Prohibitin Viral Gene Transfer Protects Hippocampal CA1 Neurons From Ischemia and Ameliorates Postischemic Hippocampal Dysfunction

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Background and Purpose—Prohibitin is a multi-functional protein involved in numerous cellular activities. Prohibitin overexpression protects neurons from injury in vitro, but it is unclear whether prohibitin can protect selectively vulnerable hippocampal CA1 neurons in a clinically relevant injury model in vivo and, if so, whether the salvaged neurons remain functional.

Methods—A mouse model of transient forebrain ischemia that mimics the brain damage produced by cardiac arrest in humans was used to test whether prohibitin expression protects CA1 neurons from injury. Prohibitin-expressing viral vector was microinjected in mouse hippocampus to upregulate prohibitin.

Results—Prohibitin overexpression protected CA1 neurons from transient forebrain ischemia. The protection was associated with dampened postischemic reactive oxygen species generation, reduced mitochondrial cytochrome c release, and decreased caspase-3 activation. Importantly, the improvement in CA1 neuronal viability translated into an improvement in hippocampal function: prohibitin expression ameliorated the spatial memory deficit induced by ischemia, assessed by the Y-maze test, and restored postischemic synaptic plasticity assessed by long-term potentiation, indicating that the neurons spared form ischemic damage were functionally competent.

Conclusions—These data demonstrate that prohibitin overexpression protects highly vulnerable CA1 neurons from ischemic injury in vivo and suggest that the effect is mediated by reduction of postischemic reactive oxygen species generation and preservation of mitochondrial outer membrane integrity that prevents activation of apoptosis. Measures to enhance prohibitin expression could have translational value in ischemic brain injury and, possibly, other forms of brain injury associated with mitochondrial dysfunction. (Stroke. 2014;45:1131-1138.)

Key Words: brain ischemia ▪ mitochondria ▪ prohibitin

Prohibitin is a highly conserved protein that participates in a wide variety of cellular processes1,2 in multiple cellular compartments.3 In mitochondria, prohibitin acts as a scaffolding protein and, as such, is essential for the formation and maintenance of mitochondrial structure and for the stability of their nucleoids.4,5 Deletion of prohibitin is lethal, highlighting its essential biological role, and its upregulation is protective for intestinal epithelial cells, cardiomyocytes, and pancreatic-β cells.6-8

Prohibitin is also emerging as a key regulator of neuronal survival. It is upregulated in models of ischemic tolerance and in exercise-induced neuroplasticity,9,10 suggesting a cytoprotective role in brain. Accordingly, prohibitin expression in neuronal cultures is protective against excitotoxicity, reactive oxygen species (ROS), and apoptosis inducers.10 Conversely, ablation of prohibitin expression in mouse forebrain results in neuronal death.9 A major mechanism of prohibitin-mediated cytoprotection involves suppression of mitochondrial ROS production. Whereas upregulation of prohibitin expression in neurons dampens complex I-dependent ROS generation, downregulation of prohibitin expression increases mitochondrial ROS formation in response to glutamate.10 Therefore, prohibitin is an important modulator of neuronal survival through mitochondrial mechanisms. However, because of the lack of pharmacological approaches to specifically upregulate prohibitin in vivo, it has not been possible to determine whether prohibitin is neuroprotective in a clinically relevant animal model of neuronal injury. Considering the central role...
that mitochondria and oxidative stress play in cerebral ischemia–reperfusion injury.12-14 It is conceivable that prohibitin could confer neuroprotection in this highly prevalent and devastating form of brain injury.

In the present study, we tested this possibility using adeno-associated viral (AAV) gene transfer to express human prohibitin in the hippocampal CA1, a region selectively vulnerable to the deleterious effects of ischemia–reperfusion. We found that prohibitin expression in CA1 neurons protects them from the delayed cell death induced by transient forebrain ischemia, a model that mimics the brain damage produced by cardiac arrest in humans.15-17 The effect was associated with suppression of the early phase of posts ischemic ROS generation, as well as cytochrome c release and caspase-3 activation. Notably, the protection of CA1 neurons resulted in a marked improvement in posts ischemic hippocampal long-term potentiation (LTP) and in Y-maze performance. The findings provide the first evidence to date that the powerful neuroprotective capacity of prohibitin can be harnessed in a clinically relevant model of delayed neuronal death in vivo and raise the possibility that prohibitin upregulation could be a novel preventive strategy in ischemic injury and neurodegeneration.

**Materials and Methods**

**AAV Production and Injection In Vivo**

Prohibitin is a nuclear encoded protein. It is translated in the cytosol and imported into mitochondria through its N-terminal mitochondrial targeting sequence (amino acids, 1–18).18 The AAV-transduced prohibitin likely follows the same mitochondrial import pathway as the endogenous prohibitin. Recombinant AAV vectors were constructed in a di-cistronic vector containing green fluorescence protein (GFP) and prohibitin expressed under the control of a CMV promoter, as previously described.10 The same AAV vector, expressing GFP but not prohibitin, was used as control. The prohibitin-expressing AAV is designated as AAV-prohibitin-GFP. The AAV without prohibitin is designated as a vector control. Serotype 2 (AAV-2) was used in all experiments. We chose AAV-2 because of its low host immune response and minimal inflammatory stimulation19,20 and its high propensity for neuronal expression.21 Furthermore, our initial screening showed that AAV-2 achieved high neuron/glia transduction ratio in mixed primary cultures (data not shown). The AAV stock was produced by the University of Iowa Vector Core Facility.

All animal procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College. Stereotaxic AAV injections into hippocampal CA1 sector were performed in 4-week-old C57BL/6J mice as previously described22 and detailed in the online-only Data Supplement. AAV injection did not cause animal fatality.

**Transient Forebrain Ischemia by Bilateral Common Carotid Artery Occlusion**

Bilateral common carotid artery occlusion (BCCAO) was performed 3 weeks after AAV injection according to a procedure described previously23 and detailed in the Methods in the online-only Data Supplement. Briefly, after anesthesia, both common carotid arteries were exposed through a midline incision of the neck and occluded for 22 minutes. Sham-operated mice underwent the same procedures, except that their arteries were not occluded. The physiological parameters of mice before and after BCCAO are shown in the online-only Data Supplement (Figure I in the online-only Data Supplement). Of the total 210 mice that were subjected to the BCCAO procedure, 24 died before various experiments were performed, resulting in 11% attrition rate.

**Immunohistochemistry**

Mice were perfused transcardially with PBS followed by 4% paraformaldehyde in PBS. The brains were removed and coronal sections (thickness 10 μm) were cut in a cryostat and collected at 100-μm intervals for immunofluorescence staining with antibodies (MAP2 from Sigma; NeuN and GFAP from Millipore). Nuclear staining was performed with TO-PRO-3 iodide (Invitrogen).

**Cell Counts in CA1**

Cell counts were performed by an operator blinded to the treatment groups. Hippocampal cell death was assessed according to a standard protocol described previously.24 Briefly, brain sections were stained with hematoxylin and eosin. One 10-μm-thick section was collected every 100 μmol/L. Injury to the CA1 subfield was stained to determine the number of viable neurons by ImageJ (National Institutes of Health, Bethesda, MD). Viable neurons were defined as those with a blue hue, an intact plasma membrane, and a hypochromatic oval or round nucleus. Eosinophilic cells with a shrunken nucleus were defined as dead cells. Cell counting was performed in 5 sections per hippocampus starting at 1.4 mm posterior to bregma encompassing the core of the lesioned area (rostrocaudal levels, −1.4, −1.5, −1.6, −1.7, −1.8 mm from bregma).

**Determination of ROS Production**

ROS production was assessed in vivo using hydroethidine microfluorography, as previously described.25 Dihydroethidine (DHE; 10 mg/kg) was injected into the jugular vein 2.5 hours before brains were removed, frozen, and cut in a cryostat (thickness, 20 μm). Images were acquired by a computer-controlled digital monochrome camera. After subtracting the camera dark current, the pixel intensity of the fluorescent-oxidized hydroethidine signal was assessed in the hippocampal CA1 region, 5 sections per animal. The sum of the fluorescence intensities for each region was divided by the total number of pixels analyzed and expressed as relative fluorescence units.24 Fluorescence intensity was assessed 0, 1, 3, 6, 12, 24, 48, and 72 hours after BCCAO/reperfusion.

**Western Blotting and Cytochrome c Release**

The CA1 subregion of hippocampus was dissected as described,25 and cytochrome c released in the cytosol was assessed by a subcellular fractionation procedure described previously.26 For prohibitin Western blotting, AAV-prohibitin or vector control–injected hippocampus was homogenized in radioimmunoprecipitation assay buffer. Equal amounts of proteins were gel separated, transferred to membranes, and incubated with anti–cytochrome c antibody (Cell Signaling) or with anti-prohibitin antibody (Neomarker) in appropriate dilutions. Protein bands were detected and intensity quantitated using a Kodak Digital Imaging Station.

**Caspase-3 Activity Assay**

Caspase-3 activity in hippocampal CA1 tissues was assayed as described previously27 and detailed in the Methods in the online-only Data Supplement.

**Hippocampal Slice Preparation and Electrophysiology**

Electrophysiology experiments were performed as described previously28 and are described in the Methods in the online-only Data Supplement.

**Y-Maze Test**

Spatial working memory in mice was evaluated by the Y-maze test, as previously described.29,30 We chose this test because it is less stressful and more consistent with the natural behavior of mice than other...
spatial memory tests, such as the Morris water maze. Furthermore, the Y-maze test does not involve learning new rules and takes advantage of the natural tendency of rodents to explore new environments. The operator was blinded to the treatment that the mice had received. Before and 7 days after BCCAO, each mouse was placed at the end of one arm and allowed to freely explore the apparatus for 8 minutes. The sequence and number of all arm entries were recorded for each animal throughout the period. Alternation rate was defined as entries into all 3 arms on consecutive occasions using the following formula: alternation rate (%) = number of alternations/number of total arm entries − 2 × 100. Trials in which the number of total arm entries was < 10 were not included in the analysis.

Statistical Analysis
Data are expressed as mean±SEM. Differences between multiple groups were statistically evaluated by the 1-way ANOVA followed by the appropriate post hoc tests.

Results

AAV-Mediated Prohibitin Gene Transfer in Mouse Hippocampus
First, we examined the regional pattern and cellular localization of gene expression in the hippocampus injected with AAV-prohibitin-GFP. Maximal levels of gene expression of prohibitin-GFP di-cistronic construct were observed 3 weeks after AAV-prohibitin-GFP injection. Prohibitin-GFP expression was restricted mostly to the injected hippocampus (Figure 1A) and observed mostly in neurons, although sparse astrocytic processes were also positive (Figure II in the online-only Data Supplement). Prohibitin expression assessed by Western blot was also increased in the hippocampus injected with AAV-prohibitin-GFP, but not vector (Figure 1B and 1C).

Prohibitin Viral Gene Transfer in CA1 Protects Against BCCAO-Induced Cell Death

Next, we tested the effect of prohibitin viral gene transfer on hippocampal CA1 injury produced by transient forebrain ischemia. In mice injected with the control vector, BCCAO caused extensive cell death in CA1 assessed by morphological criteria. However, in mice that received AAV-prohibitin-GFP injections, postischemic cell death was markedly reduced (Figure 2A). In postischemic mice treated with control vector, cell nuclei in CA1 seemed smaller and GFP expression was not observed in cells with neuronal morphology (Figure 2B, upper). In contrast, in mice treated with AAV-prohibitin-GFP, most nuclei had a normal appearance and several prohibitin-GFP positive neurons were observed in CA1 (Figure 2B, lower). Because the magnitude of cell death induced by BCCAO in CA1 differs rostrocaudally, we examined the degree of protection conferred by AAV-prohibitin-GFP gene transfer at different rostrocaudal levels of the hippocampus (Figure 2C). Three-dimensional reconstruction from serial sections demonstrated that the ischemic lesion was located entirely within the volume of tissue expressing prohibitin (Figure 2C). A reduction in cell death was observed at all rostrocaudal levels, although the protection tended to be more pronounced caudally, where the injury was less severe (Figure 2D). Collectively, these results demonstrate that AAV-prohibitin-GFP gene transfer protects CA1 cells from the deleterious effects of transient forebrain ischemia.

Prohibitin Viral Gene Transfer in CA1 Attenuates Postischemic ROS Production

Next, we sought to investigate the mechanisms of the protective effect of prohibitin gene transfer in CA1. Oxidative stress is a key factor in ischemic brain damage. Therefore, we examined whether the protective effect of prohibitin could be attributed to reduced postischemic ROS production. ROS production in mice not injected with AAV, assessed by DHE fluorimicroscopy, exhibited a biphasic time course with peaks at 3 and 72 hours after reperfusion (Figure 3A). As shown in Figure 3B and 3C, AAV-prohibitin-GFP injection significantly decreased the DHE signal in hippocampus at both time points (3 and 72 hours) compared with samples from vector-injected mice. Therefore, prohibitin viral gene transfer suppresses the postischemic increase in damaging ROS in CA1.

Prohibitin Viral Gene Transfer Attenuates Postischemic Cytochrome c Release From Mitochondria and Caspase-3 Activation

Having established that prohibitin viral gene transfer attenuated postischemic ROS production, we examined whether cytochrome c release and caspase activation, critical steps downstream of ROS in delayed postischemic hippocampal...
damage,33 were also attenuated by prohibitin expression. Released hippocampal cytochrome c was assayed in the cytosolic fraction, after subcellular fractionation. As previously reported,34 cytosolic cytochrome c increased at 6 hours, returned to baseline at 12 hours, rose again at 24 hours and reached a second peak at 48 hours, after BCCAO (Figure IIIA and IIIB in the online-only Data Supplement). Prohibitin viral gene transfer attenuated cytochrome c release at 48 hours after BCCAO (Figure 4A and 4B). Because cytochrome c release has been linked to postischemic caspase activation and apoptotic cell death in CA1,35 we next sought to determine whether prohibitin viral gene transfer reduced also postischemic caspase-3 activation. Hippocampal caspase-3 activation exhibited a biphasic pattern, which was delayed relative to that of cytochrome c release, with peaks at 8 and 72 hours after BCCAO (Figure 4C). Because the 72-hour peak in caspase activation is thought to contribute to neuronal death after forebrain ischemia,36 we assessed the effect of prohibitin viral gene transfer at this time point. As illustrated in Figure 4D, injection of AAV-prohibitin-GFP, but not vector, attenuated caspase-3 activity. Therefore, prohibitin viral gene transfer attenuated postischemic cytochrome c release from mitochondria and the ensuing caspase-3 activation in CA1.

Prohibitin Viral Gene Transfer Ameliorates Postischemic Alterations in Hippocampal LTP and Y-Maze Performance

Finally, we sought to determine whether the neuroprotection exerted by prohibitin viral gene transfer in CA1 is associated with improvement in postischemic hippocampal function. Hippocampal LTP is impaired by transient forebrain ischemia.37,38 Therefore, we examined whether prohibitin viral gene transfer could counteract the LTP alterations induced by BCCAO. Consistent with hippocampal injury, LTP was suppressed in hippocampal slices 7 days after BCCAO in mice injected with vector (Figure 5A–5C). In contrast, hippocampal LTP was well developed in mice that received AAV-prohibitin-GFP (Figure 5A–5C). In addition, prohibitin viral gene transfer did not alter LTP in hippocampal slices from vector-injected mice (Figure 5D). To provide evidence of cognitive preservation by prohibitin viral gene transfer, we examined the performance of the mice at the Y-maze (arm alternation) test, a sensitive and widely used approach to assess hippocampus-dependent spatial memory.39–41 Mice injected with vector or prohibitin-AAV were tested before and 7 days after sham operation or BCCAO. Before BCCAO, there were no differences in spontaneous arm alternations among groups (Figure 6B). Seven days after BCCAO, the vector group showed reduced spontaneous alternations (Figure 6B), suggestive of...
Prohibitin (PHB) expression attenuated cytochrome (c) release and postischemic caspase-3 activity in the hippocampus after bilateral common carotid artery occlusion (BCCAO). PHB expression reduced caspase-3 activity in hippocampal CA1 of noninjected mice. *P<0.05, compared with sham group; #P<0.05, compared with vector group, 1-way ANOVA analysis followed by Dunnett test; n=6 per group. Figure 4. Prohibitin (PHB) expression attenuated cytochrome (c) release and postischemic caspase-3 activity in the hippocampus after bilateral common carotid artery occlusion (BCCAO). PHB expression reduced caspase-3 activity in hippocampal CA1 of noninjected mice. *P<0.05, compared with sham group; #P<0.05, compared with vector group, 1-way ANOVA analysis followed by Tukey test, n=4 per group. D, PHB expression reduced caspase-3 activity in hippocampal CA1 measured at 3 days after BCCAO. *P<0.05, compared with sham group; #P<0.05, compared with vector group, n=6 per group.

Prohibitin Viral Gene Transfer Suppresses Postischemic ROS Production in CA1

The major contributing factors causing delayed neuronal death in the vulnerable hippocampal CA1 subregion in the transient forebrain ischemia model include oxidative stress and activation of the apoptotic cell death pathway.10 In agreement with previous reports,10 we observed a biphasic increase in ROS in CA1 3 hours and 3 days after BCCAO. The initial ROS peak is thought to trigger downstream events leading to delayed cell death.49 Therefore, the observation that prohibitin viral gene transfer dampens the early ROS peak suggests that this effect is involved in the attendant protection from delayed neuronal death.

The mechanisms by which prohibitin suppresses ROS production remain to be elucidated. Prohibitin reduces complex I–generated ROS because of rotenone inhibition in neuronal mitochondria and vice versa.53 Consequently, more stress could be sustained before the respiratory chain complexes succumb to oxidative damage, giving rise to a vicious cycle of ROS production. Irrespective of the mitochondrial mechanisms of the effect, the attenuation of postischemic ROS production by prohibitin is partial, suggesting that either mitochondrial radical sources are not completely stabilized or other, nonrespiratory chain dependent, ROS generating pathways are also involved. An alternative source of ROS after transient global ischemia is the superoxide-producing enzyme NADPH oxidase,54 but it remains to be established whether prohibitin overexpression dampens this ROS source as well. Given the proximity of NADPH oxidase subunits to neuronal mitochondria,55 it is likely that NADPH oxidase–generated ROS may trigger additional ROS production from neuronal mitochondria and vice versa.56,57 Therefore, inhibition of either source may lower total ROS production.
Inflammatory response is another factor contributing to postischemic brain injury. However, recent data indicate that inflammation is likely to be downstream of the early ROS production observed in transient forebrain ischemia. Therefore, it would be of interest to investigate whether prohibitin also suppresses postischemic inflammation in this model.

Prohibitin Viral Gene Transfer Reduces Postischemic Cytochrome c Release and Caspase-3 Activation

Because mitochondria play a key role in apoptotic cell death in CA1, we examined the effect of prohibitin viral gene transfer on the mitochondrial release of cytochrome c and on the attendant caspase-3 activation. Prohibitin viral gene transfer markedly attenuated cytochrome c release and caspase-3 activation in the postischemic hippocampus, implicating inhibition of the mitochondrial apoptotic pathway in the mechanisms of protection. Considering that oxidative stress leads to mitochondrial permeability transition, cytochrome c release, and apoptosis, it is conceivable that prohibitin suppresses the ROS increase at 3 hours after ischemia, thereby preventing mitochondrial permeability transition, the resulting reduction in cytochrome c release and caspase-3 activation.

Prohibitin Viral Gene Transfer Improves Postischemic Hippocampal Function and the Y-Maze Performance

The damage to CA1 induced by forebrain ischemia is associated with deficits in hippocampal function and behavioral performance. The decrease in the postischemic suppression
of LTP indicates that the CA1 neurons salvaged by prohibitin gene transfer are able to express synaptic strengthening in response to tetanic stimulation of their inputs, a physiological characteristic of these neurons thought to play a role in learning and memory.53 The improvement in LTP involved the maintenance phase and not the induction phase of the potentiation. The detailed neurophysiological and molecular mechanisms associated with the phenomenon together with the effect of prohibitin expression on the alterations in LTP induced by forebrain ischemia remain to be elucidated.

In agreement with the LTP improvement, we also observed an improvement in the cognitive performance assessed by the Y-maze test, indicating preservation of hippocampal circuits involved in spatial working memory.99 However, we cannot rule out the behavioral improvement resulted from an enhancement of recovery of function induced by prohibitin expression. We used the Y-maze to explore cognitive function because this method relies on the natural inclination of mice to explore and, as such, does not require training, is not stressful, and is a sensitive indicator of posts ischemic hippocampal dysfunction.42 Collectively, these observations provide evidence that the CA1 neurons spared from cell death by prohibitin viral gene transfer not only seem to be morphologically intact but are also functionally competent. Another issue concerns whether prohibitin expression improves the baseline performance of intact CA1 neurons. Our data suggest that this is not the case because prohibitin viral gene transfer did not enhance LTP or Y-maze performance in naïve mice. Therefore, the improvement of hippocampal function is likely the direct consequence of the protection from posts ischemic degeneration of CA1 neurons because of viral-mediated prohibitin expression.

In conclusion, we have shown for the first time that prohibitin expression mediated by viral gene transfer protects hippocampal CA1 neurons from injury and preserves neuronal function after transient forebrain ischemia. Although the full translational potential of prohibitin remains to be assessed, the present results provide proof of principle that upregulation of prohibitin is beneficial to the posts ischemic brain in vivo and, as such, could be a novel approach to protect the brain in neurologic diseases associated with acute or chronic neuronal degeneration because of mitochondrial dysfunction.

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Disclosures

None.

References


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 Supplemental Methods

AAV injection in mouse hippocampus
Stereotaxic AAV injections into the CA1 sector of the hippocampus were performed in 4-week-old C57Bl/6 mice. Under isoflurane anesthesia (1.5 % to 2.0 %), a midline skin incision was made between the bregma and interaural line. A 2 mm hole was drilled in the skull and AAV-PHB or AAV-control virus in 1 µl Ringer solution (titer: $10^{12}$-$10^{13}$ genomic copy/ml) were injected into CA1 using a glass micropipette. The stereotaxic coordinates were 1.5 mm posterior to bregma, 0.9 mm lateral to the midline and 2.1mm below the dura. The solution was slowly injected over 30 min and the needle was left in place for an additional 10 min. The needle was then slowly withdrawn and the incision closed. AAV-injected mice were used for experiments after three weeks, a period determined in pilot studies to be necessary for the full expression of viral mediated gene expression.

Transient forebrain ischemia by bilateral common carotid artery occlusion
Mice were anesthetized with a mixture of isoflurane (1.8-2%), oxygen (30%), and nitrogen (70%). Fiber-optic probes were glued to both parietal bones (3 mm lateral, 2 mm caudal to bregma) and connected to a laser-Doppler flowmeter (Periflux System 5000; Perimed, Järfälla, Sweden) for continuous monitoring of cerebral blood flow (CBF) in the neocortex. During surgery body temperature was monitored and maintained between 36.5° and 37.5°C using a thermostatically controlled heating pad. Through a midline incision of the neck, 4-0 surgical threads were loosely placed around both common carotid arteries and the arteries were tied for 22 min. Only animals that exhibited 90% reduction in CBF within the first minute of occlusion and CBF recovered by 80% after 10 min of reperfusion were included in this study. After surgery, mice were kept at 37°C for 24 hours to prevent post-ischemic hypothermia. Thereafter, mice were returned to general housing (22-24°C). Sham-operated mice underwent the same procedures, except that their arteries were not occluded. Twenty-one % of all animals used in this study were excluded because of insufficient CBF reduction. About 11% of animals died after surgery and were also excluded from the study. The percentage of excluded animals was similar in AAV-vector and AAV-PHB treated groups. Animals were randomly assigned to the treatment groups.

Caspase-3 activity assay
Caspase-3 activity in hippocampal tissues was assayed as described previously. Briefly, at the designated time points the hippocampus was quickly dissected and frozen in liquid nitrogen. The tissue was homogenized in lysis buffer (25mMol/L Hepes, pH 7.4, 0.1% Triton X-100, 5mMol/L MgCl₂, 2mMol/L DTT, 1.5mMol/L EDTA, 1mMol/L EGTA, 1x protease inhibitor cocktail). After a 10 min spin in a table top centrifuge at 10,000xg, the supernatant was mixed with equal volume of 2x assay buffer containing DEVD-afc as caspase-3 substrate in a 96 well plate. After 30 min of incubation at 37°C, the fluorescence intensity was measured in a fluorescence plate reader (405 nm excitation, 505 nm emission wavelength, respectively). The fluorescence intensity was normalized to the amount of protein in samples and expressed as relative fluorescence intensity per mg protein (RFI/mg protein).
Hippocampal slice preparation and electrophysiology.

400 µm hippocampal slices were prepared using a vibratome as described previously. The slices were maintained at room temperature in a submersion chamber with artificial cerebrospinal fluid (ACSF) containing (in mM/µL) 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 1.25 NaH2PO4, 24 NaHCO3, and 15 glucose, bubbled with 95% O2/5% CO2. Slices were incubated for at least 2 hours before the experiments and the operator was blinded to the treatment groups. For electrophysiology experiments, slices were transferred to recording chambers (preheated to 32 ºC) where they were superfused with oxygenated ACSF. Monophasic, constant-current stimuli (100 µsec) were delivered with a bipolar silver electrode placed in the stratum radiatum of area CA3, and the field excitatory postsynaptic potentials (fEPSPs) were recorded in the stratum radiatum of area CA1 with electrodes filled with ACSF (Re = 2–4 MΩ). Baseline fEPSPs were monitored by delivering stimuli at 0.033 Hz. fEPSPs were acquired, and amplitudes and maximum initial slopes measured, using pClamp 10 (Axon Instruments, Foster City, CA). LTP was induced with a high-frequency stimulation (HFS) protocol consisting of two 1-second long 100 Hz trains, separated by 60 seconds, delivered at 70-80% of the intensity that evoked spiked fEPSPs.

References for Supplemental Methods

Supplemental figure I. Physiological parameters of mice before and after BCCAO. (A). Cerebral blood flow (CBF) measurement by a laser-Doppler flowmeter before and after BCCAO. (B). Body temperature measurement of mice 24 hrs after BCCAO/reperfusion. (C). Mean blood pressure (MBP) measurements 24hrs before and after BCCAO. All measures were obtained from 4 mice and statistically not significant (n.s.).
Supplemental figure II. AAV-mediated PHB expression in the mouse hippocampus. Three weeks after AAV injection, brain sections were immunostained with the neuronal marker NeuN (red) and glial marker GFAP (red) to assess the identity of GFP positive cells. Most GFP-expressing cells co-localize with NeuN positive cells (top panels, arrows), while few GFP cells co-localize with GFAP positive processes (Lower panels, arrows). Scale bar is 75 μm.
Supplemental figure III. Biphasic cyt c cytosol release from mitochondria following BCCAO in hippocampal CA1. (A) Temporal pattern of cyt c release in hippocampus after BCCAO detected by western blotting. Hippocampal CA1 tissues were dissected and the cytosolic fractions (10µg/lane) prepared at different reperfusion time points following BCCAO were loaded on gels. (B) Protein band intensity quantitation from data in (A). *p<0.05 compared to cytosolic fractions of non-stroked mice by one-way ANOVA analysis followed by Dunnett’s test; n=4/group. The difference between the peaks at 6hrs and 48hrs after BCCAO is not statistically significant.