Tsc1 (hamartin) confers neuroprotection against ischemia by inducing autophagy

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Previous attempts to identify neuroprotective targets by studying the ischemic cascade and devising ways to suppress it have failed to translate to efficacious therapies for acute ischemic stroke. We hypothesized that studying the molecular determinants of endogenous neuroprotection in two well-established paradigms, the resistance of CA3 hippocampal neurons to global ischemia and the tolerance conferred by ischemic preconditioning (IPC), would reveal new neuroprotective targets. We found that the product of the tuberous sclerosis complex 1 gene (TSC1), hamartin, is selectively induced by ischemia in hippocampal CA3 neurons. In CA1 neurons, hamartin was unaffected by ischemia but was upregulated by IPC preceding ischemia, which protects the otherwise vulnerable CA1 cells. Suppression of hamartin expression with TSC1 shRNA viral vectors both in vitro and in vivo increased the vulnerability of neurons to cell death following oxygen glucose deprivation (OGD) and ischemia. In vivo, suppression of TSC1 expression increased locomotor activity and decreased habituation in a hippocampal-dependent task. Overexpression of hamartin increased resistance to OGD by inducing productive autophagy through an mTORC1-dependent mechanism.

Transient global ischemia can be caused by cardiac arrest or open heart surgery, resulting in selective and delayed cell death of CA1 hippocampal neurons. Notably, neighboring CA3 neurons are resistant to such an ischemic insult. This differential response remains unexplained, despite extensive research focused on understanding the vulnerability of CA1 neurons to ischemia. To identify the mechanisms responsible for the resistance of CA3 neurons to ischemia, we performed label-free proteomic analysis of CA1 and CA3 hippocampal regions from rats subjected to either sham ischemia (n = 5) or 10-min severe forebrain ischemia (n = 5), induced by bilateral occlusion of both vertebral and common carotid arteries, followed by 24 h of reperfusion (Supplementary Fig. 1). Subcellular fractionation of CA1 and CA3 regions generated membrane and cytoplasmic fractions, allowing us to enrich for membrane proteins and increase the number of proteins identified (Supplementary Fig. 2). We pooled data sets from the membrane and cytoplasmic fractions from each experimental group to increase the power of identifying relevant pathways.

We carried out a detailed comparison and ontological analyses between the different data sets (Supplementary Figs. 3–5, Supplementary Tables 1–6 and Supplementary Results and Discussion). We also conducted ingenuity pathway analysis (IPA) to identify proteins and pathways of interest in a nonbiased manner. We compared the pathways significantly altered by ischemia within CA1 and CA3 regions and identified those proteins selectively induced within CA3. In CA3 neurons, the PI3K-Akt intracellular signaling pathway was most significantly associated with the protein expression changes induced by ischemia compared to sham CA3 (P = 0.00032; Fig. 1a and Supplementary Table 7). The expression of several proteins within this pathway, including hamartin and 14-3-3 (γ, ε and θ), was higher following ischemia as compared to sham CA3. The neuroprotective properties of this pathway are diverse and well documented for global and focal ischemia, as well as for subarachnoid hemorrhage. In addition to canonical pathways, IPA identifies networks of interacting proteins. Hamartin was part of a network of proteins most significantly associated with expression changes induced by ischemia in the CA3 region compared to sham CA3 (P < 0.05; Fig. 1b and Supplementary Table 8).

Our proteomic data sets were corroborated by immunoblotting experiments (Supplementary Fig. 6) and are consistent with previous studies (Supplementary Results and Discussion). The identification of hamartin at all levels of our analysis (pathways, networks and individual proteins; Supplementary Fig. 7, Supplementary Table 9)

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Received 13 August 2012; accepted 18 January 2013; published online 24 February 2013; doi:10.1038/nm.3097
Hamartin expression is selectively induced in the CA3 hippocampal area following ischemia. (a) Illustration of the PI3K-Akt pathway, which was significantly associated with and selectively induced in the CA3 region following ischemia ($P = 0.000319$, right-tailed Benjamini-Hochberg multiple testing correction). Proteins are described in Supplementary Table 7. (b) Diagram of a protein network showing changes in expression levels significantly associated with ischemia in the CA3 region. In a and b, proteins in pink and yellow were upregulated and downregulated, respectively, by ischemia. Proteins in gray were detected by the proteomic analysis, but their expression was unaffected. Proteins in white participate in the pathway significantly associated with ischemia in the CA3 region. In

Figure 1

Hamartin expression is selectively induced in the CA3 hippocampal area following ischemia. (a) Illustration of the PI3K-Akt pathway, which was significantly associated with and selectively induced in the CA3 region following ischemia ($P = 0.000319$, right-tailed Benjamini-Hochberg multiple testing correction). Proteins are described in Supplementary Table 7. (b) Diagram of a protein network showing changes in expression levels significantly associated with ischemia in the CA3 region. In a and b, proteins in pink and yellow were upregulated and downregulated, respectively, by ischemia. Proteins in gray were detected by the proteomic analysis, but their expression was unaffected. Proteins in white participate in the pathway significantly associated with ischemia in the CA3 region. In

Hamartin was also upregulated in the CA1 region following IPC, where a short duration of ischemia protects these otherwise vulnerable cells from neuronal death induced by severe forebrain ischemia. After ischemia, hamartin protein expression was significantly higher ($P < 0.01$) in the CA1 region of rats subjected to 2 min IPC compared to sham ischemia. To confirm whether hamartin expression alters neuronal vulnerability to ischemia, we carried out OGD studies in rat hippocampal neurons. We silenced hamartin expression using a lentiviral vector expressing an shRNA targeting TSC1 (TSC1 shRNA;
Figure 2 Hamartin regulates neuronal susceptibility to OGD-induced cell death. (a) Schematic of in vitro experiments on hippocampal cultures transduced with shRNA vectors and exposed to OGD. DIV, days in vitro. (b,c) Representative phase photomicrographs (b) and quantification of cell death (c) following OGD and normoxia by lactate dehydrogenase (LDH). Cells were untransduced or treated with either TSC1 or control shRNA vectors (n = 8–13; one-way ANOVA with Bonferroni’s post hoc test, ****P < 0.0001). (d) Merged fluorescent images from cultures treated as shown, stained with ethidium homodimer III (red) and Hoechst 33342 (blue). (e) Quantification of the necrotic neurons in d as a percentage of total cells (n = 4–8 one-way ANOVA with Bonferroni’s post hoc test, ****P < 0.0001). (f) Experimental design of in vitro experiments for hippocampal cultures transduced with rat TSC1 vectors and exposed to OGD. (g) Immunofluorescent images of cultures transduced with GFP or rat TSC1 (left). Hoechst 33342 was used for nuclear staining (middle). Merged images are shown on the right. (h) Representative immunoblots of cultures transduced with Myc-tagged rat TSC1 (n = 3). (i) Quantification of neurons surviving OGD and 24 h of reperfusion, normalized to intact nuclei counts of OGD to normoxia for GFP-transduced cultures (n = 12; two-tailed t-test, **P < 0.01). (j) Representative phase photomicrographs of hippocampal cultures from the experiments quantified in i. Scale bars, 50 µm. All data are expressed as mean ± s.e.m.

Supplementary Fig. 9), and subjected cultures treated with a TSC1 shRNA vector to 3 h OGD and 24 h reperfusion (Fig. 2a). Viability assays revealed that TSC1 shRNA–transduced cultures showed 34 ± 6.7% higher cell death relative to control shRNA–transduced cultures (P < 0.0001; Fig. 2b,c). We observed a similar effect with rat cortical neurons (Supplementary Fig. 10). To control for TSC1 shRNA off-target effects, we rescued hamartin expression in TSC1 shRNA–treated hippocampal cultures with a lentiviral vector expressing human TSC1 (Supplementary Fig. 11) and found that overexpression of hamartin reduced cell death after OGD to control levels, suggesting the knockdown is specific (Fig. 2d,e). Our results are consistent with studies on Tsc1−/− mouse embryonic fibroblasts showing increased apoptosis after glucose deprivation15. Additionally, Tsc1−/− hippocampal neurons show high susceptibility to oxidative and endoplasmic reticulum stress16 and have high sensitivity to spontaneous glutamate release17. As oxidative stress and glutamate-induced excitotoxicity are major components of ischemic cell death, our findings suggest Tsc1–conditional-knockout mice may be very sensitive to ischemia.

As hamartin suppression in vitro rendered neurons more vulnerable to ischemia, we investigated whether hamartin is sufficient to protect neurons from ischemic insults (Fig. 2f). Rat hippocampal neurons transduced with a lentiviral vector expressing rat Tsc1-eGFP (rat TSC1) overexpressed hamartin compared to neurons treated with a control vector expressing eGFP alone (GFP) (Fig. 2g,h). To detect exogenous expression of full-length hamartin, the rat TSC1 lentiviral vector contained a c-Myc tag at the 3′ end of the Tsc1 sequence (Fig. 2h). Transduction efficiency assessed by EGF expression was ~50% (Fig. 2g). Hippocampal neurons transduced with rat TSC1 showed significantly higher resistance to OGD and reperfusion compared to GFP-transduced neurons. The number of cells surviving OGD versus normoxia was 31 ± 8.6% higher in rat TSC1– compared to GFP-transduced cells, (P = 0.0066; Fig. 2j). These results provide direct evidence that hamartin is neuroprotective.

To translate our findings to an in vivo paradigm, we used the same shRNA lentiviral vectors to suppress hamartin expression in rat CA3 neurons. Hamartin expression was significantly reduced (P < 0.01) in CA3 neurons 14 d after unilateral administration of the shRNA vector targeting TSC1 (Supplementary Fig. 12). The magnitude of suppression was less than that achieved in vitro, perhaps as a result of there being both transduced and untransduced cells in the microdissected tissue. Fourteen days after administration of TSC1 shRNA and control shRNA into the ipsilateral and contralateral hippocampus, respectively, we subjected rats to ischemia or sham ischemia (Fig. 3a). Because global ischemia produces a uniform bilateral insult, we used the contralateral side as control. After ischemia, the ipsilateral TSC1
shRNA–treated side had 105.9 ± 4.0 neurons per mm in the CA3 region compared to 139.2 ± 3.2 neurons per mm on the contralateral control shRNA–treated side (P < 0.01; Fig. 3b,c). Activation of compensatory mechanisms in some CA3 cells with suppressed Tsc1 expression, as well as a nonlinear relationship between shRNA knockdown efficiency and loss of CA3 neuronal resistance, could explain the modest effect of TSC1 shRNA. Tsc1 knockdown did not alter neuronal cell number in the CA3 region of sham-operated rats (Fig. 3b,c). These data indicate that the resistance of CA3 neurons to ischemia is mediated by upregulation of hamartin.

We also examined whether hippocampal function was affected by Tsc1 suppression in CA3 neurons using an open-field test18, as these neurons participate in acquisition and encoding of spatial information19. We quantified horizontal and vertical locomotor activity by measuring the number of boxes crossed and rears performed. Naive rats subjected to sham ischemia showed the expected pattern of habituation after repeated testing, with a significant decrease in both the number of boxes crossed and rears performed (Fig. 3d,e). Ischemia results in loss of habituation manifested by increased locomotor activity, which is consistent with the extent of neuronal loss in the pyramidal layer of the hippocampus20. Rats injected with either TSC1 shRNA or control shRNA bilaterally in the CA3 region showed a significant increase in locomotor activity after ischemia (Fig. 3d,e). Notably, TSC1 shRNA–treated rats had a significantly higher increase in both parameters compared to control shRNA–treated rats (P < 0.05). Quantification of CA3 neurons after ischemia (Supplementary Fig. 13) revealed similar results to the unilateral experiments, indicating that the increased locomotor activity and loss of habituation were associated with a loss of CA3 neurons.

An important downstream function of hamartin and its partner tuberin is mTORC1 suppression via direct inhibition of the small GTPase Rheb21. To examine the role of mTORC1 in the vulnerability of hippocampal neurons to OGD after hamartin suppression, we monitored mTORC1 activity by assaying phosphorylation levels of S6 ribosomal protein (S6RP). S6RP phosphorylation was higher in TSC1 shRNA–treated rat hippocampal cells compared to control shRNA–transduced cells, suggesting increased mTORC1 activity (Fig. 4a). Treatment of TSC1 shRNA–transduced cultures with the mTORC1 inhibitor rapamycin prevented S6RP phosphorylation and increased neuronal survival in response to OGD to 70.9 ± 4.71% from 45.9 ± 3.19% for cultures treated with TSC1 shRNA alone (Fig. 4a,b). Therefore, silencing of hamartin exacerbates OGD-induced injury by activating mTORC1. Of note, rapamycin has been a promising candidate for treatment in Parkinson’s disease22, cardioprotection23 and focal cerebral ischemia24.

mTORC1 is an inhibitor of autophagy, a catabolic process implicated in ischemic pathophysiology25. Autophagy degrades damaged organelles and protein aggregates, enclosing them inside autophagosomes and digesting them with hydrolases after autophagosome fusion with lysosomes. Efficient completion of this cascade is termed productive autophagy25. Because hamartin indirectly inhibits mTORC1 activity, we deciphered its neuroprotective mechanism by assessing autophagy. Upon induction of autophagy, microtubule-associated protein 1 light chain 3 (LC3) is processed from a 16-kDa form (LC3-I) to a 14-kDa form (LC3-II), which is recruited to autophagosomes and is an indicator of autophagosome formation26. LC3-II expression was significantly higher in TSC1 shRNA–transduced compared to control shRNA–transduced rat hippocampal cultures following...
Hamartin promotes neuronal survival by inhibiting mTORC1 and inducing productive autophagy. (a) Representative immunoblots of phosphorylated S6RP expression in control shRNA– or TSC1 shRNA–transduced rat hippocampal neuronal cultures (under normoxia or OGD) in the presence or absence of 10 nM rapamycin (n = 3). (b) Quantification of percentage survival (OGD to normoxia nuclear counts ratio) from control shRNA– or TSC1 shRNA–transduced cultures, with or without 10 nM rapamycin (n = 3). (c,d) Representative immunoblots depicting LC3-I, LC3-II and p62 expression (c) and quantification of LC3-II and p62 levels (d) in the hippocampal cultures described in a (n = 3). (e) Quantification of macroautophagy-dependent (3MA-sensitive) lysosomal degradation in control transduced– and TSC1 shRNA–transduced cultures (n = 3). (f,g) Representative immunoblots of LC3-I, LC3-II and p62 expression (f) and quantification of LC3-I and p62 expression (g) from GFP– and rat TSC1–transduced cultures exposed to normoxia or OGD (n = 3). (h) Quantification of macroautophagy-dependent lysosomal degradation in GFP– and rat TSC1–transduced cultures (n = 3). (i) Quantification of percentage survival (OGD to normoxia nuclear count ratio) of GFP– and rat TSC1–transduced cultures, described in Figure 2i with or without 3MA (10 mM). For d and g, data are normalized to control shRNA–treated levels. Sypro staining was used as loading control. For e and h, data are normalized to 3MA-sensitive degradation from control shRNA–transduced cultures following normoxia. Values are mean ± s.e.m. of n = 3 independent experiments, and within each experiment at least triplicate samples per condition were assessed (two-way ANOVA with Bonferroni’s post hoc test (b,d,e,h,i) or two tailed t-test (g), *P < 0.05, **P < 0.01, ***P < 0.001).

As suppression of hamartin expression activated mTORC1, these results suggested that autophagosome accumulation resulted from impaired autophagic flux (fusion and degradation of autophagosomes with lysosomes). Expression of sequestosome-1 (p62) protein, which is degraded by lysosomes and accumulates following impairments in autophagy, was significantly increased by suppression of hamartin compared to control shRNA–transduced rat hippocampal cultures following both normoxia and OGD (Fig. 4c,d). We found further evidence for impaired autophagy by measuring lysosomal degradation of long-lived proteins during macroautophagy. We used the autophagy inhibitor 3-methyladenine (3MA) to dissect the macroautophagy component of lysosomal activity. During normoxia, TSC1 shRNA–transduced neurons showed significantly less macroautophagy-dependent

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**Figure 4** Hamartin promotes neuronal survival by inhibiting mTORC1 and inducing productive autophagy. (a) Representative immunoblots of phosphorylated S6RP expression in control shRNA– or TSC1 shRNA–transduced rat hippocampal neuronal cultures (under normoxia or OGD) in the presence or absence of 10 nM rapamycin (n = 3). (b) Quantification of percentage survival (OGD to normoxia nuclear counts ratio) from control shRNA– or TSC1 shRNA–transduced cultures, with or without 10 nM rapamycin (n = 3). (c,d) Representative immunoblots depicting LC3-I, LC3-II and p62 expression (c) and quantification of LC3-II and p62 levels (d) in the hippocampal cultures described in a (n = 3). (e) Quantification of macroautophagy-dependent (3MA-sensitive) lysosomal degradation in control transduced– and TSC1 shRNA–transduced cultures (n = 3). (f,g) Representative immunoblots of LC3-I, LC3-II and p62 expression (f) and quantification of LC3-I and p62 expression (g) from GFP– and rat TSC1–transduced cultures exposed to normoxia or OGD (n = 3). (h) Quantification of macroautophagy-dependent lysosomal degradation in GFP– and rat TSC1–transduced cultures (n = 3). (i) Quantification of percentage survival (OGD to normoxia nuclear count ratio) of GFP– and rat TSC1–transduced cultures, described in Figure 2i with or without 3MA (10 mM). For d and g, data are normalized to control shRNA–treated levels. Sypro staining was used as loading control. For e and h, data are normalized to 3MA-sensitive degradation from control shRNA–transduced cultures following normoxia. Values are mean ± s.e.m. of n = 3 independent experiments, and within each experiment at least triplicate samples per condition were assessed (two-way ANOVA with Bonferroni’s post hoc test (b,d,e,h,i) or two tailed t-test (g), *P < 0.05, **P < 0.01, ***P < 0.001).
degradation (down 53 ± 10% as compared to control shRNA-transduced cultures), which was completely diminished after OGD (Fig. 4c). Therefore, suppression of hamartin expression results in the accumulation of autophagosomes and impaired autophagic flux both in normoxia and OGD in hippocampal neurons.

Overexpression of Tsc1 in rat hippocampal neurons upregulated LC3-II expression, suppressed p62 expression by 44 ± 7% and increased 3MA-sensitive degradation 340 ± 40% compared to GFP-transduced cultures after OGD (Fig. 4f–h). Inhibition of autophagy in rat TSC1-transduced cultures with 3MA abolished the protection conferred by overexpression of rat TSC1, reducing neuronal survival to 23 ± 2% from 47 ± 3% for untreated cultures overexpressing hamartin (P < 0.001; Fig. 4i). Taken together, these findings suggest that overexpression of hamartin conferred protection of hippocampal neurons to OGD by inducing efficient autophagic flux.

The role of autophagy in cerebral ischemia is controversial, with some studies showing that activation of autophagy is detrimental27,28 and others supporting a neuroprotective role29,30. In keeping with our findings, it was previously31 reported that global ischemia increased the number of autophagosomes in CA1 neurons as a result of decreased autophagosome degradation, which indicates impaired autophagy. Moreover, autophagy was implicated in mediating neuroprotection in response to IPC32, which is consistent with our finding that hamartin upregulation in CA1 neurons after IPC. All these observations highlight the key role of both the extent and time point of autophagy induction in determining the outcome after ischemia.

Hamartin has been studied in the context of cancer, epilepsy and more specifically tuberous sclerosis (which results from TSC1 mutations)33. This study shows the importance of examining endogenous neuroprotection in identifying new targets and suggests that hamartin confers resistance against ischemia by inducing productive autophagy. Although the resistive properties of CA3 neurons to ischemia are absent in other paradigms, such as traumatic brain injury, our finding that hamartin alters susceptibility of cortical neurons conventionally affected by paradigms, such as traumatic brain injury, our finding that hamartin confers by overexpression of rat TSC1, reducing neuronal survival in rat TSC1-transduced cultures. Inhibition of autophagy in rat TSC1-transduced cultures with 3MA abolished the protection conferred by overexpression of rat TSC1, reducing neuronal survival to 23 ± 2% from 47 ± 3% for untreated cultures overexpressing hamartin (P < 0.001; Fig. 4i). Taken together, these findings suggest that overexpression of hamartin conferred protection of hippocampal neurons to OGD by inducing efficient autophagic flux.

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Methods

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

Acknowledgments
This work was supported by the UK Medical Research Council grant G0500495 and by the Dunhill Medical Trust. A.M.B. is a senior investigator of the UK National Institute for Health Research (NIHR) and received funding from Fondation Leducq for neurovascular coupling. G.H. was funded through the NIHR Integrated Academic Training Programme and Oxford University Clinical Academic Graduate School. B.K. and C.W.D. were supported by the NIHR Biomedical Research Centre. S.N. was supported by the Deutsche Forschungsgemeinschaft. G.T. and S.M.W. received funding from National Health Service Blood and Transplant (NIHR Programmes RP-PG-0310-10001 and -10003). We would also like to thank R. Deacon from the Department of Experimental Psychology, University of Oxford, for providing us with the open-field apparatus and for his guidance with the behavioral experiments. We thank E. Martin Rendon for her input concerning the lentiviral vectors and J. Peeling for his evaluation of the manuscript. We dedicate this paper to the memory of our colleague and mentor, John P. MacManus.

Author Contributions
M.P. initiated and designed the study, carried out the proteomic and biochemical analyses, performed part of the in vitro and in vivo shRNA lentiviral studies and part of the in vitro overexpression studies and their analysis, and wrote the manuscript. G.H. carried out the subcellular fractionation and immunoblotting experiments for Figures 1 and 4. M.X. produced the overexpression lentiviral particles and helped with the analysis of long-lived protein degradation assays. L.C.H. carried out the rat surgeries for the IPC studies and contributed to the development of the subcellular fractionation protocol. S.N. assisted with the rat surgeries and immunofluorescence experiments and contributed in the interpretation of the proteomic data sets. G.T. assisted with the cortical culture experiments. S.M.W. supervised the National Health Service Blood and Transplant collaborative studies and critically reviewed and edited the manuscript. C.W.D. was involved with the IPC in the behavioral testing. Z.Z. carried out the rat surgeries to generate the tissue for the proteomic experiments and time course studies. M.M.M. and M.I.A.W. assisted with the design and conduct of the lentiviral overexpression experiments. B.K. supervised and helped with the proteomic analysis. K.V. contributed to the primary culture lentiviral studies, assisted with the in vivo shRNA experiments and designed, carried out and assisted in the analysis of protein degradation, autophagy and necrosis assays and supervised the collaboration. A.M.B. initiated and supervised the whole project. All authors edited the manuscript.

Competing Financial Interests
The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nm.3097.
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ONLINE METHODS

Rats. All procedures were conducted in accordance with regulations of the Animal Care Committee at the University of Calgary, or with the 1986 Animals Act (Scientific Procedures) under project license from the UK Home Office or with the Institutional Animal Care and Use Committee of the Biomedical Research Foundation Academy of Athens. Experiments were approved by the clinical medicine ethical review committee of the University of Oxford. Male adult Wistar rats (200 g ± 10%) were obtained from Charles River Laboratories (Calgary) or from Harlow, UK.

Global forebrain ischemia. We subjected rats either to 10 min of severe ischemia or to 2 min of IPC, followed by 72 h of reperfusion and 10 min of ischemia using a modified four-vessel occlusion (4-VO) method34. Rats were anesthetized with isoflurane (1% to 1.5% (v/v) maintenance) in 30% (v/v) O2 and 70% (v/v) N2O. Common carotid arteries were dissected and exposed, and a 3-O silk suture was looped around them. The vertebral arteries were electrocauterized. Rats were fastened overnight, with free access to water, to ensure low serum glucose levels. Twenty-four hours after the preparatory surgery, rats were briefly anesthetized, and both common carotid arteries were temporarily occluded with aneurysm clips for 2 min (IPC) or 10 min (ischemia). A 2-O silk suture, previously inserted through the neck, posterior to the trachea, esophagus, external jugular veins and common carotid arteries but anterior to the cervical and paravertebral muscles, was tightened to prevent any collateral blood flow. We further used rats if they showed complete loss of consciousness, loss of tail and foot pad response and loss of righting and corneal reflex. We regulated core temperature at 37.0 ± 0.5°C in all rats during surgery using a rectal thermometer connected to a feedback-controlled heating pad, and for 24 h after ischemia by a telemetry probe previously inserted in the peritoneal space controlling an infrared lamp.

For sham ischemia and sham IPC, the vertebral arteries were electrocauterized and the carotid arteries were manipulated but not occluded. We killed rats either immediately after 10 min ischemia or 12 h, 24 h or 7 d after reperfusion.

Core temperature telemetry probe implantation and regulation. We implanted sterilized core temperature telemetry probes (TA-F40, Data Sciences International) into the peritoneal cavity 7 d before any further surgery under isoflurane anaesthesia (3% (v/v) induction, 1% (v/v) to 1.5% (v/v) maintenance) in 30% (v/v) O2 and 70% (v/v) N2O. Following ischemia, rats were placed on receivers (RLA-1020, Data Sciences International) connected to infrared lamps. Temperature was sampled in the freely moving rats every ±0.5°C using the computerized temperature control system ART-2.2.

Subcellular fractionation. We microdissected rat brains on ice, sectioned them at 500 m, and punched-separated the CA1 and CA3 regions on the basis of their visual boundaries under a surgical microscope. We carried out subcellular fractionation using a modified version of Guillen35. We conducted all steps at 4°C, and all buffers were supplemented with 1 mM PMSE, 10 mM NaVO3 and a protease inhibitor cocktail (Roche Diagnostics). We homogenized tissues in 1 ml CLB buffer (10 mM HEPES, 10 mM NaCl, 1 mM KH2PO4, 5 mM NaHCO3, 5 mM EDTA, 1 mM CaCl2, 0.5 mM MgCl2, 250 mM sucrose, pH 7.4) with a glass/wall homogenizer. We centrifuged the homogenate at 1,000g for 10 min. The pellet was resuspended in 1 ml CLB buffer and centrifuged at 1,000g for 10 min. We combined the resulting supernatants to derive a mixed cytoplasmic and membrane fraction. After sedimentation at 107,000g for 30 min, the resulting supernatant (cytoplasmic fraction) was stored at −80°C. We resuspended the pellet in 300 µl solubilization buffer (20 mM Tris, 7 M urea, 2 M thiourea, 4% (v/v) CHAPS, pH 7.4) and incubated for 1 h at 4°C. Samples were centrifuged at 120,000g for 3 h, and the supernatant (membrane fraction) was stored at −80°C.

Sample preparation and analysis by tandem mass spectrometry. We analyzed digested, desalted and concentrated protein samples (75 µg) by nanoUPLC-MS5 as described previously36. We determined protein content for the cytoplasmic fraction using the Dc Protein assay (BioRad) and for the membrane fraction using the EZQ protein assay (Invitrogen). We precipitated protein (75 µg) using the methanol/chloroform method, evaporated, resuspended in 100 mM Tris-pH 7.8, 6 M urea buffer, reduced for 30 min (100 mM Tris pH 7.8, 195 mM DTT), alkylated for 30 min (100 mM Tris pH 7.8, 195 mM iodoacetamide), diluted with 100 mM Tris pH 7.8 (1:5) and digested with sequence-grade modified trypsin (Promega) (200 ng µl−1) at 37°C overnight. Digested peptides were desalted and concentrated using Sep Pak C18 column cartridges (Waters) according to the manufacturer’s instructions. We performed sample analysis using a 75 µm i.D. × 25 cm C18 nanoAcquity UPLC column (Waters) and a 90-min gradient: 2% (v/v) to 45% (v/v) solvent B (solvent A: 99.9% (v/v) H2O, 0.1% (v/v) formic acid; solvent B: 99.9% (v/v) acetonitrile, 0.1% (v/v) formic acid) on a Waters nanoAcquity UPLC system (final flow rate 250 nl min−1, 7000 p.s.i.) coupled to a Waters Q-TOF Premier tandem mass spectrometer (Waters)36. Data were acquired in high-definition MS5 mode and processed with ProteinLynx Global Server (PLGS version 2.2.5, Waters) to rebuild MS/MS spectra by combining all masses with identical retention times. We calibrated the raw data using glu-fibrinopeptide (200 fmol µl−1, 700 nl min−1 flow rate, 785.8426 Da [M+2H]2+) as a lock mass. We processed the raw data sets including deisotoping, deconvolution and peak lists generated on the basis of assigning precursor ions and fragments based on similar retention times. We used a SwissProt database (release 51.0, 10/2006, 241,242 entries) to identify the protein identities with the following parameters: peptide tolerance 15 p.p.m., fragment tolerance 0.015 Da, trypsin missed cleavages 1, variable modifications: carbamidomethylation, M oxidation.

Quantitative analysis of the proteomic data set. We analyzed quantitative changes in protein expression on the basis of mass spectrometry peptide ion peak intensities using the Waters Expression Analysis Software (WEPS). For normalization, the ‘auto-normalization’ function was used. Included protein hits were identified with a confidence of >95%. Identical peptides across samples were clustered on the basis of mass precision (<15 p.p.m., typically 5 p.p.m.) and a retention time tolerance of <0.25 min, using the clustering software included in PLGS 2.2.5. To avoid potential errors, if two or more distinct proteins shared an identical peptide but were found to be regulated differently, then the quantification algorithm did not include the peptide in question. Only proteins with a score higher than 45 were included for further analysis. The associated P value of protein level changes between samples was calculated by the PLGS 2.2.5 software using a Monte Carlo algorithm. Proteins that were detected in all experimental groups were selected for further analysis. Quantitative differences were used in subsequent analysis only for proteins with a P value for the change less than 0.05 and with a minimum 10% difference in their expression levels between the corresponding groups.

Ingenuity pathway analysis. We analyzed identified proteins together with the quantitative data using the IPA software and database (IPA, Ingenuity Systems). Pathway analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. Proteins from the data set that met the expression change cut off between conditions of 10% and were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured using the right-tailed Benjamini-Hochberg multiple testing correction. The calculated P value determined the probability that the association between the proteins in the data set and the canonical pathway is explained by chance alone.

For protein network generation, the data set containing protein identifiers and corresponding expression values was uploaded into the IPA application. Each protein was mapped to its corresponding object in the Ingenuity Knowledge Base. A protein expression change cut off of 10% between conditions was set to identify proteins whose expression was significantly differentially regulated. These proteins, called network-eligible proteins, were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks of network-eligible proteins were then algorithmically generated based on their connectivity.

Ontological analysis. In addition to IPA, we analyzed individual data sets using the online panther classification system (http://www.pantherdb.org/).
which classifies proteins ontologically according to their molecular function or their biological process.

**Primary hippocampal cultures and lentiviral transduction.** We prepared hippocampal and cortical cultures from E18 rat embryos, as previously described. We plated dissociated hippocampal or cortical cells onto 12- or 24-well plates at a density of 200,000 cells per cm. We maintained cells in Neurobasal medium (Invitrogen), with 2% B27 supplement (Invitrogen). We replaced half the medium with fresh medium every 3 d. We supplemented hippocampal cells for the first 3 d with 25 μM l-glutamate to promote cell sprouting and improve neuronal viability. We transduced cells with lentiviral vectors at a multiplicity of infection of 10–20, 7 days in vitro (DIV) for shRNA studies and at 11 DIV for overexpression studies. We added rapamycin (10 nM) or 3MA (10 μM) or vehicle 24 h before normoxia or OGD.

**Oxygen glucose deprivation.** We induced OGD in hippocampal cultures by washing twice and immersing in 500 μl deoxygenated custom Neurobasal medium without glucose, aspartate, glutamate, glutamine or pyruvate (Invitrogen). We removed oxygen by bubbling the immersion solution for 30 min with a premixed gas (85% N₂, 10% H₂, 5% CO₂). We sealed cultures inside a modular chamber (Billups-Rothenberg) flushed for 10 min with the same premixed gas and placed inside an incubator for 3 h. Anoxia was confirmed using disposable anaerobic indicator strips (GasPak, BD Biosciences). We treated control cultures similarly but with normoxic custom Neurobasal medium supplemented with 3 mM l-glucose in a normoxic incubator.

**Primary hippocampal cultures and lentiviral transduction.** We prepared hippocampal cultures and lentiviral transduction. We induced OGD in cortical cultures by washing twice and immersing in 500 μl deoxygenated glucose-free balanced salt solution (BSS; 20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 0.03 mM glycine, pH 7.4) in a CellHouse 170 hypoxic incubator (Heto Holten) under 95% N₂ and 5% CO₂ (<0.1% O₂). The immersion solution was preoxygenated overnight in the hypoxic incubator, resulting in a PO₂ less than 10 mm Hg, measured with an ABL-77 gas analyzer (Radiometer). We treated control cultures in a similar fashion but with normoxic BSS solution containing 3 mM l-glucose in a normoxic incubator. Following OGD, both hippocampal and cortical cultures were fed with their original medium, which was replaced 1:1 with fresh medium and incubated for 24 h in a normoxic incubator.

**Cell death assays.** We assessed cell death in primary neuronal cultures quantitatively using the LDH release Cytotox96 assay (Promega) and the necrotic/ healthy cells detection kit (PromoKine).

For both assays, we carried out measurements in triplicate for at least n = 3 independent experiments in a blinded fashion. For the LDH assay, the results were expressed as a ratio of the amount of LDH released in the cell culture medium to the total LDH content, measured in lysed sister cultures.

For the necrotic/healthy cells detection kit, cells were co-stained with Hoechst 33342 and ethidium homodimer III to the number stained with Hoechst 33342 and ethidium homodimer III. We visualized fluorescence using a fluorescence microscope, and images were processed for background subtraction using the NIS elements imaging software (Nikon).

**Lentiviral vectors.** Lentiviral vectors (Sigma-Aldrich) used were pLKO.1 TSC1 shRNA (5′- CGC GCC GGA AGC TGT TCC GTA ATA ACT CGA GTT ATT ACC GAA CAG CTC CGG GTT TTT G3′), which targets nucleotides 2,262–2,282 of the rat TSC1 mRNA (GenBank accession number NM021854); pLKO.1 control shRNA (5′-CGG CCA ACA AGA TGA AGA CCA ACT CGA GTT GGT GCT CAT CTG GTT GTT TTT T3′), which contains a scrambled sequence that does not target any known rat genes; pLKO.1 TurboGFP, which contains the TurboGFP gene.

Recombinant lentiviral particles encoding rat Tsc1-eGFP, human TSC1, or a control eGFP plasmid were generated by co-transfection of human embryonic kidney (HEK) 293T cells with each expression plasmid (pE2-Lv201 vector) together with the GeneCopoeia Lenti-Pac HIV Expression Packaging Kit, according to manufacturer’s instructions (Lifesiences, Source Bioscience).

We cultured HEK 293T cells in DMEM (Invitrogen) supplemented with 10% FBS and 1% penicillin-streptomycin. Two days before transfection, we plated 1.5 × 10⁶ cells onto 10-cm dishes. For transfection, 2.5 μg of vector DNA (rat or human TSC1, or control eGFP plasmid) and 2.5 μg of Lenti-Pac HIV mix were added in 293T cells in the presence of Endofectin Lenti transfection reagent (GeneCopoeia). After 16 h, we removed the medium, and the cells were washed twice with PBS and returned to the normal culture medium supplemented with 1:500 volume of the Titer Boost reagent. We collected medium containing recombinant lentiviral particles 48 h after transfection and centrifuged (400g, 10 min, 4 °C) to remove debris. After filtration through a 0.45-μm filter unit (Millipore, USA) we centrifuged the supernatant at 46,100 g at 4 °C in a SS34 rotor (Sorvall). We suspended the viral particles in 50 µl per tube of PBS supplemented with 0.5% BSA, aliquoted them and stored them at −80 °C. We determined lentiviral titers by seeding HeLa cells in 12-well plates at 5 × 10⁵ cells per well, 3–4 h before infection with serial dilutions of the concentrated viral stock. After incubation for 2 d, the medium was removed and the eGFP-expressing cells were identified by FACS. Titers ranged from 4 × 10⁸ to 2.5 × 10⁹ infectious units (IU ml⁻¹). The human TSC1 mRNA sequence contains six nucleotide mismatches compared to the rat Tsc1 mRNA base pairs 2,262–2,282, making it resistant to TSC1 shRNA knockdown.

**Stereochemical administration of lentiviral vectors.** We administered lentiviral vectors into the hippocampus of live rats by stereotaxic injection 14 d before any further treatment. A 5.0-μl volume containing 5 × 10⁸ particles ml⁻¹ was injected into the hippocampus (3.8 mm posterior to bregma, 3.8 mm lateral to bregma, 3.0 mm below the dura) using 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 withdrawn at rate of 1 mm min⁻¹. We stereotaxically administered the control pLKO.1 TurboGFP vector to determine the extent of gene expression in the hippocampus. GFP immunoreactivity was detected ±0.5 mm anterior–posteriorly from the injection site.

**Immunoblotting.** We precipitated cytoplasmic and membrane fractions or whole-cell homogenates (20–50 μg) using methanol/chloroform resolved by SDS-PAGE and transferred to PVDF membranes, stained with Sypro or Ponceau S to determine loading (total density of eight most prominent bands per lane; representative part of the lane shown in Figures 1 and 4), and probed with the antibodies indicated in Figures 1.2 and 4 and Supplementary Figures 2,6,8–12. We developed immunoreactive species using enhanced chemoluminescence (Amersham), quantified by densitometry (VisionWorks Analysis Software, UVP Biospectrum AC imaging system) and corrected for loading using the quantified data from loading controls.

**Immunofluorescence.** We cut coronal sections (6 μm) of paraffin-embedded brains at 3.6–4.16 mm posterior to bregma. After deparaffinization, brain sections were rehydrated, treated for antigen retrieval (DAKO) and blocked with 10% (v/v) goat serum in PBS. We incubated sections with the primary antibodies indicated in Figure 1 and Supplementary Figure 12 overnight at 4 °C, followed by Cy3- or Cy5-labeled secondary antibodies. Mounting medium contained DAPI (Vector Laboratories) as a counterstain. We detected fluorescence with fluorescent microscope, and images were processed for background subtraction using the NIS elements imaging software (Nikon).
Perfusion-fixation. Rats were killed by transcardiac perfusion with chilled (4 °C) PBS solution and 4% (v/v) formaldehyde in PBS. Brains were removed and incubated overnight in chilled 10% (v/v) formaldehyde in PBS, before dehydration and embedding in paraffin wax.

Hematoxylin and eosin staining. We cut coronal sections (10 µm) of paraffin-embedded brains at 3.6–4.16 mm posterior to bregma. After deparaffinization in Clearene and rehydration, sections were stained with H&E.

CA1 and CA3 cell counting. We counted healthy, viable cells in the entire CA3 molecular layer of the hippocampus in H&E-stained coronal sections at 3.6, 3.8 and 4.16 mm posterior to bregma. Three sections per rat were examined. Averaged measurements were expressed as number of neurons mm⁻¹ ± s.e.m. Measurements were done in a blinded fashion.

Preparation of whole-cell homogenates. We prepared whole-cell homogenates from microdissected CA3 tissue and primary cortical cultures. We harvested hippocampal or cortical cultures in homogenization buffer (50 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, pH 7.4, 4 °C) supplemented with protease inhibitors, 1 mM PMSF and phosphatase inhibitors. We centrifuged homogenates at 1,000 g for 10 min. The pellet was resuspended in homogenization buffer and centrifuged at 1,000g for 10 min. The resulting supernatants were combined. For tissue, the same procedure was followed, but homogenization was done using a polytron homogenizer (Omni International).

Protein degradation assay. We measured total protein degradation by radioactive labeling as previously described, determined as the percentage of the initial total acid precipitable radioactivity (protein) in the cell lysates transformed to acid-soluble radioactivity (amino acids and small peptides) in the medium during the incubation. We quantified total lysosomal degradation as the NH₄Cl-inhibited degradation, whereas macroautophagic degradation was quantified using the 3MA-inhibited proteolysis.

Open-field test. The open-field test was conducted by an investigator blinded to treatment, as previously described. Rats used in open-field testing were grouped according to the boxes crossed and rears performed at baseline and randomly assigned to different experimental treatments so there was no significant difference between groups before further treatments. The open field apparatus was a 60 cm × 100 cm black wooden box with walls 18 cm high. The floor was divided into fifteen 20 cm × 20 cm identical squares. We recorded the activity of each rat for a 3-min session. We manually counted and recorded in a blinded fashion the numbers of squares crossed and rears performed.

Statistical analyses. Data are shown as mean ± s.e.m. Unless stated otherwise, we carried out statistical analysis with GraphPad Prism 5 using a two-tailed Student’s t-test if two groups were compared, one-way ANOVA with Bonferroni’s multiple comparisons post hoc test for comparisons of more than two groups and two-way ANOVA with Bonferroni’s post hoc test when two independent variables were assessed. Differences were considered significant for P < 0.05.

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