt (PKB) at Ser473 and phosphorylation/inactivation of downstream targets implicated in both caspase-dependent and independent mechanisms of cell death; moreover, Akt signaling is essential to preconditioning-induced neuroprotection14–16.

Akt plays a critical role in mediating neuronal survival of a wide range of neuronal cell types16–20. During brain development, newly formed synaptic contacts strategically position neurons in contact with target-derived trophic factors that suppress apoptosis, enabling neuronal survival. Trophic factors such as NGF (neurotrophic growth factor), BDNF (brain-derived neurotrophic factor), insulin and IGF-I (insulin-like growth factor I) recruit Akt to the inner face of the plasma membrane in close proximity to upstream activators. At the membrane, upstream activators such as 1) PDK1 (phosphoinositide-dependent protein kinase-1) activate Akt by phosphorylation of Thr308 in the activation loop of the Akt kinase domain and 2) the mammalian target of rapamycin (mTOR) complex-2, which phosphorylates Ser473 in the carboxy-terminal regulatory domain of Akt21–23. Upon phosphorylation, Akt promotes neuronal survival via phosphorylation (and inactivation) of downstream targets such as the Ser/Thr kinase GSK-3β24 and pro-apoptotic Bcl-2 family member Bad19 and caspase-920,25. In addition to promoting neuronal survival via transcription-independent mechanisms, Akt phosphorylates and inactivates the FOXO subfamily of Forkhead box transcription factors which promote transcription of pro-death genes, and positively regulates CREB and NF-KB, which orchestrate expression of an array of pro-survival genes19. Paradoxically, ischemic insults elicit a marked, albeit transient increase in p-Akt in neurons destined to die26,27.

Akt is negatively regulated by endogenous inhibitors which serve as brakes on Akt signaling28. CTMP is a novel binding partner and endogenous inhibitor of p-Akt, which binds the carboxyl-terminal regulatory domain of p-Akt at the plasma membrane and suppresses Akt activity29. CTMP reverts the phenotype of Akt-expressing cells by increasing cell size and inhibiting cell proliferation. Suppression of CTMP and overstimulation of Akt are implicated in the progression of glioblastomas30. PTEN (phosphatase and tensin homolog deleted on chromosome 10) is another negative regulator of Akt. Upon dephosphorylation, PTEN becomes biologically activated (and destabilized) and dephosphorylates its downstream target PIP3 (phosophatidylinositol 3, 4, 5-triphosphate), converting it to PIP2 (phosphatidylinositol 4, 5-biphosphate), which, in turn, inhibits Akt phosphorylation and activation31.

Dysregulation of Akt is implicated in the pathogenesis of a wide range of disorders including cancer32,33, type-2 diabetes34 and heart disease35. The relation of Akt

deregulation to neurodegenerative disorders is, however, less clear. The present study was undertaken to examine a possible causal role for CTMP in ischemia-induced neuronal death. Here we show that global ischemia markedly promotes Akt phosphorylation, but not kinase activity or phosphorylation of downstream targets in neurons destined to die. We show that ischemia promotes CTMP induction and that CTMP expression mirrors the loss of Akt activity in postischemic neurons. Direct delivery of CTMP micro RNA (miRNA) to the hippocampal CA1 of living animals by means of the lentiviral system affords robust protection against ischemia induced neuronal death. These findings document a causal role for CTMP in ischemia-induced neuronal death and implicate CTMP as an important therapeutic target for intervention in the neurodegeneration and neurological sequelae of cardiac arrest in humans.

Results

Akt phosphorylation and nuclear translocation

Injurious stimuli such as global ischemia promote Akt phosphorylation in selectively vulnerable CA1 neurons. To examine a possible role for Akt phosphorylation/activation in the selective, delayed death of CA1 neurons following ischemia, we subjected rats to sham operation (control), preconditioning; 4 min 4-vessel occlusion (4-VO), ischemia (10 min 4-VO), or preconditioning followed by ischemia (preconditioning+ischemia; 4 min 4-VO, followed 48 h later by 10 min 4-VO), and examined Akt abundance and phosphorylation status at times after ischemia. Global ischemia in this model promotes highly selective, delayed death of hippocampal CA1 pyramidal neurons (Supplementary Fig. 1; see also2,4). Inhibitory interneurons, pyramidal neurons of the nearby CA2 or transition zone and glial cells survive2,4. Whereas the onset of histologically detectable cell death is not evident until at least 48 h after ischemia, by 7 days, the CA1 pyramidal cell layer is largely ablated2,4. Preconditioning itself does not elicit cell death, but affords robust protection of CA1 neurons against a subsequent severe ischemic challenge (Supplementary Fig. 1). Global ischemia induced a marked, but transient increase in phosphorylation of Akt at Ser473 (p-Ser473-Akt) in the cytosol (Fig. 1a) and nucleus of CA1, as assessed by Western blot analysis (Fig. 1b; for this and all other Westerns, see also Supplementary Fig. 2). p-Ser473-Akt was maximal in the cytosol at 1 h (331 ± 31% of control; Fig. 1a) and declined to control levels by 24 h after ischemia; p-S473-Akt in the nucleus was maximal at 3 h (348 ± 67% of control; Fig. 1b) and remained elevated at 24 h. Preconditioning increased p-Ser473-Akt in the cytosol (161 ± 23% of control; Fig. 1a) but not significantly in the nucleus (135 ± 10% of control; Fig. 1b) and markedly attenuated the ischemia-induced increase in cytosolic (Fig. 1a) and nuclear (Fig. 1b) p-Ser473-Akt at all times examined. Alterations in p-Ser473-Akt were subfield-specific, in that neither ischemia nor preconditioning significantly altered phosphorylation status of Ser473-Akt in CA3 (Fig. 1c).

We next examined the impact of ischemia and preconditioning on phosphorylation of Akt at Thr308 (p-Thr308-Akt), a second site implicated in Akt kinase activity. Ischemia induced an increase in p-Thr308-Akt (177 ± 27% of control at 3 h; Fig. 1d), but less so than p-Ser473-Akt. Preconditioning-induced phosphorylation of Akt was site-specific, in that it did not

detectably alter the phosphorylation status or abundance of p-Thr308-Akt in either the cytosol (Fig. 1d) or nucleus (data not illustrated).

To directly visualize nuclear translocation of p-Akt, we performed immunolabeling on brain sections from control, preconditioning, preconditioning+ischemia and ischemic animals and probed with a phospho-specific antibody against p-Ser473-Akt. Ischemia induced a pronounced increase in p-Ser473-Akt selectively in the nucleus of CA1 neurons, evident at 1 h after reperfusion (ischemia, 62 ± 5% *vs*. control, 41 ± 3% colocalization, Fig. 1e; Fig. 1f). Preconditioning alone did not alter colocalization, but blunted the ischemia-induced increase in nuclear p-Akt (preconditioning+ischemia, 53 ± 2% *vs*. control, 41 ± 3% colocalization, Fig. 1e; Fig. 1f). Thus, ischemia promotes, and preconditioning slightly blunts, phosphorylation and nuclear translocation of the pro-survival kinase Akt in neurons destined to die.

Preconditioning but not ischemia promotes Akt activity

The results thus far demonstrate that ischemia enhances phosphorylation and nuclear localization of Akt in neurons destined to die, but do not address Akt functional activity. Toward this end, we undertook two experimental strategies. First, we performed Akt kinase assays on samples of CA1 (Fig. 2a,b) and CA3 (Fig. 2c) of control, preconditioning, preconditioning+ischemia and ischemic animals. Whereas preconditioning or preconditioning+ischemia, markedly increased kinase activity in CA1 (preconditioning, 137 ± 8% of control; preconditioning+ischemia, 139 ± 6% of control; Fig. 2a,b), ischemia did not significantly alter Akt kinase activity relative to that of control (Fig. 2a,b), as assessed by real-time kinase activity assays. The effects were subfield-specific in that neither preconditioning nor ischemia significantly altered Akt kinase activity in the resistant CA3. These findings indicate that although ischemia enhances the phosphorylation status of Akt in CA1 (Fig. 1), it does not significantly alter Akt activity.

Second, we examined the phosphorylation status of Akt downstream targets. Akt phosphorylation promotes cell survival via phosphorylation and inactivation of downstream targets such as GSK-3β and FOXO3A. We performed Western blot analysis of samples from the CA1 of control, preconditioning, preconditioning+ischemia and ischemia and probed for GSK-3β and FOXO3A. Whereas preconditioning alone or preconditioning, followed by ischemia, markedly enhanced p-Ser9-GSK-3β (to 295 ± 52% at 3 h; Fig. 2d), a mark of inactivation; ischemia did not significantly alter p-Ser9-GSK-3β from 0–12 h after reperfusion, although it markedly enhanced p-Ser9-GSK-3β by 24 h (to 190 ± 19%; Fig. 2d). Whereas preconditioning markedly enhanced p-Ser256-FOXO3A in the cytosol (maximal increase to 188 ± 37% at 3 h; Fig. 2e) and nucleus (maximal increase to 156 ± 24% at 12 h; Fig. 2f), a mark of inactivation; ischemia did not significantly alter FOXO3A phosphorylation in the cytosol from 0–12 h, but modestly enhanced FOXO3A phosphorylation at 24 h after reperfusion (to 148 ± 15%; Fig. 2e) in the cytosol. Ischemia induced a transient decrease in decrease in p-S256-FOXO3A in the nucleus (to 58 ± 16% of control at 1 h; Fig. 2f), after which it returned to and remained at control levels (Fig. 2f). Collectively, these results indicate that preconditioning induces phosphorylation and activation of Akt, which phosphorylates and inactivates downstream targets and thereby

promotes neuronal survival. In contrast, p-Akt activity was unaltered in postischemic CA1 neurons and Akt targets were not phosphorylated in the first 12 h after ischemia.

Ischemia induces expression of the Akt inhibitor CTMP

CTMP is a novel binding partner and endogenous inhibitor of Akt, which negatively regulates Akt activity by tightly binding p-Akt and preventing p-Ser473-Akt and p-Thr308-Akt29. Whereas CTMP reverses the cellular phenotype of Akt-expressing mammalian cells in culture, little is known about the impact of CTMP on neurons. We hypothesized that overstimulation of p-Akt might induce expression of its endogenous inhibitor CTMP in postischemic CA1 neurons. Toward this end, we performed Western blot analysis of samples of total cellular protein from the CA1 (Fig. 3a,b) and CA3 (Fig. 3c) of control, preconditioning, preconditioning+ischemia or ischemia and probed for CTMP. Ischemia induced a modest, but sustained, increase in CTMP abundance in selectively vulnerable CA1, evident at 1 and 3 h after reperfusion (252 ± 32% of control at 3 h; Fig. 3a). The ischemia-induced increase in CTMP was long-lasting in that it was evident as late as 24 h after reperfusion (243 ± 29% of control at 24 h; Fig. 3b). Alterations in CTMP were restricted to the cytoplasm, in that little or no CTMP was detected in the nucleus or mitochondria of control or postischemic CA1 (Supplementary Fig. 3), and was subfield-specific, in that neither ischemia nor preconditioning significantly altered CTMP abundance in CA3 (Fig. 3c). Preconditioning alone modestly enhanced CTMP abundance (175 ± 19% of control; Fig. 3a) and markedly attenuated ischemia-induced CTMP upregulation in CA1 after 3 h reperfusion (Fig. 3a).

We next examined whether CTMP assembles with Akt and/or p-Akt in postischemic CA1 neurons. Ischemia promoted assembly of CTMP and Akt, as assessed by co-immunoprecipitation with an anti-Akt antibody and probed for CTMP (Fig. 3d, upper panel) and by an antibody to CTMP and probed for p-dSer473-Akt and Akt (Fig. 3d, middle panel). Preconditioning attenuated formation of the Akt-CTMP complex in postischemic neurons (Fig. 3d), consistent with the role of preconditioning in neuroprotection. Thus, ischemia promotes expression of CTMP, which binds Akt and inhibits Akt activity in neurons destined to die; preconditioning modestly enhances CTMP expression, but attenuates ischemia-induced CTMP upregulation and assembly with p-Akt.

To directly examine the impact of CTMP on Akt function in cells with a neuronal phenotype, we overexpressed CTMP and assessed Akt kinase activity in Neuro 2A (N2A) cells by kinase assays. Overexpression of CTMP markedly reduced Akt kinase activity (Supplementary Fig. 4.To examine whether interaction of Akt with CTMP required Akt phosphorylation, we examine association of CTMP with Akt in N2A cells expressing wild-type or mutant, nonphosphorylatable Akt by reciprocal co-immunoprecipitation. In cells expressing wild-type Akt, an antibody to Akt pulled down CTMP; stimulation with insulin, which promotes PI3K-Akt signaling and Akt phosphorylation, increased CTMP in the immunoprecipitate (Fig. 3e, **lanes 1 and 2**). In contrast, in cells expressing mutant Akt (Ser473A/T308A), there was little or no CTMP in the immunoprecipitate in the absence or presence of insulin stimulation (Fig. 3e, **lanes 3 and 4**). Similar results were obtained with the reverse co-immunoprecipitation using antibody to CTMP (Fig. 3e).

To determine whether additional regulators of Akt are activated in response to global ischemia, we examined the impact of ischemia on PTEN abundance and phosphorylation status in vulnerable CA1 by Western blot analysis and probed with a broad-spectrum phospho-specific antibody directed to p-PTEN, but which does not discriminate phosphorylation at residues Ser380, Thr382 and/or Thr383. Whereas ischemia did not detectably alter PTEN abundance at any time examined, it modestly, but significantly increased PTEN dephosphorylation/activation, evident at 3 h after reperfusion (to 78 ± 4% of control; $P < 0.01$ *vs*. control; Fig. 3f). The increase in PTEN dephosphorylation was transient in that it had declined to basal levels by 12 h. Preconditioning itself did not significantly alter PTEN abundance, but maintained PTEN phosphorylation/inactivation in the face of ischemia (Fig. 3f). These findings are consistent with a model whereby (with a slight delay) ischemia modestly promotes PTEN activation/destabilization and PTEN acts upstream of PI3K to promote Akt dephosphorylation and inhibit Akt activity. In contrast, preconditioning maintains PTEN in a phosphorylated, inactive (but stable) state in the face of ischemic insults.

RNAi-mediated CTMP silencing enables CA1 cells to survive

The results thus far demonstrate that ischemia promotes marked phosphorylation and nuclear translocation (but not activity) of the pro-survival kinase Akt and expression of CTMP in selectively vulnerable CA1 with a time course that mirrors Akt inactivation, but do not address a causal relation between CTMP and either inhibition of Akt or delayed death of hippocampal neurons. To address this issue in a clinically relevant model, we took advantage of RNA interference-mediated gene silencing and the lentivirus expression system for *in vivo* delivery of CTMP miRNA into the hippocampus of adult animals (Fig. 4a). The lentiviral system allows stable long-lasting expression of engineered miRNA sequences, which are processed *in vivo*, and is a useful method for delivery of DNA and RNA to post-mitotic mammalian cells such as neurons with exceedingly low incidence of toxicity36–38.

We designed two miRNA target sequences directed to CTMP cDNA and evaluated them separately and in combination for their ability to abrogate CTMP expression in N2A cells expressing CTMP. A generic negative miRNA that does not target any known vertebrate gene (nontargeting miRNA), served as a negative control miRNA. To validate the specificity and effectiveness of the CTMP miRNA-1/2 sequences, we undertook four experimental strategies. First, we examined its impact on CTMP abundance and Akt abundance and phosphorylation status at Ser 473 in N2A cells. Whereas CTMP miRNA-1 (directed to bps 144–164 of CTMP) and CTMP miRNA-2 (directed to bps 453–473 of CTMP) each modestly attenuated (by ∼40%), but did not silence CTMP expression (data not illustrated), chaining of CTMP miRNA-1 and CTMP miRNA-2 in the same vector more effectively attenuated CTMP protein expression (35 ± 5% of control; $P < 0.05$ *vs*. nontargeting miRNA; $P < 0.01$ *vs*. nontransfected cells; Fig. 4b) and increased p-Ser473-Akt (198 ± 15% of control; $P < 0.01$ *vs*. nontargeting miRNA; $P < 0.01$ *vs*. nontransfected cells; Fig. 4b), as assessed by Western blot analysis. In contrast, nontargeting miRNA did not detectably alter CTMP expression or Akt phosphorylation, relative to that of nontransfected cells (n.s. *vs*. nontransfected cells; Fig. 4b). Second, we examined the ability of a CTMP

construct resistant to CTMP miRNA-1/2 to rescue CTMP expression. hCTMP differs in sequence from mCTMP in the region targeted by CTMP miRNA and is therefore RNAi resistant (rescue construct). CTMP miRNA markedly attenuated CTMP expression (to 59 ± 6% control (nontransfected); $n = 4–5$; $P < 0.01$ *vs*. CTMP miRNA alone; Supplementary Fig. 5, **lane 3**) and enhanced 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). Expression of hCTMP markedly reduced pAkt, with little or no change in total Akt abundance (compare Supplementary Fig. 5, **lanes 1** and **4**). In cells expressing hCTMP, CTMP expression remained high after transfection with nontargeting (Supplementary Fig. 5, **lane 5**) or CTMP miRNA ($P < 0.01$ *vs*. mCTMP; Supplementary Fig. 5, **lane 6**). Third, we examined the impact of GFP miRNA on CTMP expression. GFP miRNA engages the endogenous RISC complex and activates endogenous miRNA formation, but does not target CTMP. In cells co-cistronically expressing CTMP and GFP, GFP miRNA reduced GFP, but not CTMP, expression (Supplementary Fig. 6). Thus, activation of the miRNA silencing machinery by GFP miRNA does not alter CTMP expression.

Fourth, we injected lentiviral vector encoding eGFP with CTMP miRNA-1/2 (CTMP miRNA-1/2) or nontargeting miRNA (nontargeting miRNA) directly into the hippocampal CA1 of intact rats and 14 days later, subjected animals to sham surgery. In animals injected with nontargeting miRNA, followed by sham surgery, CTMP, p-S473-Akt and Akt did not significantly differ in the ipsilateral relative *vs*. contralateral CA1 (Fig. 4c). In animals injected with CTMP miRNA-1/2, followed by sham surgery, CTMP was attenuated and p-S473-Akt was markedly increased in ipsilateral relative to that of contralateral hippocampus (Fig. 4c). These results validate the effectiveness and specificity of CTMP *in vivo*.

We next injected lentiviral vector encoding eGFP with CTMP miRNA-1/2 or nontargeting miRNA unilaterally directly into the right hippocampal CA1 of intact rats and subjected rats to bilateral global ischemia or sham operation 14 d later. CTMP miRNA-1/2 (Fig. 4d) and nontargeting miRNA (not illustrated) were robustly expressed in the ipsilateral hippocampus, as evidenced by intense eGFP fluorescence at 6 d after surgery. Within the CA1 pyramidal cell layer, not all cells were GFP positive, indicating that they did not all express miRNA. In animals expressing nontargeting miRNA and subjected to sham operation, CTMP expression was relatively low in CA1. Ischemia markedly increased CTMP expression, assessed 3 h after reperfusion (to 182 ± 13% of control; $n = 4$ per group; $P < 0.01$ *vs*. control nontargeting miRNA; Supplementary Fig. 7a,b, compare **lane 3** with **lane 1**) with little or no effect on Akt kinase activity, as assessed by kinase assay at 3 h after ischemia (103 ± 4% of control; $n = 4$ per group; n.s.) relative to that of control animals (Supplementary Fig. 7c,d). CTMP miRNA-1/2 reduced CTMP expression (115% of control; $n = 4–6$; $P < 0.01$ *vs*. nontargeting miRNA animals; Supplementary Fig. 7a,b) and increased Akt kinase activity in the ipsilateral *vs*. contralateral CA1 of ischemic animals (to 117 ± 7% of control; $P < 0.01$ *vs*. nontargeting miRNA animals; Supplementary Fig. 7c,d). In that the Akt activity relevant to neuronal survival might be localized to a specific compartment within CA1 pyramidal neurons, the observed changes measured in whole-cell lysates might represent an underestimate of the true increase in functionally relevant kinase activity. Moreover, the hippocampal CA1 contains a mixture of neurons and glia, further diluting an

effect of functionally relevant kinase activity in neurons. These findings indicate that CTMP miRNA restores, at least in part, Akt functional outcome in neurons destined to die.

We next examined the impact of CTMP miRNA-1/2 on neuronal survival. Toward this end, we performed two additional experiments. First, we assessed surviving neurons by histological analysis. CTMP miRNA-1/2 promoted neuronal survival in the ipsilateral (but not contralateral) hippocampus of postischemic animals, as assessed by Nissl staining (Fig. 4d, upper panels). In contrast, in animals injected with nontargeting miRNA, there was little or no neuronal survival in the ipsilateral and contralateral hemispheres at 6 d after ischemia (Fig. 4d, lower panels). Second, we evaluated ischemia-induced neurodegeneration by Fluoro-Jade staining. In animals injected with CTMP miRNA-1/2, neuronal death was dramatically reduced in the ipsilateral (but not contralateral) hemisphere at 6 d after ischemia (to 31 ± 4% of contralateral CA1; Fig. 4e upper panels,f). In contrast, in animals injected with nontargeting miRNA, Fluoro-Jade staining was prominent in the CA1 pyramidal cell layer of the ipsilateral and contralateral hemispheres (Fig. 4e lower panels,f). The area showing protection, as assessed by lack of Fluoro-Jade label, appeared greater than the area that was infected, as assessed by GFP expression (compare Fig. 4e,d), consistent with a possible “bystander effect”, in which cells not expressing CTMP miRNA are protected indirectly by contact with neighboring cells in which CTMP is suppressed39. Thus, neuronal survival may not be entirely cell-autonomous. These findings demonstrate that CTMP silencing rescues Akt kinase activity and promotes survival of neurons destined to die and implicate CTMP as causally related to the neuronal death associated with ischemic stroke.

The results thus far show that CTMP is critical to ischemia-induced neuronal death, but do not address the issue of whether CTMP expression, even in the absence of a neuronal insult, is sufficient to induce neuronal death. We next over-expressed CTMP in hippocampal neurons. Neurons expressing CTMP co-cistronically with GFP exhibited little or no neuronal death, relative to cells expressing GFP alone, as assessed by TUNEL positivity and neuronal loss (Supplementary Fig. 8). Thus, CTMP, which binds and extinguishes Akt pro-survival activity, is necessary, but not sufficient, to induce neuronal death.

Discussion

Transient global or forebrain ischemia arising as a consequence of cardiac arrest or open heart surgery elicits selective, delayed death of hippocampal CA1 neurons and cognitive deficits1,2,4. Although the consequences of global ischemia are well established, the molecular and cellular mechanisms underlying ischemia-induced neuronal death are less clear. Here we show that global ischemia triggers expression and activation of CTMP in selectively vulnerable hippocampal CA1 neurons, that CTMP binds and extinguishes Akt activity and that CTMP is essential to ischemia-induced neuronal death. Whereas ischemia induces a dramatic phosphorylation and nuclear translocation of the pro-survival kinase Akt in neurons destined to die, p-Akt in postischemic neurons is not active, as assessed by real-time kinase assays and phosphorylation/inactivation of downstream targets GSK-3β and FOXO3A. The time course of CTMP induction in selectively vulnerable CA1 neurons mirrors loss of Akt phosphorylation and kinase activity. RNA-interference-mediated

depletion of CTMP in intact animals prior to global ischemia restores Akt activity and phosphorylation at least in part and rescues hippocampal neurons destined to die. These findings document a role for CTMP as a critical player in ischemic cell death and identify CTMP as a novel therapeutic target for amelioration of hippocampal injury and cognitive deficits associated with global ischemia.

A novel (and at first glance paradoxical) observation of the present study is that Akt phosphorylation status and nuclear localization are markedly enhanced, but p-Akt is inactive, in postischemic neurons. An implication of our findings is that Akt phosphorylation status is not necessarily an indication of kinase activity. Possible scenarios are that phosphorylation of Akt at Ser473 does not always correlate with kinase activity and/or that Akt is phosphorylated and activated in response to neuronal insults and its activity is squelched by binding an endogenous inhibitor. To date, at least two endogenous regulators of Akt have been identified, CTMP and PTEN. On the one hand, CTMP directly binds and inhibits Akt. However, the relation of CTMP to Akt phosphorylation status and functional activity has recently been challenged40. Findings in the present study demonstrate that CTMP preferentially binds p-Akt and extinguishes Akt activity and phosphorylation. Moreover, whereas ischemia induces marked and sustained expression and activation of CTMP, it has a relatively small and transient impact on PTEN dephosphorylation and activation and/or stability (present study). We therefore suggest that CTMP is the major functionally relevant inhibitor of p-Akt in postischemic CA1 neurons. Our finding that global ischemia induces a marked, but transient phosphorylation of Akt at Ser473 is consistent with findings of others in models of ischemic injury26,41.

A second novel finding of the present study is that ischemia induces expression and activation of the endogenous inhibitor of Akt, CTMP, and that CTMP is critical to neurodegeneration following global ischemia. Our findings are consistent with a model whereby ischemia triggers hyperphosphorylation and activation of Akt, which in turn promotes induction of CTMP in selectively vulnerable CA1 neurons. An attractive scenario is that upon activation, Akt phosphorylates and activates a factor that promotes expression and/or stabilization of CTMP. Upon activation, CTMP binds Akt leading to Akt dephosphorylation and inactivation. Thus, in postischemic CA1 there are two opposing forces, ischemia, which drives overactivation of Akt and (with a slight delay) CTMP, which suppresses Akt activity and (with a delay) phosphorylation. An additional factor in postischemic CA1 neurons is PTEN activity, which increases transiently at 3 h after ischemia. Whereas CTMP acts downstream of activated p-Akt to temper Akt activity, PTEN acts upstream to inhibit Akt activity31. Under physiological conditions hippocampal CA1 exhibits relatively low levels of CTMP and high levels of p-Akt, whereas N2A cells exhibit high CTMP and low p-Akt (data not illustrated). Under these conditions, endogenous inhibitors of Akt such as CTMP may serve to suppress aberrant Akt hyperactivity and thereby promote cellular survival42. Downregulaton of CTMP and aberrant hyperactivity of Akt are implicated in gliomas30. CTMP overexpression *in vivo* prevents tumor growth in nude mice29. Findings in the present study show that CTMP overexpression in the absence of a neuronal insult does not kill neurons. However, in neurons subjected to ischemia, caspase-dependent and independent pro-apoptotic Akt targets are upregulated, and thus,

high Akt activity is essential to enable neuronal survival. Under these conditions, upregulation of CTMP causes neuronal death. Consistent with this, CTMP miRNA, administered directly into the hippocampal CA1 of intact rats prior to induction of ischemia, enhances Akt activity in postischemic CA1 neurons and affords protection against ischemic cell death. To our knowledge, these findings represent the first demonstration that a neuronal insult in a clinically relevant *in vivo* model of neurodegeneration induces CTMP expression and that CTMP is critical to the neuronal death associated with ischemic stroke or any brain disorder.

A third novel finding of the present study is that a brief preconditioning stimulus that affords neuroprotection (Supplementary Fig. 1) blunts Akt phosphorylation, but promotes Akt activity and phosphorylation/inactivation of GSK-3β and FOXO3a. Our finding that preconditioning promotes sustained Akt phosphorylation and kinase activity, even in the face of severe ischemia, is consistent with findings of others26 and with the observation that PI3K/Akt signaling is critical to ischemic tolerance26,43. A novel and unexpected finding of the present study is that preconditioning blunts ischemia-induced expression and activation of CTMP and thereby enables sustained activation of p-Akt and phosphorylation/inactivation of its downstream targets in postischemic neurons. These findings are consistent with a model whereby there is a critical set-point for Akt phosphorylation and activation. Whereas ischemia drives hyperphosphorylation and overactivation of Akt, which induces high CTMP, loss of Akt activity and neuronal death, preconditioning induces a more modest phosphorylation of Akt, blunting CTMP and enabling neurons to survive. In addition to PI3K/Akt, a number of other effector proteins, including signal transduction factors, mitochondrial effectors, transcription factors and chaperone proteins, are directly implicated in the neuronal survival afforded by ischemic preconditioning11,13.

In summary, findings from the present study demonstrate that ischemic insults trigger induction of CTMP in neurons destined to die. CTMP acts downstream of Akt phosphorylation to blunt activity of this important pro-survival pathway and is causally related to ischemic cell death. These findings identify CTMP as a novel therapeutic target for amelioration of the hippocampal injury and cognitive deficits associated with ischemic stroke. Dysregulation of Akt signaling is a critical player in a broad range of diseases and disorders including cancer, type-2 diabetes and heart disease. Our study adds neurodegenerative brain disorders to the growing list of diseases and disorders associated with Akt dysregulation.

METHODS

Animals

Age-matched adult male Sprague Dawley rats weighing 100–150 gm (Charles River) were maintained in a temperature- and light-controlled environment with a 14/10 h light/dark cycle and were treated according to the protocols approved by the Albert Einstein College of Medicine Animal Care and Use Committee.

Ischemic preconditioning and global ischemia

Animals were subjected to preconditioning, global ischemia, or preconditioning followed by ischemia, by the 4-vessel occlusion paradigm (preconditioning, 4 min; ischemia, 10 min), followed by reperfusion, as described7. For sham surgery, animals were subjected to the same anesthesia and surgical procedures, except that the carotid arteries were not occluded. Body temperature was monitored and maintained at 37.5 ± 0.5°C with a rectal thermistor and heat lamp until the animal had fully recovered from anesthesia. Animals that failed to show complete loss of righting reflex 2 min after occlusion was initiated and the rare animals that exhibited obvious behavioral manifestations (abnormal vocalization when handled, generalized convulsions, loss of > 20% body weight by 3–7d or hypoactivity) were excluded from the study.

Western blotting

Westerns were performed as described44. The CA1 and CA3 subfields were microdissected, placed in ice-cold saline supplemented with a 1% cocktail of protease and phosphatase inhibitors (Sigma). Tissue was homogenized in lysis buffer and centrifuged at 3200 rpm (10 min at 4°C) to separate cytosolic (supernatant) from nuclear (pellet) fraction. Aliquots of protein (30–40 μg) were subjected to SDS-PAGE (4–12%) and probed with antibody. Band density values were normalized to β-actin (whole cell and cytosolic samples) or histone H3 (nuclear samples). Mean band densities for samples from experimental animals were normalized to corresponding samples from control animals.

Co-immunoprecipitation

Co-immunoprecipitation was performed on lysates of microdissected CA1 from control and experimental animals at 3 h after the last surgery. In brief, CA1 tissue was homogenized in lysis buffer containing (in mM): 25 Tris-HCl, 100 NaCl, 5 EDTA, 5 EGTA, 1% Triton X-100, 1% protease and 1% phosphatase inhibitors (Sigma), pH 7.40 (30 min at 4°C). Insoluble material was centrifuged at 16,000 x *g* for 15 min. Lysates (400 μg protein) were precleared by addition of protein G agarose (25 μl), immunoprecipitated (overnight at 4°C) with mouse antibody to Akt or rabbit antibody to CTM, pulled down with protein G agarose beads (50 μl, Millipore) and eluted with Laemmli buffer containing 100 mM DTT. Eluted proteins (40 μg) were subjected to SDS-PAGE (4–12%) and probed with antibodies to Akt, p-Akt or CTMP.

Immunofluorescence

Immunolabeling was performed on sections of rat brain at 1 h after the last surgery as described7,44. In brief, rats were killed under deep anesthesia by transcardial perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were postfixed in 4% paraformaldehyde in PBS and sectioned at 40 μm. Sections were blocked and incubated overnight at 4°C with antibody to p-S473-Akt (1:200, R&D Systems), followed by goat anti-rabbit secondary antibody. To amplify fluorescence signal, sections were processed with avidin–biotin fluorescein complex (Vector Laboratories). Specificity of immunolabeling was confirmed by incubation of sections with pre-immune rabbit IgG in place of primary antibody; under these conditions, no detectable labeling was observed.

Images were viewed through a Nikon ECLIPSE TE300 inverted fluorescence microscope and acquired with a SPOT RT CCD-cooled camera equipped with diagnostic software version 3.0. Images were processed in Adobe Photoshop.

Antibodies and plasmids

For primary antibodies, we used rabbit antibody to Akt (1:1000), mouse antibody to p-S473-Akt (1:1000), mouse antibody to p-T308-Akt (1:1000), rabbit antibody to CTMP (1:1000), rabbit antibody to p-S9-GSK-3β (1:1000), mouse antibody to GSK-3β (1:1000), rabbit antibody to p-S256-FOXO3A (1:1000), rabbit antibody to FOXO3A (1:1000), rabbit antibody to p-S380/T382/T383-PTEN (1:1000), rabbit antibody to PTEN (1:1000), rabbit antibody to H3 (1:1000), mouse antibody to eGFP (1:1000; Abcam), mouse antibody to β-actin (1:10000; Sigma). Antibodies were from Cell Signaling unless otherwise indicated. Secondary antibodies were goat anti-rabbit IgG (1:3000; Vector Laboratories) except for β-actin, in which case horse anti-mouse IgG (1:5000; Vector Laboratories) was used. pcDNA3 T7 Akt1 (plasmid 9003)45 and pcDNA3 T7 Akt1(T308A)(S473A) (plasmid 9030)45 were obtained from Addgene plasmid repository). pcDNA4-CTMP-IRES-GFP and pcDNA3.1-Myc-RFP-CTMP were cloned as described29.

Akt kinase assay

Akt kinase activity assays were performed on lysates of microdissected CA1 tissue from control and experimental animals at 1 h after the last surgery by means of a kinase activity assay kit according to the manufacturer's instructions (Omnia™ Lysate Assay for Akt/PKB; Biosource International).

CTMP miRNAs

CTMP and nontargeting miRNA sequences were engineered in the pcDNATM 6.2-GW/EmGFP miRNA expression vector (Invitrogen): CTMP miRNA-1: (5'–**ATAACCTCCTAGCTGCTCCTGGTTTTGGCCACTGACTGACCAGGAGCATAGGAGGTTAT**–3'); CTMP miRNA-2: (5'–**GAAACAAGGAGACCGTCCTCTGTTTTGGCCACTGACTGACAGAGGACGCTCCTTGTTTC**–3'); and nontargeting-miRNA, a silencer-resistant miRNA sequence that does not target any known eukaryotic gene (5'–**AAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT**–3'). Bold-face denotes sense and antisense strands; normal text denotes loop regions. The efficacy of CTMP miRNA-1/2 was evaluated by Westerns 4–5 d after transduction of N2A cells (which express endogenous CTMP) with serial dilutions of concentrated CTMP miRNA1/2 by lipofectamine LTX (Invitrogen).

Lentiviral vectors

For RNAi-mediated CTMP silencing in neurons of intact animals, miRNAs in the pcDNATM 6.2-GW/EmGFP expression vector were subcloned into the self-inactivating lentiviral pRRLsin.cPPT.CMV.eGFP.Wpre46 to generate CTMP miRNA-1 and -2 and nontargeting miRNA transfer constructs. To enhance CTMP silencing, miRNA-1 and 2 were chained into the same lentiviral vector. High-titer vesicular stomatitis virus (VSV)-

pseudotyped lentiviral stocks were produced in HEK-293T cells47. In brief, cells were transfected with transfer constructs pRRL.PPT.hCMV.miRNA1/2.GFP.Wpre or pRRL.PPT.hCMV.nontargeting-miRNA.GFP.Wpre, packaging constructs pMDLg/pRRE and pRSV-REV and envelope protein construct pMD2.G by means of calcium phosphate46. To determine viral titers, N2A cells were transduced with serial dilutions of concentrated lentivirus and GFP fluorescence evaluated by flow cytometry at 48 h47. Titers were 1×108 (CTMP miRNA1/2) and 1.8×108 (nontargeting miRNA) transducing units per ml.

RNAi-mediated CTMP silencing in intact animals

For *in vivo* experiments, CTMP miRNA-1/2 or nontargeting miRNA was delivered into the hippocampus of live rats by stereotaxic injection 14 d prior to global ischemia or sham surgery as described44. In brief, animals were placed in a stereotaxic frame, anesthetized with 4% isoflurane and maintained on 1.5% isoflurane anesthesia. Concentrated viral solution (4.0 μl) was injected into the right hippocampus (3.0 mm posterior to bregma, 2.0 mm lateral to bregma, 4.0 mm below the skull surface) by means of a 10 μl Hamilton syringe with a 34-guage needle at a flow rate of 0.2 μl/min. The needle was left in place for an additional 3 min and gently withdrawn. Animals were injected subcutaneously with a single dose of flunixin (2.2 mg/kg) to limit inflammation. Fourteen days later, animals were subjected to global ischemia or sham surgery and used for histology experiments as described below. To evaluate spread of the virus, lentiviral-GFP was delivered into the right hippocampus by stereotaxic injection. Fourteen days later, animals were sacrificed and GFP fluorescence assessed in brain sections at the level of the dorsal hippocampus. Sections were viewed and images acquired as above. GFP-positive neurons were prominent in brain sections ranging from 0.60 mm rostral to +0.60 mm caudal to the injection site.

Histology and Fluoro-Jade labeling

Histological analysis of Nissl or Fluoro-Jade stained brain sections at the level of dorsal hippocampus was performed at 6 d after ischemia. Coronal sections (30 μm) were cut at the level of the dorsal hippocampus with a cryotome and processed for staining with Nissl or Fluoro-Jade. The processing of brain sections for Fluoro-Jade labeling involves potassium permanganate, which quenches all fluorescence including GFP. Thus, eGFP does not interfere with Fluoro-Jade signal48.The number of Nissl- or Fluoro-Jade-positive cells per 250 μm length of the medial sector of the CA1 pyramidal cell layer from 9–10 rats per treatment group (4 sections per rat) were counted.

TUNEL

The number of cells undergoing apoptosis was assessed in primary cultures of hippocampal neurons by means of *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using the ApoTag Red *in situ* apoptosis detection kit (Millipore) according to the manufacturer's instructions.

Hippocampal cell culture and transfection

Primary cultures of hippocampal neurons were prepared from embryonic day 18 (E18) Sprague Dawley rat brains and plated on poly-L-lysine-coated coverslips (18 mm) at low

density (100,000 cells/dish) as described49. Neurons were transfected by means of the calcium phosphate method.

Statistical analysis

All results are reported as mean ± s.e.m and analyses were performed using Origin software. Three experimental groups were compared by one-way analysis of variance (ANOVA) with Scheffe's *post hoc* pair-wise analyses and two experimental groups were compared with Student's unpaired, two-tailed *t*-test. Statistical significance was defined as $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference List

1. Graham SH, Chen J. Programmed cell death in cerebral ischemia. J. Cereb. Blood Flow Metab. 2001; 21:99–109. [PubMed: 11176275]
2. Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. Nat. Rev. Neurosci. 2003; 4:399–415. [PubMed: 12728267]
3. Liou AK, Clark RS, Henshall DC, Yin XM, Chen J. To die or not to die for neurons in ischemia, traumatic brain injury and epilepsy: a review on the stress-activated signaling pathways and apoptotic pathways. Prog. Neurobiol. 2003; 69:103–142. [PubMed: 12684068]
4. Zukin, RS., et al. Molecular and Cellular Mechanisms of Ischemia-Induced Neuronal Death in. In: Mohr, JP.; Choi, DW.; Grotta, JC.; Weir, B.; Wolf, PA., editors. Stroke: Pathophysiology, Diagnosis, and Management. Philadelphia: Churchill Livingstone; 2004. p. 829-854.
5. Ouyang YB, et al. Survival- and death-promoting events after transient cerebral ischemia: phosphorylation of Akt, release of cytochrome C and Activation of caspase-like proteases. J. Cereb. Blood Flow Metab. 1999; 19:1126–1135. [PubMed: 10532637]
6. Krajewski S, et al. Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. Proc. Natl. Acad. Sci. U. S. A. 1999; 96:5752–5757. [PubMed: 10318956]
7. Tanaka H, et al. Ischemic preconditioning: neuronal survival in the face of caspase-3 activation. J. Neurosci. 2004; 24:2750–2759. [PubMed: 15028768]
8. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell. 1997; 90:405–413. [PubMed: 9267021]
9. Colbourne F, Sutherland GR, Auer RN. Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. J. Neurosci. 1999; 19:4200–4210. [PubMed: 10341224]
10. Kirino T. Ischemic tolerance. J Cereb. Blood Flow Metab. 2002; 22:1283–1296. [PubMed: 12439285]
11. Gidday JM. Cerebral preconditioning and ischaemic tolerance. Nat. Rev. Neurosci. 2006; 7:437–448. [PubMed: 16715053]
12. Obrenovitch TP. Molecular physiology of preconditioning-induced brain tolerance to ischemia. Physiol Rev. 2008; 88:211–247. [PubMed: 18195087]

13. Pignataro G, Scorziello A, Di RG, Annunziato L. Post-ischemic brain damage: effect of ischemic preconditioning and postconditioning and identification of potential candidates for stroke therapy. FEBS J. 2009; 276:46–57. [PubMed: 19087199]
14. Yano S, Tokumitsu H, Soderling TR. Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. Nature. 1998; 396:584–587. [PubMed: 9859994]
15. Hashiguchi A, et al. Up-regulation of endothelial nitric oxide synthase via phosphatidylinositol 3-kinase pathway contributes to ischemic tolerance in the CA1 subfield of gerbil hippocampus. J. Cereb. Blood Flow Metab. 2004; 24:271–279. [PubMed: 15091107]
16. Zhao H, Sapolsky RM, Steinberg GK. Phosphoinositide-3-kinase/akt survival signal pathways are implicated in neuronal survival after stroke. Mol. Neurobiol. 2006; 34:249–270. [PubMed: 17308356]
17. Yuan J, Yanker BY. Apoptosis in the nervous system. Nature. 2000; 407:802–809. [PubMed: 11048732]
18. Dudek H, et al. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science. 1997; 275:661–665. [PubMed: 9005851]
19. Brunet A, Datta SR, Greenberg ME. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr. Opin. Neurobiol. 2001; 11:297–305. [PubMed: 11399427]
20. Parcellier A, Tintignac LA, Zhuravleva E, Hemmings BA. PKB and the mitochondria: AKTing on apoptosis. Cell Signal. 2008; 20:21–30. [PubMed: 17716864]
21. Coffer PJ, Jin J, Woodgett JR. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem. J. 1998; 335((Pt 1)):1–13. [PubMed: 9742206]
22. Hresko RC, Mueckler M. mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. J. Biol. Chem. 2005; 280:40406–40416. [PubMed: 16221682]
23. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005; 307:1098–1101. [PubMed: 15718470]
24. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature. 1995; 378:785–789. [PubMed: 8524413]
25. Cardone MH, et al. Regulation of cell death protease caspase-9 by phosphorylation. Science. 1998; 282:1318–1321. [PubMed: 9812896]
26. Yano S, et al. Activation of Akt/protein kinase B contributes to induction of ischemic tolerance in the CA1 subfield of gerbil hippocampus. J. Cereb. Blood Flow Metab. 2001; 21:351–360. [PubMed: 11323521]
27. Endo H, Nito C, Kamada H, Nishi T, Chan PH. Activation of the Akt/GSK3beta signaling pathway mediates survival of vulnerable hippocampal neurons after transient global cerebral ischemia in rats. J. Cereb. Blood Flow Metab. 2006; 26:1479–1489. [PubMed: 16538228]
28. Franke TF, et al. Intracellular signaling by Akt: bound to be specific. Sci. Signal. 2008; 1:e29.
29. Maira SM, et al. Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane. Science. 2001; 294:374–380. [PubMed: 11598301]
30. Knobbe CB, Reifenberger J, Blaschke B, Reifenberger G. Hypermethylation and transcriptional downregulation of the carboxyl-terminal modulator protein gene in glioblastomas. J. Natl Cancer Inst. 2004; 96:483–486. [PubMed: 15026474]
31. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. 1998; 273:13375–13378. [PubMed: 9593664]
32. Shaw J, Kirshenbaum LA. Prime time for JNK-mediated Akt reactivation in hypoxia-reoxygenation. Circ. Res. 2006; 98:7–9. [PubMed: 16397151]
33. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat. Rev. Cancer. 2002; 2:489–501. [PubMed: 12094235]
34. Manning BD. Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. J. Cell Biol. 2004; 167:399–403. [PubMed: 15533996]

35. Hausenloy DJ, Mocanu MM, Yellon DM. Cross-talk between the survival kinases during early reperfusion: its contribution to ischemic preconditioning. Cardiovasc. Res. 2004; 63:305–312. [PubMed: 15249188]

36. Naldini L, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science. 1996; 272:263–267. [PubMed: 8602510]

37. Van den HC, Eggermont K, Nuttin B, Debyser Z, Baekelandt V. Lentiviral vector-mediated delivery of short hairpin RNA results in persistent knockdown of gene expression in mouse brain. Hum. Gene Ther. 2003; 14:1799–1807. [PubMed: 14670130]

38. Dittgen T, et al. Lentivirus-based genetic manipulations of cortical neurons and their optical and electrophysiological monitoring in vivo. Proc. Natl. Acad. Sci. USA. 2004; 101:18206–18211. [PubMed: 15608064]

39. Lin JH, et al. Gap-junction-mediated propagation and amplification of cell injury [see comments] [published erratum appears in Nat Neurosci 1998 Dec;1(8):743]. Nat. Neurosci. 1998; 1:494–500. [PubMed: 10196547]

40. Ono H, et al. Carboxy-terminal modulator protein induces Akt phosphorylation and activation, thereby enhancing antiapoptotic, glycogen synthetic, and glucose uptake pathways. Am. J. Physiol Cell Physiol. 2007; 293:C1576–C1585. [PubMed: 17615157]

41. Noshita N, Lewen A, Sugawara T, Chan PH. Evidence of phosphorylation of Akt and neuronal survival after transient focal cerebral ischemia in mice. J. Cereb. Blood Flow Metab. 2001; 21:1442–1450. [PubMed: 11740206]

42. Hillion JA, et al. Involvement of Akt in preconditioning-induced tolerance to ischemia in PC12 cells. J. Cereb. Blood Flow Metab. 2006; 26:1323–1331. [PubMed: 16511503]

43. Miyawaki T, et al. Ischemic preconditioning blocks BAD translocation, Bcl-xL cleavage, and large channel activity in mitochondria of postischemic hippocampal neurons. Proc. Natl. Acad. Sci. USA. 2008; 105:4892–4897. [PubMed: 18347331]

44. Calderone A, et al. Late calcium EDTA rescues hippocampal CA1 neurons from global ischemia-induced death. J. Neurosci. 2004; 24:9903–9913. [PubMed: 15525775]

45. Ramaswamy S, et al. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. Proc. Natl. Acad. Sci. USA. 1999; 96:2110–2115. [PubMed: 10051603]

46. Follenzi A, Naldini L. Generation of HIV-1 derived lentiviral vectors. Methods Enzymol. 2002; 346:454–465. [PubMed: 11883085]

47. Follenzi A, Sabatino G, Lombardo A, Boccaccio C, Naldini L. Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. Hum. Gene Ther. 2002; 13:243–260. [PubMed: 11812281]

48. Liu S, et al. Expression of Ca(2+)-permeable AMPA receptor channels primes cell death in transient forebrain ischemia. Neuron. 2004; 43:43–55. [PubMed: 15233916]

49. Skeberdis VA, et al. Protein kinase A regulates calcium permeability of NMDA receptors. Nat. Neurosci. 2006; 9:501–510. [PubMed: 16531999]

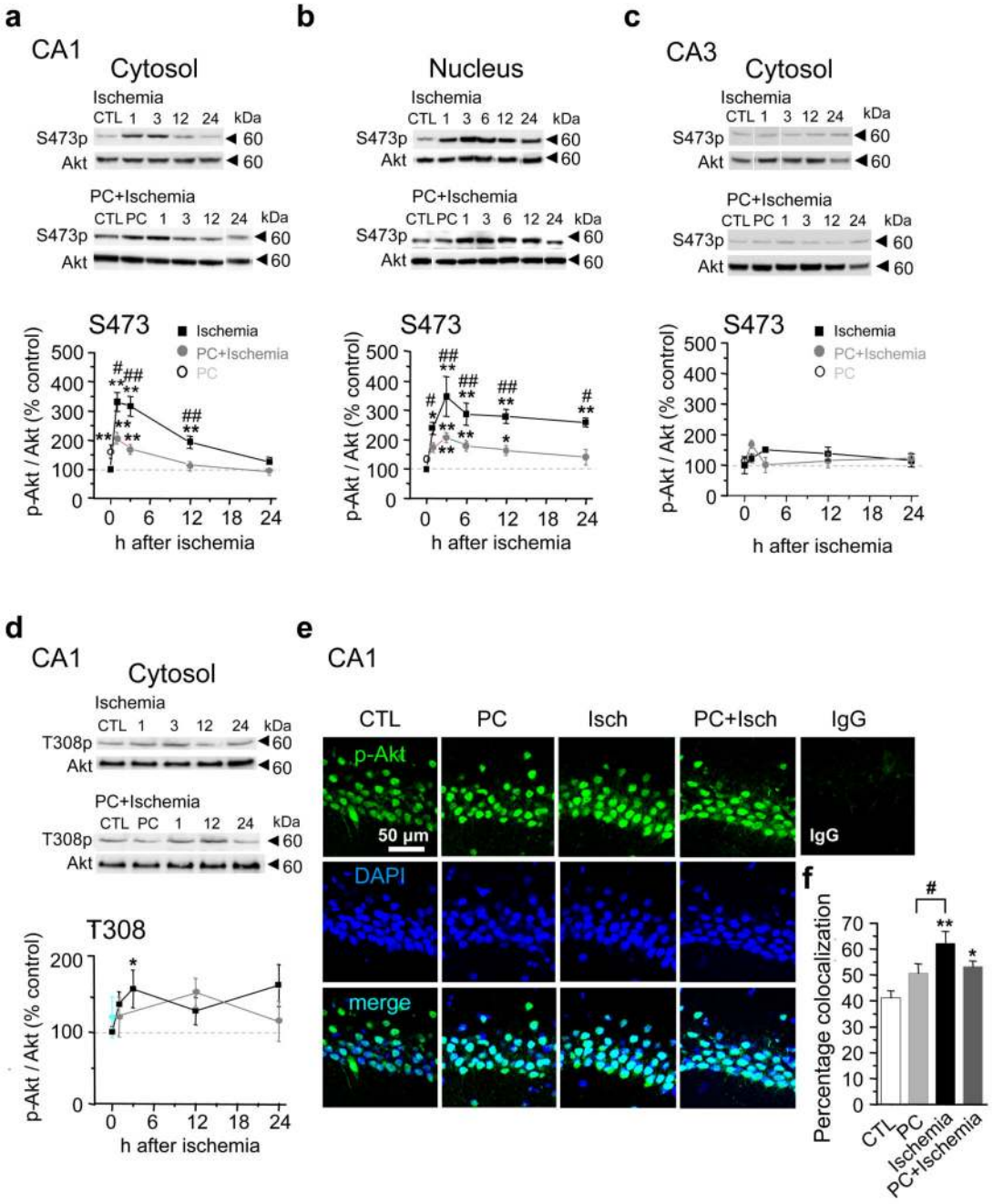

Figure 1.

Global ischemia promotes marked phosphorylation and nuclear translocation of the pro-survival kinase Akt in CA1 neurons destined to die. (**a**) Western blot for p-Akt at Ser 473 in the cytosol. *n* = 7 animals per treatment group. (**b**) Western blot for p-Akt at Ser 473 in the nucleus. *n* = 4 animals per group. (**c**) Western blot for p-Akt at Ser 473 in the cytosol in CA3. *n* = 4 animals per group. Protein samples isolated from animals subjected to sham operation, global ischemia (Ischemia), preconditioning (PC), preconditioning followed by global ischemia. Control animals (denoted as 0 h after ischemia) were sacrificed at 12 h after

sham operation; preconditioned animals not subjected to ischemia (denoted as 0 h preconditioning) were sacrificed 48 h after preconditioning. Ischemia and preconditioning +ischemia animals were sacrificed 1, 3, 6, 12, 24 h after reperfusion. (**d**) Western blot for p-Akt at Thr 308 in the cytosol. $n = 5$ animals per group. (**e**) Double-labeling of p-Akt (green) and DAPI (blue) in the CA1 at 1 hr after the last surgery. IgG labeling of control tissue reveals little or no background signal. (**f**)Quantification of colocalization (p-Akt and DAPI). $n = 4–5$ animals per group. Scale bar = 50 μm. Error bars represent means ± SEM. Significance of experimental *vs*. control animals is denoted as (*); preconditioning+ischemia *vs*. Ischemia is denoted as ($^{\#}$). $^{*/\#} P < 0.05$, $^{**/\#\#} P < 0.01$.

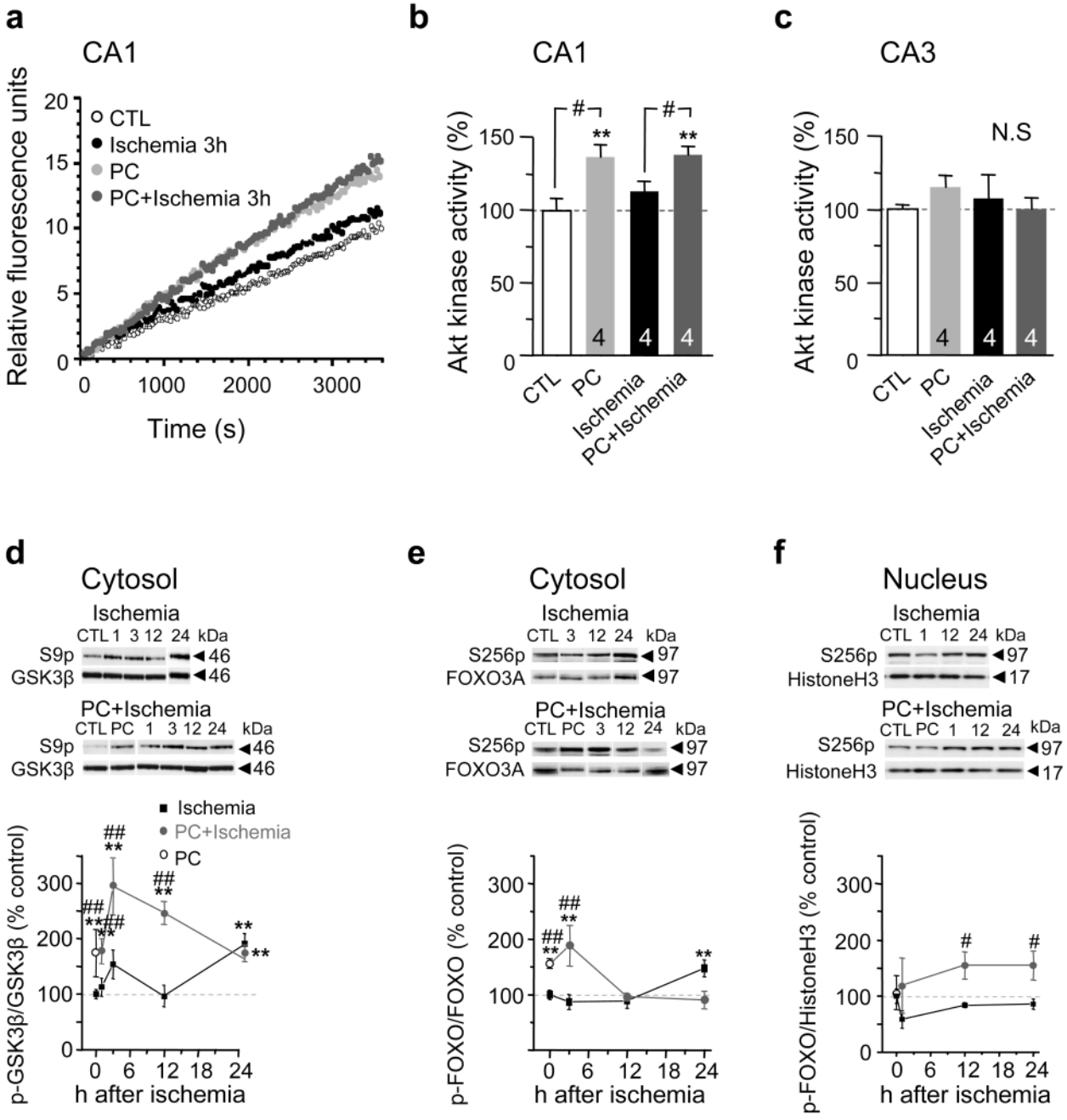

Figure 2.

Preconditioning but not ischemia promotes Akt kinase activity and phosphorylation of Akt targets. (**a**) Akt activity plots of relative fluorescence units *vs*. time. Experimental animals were sacrificed 3 h after reperfusion. (**b**) Mean Akt kinase activity in CA1. *n* = 4 animals per group. (**c**) Mean Akt kinase activity in CA3. *n* = 4 animals per group. (**d**) Western blot for p-GSK-3β at Ser 9 in the cytosol. *n* = 4–6 animals per group. (**e**) Western blot for p-FOXO3A at Ser 256 in the cytosol. *n* = 4–6 animals per group. (**f**) Western blot for p-FOXO3A at Ser

256 in the nucleus. $n = 4–6$ animals per group. Error bars represent means ± SEM. Significance is as described in the legend to Fig. 1.

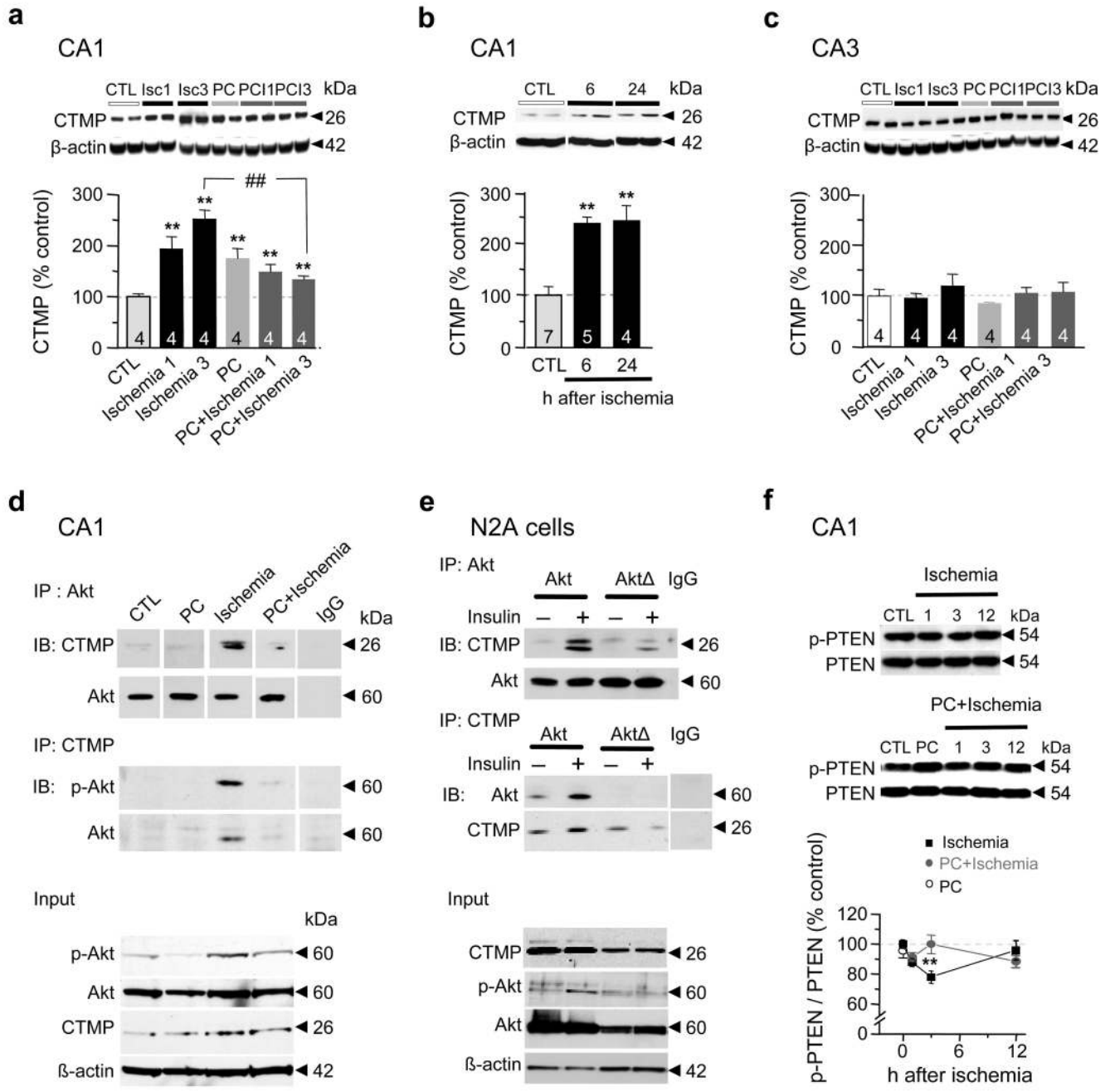

Figure 3.

Ischemia promotes CTMP expression and Akt-CTMP assembly in CA1. (**a—c**) Westerns of CTMP in CA1 (**a,b**) and CA3 (**c**) of control, ischemia, preconditioning (PC) and preconditioning+ischemia animals at times after last surgery; CA1, $n = 5–7$; CA3, $n = 4$ animals per group and time point. (**d**) Ischemia promotes and preconditioning attenuates Akt-CTMP assembly in CA1. *Upper*, co-immunoprecipitation (IP) with anti-Akt and immunoblot (IB) for CTMP. *Middle*, co-immunoprecipitation with CTMP and immunoblot for p-Akt and Akt. *Lower*, Input. $n = 5–7$ animals per group; $P < 0.01$ *vs*. control and *vs*. preconditioning+ischemia). Input shows equal Akt in all groups. (**e**) Akt-CTMP assembly

requires Akt phosphorylation. *Upper*, co-immunoprecipitation with anti-Akt and immunoblot for CTMP. *Middle*, co-immunoprecipitation with anti-CTMP and immunoblot for Akt. *Lower*, Input. N2A cells expressing wild-type (*left*) or mutant (nonphosphorylatable) Akt(T308A/S473A) (*right*) under basal (−) or insulin-stimulated (+) conditions were processed for co-immunoprecipitation. In cells expressing WT Akt, Akt/CTMP association is modest under basal (**lane 1**) and marked under stimulated (**lane 2**) conditions. In cells expressing mutant Akt(T308A/S473A), Akt/CTMP association is near background under basal (**lane 3**) and stimulated (**lane 4**) conditions. Input shows equal CTMP and Akt in all groups, and higher p-Akt in stimulated cells expressing wild-type Akt. $n = 3$ independent experiments. (**f**) Ischemia (but not preconditioning) dephosphorylates and activates (destabilizes) PTEN in CA1. $n = 5–7$ animals per group and time point. Error bars represent means ± SEM. Significance is as described in the legend to Fig. 1.

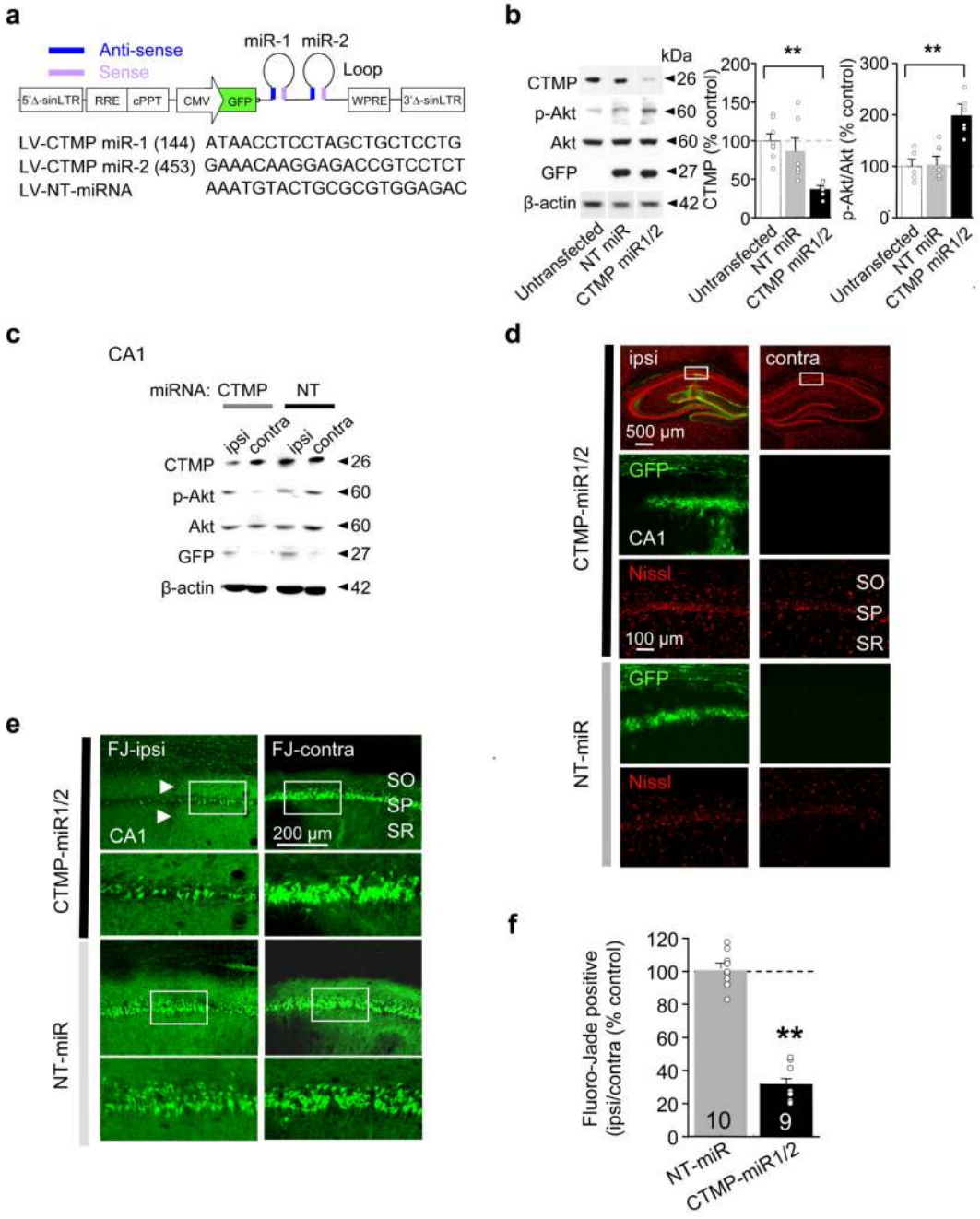

Figure 4.

CTMP is critical to ischemia-induced neuronal death. (**a**) CTMP miRNA-1 (directed to bps 144–164 of CTMP) and CTMP miRNA-2 (directed to bps 453–473 of CTMP) were chained in the lentiviral vector; nontargeting (nontargeting)-miRNA. (**b**) miRNA was transfected into N2A cells with lipofectamine, for 3–5 days. *Left*, Westerns of CTMP miRNA-1/2-transfected, nontargeting-miRNA-transfected or untransfected N2A cells were probed for CTMP, p-Akt (Ser 473), Akt, GFP and β-actin. *Right*, summary of Western data on *left*. (**c**) miRNA was unilaterally transduced directly into the right CA1 of intact rats. Westerns of

ipsilateral CA1 of CTMP miRNA-1/2 or nontargeting-miRNA-transduced rats were probed for CTMP, p-Ser473-Akt and GFP. **c** indicates sample from contralateral hippocampus and **i** indicates sample from ipsilateral hippocampus. (**d**) GFP expression and Nissl staining of brain sections at the level of the dorsal hippocampus from rats unilaterally transduced in the right hippocampus with CTMP miRNA-1/2 (*upper*) or nontargeting-miRNA (*lower*). (**e**) Fluoro-Jade (FJ) staining of adjacent sections from rats unilaterally transduced in the right hippocampus with CTMP miRNA-1/2 (*upper*) or nontargeting-miRNA (*lower*). Arrow shows a needle track. (**f**) Summary of data in (**e**); $n = 9–10$ animals per group. Errors represent means ± SEM. $^{**}P < 0.01$.