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

Takahiro Miyawaki[1,2,6], Dimitry Ofengeim[1,6], Kyung-Min Noh[1], Adrianna Latuszek-Barrantes[1], Brian A Hemmings[3], Antonia Follenzi[4,5] & R Suzanne Zukin[1]

Dysregulation of Akt signaling is important in a broad range of diseases that includes cancer, diabetes and heart disease. The role of Akt signaling in brain disorders is less clear. We found that global ischemia in intact rats triggered expression and activation of the Akt inhibitor CTMP (carboxyl-terminal modulator protein) in vulnerable hippocampal neurons and that CTMP bound and extinguished Akt activity and was essential to ischemia-induced neuronal death. Although ischemia induced a marked phosphorylation and nuclear translocation of Akt, phosphorylated Akt was not active in post-ischemic neurons, as assessed by kinase assays and phosphorylation of the downstream targets GSK-3β and FOXO3A. RNA interference–mediated depletion of CTMP in a clinically relevant model of stroke restored Akt activity and rescued hippocampal neurons. Our results indicate that CTMP is important in the neurodegeneration that is associated with stroke and identify CTMP as a therapeutic target for the amelioration of hippocampal injury and cognitive deficits.

Transient global or forebrain ischemia that arises as a consequence of cardiac arrest or open heart surgery elicits selective, delayed death of hippocampal CA1 neurons and cognitive deficits[1–4]. The relative contributions of apoptosis and necrosis remain controversial. Post-ischemic neurons show 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 before the onset of neuronal death[5–8], but also show morphologic hallmarks of neuronal necrosis, including dilated organelles and intranuclear vacuoles concurrent with cell death[9]. Ischemic tolerance is a well-established phenomenon in which brief ischemic insult (or preconditioning) acts downstream of caspase-3 to suppress apoptosis and protect CA1 neurons against a subsequent severe ischemic challenge[4,10–13]. Neuroprotective strategies such as ischemic preconditioning promote phosphorylation of Akt (PKB) at Ser473 and phosphorylation/inactivation of downstream targets that have been implicated in both caspase-dependent and caspase-independent mechanisms of cell death; moreover, Akt signaling is essential to preconditioning-induced neuroprotection[14–16].

Akt is important in mediating neuronal survival of a wide range of neuronal cell types[16–20]. 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 neurotrophic growth factor, brain-derived neurotrophic factor, insulin and 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 phosphoinositide-dependent protein kinase 1 activate Akt by phosphorylation of Thr308 in the activation loop of the Akt kinase domain and the mammalian target of rapamycin complex 2 phosphorylates Ser473 in the carboxy-terminal regulatory domain of Akt[21–23]. On phosphorylation, Akt promotes neuronal survival via phosphorylation (and inactivation) of downstream targets such as the Ser/Thr kinase GSK-3β[24] and pro-apoptotic Bcl-2 family members Bad[19] and caspase-9 (refs. 20,25). 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-κB, which orchestrate the expression of an array of pro-survival genes[19]. Paradoxically, ischemic insults elicit a marked, albeit transient, increase of phosphorylated Akt (p-Akt) in neurons that are destined to die[26,27].

Akt is negatively regulated by endogenous inhibitors that serve as brakes on Akt signaling[28]. CTMP is a previously unknown binding partner and endogenous inhibitor of p-Akt that binds the carboxyl-terminal regulatory domain of p-Akt at the plasma membrane and suppresses Akt activity[29]. 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 glioblastomas[30]. PTEN (phosphatase and tensin homolog deleted on chromosome 10) is another negative regulator of Akt. On dephosphorylation, PTEN becomes biologically activated (and destabilized) and dephosphorylates its downstream target phosophatidylinositol-3,4,5-triphosphate,

[1]Dominick P. Purpura Department of Neuroscience, Rose F. Kennedy Center, Albert Einstein College of Medicine, Bronx, New York, USA. [2]Department of Neurosurgery, Jichi Medical University, Shimotsuke-shi, Tochigi-ken, Japan. [3]Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland. [4]Department of Pathology, Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, New York, USA. [5]University of Piemonte Orientale, School of Medicine, Novara, Italy. [6]These authors contributed equally to this work. Correspondence should be addressed to R.S.Z. (zukin@aecom.yu.edu).

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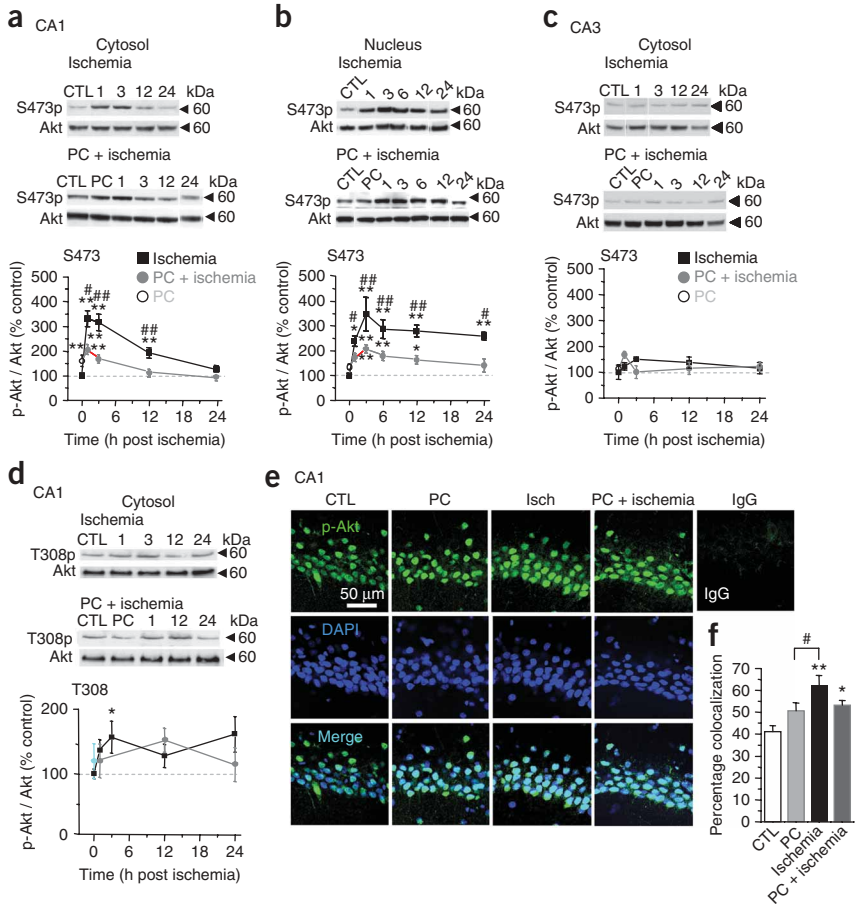

Figure 1 Global ischemia promotes marked phosphorylation and nuclear translocation of the pro-survival kinase Akt in CA1 neurons that are destined to die. (**a**) Western blot examining Akt phosphorylation at Ser473 in the cytosol ($n = 7$ rats per treatment group). (**b**) Western blot examining Akt phosphorylation at Ser473 in the nucleus ($n = 4$ rats per group). (**c**) Western blot examining Akt phosphorylation at Ser473 in the cytosol in CA3 ($n = 4$ rats per group). Protein samples were isolated from rats that were subjected to sham operation, global ischemia (ischemia), preconditioning (PC) or preconditioning followed by global ischemia. Control (CTL) rats (denoted as 0 h after ischemia) were killed 12 h after the sham operation and preconditioned rats that were not subjected to ischemia (denoted as 0 h preconditioning) were killed 48 h after preconditioning. Ischemia and preconditioning and ischemia rats were killed 1, 3, 6, 12 and 24 h after reperfusion. (**d**) Western blot examining Akt phosphorylation at Thr308 in the cytosol ($n = 5$ rats per group). (**e**) Double-labeling of p-Akt (green) and DAPI (blue) in the CA1 1 h after the last surgery. IgG labeling of control tissue revealed little or no background signal. (**f**) Quantification of colocalization (p-Akt and DAPI, $n = 4$–5 rats per group). Scale bar indicates 50 μm. Error bars represent mean ± s.e.m. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, for experimental versus control animals. # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, for preconditioning and ischemia versus ischemia.

converting it to phosphatidylinositol-4,5-biphosphate, which in turn inhibits Akt phosphorylation and activation[31].

Dysregulation of Akt is implicated in the pathogenesis of a wide range of disorders, including cancer[32,33], type-2 diabetes[34] and heart disease[35]. The relationship between Akt deregulation and neurodegenerative disorders is, however, less clear. We sought to determine whether there was a causal role for CTMP in ischemia-induced neuronal death. We found that global ischemia markedly promoted Akt phosphorylation, but not kinase activity or phosphorylation of downstream targets in neurons that were destined to die. We found that ischemia promoted CTMP induction and that CTMP expression mirrored the loss of Akt activity in postischemic neurons. Direct delivery of CTMP microRNA (miRNA) to the hippocampal CA1 of living animals by means of a lentiviral system afforded robust protection against ischemia-induced neuronal death. Our findings indicate that there is a causal role for CTMP in ischemia-induced neuronal death and that CTMP is 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 four-vessel occlusion), ischemia (10-min four-vessel occlusion) or preconditioning followed by ischemia (preconditioning and ischemia, 4-min four-vessel occlusion followed 48 h later by 10-min four-vessel occlusion) and examined Akt abundance and phosphorylation status at times after ischemia. Global ischemia in this model promoted highly selective, delayed death of hippocampal CA1 pyramidal neurons[2,4] (**Supplementary Fig. 1** online). Inhibitory interneurons, pyramidal neurons of the nearby CA2 or transition zone, and glial cells survive[2,4]. Although the onset of histologically detectable cell death is not evident until at least 48 h after ischemia, the CA1 pyramidal cell layer is largely ablated by 7 d[2,4]. Preconditioning itself did not elicit cell death, but afforded 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 western blots, see also **Supplementary Fig. 2** online). 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 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 of the 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 ($P > 0.05$; **Fig. 1c**).

We next examined the effect of ischemia and preconditioning on phosphorylation of Akt at Thr308 (p-Thr308–Akt), a second site that

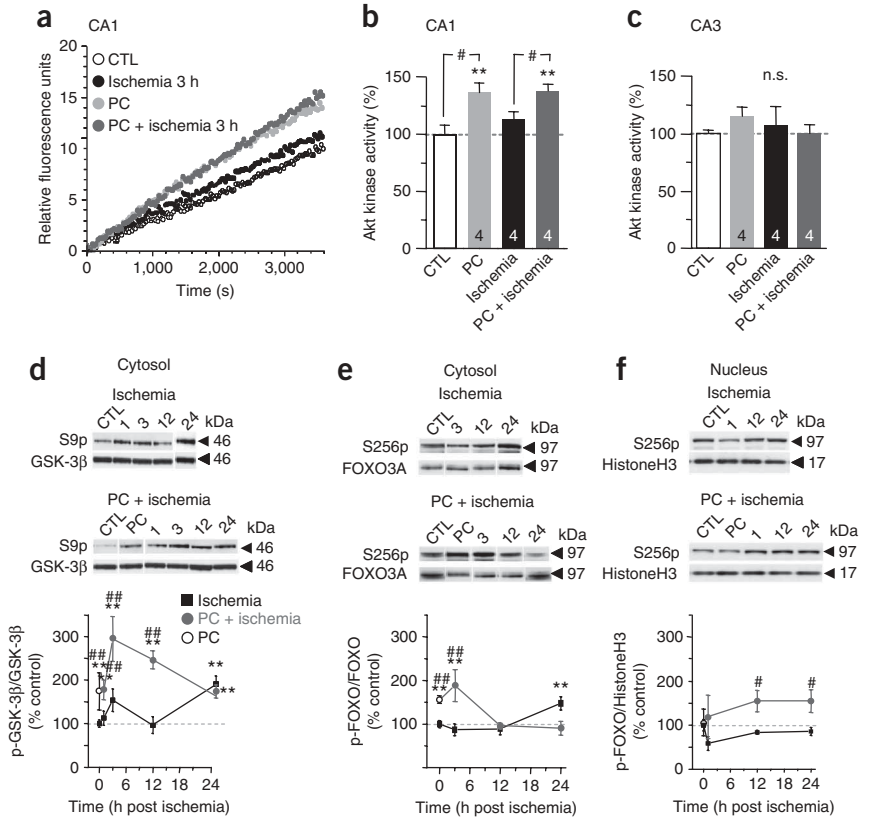

Figure 2 Preconditioning, but not ischemia, promotes Akt kinase activity and phosphorylation of Akt targets. (**a**) Akt activity plots of relative fluorescence units versus time. Experimental rats were killed 3 h after reperfusion. (**b**) Mean Akt kinase activity in CA1 ($n = 4$ rats per group). (**c**) Mean Akt kinase activity in CA3 ($n = 4$ rats per group). (**d**) Western blot examining GSK-3β phosphorylation at Ser9 in the cytosol ($n = 4$–6 rats per group). (**e**) Western blot examining FOXO3A phosphorylation at Ser256 in the cytosol ($n = 4$–6 rats per group). (**f**) Western blot examining FOXO3A phosphorylation at Ser256 in the nucleus ($n = 4$–6 rats per group). Error bars represent mean ± s.e.m. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, for experimental versus control animals. # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, for preconditioning and ischemia versus ischemia.

has been implicated in Akt kinase activity. Ischemia induced an increase in p-Thr308–Akt (177 ± 27% of control at 3 h; **Fig. 1d**), but less of an increase than that of 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 shown).

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

Preconditioning, but not ischemia, promotes Akt activity

Thus far, our results indicate that ischemia enhances phosphorylation and nuclear localization of Akt in neurons that are destined to die, but they do not address Akt functional activity. Toward this end, we undertook two experimental strategies. First, we carried out Akt kinase assays on samples of CA1 (**Fig. 2a,b**) and CA3 (**Fig. 2c**) neurons of control, preconditioning, preconditioning and ischemia, and ischemic rats. Although preconditioning or preconditioning and ischemia markedly increased kinase activity in CA1 neurons (preconditioning, 137 ± 8% of control, $P < 0.01$; preconditioning and ischemia, 139 ± 6% of control, $P < 0.01$; **Fig. 2a,b**), ischemia did not significantly alter Akt kinase activity relative to that of control ($P > 0.05$; **Fig. 2a,b**), as assessed by real-time kinase activity assays. The effects were subfield specific in that neither preconditioning nor ischemia 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 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 and ischemia, and ischemia and probed for GSK-3β and FOXO3A. Although preconditioning alone or preconditioning followed by ischemia markedly enhanced the amount of p-Ser9–GSK-3β (to 295 ± 52% at 3 h; $P < 0.01$; **Fig. 2d**), a marker of inactivation, ischemia did not significantly alter p-Ser9–GSK-3β from 0–12 h after reperfusion, but it markedly enhanced p-Ser9–GSK-3β by 24 h (to 190 ± 19%, $P > 0.05$; **Fig. 2d**). Although preconditioning markedly enhanced the amount of p-Ser256–FOXO3A in the cytosol (maximal increase to 188 ± 37% at 3 h, $P < 0.01$; **Fig. 2e**) and nucleus (maximal increase to 156 ± 24% at 12 h, $P < 0.01$; **Fig. 2f**), a mark of inactivation, ischemia did not significantly alter FOXO3A phosphorylation in the cytosol from 0–12 h ($P > 0.05$), but modestly enhanced FOXO3A phosphorylation at 24 h after reperfusion (to 148 ± 15%; **Fig. 2e**) in the cytosol. Ischemia induced a transient decrease in the amount of 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 post-ischemic 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 binding partner and endogenous inhibitor of Akt that negatively regulates Akt activity by tightly binding p-Akt and preventing phosphorylation of Akt at Ser473–Akt and Thr308–Akt[29]. Although CTMP reverses the cellular phenotype of Akt-expressing mammalian cells in culture, little is known about the effect of CTMP on

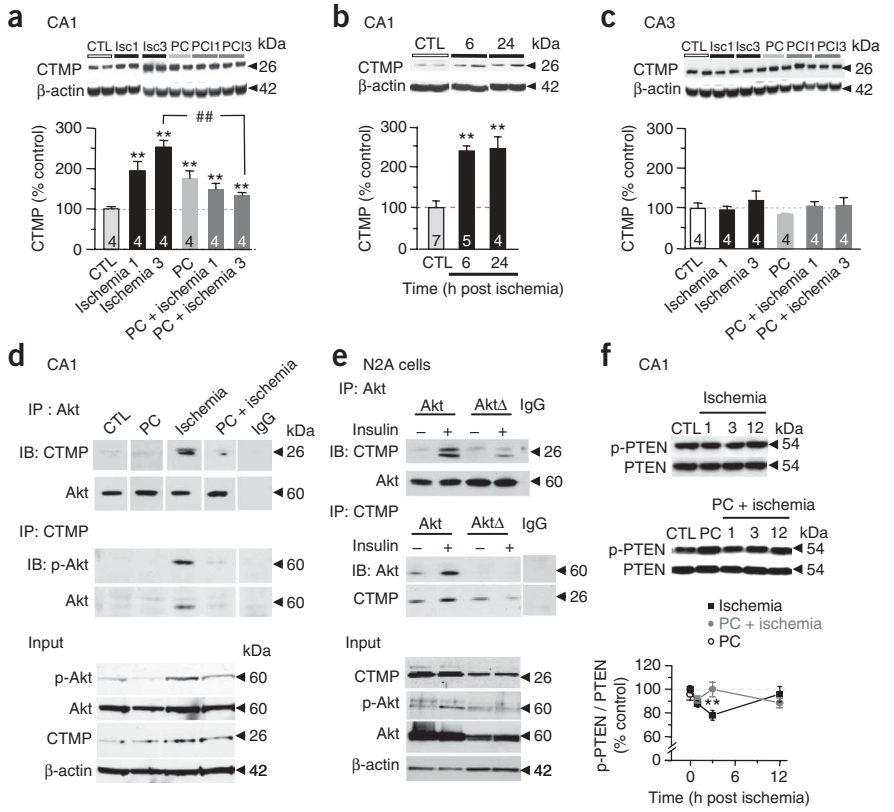

Figure 3 Ischemia promotes CTMP expression and Akt-CTMP assembly in CA1. (**a**–**c**) Western blots examining CTMP expression in CA1 (**a**,**b**) and CA3 (**c**) of control, ischemia, preconditioning, and preconditioning and ischemia rats at times after the last surgery (CA1, $n = 5$–7; CA3, $n = 4$ rats per group and time point). (**d**) Ischemia promoted and preconditioning attenuated Akt-CTMP assembly in CA1. Top, co-immunoprecipitation (IP) with antibody to Akt and immunoblot (IB) with antibody to CTMP. Middle, co-immunoprecipitation with antibody to CTMP and immunoblot with antibodies to p-Akt and Akt. Bottom, input ($n = 5$–7 animals per group, $P < 0.01$ versus control and versus preconditioning and ischemia). Cellular lysates (input) showed equal Akt protein in each group. (**e**) Akt-CTMP assembly requires Akt phosphorylation. Top, co-immunoprecipitation with antibody to Akt and immunoblot with antibody to CTMP. Middle, co-immunoprecipitation with antibody to CTMP and immunoblot with antibody to Akt. Bottom, 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 wild-type Akt, Akt/CTMP association was modest under basal (lane 1) and marked under stimulated (lane 2) conditions. In cells expressing mutant Akt(T308A/S473A), Akt/CTMP association was near background under basal (lane 3) and stimulated (lane 4) conditions. Input showed equal CTMP and Akt in all group, and higher p-Akt in stimulated cells expressing wild-type Akt ($n = 3$ independent experiments). (**f**) Ischemia (but not preconditioning) dephosphorylated and activated (destabilized) PTEN in CA1 neurons ($n = 5$–7 animals per group and time point). Error bars represent means ± s.e.m. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, for experimental versus control animals. # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, for preconditioning and ischemia versus ischemia.

neurons. We hypothesized that overstimulation of p-Akt might induce expression of its endogenous inhibitor CTMP in post-ischemic CA1 neurons. Toward this end, we carried out western blot analysis of samples of total cellular protein from the CA1 (**Fig. 3a**,**b**) and CA3 (**Fig. 3c**) neurons of control, preconditioning, preconditioning and ischemia, or ischemia rats and probed for CTMP. Ischemia induced a modest, but sustained, increase in CTMP abundance in selectively vulnerable CA1 neurons, 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, as little or no CTMP was detected in the nucleus or mitochondria of control or post-ischemic CA1 neurons (**Supplementary Fig. 3** online), and were subfield specific, in that neither ischemia nor preconditioning significantly altered CTMP abundance in CA3 neurons (**Fig. 3c**). Preconditioning alone modestly enhanced CTMP abundance (175 ± 19% of control; **Fig. 3a**) and markedly attenuated ischemia-induced CTMP upregulation in CA1 neurons after 3 h reperfusion (**Fig. 3a**).

We next examined whether CTMP assembles with Akt and/or p-Akt in post-ischemic CA1 neurons. Ischemia promoted assembly of CTMP and Akt, as assessed by co-immunoprecipitation with an antibody to Akt (probed for CTMP; **Fig. 3d**) and an antibody to CTMP (probed for p-dSer473–Akt and Akt; **Fig. 3d**). Preconditioning attenuated the formation of the Akt-CTMP complex in post-ischemic 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 that are destined to die, and preconditioning modestly enhances CTMP expression, but attenuates ischemia-induced CTMP upregulation and assembly with p-Akt.

To directly examine the effect of CTMP on Akt function in cells with a neuronal phenotype, we overexpressed CTMP and assessed Akt kinase activity in Neuro2A (N2A) cells by kinase assays. Overexpression of CTMP markedly reduced Akt kinase activity (**Supplementary Fig. 4** online). To determine whether the interaction of Akt with CTMP required Akt phosphorylation, we examined the 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**). 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**). 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 effect of ischemia on PTEN abundance and phosphorylation status in vulnerable CA1 neurons by western blot analysis and probed with a broad-spectrum phosphospecific antibody directed to phosphorylated PTEN that does not discriminate between phosphorylation at residues Ser380, Thr382 and/or Thr383. Although ischemia did not detectably alter PTEN abundance at any time examined, it modestly, but significantly, increased PTEN dephosphorylation/activation, evident at 3 h after

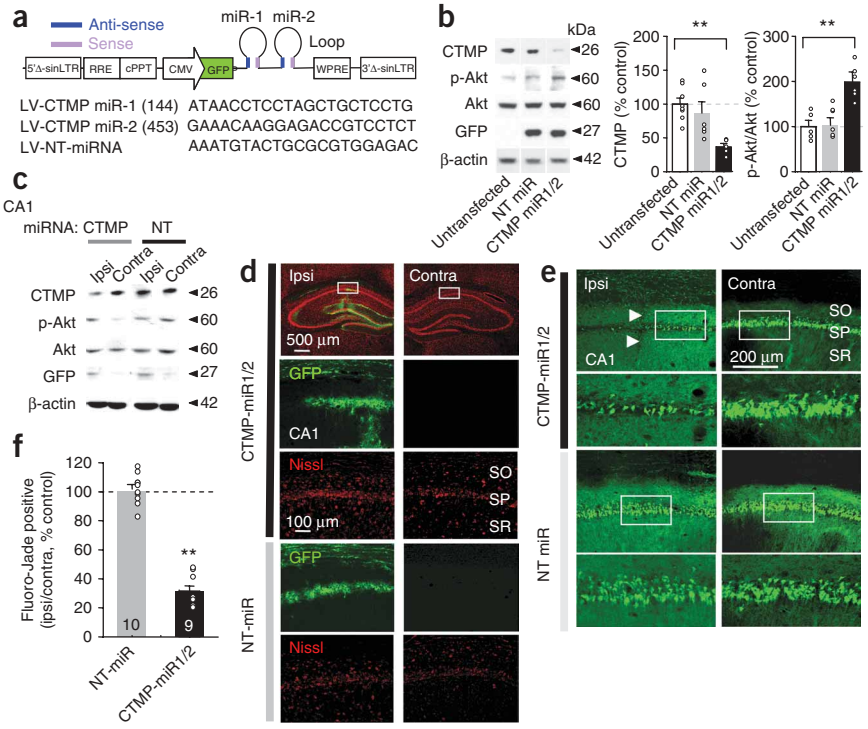

Figure 4 CTMP is critical to ischemia-induced neuronal death. (**a**) CTMP miRNA-1 (directed to base pairs 144–164 of CTMP) and CTMP miRNA-2 (directed to base pairs 453–473 of CTMP) were chained in a lentiviral vector (NT represents nontargeting miRNA). (**b**) miRNA was transfected into N2A cells with lipofectamine for 3–5 d. Left, western blots of CTMP miRNA-1/2–transfected, nontargeting miRNA–transfected or nontransfected N2A cells were probed for CTMP, p-Akt (Ser473), Akt, GFP and β-actin. Right, summary of western blot data. (**c**) miRNA was unilaterally transduced directly into the right CA1 of intact rats. Western blots of ipsilateral CA1 of CTMP miRNA-1/2– or nontargeting miRNA–transduced rats were probed for CTMP, p-Ser473-Akt and GFP. (**d**) GFP expression and Nissl staining of brain sections at the level of the dorsal hippocampus from rats that were unilaterally transduced in the right hippocampus with CTMP miRNA-1/2 (top) or nontargeting miRNA (bottom). (**e**) Fluoro-Jade staining of adjacent sections from rats that were unilaterally transduced in the right hippocampus with CTMP miRNA-1/2 (top) or nontargeting miRNA (bottom). Arrows indicate a needle track. (**f**) Summary of data in **e** ($n = 9$–10 animals per group). Error bars represent mean ± s.e.m. ** $P < 0.01$.

reperfusion (to 78 ± 4% of control, $P < 0.01$ versus 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 detectably 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

Our results thus far indicate 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 neurons 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 (RNAi)-mediated gene silencing and a lentivirus expression system for *in vivo* delivery of CTMP miRNA into the hippocampus of adult rats (**Fig. 4a**). The lentiviral system allows for 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 postmitotic mammalian cells such as neurons with exceedingly low incidence of toxicity[36–38].

We designed two miRNA target sequences that were 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 the effects of the miRNAs on CTMP and Akt abundance and phosphorylation status at Ser473 in N2A cells. Although CTMP miRNA-1 (directed to base pairs 144–164 of CTMP) and CTMP miRNA-2 (directed to base pairs 453–473 of CTMP) each modestly attenuated (by ~40%), but did not silence, CTMP expression (data not shown), chaining of CTMP miRNA-1 and CTMP miRNA-2 in the same vector more effectively attenuated CTMP protein (35 ± 5% of control, $P < 0.05$ versus nontargeting miRNA, $P < 0.01$ versus nontransfected cells; **Fig. 4b**) and increased the amount of p-Ser473–Akt that was present (198 ± 15% of control, $P < 0.01$ versus nontargeting miRNA, $P < 0.01$ versus 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 nontransfected cells ($P > 0.05$ versus nontransfected cells; **Fig. 4b**).

Second, we examined the ability of a CTMP construct that was 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 resistant to RNAi (rescue construct). CTMP miRNA markedly attenuated CTMP expression (to 59 ± 6% control (nontransfected), $n = 4$–5, $P < 0.01$ versus CTMP miRNA alone; **Supplementary Fig. 5** online) and enhanced Akt phosphorylation with little or no change in total Akt abundance (p-Akt/Akt = 188 ± 5% control, $n = 4$–5, $P < 0.01$ versus CTMP miRNA alone). Expression of hCTMP markedly reduced the amount of p-Akt that was present, with little or no change in total Akt abundance (**Supplementary Fig. 5**). In cells expressing hCTMP, CTMP expression remained high after transfection with nontargeting (**Supplementary Fig. 5**) or CTMP miRNA ($P < 0.01$ versus mCTMP; **Supplementary Fig. 5**).

Third, we examined the effect of green fluorescent protein (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** online). Thus, activation of the miRNA silencing machinery by GFP miRNA does not alter CTMP expression.

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

We next injected a lentiviral vector encoding eGFP with CTMP miRNA-1/2 or nontargeting miRNA directly into the right hippocampal CA1 of intact rats and subjected the rats to bilateral global ischemia or sham operation 14 d later. CTMP miRNA-1/2 (**Fig. 4d**) and nontargeting miRNA (data not shown) were robustly expressed in the ipsilateral hippocampus, as evidenced by intense eGFP fluorescence 6 d after surgery. In the CA1 pyramidal cell layer, not all cells were GFP positive, indicating that they did not all express the miRNA. In rats that expressed nontargeting miRNA and were subjected to sham operation, CTMP expression was relatively low in CA1 neurons. Ischemia markedly increased CTMP expression, which was assessed 3 h after reperfusion (to 182 ± 13% of control, $n = 4$ per group, $P < 0.01$ versus control nontargeting miRNA; **Supplementary Fig. 7** online), and had 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, $P > 0.05$), relative to that of control rats (**Supplementary Fig. 7**). CTMP miRNA-1/2 reduced CTMP expression (115% of control, $n = 4$–6, $P < 0.01$ versus nontargeting miRNA animals; **Supplementary Fig. 7**) and increased Akt kinase activity in the ipsilateral versus contralateral CA1 of ischemic rats (to 117 ± 7% of control, $P < 0.01$ versus nontargeting miRNA rats; **Supplementary Fig. 7**). In that the Akt activity relevant to neuronal survival might be localized to a specific compartment in CA1 pyramidal neurons, the changes that we observed 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 that are destined to die.

We next examined the effect of CTMP miRNA-1/2 on neuronal survival. Toward this end, we carried out 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 post-ischemic rats, as assessed by Nissl staining (**Fig. 4d**). In contrast, in rats injected with nontargeting miRNA, there was little or no neuronal survival in the ipsilateral and contralateral hemispheres at 6 d after ischemia (**Fig. 4d**). Second, we evaluated ischemia-induced neurodegeneration by Fluoro-Jade staining. In rats injected with CTMP miRNA-1/2, neuronal death was markedly reduced in the ipsilateral (but not contralateral) hemisphere at 6 d after ischemia (to 31 ± 4% of contralateral CA1; **Fig. 4e,f**). In contrast, in rats injected with nontargeting miRNA, Fluoro-Jade staining was prominent in the CA1 pyramidal cell layer of the ipsilateral and contralateral hemispheres (**Fig. 4e,f**). The area showing protection, as assessed by a lack of Fluoro-Jade label, appeared to be greater than the area that was infected, as assessed by GFP expression (**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 suppressed[39]. Thus, neuronal survival may not be entirely cell autonomous. These findings demonstrate that CTMP silencing rescues Akt kinase activity and promotes the survival of neurons that are destined to die and implicate CTMP as being causally related to the neuronal death that is associated with ischemic stroke.

Thus far, our results indicate 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 overexpressed CTMP in hippocampal neurons. Neurons expressing CTMP co-cistronically with GFP showed little or no neuronal death relative to cells expressing GFP alone, as assessed by TUNEL staining and neuronal loss (**Supplementary Fig. 8** online). Thus, CTMP, which binds Akt 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 deficits[1,2,4]. Although the consequences of global ischemia are well established, the molecular and cellular mechanisms underlying ischemia-induced neuronal death are less clear. We found that global ischemia triggered the 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. Although ischemia induces a marked phosphorylation and nuclear translocation of the pro-survival kinase Akt in neurons that are destined to die, p-Akt in post-ischemic neurons was 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 mirrored the loss of Akt phosphorylation and kinase activity. RNAi-mediated depletion of CTMP in intact rats before global ischemia restored Akt activity and phosphorylation, at least in part, and rescued hippocampal neurons that were destined to die. These findings indicate that CTMP is important in ischemic cell death and identify CTMP as a therapeutic target for amelioration of hippocampal injury and cognitive deficits associated with global ischemia.

One of our observations (and a paradoxical one at first glance) was that Akt phosphorylation status and nuclear localization were markedly enhanced, but p-Akt was inactive in post-ischemic neurons. An implication of our findings is that Akt phosphorylation status is not necessarily an indication of kinase activity. It is possible 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. CTMP directly binds and inhibits Akt; however, the relationship of CTMP to Akt phosphorylation status and functional activity has recently been challenged[40]. Our results indicate that CTMP preferentially binds p-Akt and extinguishes Akt activity and phosphorylation. Moreover, although ischemia induced marked and sustained expression and activation of CTMP, it had a relatively small and transient effect on PTEN dephosphorylation and activation and/or stability. We therefore suggest that CTMP is the major functionally relevant inhibitor of p-Akt in post-ischemic CA1 neurons. Our finding that global ischemia induced a marked, but transient, phosphorylation of Akt at Ser473 is consistent with the results of other models of ischemic injury[26,41].

A second finding of our study was that ischemia induced expression and activation of the endogenous inhibitor of Akt, CTMP, and that CTMP was critical to neurodegeneration following global ischemia. Our findings are consistent with a model in which ischemia triggers

hyperphosphorylation and activation of Akt, which in turn promotes induction of CTMP in selectively vulnerable CA1 neurons. An attractive scenario is that, after activation, Akt phosphorylates and activates a factor that promotes the expression and/or stabilization of CTMP. On activation, CTMP binds Akt, leading to Akt dephosphorylation and inactivation. Thus, in post-ischemic CA1 neurons, there are two opposing forces: ischemia, which drives overactivation of Akt (with a slight delay), and CTMP, which suppresses Akt activity and (with a delay) phosphorylation. An additional factor in post-ischemic CA1 neurons is PTEN activity, which increases transiently at 3 h after ischemia. CTMP acts downstream of activated p-Akt to temper Akt activity, whereas PTEN acts upstream to inhibit Akt activity[31].

Under physiological conditions, hippocampal CA1 neurons show relatively low levels of CTMP and high levels of p-Akt, whereas N2A cells have high CTMP and low p-Akt (data not shown). Under these conditions, endogenous inhibitors of Akt, such as CTMP, may serve to suppress aberrant Akt hyperactivity and thereby promote cellular survival[42]. Downregulation of CTMP and aberrant hyperactivity of Akt are implicated in gliomas[30]. CTMP overexpression *in vivo* prevents tumor growth in nude mice[29]. We found that CTMP overexpression in the absence of a neuronal insult did not kill neurons. In neurons subjected to ischemia, however, caspase-dependent and caspase-independent pro-apoptotic Akt targets were upregulated, and high Akt activity is therefore essential for 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 before induction of ischemia, enhanced Akt activity in post-ischemic CA1 neurons and afforded protection against ischemic cell death. To the best of 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 finding of our study is that a brief preconditioning stimulus that affords neuroprotection (**Supplementary Fig. 1**) blunted Akt phosphorylation, but promoted Akt activity and phosphorylation/inactivation of GSK-3β and FOXO3a. Our finding that preconditioning promoted sustained Akt phosphorylation and kinase activity, even in the face of severe ischemia, is consistent with the results of other studies[26] and with the observation that PI3K/Akt signaling is critical to ischemic tolerance[26,43]. An unexpected result of our study is that preconditioning blunted ischemia-induced expression and activation of CTMP and thereby enabled sustained activation of p-Akt and phosphorylation/inactivation of its downstream targets in post-ischemic neurons. These findings are consistent with a model in which there is a critical set point for Akt phosphorylation and activation. Ischemia drives hyperphosphorylation and overactivation of Akt, which induces high CTMP, loss of Akt activity and neuronal death, whereas preconditioning induces a more modest phosphorylation of Akt, blunting CTMP and enabling neuronal survival. 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 that is afforded by ischemic preconditioning[11,13].

In summary, our results indicate that ischemic insults trigger induction of CTMP in neurons that are 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 therapeutic target for amelioration of the hippocampal injury and cognitive deficits that are associated with ischemic stroke. Dysregulation of Akt signaling is important 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 that are associated with Akt dysregulation.

METHODS

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

Ischemic preconditioning and global ischemia. Rats were subjected to preconditioning, global ischemia or preconditioning followed by ischemia via the four-vessel occlusion procedure (preconditioning, 4 min; ischemia, 10 min), followed by reperfusion, as described[7]. For sham surgery, rats 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 rat had fully recovered from anesthesia. Rats 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 3–7 d or hypoactivity) were excluded from the study.

Western blotting. Western blots were carried out as described[44]. The CA1 and CA3 subfields were microdissected and placed in ice-cold saline supplemented with a 1% cocktail of protease and phosphatase inhibitors (Sigma). Tissue was homogenized in lysis buffer (5 mM HEPES, 1 mM MgCl2, 2 mM EGTA, 1 mM DTT and 320 mM sucrose, supplemented with a 1% (vol/vol) cocktail of protease and phosphatase inhibitors). The resulting lysate was centrifuged at 0.8*g* (10 min at 4 °C) to separate the cytosolic (supernatant) and nuclear (pellet) fractions. To further purify the cytosolic fraction and to isolate the mitochondrial fraction, we further centrifuged the supernatant at 16,000*g* (10 min at 4 °C). Aliquots of protein (30–40 μg) were run on SDS-PAGE gels (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 rats were normalized to corresponding samples from control rats.

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

Immunofluorescence. Immunolabeling was performed on sections of rat brain 1 h after the last surgery as described previously[7,44]. Briefly, rats were killed under deep anesthesia by transcardial perfusion with 4% paraformaldehyde (wt/vol) in phosphate-buffered saline. Brains were postfixed in 4% paraformaldehyde in phosphate-buffered saline 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 a goat secondary antibody to rabbit. To amplify fluorescence signal, sections were processed with avidin–biotin fluorescein complex (Vector Laboratories). The 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 (1:1,000), mouse antibody to p-S473–Akt (1:1,000), mouse antibody to p-T308–Akt (1:1,000), rabbit antibody to CTMP (1:1,000), rabbit antibody to p-S9–GSK-3β (1:1,000), mouse antibody to GSK-3β (1:1,000), rabbit antibody to p-S256–FOXO3A (1:1,000), rabbit antibody to FOXO3A (1:1,000), rabbit antibody to p-S380/T382/T383–PTEN (1:1,000), rabbit antibody to PTEN (1:1,000), rabbit antibody to H3 (1:1,000), mouse antibody to eGFP (1:1,000, Abcam) and mouse antibody to β-actin (1:10,000, Sigma). All antibodies were from Cell Signaling unless otherwise indicated. For secondary antibodies, we used goat antibody to rabbit IgG (1:3,000, Vector Laboratories) except for β-actin, in which case we used horse antibody to mouse IgG (1:5,000, Vector Laboratories). pcDNA3 T7 Akt1 (plasmid 9003)[45] and pcDNA3 T7 Akt1 (T308A)(S473A) (plasmid 9030)[45] were obtained from the Addgene plasmid repository. pcDNA4-CTMP-IRES-GFP and pcDNA3.1-Myc-RFP-CTMP were cloned as described previously[29].

Akt kinase assay. Akt kinase activity assays were performed on lysates of microdissected CA1 tissue from control and experimental rats 1 h after the last surgery by means of a kinase activity assay kit (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′-ATA ACC TCC TAG CTG CTC CTG GTT TTG GCC ACT GAC TGA CCA GGA GCA TAG GAG GTT AT-3′), CTMP miRNA-2 (5′-GAA ACA AGG AGA CCG TCC TCT GTT TTG GCC ACT GAC TGA CAG AGG ACG CTC CTT GTT TC-3′) and nontargeting miRNA, a silencer-resistant miRNA sequence that does not target any known eukaryotic gene (5′-AAA TGT ACT GCG CGT GGA GAC GTT TTG GCC ACT GAC TGA CGT CTC CAC GCA GTA CAT TT-3′). The efficacy of CTMP miRNA-1/2 was evaluated by western blot 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, we cloned miRNAs in the pcDNATM 6.2-GW/EmGFP expression vector into self-inactivating lentiviral pRRLsin.cPPT.CMV.eGFP.Wpre[46] to generate CTMP miRNA-1 and miRNA-2 and nontargeting miRNA transfer constructs. To enhance CTMP silencing, we chained miRNA-1 and miRNA-2 into the same lentiviral vector. High-titer vesicular stomatitis virus–pseudotyped lentiviral stocks were produced in HEK-293T cells[47]. In brief, cells were transfected with the pRRL.PPT.hCMV.miRNA1/2.GFP.Wpre or pRRL.PPT.hCMV.nontargeting-miRNA.GFP.Wpre transfer constructs, the pMDLg/pRRE and pRSV-REV packaging constructs, and the envelope protein construct pMD2.G by means of calcium phosphate[46]. To determine viral titers, we transduced N2A cells with serial dilutions of concentrated lentivirus and evaluated GFP fluorescence by flow cytometry at 48 h and concentrated by ultracentrifugation as described previously[47]. Titers were 1×10^8 (CTMP miRNA1/2) and 1.8×10^8 (nontargeting miRNA) transducing units per ml after 200-fold concentration of vector supernatant.

RNAi-mediated CTMP silencing in intact rats. For *in vivo* experiments, CTMP miRNA-1/2 or nontargeting miRNA was delivered into the hippocampus of live rats by stereotaxic injection 14 d before global ischemia or sham surgery as described previously[44]. In brief, rats were placed in a stereotaxic frame, anesthetized with 4% isoflurane (vol/vol) and maintained on 1.5% isoflurane (vol/vol) 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-gauge needle at a flow rate of 0.2 μl min^{-1}. The needle was left in place for an additional 3 min and then gently withdrawn. Rats were injected subcutaneously with a single dose of flunixin (2.2 mg per kg of body weight) to limit inflammation. We subjected rats to global ischemia or sham surgery 14 d later and used them for histology experiments as described below. To evaluate the spread of the virus, we delivered lentiviral-GFP into the right hippocampus by stereotaxic injection. We killed the rats 14 d later and assessed GFP fluorescence in brain sections at the level of the dorsal hippocampus. Sections were viewed and images were acquired as described 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 post 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 signal[48]. The numbers 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* TUNEL staining 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 Sprague Dawley rat brains and plated on poly-L-lysine–coated coverslips (18 mm) at low density (100,000 cells per dish), as described previously[49]. Neurons were transfected by the calcium phosphate method.

Statistical analysis. All results are reported as mean ± s.e.m. and analyses were performed using Origin software (Origin Lab). Three experimental groups were compared by one-way analysis of variance 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 $P < 0.05$.

Note: Supplementary information is available on the Nature Neuroscience website.

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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. carried out western blot 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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