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Tuberous Sclerosis Complex Activity Is Required to Control Neuronal Stress Responses in an mTOR-Dependent Manner

Alessia Di Nardo, Ioannis Kramvis, Namjik Cho, Abbey Sadowski, Lyny Meikle, David J. Kwiatkowski, and Mustafa Sahin

Tuberous sclerosis complex (TSC) is a neurogenetic disorder caused by loss-of-function mutations in either the TSC1 or TSC2 genes and frequently results in prominent CNS manifestations, including epilepsy, mental retardation, and autism spectrum disorder. The TSC1/TSC2 protein complex plays a major role in controlling the Ser/Thr kinase mammalian target of rapamycin (mTOR), which is a master regulator of protein synthesis and cell growth. In this study, we show that endoplasmic reticulum (ER) stress regulates TSC1/TSC2 complex to limit mTOR activity. In Tsc2-deficient neurons, the expression of stress markers such as CHOP and HO-1 is increased, and this increase is completely reversed by the mTOR inhibitor rapamycin both in vitro and in vivo. Neurons lacking a functional TSC1/TSC2 complex have increased vulnerability to ER stress-induced cell death via the activation of the mitochondrial death pathway. Importantly, knockdown of CHOP reduces oxidative stress and apoptosis in Tsc2-deficient neurons. These observations indicate that ER stress modulates mTOR activity through the TSC protein complex and that ER stress is elevated in cells lacking this complex. They also suggest that some of the neuronal dysfunction and neurocognitive deficits seen in TSC patients may be attributable to ER and oxidative stress and therefore potentially responsive to agents moderating these pathways.

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by the growth of benign tumors called hamartomas in multiple organs, including the brain (Crimo et al., 2006). TSC patients suffer from epilepsy, autism, and developmental delay. Within the CNS, TSC is associated with cortical hamartomas, made up of giant cells, dysmorphic neurons, and astrocytes. TSC is caused by mutations in either the TSC1 or TSC2 genes. Proteins encoded by TSC1 or TSC2 genes interact with each other to form the TSC1/TSC2 complex. One of the major cellular functions of the TSC1/TSC2 complex is to limit protein synthesis and regulate cell size by inhibiting the Rheb–mamman- tian target of rapamycin (mTOR) pathway (Kwiatkowski and Manning, 2005). Mutations in either TSC1 or TSC2 lead to constitutive activation of mTOR, which phosphorylates substrates such as S6 kinase (S6k) and 4E-BP1, ultimately increasing protein synthesis.

Recently, embryonic fibroblasts and kidney tumors from Tsc2-deficient mice were shown to have increased endoplasmic reticulum (ER) stress (Ozcan et al., 2008). ER stress can be caused by excessive protein synthesis, perturbation in calcium homeostasis, or nutrient deprivation (Ron and Walter, 2007). Under normal conditions, the ER stress sensor GRP78 has an inhibitory role on the effectors [PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1)] of the unfolded protein response (UPR), which is the cellular response to ER stress (Dorner et al., 1992; Liu et al., 2000). During ER overload, GRP78 releases its inhibition of PERK, ATF6, and IRE1 (Mori, 2000) and activates the UPR. The UPR leads to three distinct specific cascades: (1) the PERK/eIF2α pathway reduces protein synthesis by inhibiting translation; (2) the ATF6 pathway activates transcription of chaperone proteins increasing folding capacity; (3) the IRE/XBP-1 pathway promotes proteasome-dependent protein degradation to remove proteins from the ER (Bertolotti et al., 2000; Mori, 2000; Liu et al., 2003; Rutkowski and Kaufman, 2004). Ultimately, the UPR response results in either the successful elimination of ER overload or, if unsuccessful, in ER stress–induced cell death via caspase activation and induction of the proapoptotic transcription factor CHOP (C/EBP homologous protein, GADD153) (Oyadomari and Mori, 2004).

Although ER stress has been demonstrated in Tsc-deficient mouse embryonic fibroblasts (MEFs) and kidney tumors (Ozcan et al., 2008), it remains unclear whether TSC deficiency leads to ER stress in neurons, what role mTOR pathway plays in neuronal stress response, and whether similar dysfunctions are present in seizure models of Tsc in vivo. To address these questions, we...
investigated the role of the TSC1/TSC2 complex during ER stress in greater detail and examined the effects of TSC deficiency on neuronal stress pathways. We demonstrate that TSC2 is initially inactivated in neurons during ER stress and later activated, as part of an apparent regulatory mechanism to limit mTOR activity. Lack of a functional TSC1/TSC2 complex abolishes this regulation, resulting in increased ER stress and vulnerability to neuronal damage. Furthermore, Tsc-deficient neurons have increased accumulation of reactive oxygen species (ROS) and oxidative stress. Similar dysfunctions were identified in TSC brain lesions in vivo, identifying a new role for the TSC1/TSC2 complex in the neuronal stress response.

Materials and Methods

**Animals.** All experimental procedures were performed in compliance with animal protocols approved by the Institutional Animal Care and Use Committee at Children's Hospital (Boston, MA). The Tsc1+/−/Syn-Cre+ mice used in this study were described previously (Meikle et al., 2007). For rapamycin treatment, mice were injected intraperitoneally at 6 mg/kg every other day from postnatal day 9 (P9) to P33. Mice subjected to the On/Off treatment were on rapamycin treatment (6 mg/kg) every other day from P9 to P30, followed by no treatment until P45 (On/Off) (Meikle et al., 2008).

**Neuronal cultures.** Neuronal cultures were prepared as published previously (Sahin et al., 2005). Briefly, hippocampi from 18-d-old rat embryos (CD1; Charles River) were isolated under the microscope and collected in HBSS containing 10 mM MgCl2, 1 mM kynurenic acid, 10 mM HEPES, and penicillin/streptomycin. After 5 min dissociation at 37°C in 30 U/ml papain (Worthington), neurons were mechanically triturated and plated in Neurobasal (NB) medium containing B27 supplement, 2 mM l-glutamine, and penicillin/streptomycin (Invitrogen). For biochemical analysis, cells were plated at 1 × 105 cells per well onto six-well plates coated with 20 μg/ml poly-D-lysine (PDL) and 2.5 × 103 cells per plate for immunofluorescent (IF) studies onto PDL–laminin-coated glass coverslips in 24-well plates.

**Lentivirus infection.** Viral stocks for lentiviral infection were prepared as described previously (Mostoslavsky et al., 2005), except that the four packaging vectors (kindly provided by Dr. R. C. Mulligan, Department of Genetics, Harvard Medical School, Boston, MA) were cotransfected into HEK293 T cells with the plasmid to be coexpressed using Lipofectamine 2000 according to the instructions of the manufacturer. Viral particles were collected 48 and 72 h after transfection and filtered through a 0.45 μm membrane. Hippocampal neurons were infected at 1 day in vitro (1 DIV) in the presence of polybrene at 0.6 μg/ml. Six hours after infection, the virus-containing medium was replaced by fresh NB/B27 medium. After infection, neurons were kept in culture for an additional 10 d. Control short hairpin RNA (ShRNA) construct against the luciferase gene targeting was the following: 5′-GCTGTAAGAGAGGCGGTATCACA-3′.

**Semiquantitative and real-time quantitative PCR.** Total RNA was prepared with an RNAeasy kit (Qiagen) following the instructions of the manufacturer and quantified by a spectrophotometer. A total of 2 μg of poly(A) mRNA was used for reverse transcription using the SuperScript RT system (Invitrogen). Semiquantitative PCR reactions were performed using Taq Polymerase (PerkinElmer Life and Analytical Sciences). Quantification of the semiquantitative PCR was performed by densitometry scans, and values were normalized against total β-actin. Real-time PCRs were performed using SYBR Green PCR Master Mix (Applied Biosystems). All quantitative PCR (qPCR) reactions were performed in triplicate and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Analysis was performed using 7300 System (Applied Biosystems). All quantitative PCR (qPCR) reactions were performed in triplicate and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Analysis was performed using 7300 System (Applied Biosystems).

**Flow cytometric analysis.** Neurons cultured in NB media for 24 h were harvested by 5 min incubation at 37°C with 15 U/ml papain (Worthington) made in HBSS containing 10 mM MgCl2, 1 mM kynurenic acid, 10 mM HEPES, and penicillin/streptomycin. Before dissociation, a solution of 7 mg/ml trypsin inhibitor (Sigma) was added to stop the reaction.

**CHOP knockdown.** CHOP ShRNA (CHOP-Sh) and control CHOP RNAi (CHOP-C) were purchased from Sigma, and the sequences are as follows: CHOP-Sh, 5′-GAAAAGAAGGAGAATCA-3′; CHOP-C 5′-CGGAATGTGACCAGCCAC-3′.

**Antibodies and reagents.** Antibodies used for this study included the following: rabbit polyclonal anti-phospho-S6 (Ser235/236) (catalog #2211), mouse monoclonal anti-total S6 (catalog #2317), rabbit polyclonal anti-phospho-Akt (Ser473) (catalog #9271), rabbit polyclonal anti-S6K (catalog #9202), rabbit polyclonal anti-phospho-S6K (Thr389) (catalog #9234), rabbit polyclonal anti-Tsc1 (catalog #4906), and rabbit polyclonal anti-Tsc2 (Thr1462) (catalog #3611) (all from Cell Signaling Technology); rabbit polyclonal anti-Tsc2 (sc-893), mouse monoclonal anti-GADD153 (CHOP) (sc-7351), and goat polyclonal anti-Akt (sc-1618) (all from Santa Cruz Biotechnology); and rabbit polyclonal anti-GRP78 (SPA-826) and mouse monoclonal anti-heme oxygenase-1 (HO-1) (OSA-110) (from Stressgen). HRP-conjugated secondary antibodies were from VWR.

**Western blot.** Details can be found in the supplemental data (available at www.jneurosci.org as supplemental material).

**ER stress induction.** Thapsigargin (Tg) and Tunicamycin (Tn) were purchased from Sigma and used at a final concentration of 0.5 μM and 4 μg/ml, respectively. Stocks of drugs were made in DMSO and freshly diluted in NB media at 20% of the final concentration before performing each experiment. The same amount of DMSO was used as vehicle-only control. Before ER stress induction, NB/B27 media was replaced with NB in the presence of penicillin/streptomycin for 4 h, and drugs were then added for an additional 3, 4, and 24 h. When indicated, rapamycin was used for 24 h in NB media at a final concentration of 20 nM.

**Apoptosis quantification.** The number of apoptotic cells was determined by Hoechst staining and trypan blue exclusion test. Embryonic day 17 rat neurons were plated on coverslips at a density of 10 × 104 cells/ml and infected with lentivirus as described above. After 10 DIV, neurons were left untreated or treated for ER stress induction. For Hoechst quantification, neurons were fixed and stained with 5 μg/ml Hoechst (Invitrogen) for 5 min at room temperature. Neurons were then washed in PBS, mounted, and analyzed with a Leica DM RNA microscope equipped with epifluorescence. Apoptotic nuclei were counted under a 20× objective and expressed as the percentage of the total number of infected cells in the same field. Data are expressed as means ± SE from at least three different experiments, and statistical analysis was performed by Student’s t test. For trypan blue exclusion test, neurons were harvested as described in flow cytometric analysis and resuspended in a 0.2% trypan blue solution (Sigma) prepared in HBSS for 5 min at room temperature. Apoptotic cells were evaluated under bright-field microscopy by counting nonviable cells (dye-positive) and viable cells (dye-negative) within hemocytometer fields.

For quantification of cell death at the single-cell level, the number of apoptotic cells was determined by counting cleaved caspase 3 (cc3) (Cell Signaling Technology catalog #9664) positively stained neurons after immunofluorescent microscopy using a 20× objective. Data were expressed as a percentage of the total number of infected cells. The experiment was performed in triplicate, and at least 300 cells per experiment were counted. Statistical analysis was performed by unpaired two-tailed Student’s t test and considered significant at p < 0.05.

**Immunochemical analysis.** Details can be found in the supplemental data (available at www.jneurosci.org as supplemental material).

**Mitochondrial ROS.** Rat hippocampal neurons were cultured in NB media for 24 h, followed by incubation with 100 nM MitoTracker Red CM-H2XRos dye (MT-Red) (Invitrogen) for 30 min before being processed for immunofluorescence and stained with Hoechst. Oxidative stress was quantified by counting the number of MT-Red-labeled cells under an epifluorescent microscope with a rhodamine filter and expressed as the average percentage of MT-Red-labeled cells from three independent experiments.

**Flow cytometric analysis.** Neurons cultured in NB media for 24 h were harvested by 5 min incubation at 37°C with 15 U/ml papain (Worthington) made in HBSS containing 10 mM MgCl2, 1 mM kynurenic acid, 10 mM HEPES, and penicillin/streptomycin. Before dissociation, a solution of 7 mg/ml trypsin inhibitor (Sigma) was added to stop the reaction.
Neurons were then collected by centrifugation, washed, and resuspended in NB media at 2 × 10^6 cells/ml. Neurons were divided into two aliquots, which were incubated in the absence or presence of 100 nM MitoTracker Red CM-H2XRos dye (Invitrogen). After 20 min at 37°C, neurons were collected by centrifugation, rinsed, and fixed in 4% paraformaldehyde made in PBS for 15 min at room temperature. After fixation, neurons were washed and resuspended in 200 μl for analysis. Flow cytometric analysis was performed with Dako MoFlo equipped with Spectra-physics laser model 177 with an emission at 488 and a strength of 100 mW. Data were analyzed with Summit 4.3 software (Dako). Gating was performed before the collection of data to remove apoptotic cells and cellular debris. Mean fluorescence intensity of MT-Red was calculated by subtracting for each sample the fluorescence-activated cell sorting (FACS) measurement obtained in the absence of the dye (background) to the measurement obtained in the presence of the dye.

**Results**

**TSC and mTOR are dynamically regulated under ER stress**

Because some of the most severe manifestations of TSC disease are in the CNS, we investigated the role of the TSC1/TSC2 complex in the neuronal response to ER stress. We treated rat hippocampal neurons with two widely used ER stress inducers: the ER-Ca^2+^-ATPase blocker Tg and the N-glycosylation inhibitor Tn (Li et al., 2000; Urano et al., 2000). To determine the optimal doses for these ER stress-inducing chemicals in neurons, we performed dose/response curves (0.1–5 μM for Tg; 1–12 μg/ml for Tn) using wild-type rat hippocampal neurons and assessed cell death as the outcome. Expression of UPR-regulated genes GRP78 and CHOP confirmed ER stress induction already at the lowest concentrations used for both drugs (supplemental Fig. 1A–C, available at www.jneurosci.org as supplemental material). As expected, cell death assessed by Hoechst staining (supplemental Fig. 1D,E, available at www.jneurosci.org as supplemental material) and trypan blue exclusion assay (supplemental Fig. 1F, available at www.jneurosci.org as supplemental material) showed that the percentage of apoptotic neurons increased in a dose-dependent manner for both Tg and Tn. For the purpose of this study, we decided to use 0.5 μM for Tg and 4 μg/ml for Tn because, at these doses, we observed a robust ER-stress-induced UPR activation and ER-stress-induced cell death of at least 50–60% neuronal cells.

When assessing the effect of ER stress on the Akt/mTOR pathway, we found a response that varied with duration of treatment. Tg treatment led to an initial activation of mTOR as evidenced by increased phosphorylation of S6 ribosomal protein (phospho-S6 Thr1462) and in the phosphorylation of Tsc2 at Thr1462 (Fig. 1A), whereas the time-dependent increase in the expression of the UPR-regulated gene, GRP78, was blocked (Fig. 1B). Prolonged ER stress (24 h) correlated with a progressive decrease in Akt activity (phospho-Akt Ser473) and in the phosphorylation of Tsc2 at Thr1462 (Fig. 1C, D). Akt phosphorylation is known to inhibit TSC complex activity (Inoki et al., 2002; Potter et al., 2002). Thr1462 phosphorylation is known to inhibit TSC complex activity (Inoki et al., 2002; Manning et al., 2002). Accordingly, we observed inhibition of the downstream mTOR pathway, as indicated by decreased phosphorylation of S6 at 24 h (Fig. 1A, D). ER stress was confirmed by the time-dependent increase in the expression of the UPR-regulated gene, GRP78. Prolonged ER stress (24 h) correlated with expression of the proapoptotic UPR regulated gene CHOP and with apoptosis as shown by activated (cleaved) caspase 3. Tunicamycin treatment, an alternative method of inducing ER stress, produced similar results in neurons (Fig. 1B–E). To investigate whether regulation of TSC1/TSC2 complex activity under ER stress also occurred in non-neuronal cells, we treated HEK293T and MEFs with ER stress inducers. We found that ER stress also induced transient mild activation of Akt, phosphorylation of Tsc2, and phosphorylation of S6, followed by inhibition of this pathway at 24 h (supplemental Fig. 2A, B, available at www.jneurosci.org as supplemental material).

**Loss of Tsc correlates with an mTOR-dependent ER stress response in neurons**

Having identified dynamic regulation of the TSC1/TSC2 complex activity under ER stress, we hypothesized that neurons lacking TSC activity would display features of ER stress at baseline attributable to constitutive activation of mTOR and high levels of protein synthesis. Therefore, we investigated ER stress response in neurons after RNAi-mediated knockdown of the Tsc2 gene. Rat hippocampal neurons were infected after 1 DIV with a lentivirus expressing Tsc2 gene plasmid-based short hairpin RNA (Tsc2-Sh) or a control lentivirus expressing shRNA against the luciferase gene (GL3-Sh). Because we have shown previously that knockdown of Tsc2 produces the same morphological and biochemical changes as Tsc1 knock-out in dissociated cells (Choi et al., 2008), we used Tsc2 knockdown neurons as an *in vitro* model to examine neuronal ER stress.

In Tsc2 knockdown neurons, there was a significant increase (3.5-fold) in the mRNA of CHOP as assessed by real-time qRT-PCR (Fig. 2A). Additionally, upregulated was the CHOP upstream regulator ATF4 (2.5-fold) (Fawcett et al., 1999; Harding et al., 2000; Ma et al., 2002) and to some extent (1.5-fold) ER stress sensor GRP78. Treatment of Tsc2 knockdown hippocampal neurons with rapamycin reduced CHOP, ATF4, and GRP78 mRNA levels to baseline, indicating that the increase was mTOR dependent. Consistent with the increased CHOP mRNA levels in the Tsc2-deficient hippocampal cultures, immunocytochemical analysis revealed that, in the Tsc2-Sh infected cultures, 13.3% of neurons had CHOP nuclear expression and 6.1% had CHOP cytosolic expression compared with 0.7% CHOP nuclear expression and 0.1% CHOP cytosolic expression for control infected neurons (Fig. 2B, C).

CHOP is a proapoptotic transcription factor that is expressed and translocated into the nucleus under ER stress (Oyadomari and Mori, 2004). Because Tsc2 knockdown neurons demonstrated elevated CHOP expression at baseline, we investigated its expression and localization in Tsc-deficient neurons treated with Tg. Exposure to Tg significantly increased CHOP protein nuclear expression in Tsc2-deficient neurons compared with control cultures as assessed by immunocytochemical analysis (Fig. 2D, E). Together, these data show that loss of a functional TSC1/TSC2 complex in hippocampal neurons lowers the threshold for activation of UPR-regulated genes.

**Tsc2-deficient neurons show increased basal and ER-stress-induced cell death**

Although ER-stress-activated signaling is a protective cellular response to reduce ER load, prolonged ER stress often leads to cell death by apoptosis (Rao et al., 2001). To assess the effects of activating UPR response, we treated Tsc2 knockdown and control neuronal cultures with the ER stress inducers Tg or Tn for 3, 6, and 24 h. Knockdown of Tsc activity was confirmed by the reduced Tsc2 protein level and constitutively high S6 and S6K phosphorylation (Fig. 3A, B). Exposure to either Tg or Tn induced cellular ER stress as seen by the gradual increase in the expression of the ER stress sensor GRP78. Western blot analysis showed that Tsc2 knockdown neurons had a small but significant
increase in the levels of CHOP protein expression at baseline compared with control-infected neurons, which was consistent with the observed increased basal transcription of CHOP mRNA. No differences were observed in the baseline and in the ER-stress-induced GRP78 levels between the control and Tsc-deficient neurons (supplemental Fig. 3A, B, available at www.jneurosci.org as supplemental material).

The effects of ER stress induction on neuronal viability were then monitored using an antibody for cleaved (active) caspase 3 on Western blots. In control neurons, Tg or Tn treatments induced cleavage of caspase 3 only after 24 h, whereas in Tsc2-deficient neurons, cc3 was already detectable at baseline and further increased shortly after (3 h) ER stress induction (Fig. 3A, B). Similarly, as assessed by Hoechst staining, a higher proportion of Tsc2 knockdown neurons showed a significant increase in apoptotic nuclei at baseline and after short Tg (3 h) (Fig. 3C) or Tn (3 and 6 h) treatment (Fig. 3E). Similar results were obtained when assessing baseline cell death by trypan blue exclusion assay for Tg (Fig. 3D) and Tn (Fig. 3F). To further confirm induction of apoptosis in Tsc2 knockdown cells, we assessed cytoplasmic levels of cytochrome c, a marker of early apoptosis (Ferri and Kroemer, 2001; Rao et al., 2001). Consistent with the higher level of baseline apoptosis, we detected cytochrome c release in the cytosolic fraction of Tsc2-deficient neurons only (Fig. 3G). Den- sitometric quantification of cytosolic and mitochondrial cytochrome c levels was performed on three independent experiments and revealed a 4.9-fold increase in the cytosolic cytochrome release in Tsc2-deficient neurons compared with controls (*p < 0.01 by t test). In cells under ER stress, the inositol requiring enzyme 1 (IRE1) pathway is responsible for the alternative splicing of the XBP-1 transcript (Lee et al., 2002). Interestingly, compared with neurons infected with control virus, Tsc2-deficient neurons had a more robust ER stress-induced activation of the IRE1 pathway during both Tg and Tn treatment, as shown by XBP-1 splicing (supplemental Fig. 4A, B, available at www.jneurosci.org as supplemental material). Together, these findings suggest that lack of Tsc activity in cultured hippocampal neurons correlates with increased ER-stress-induced cell death via activation of the mitochondrial death pathway.

**Figure 1.** Regulation of the Tsc1/Tsc2 complex by ER stress. **A, B,** Rat hippocampal neurons were treated with vehicle (DMSO) or with 0.5 μM Tg (A) or with 4 μg/ml Tn (B) for 3, 6, and 24 h to induce ER stress. Western blot analysis shows that ER stress induction modulates activity of the mTOR pathway (phospho-S6 Ser235/236). The regulation of the mTOR pathway closely parallels the decreased phosphorylation of Tsc2 (on Thr1462) by Akt. Equal loading is shown by probing with the antibodies against total Tsc2, total S6, and total Akt. **C–E,** Quantification of fold change of phospho-Tsc2 (Thr1462) (**C**), phospho-S6 (Ser235/236) (**D**), and phospho-Akt (Ser473) (**E**) after ER stress induction by Tg and Tn. Values were normalized against total Akt level and are represented as mean fold change relative to DMSO from at least three independent experiments. Statistical analysis was performed by a two-tailed t test with an adjusted significant (*) p value < 0.017 after Bonferroni’s correction for multiple pairwise comparisons.

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Tsc-deficient neurons have increased oxidative stress and undergo cell death via a CHOP-dependent mechanism

CHOP is a proapoptotic transcription factor that promotes apoptosis by modulat-
ing the expression of proteins that regulate cell survival and death pathways (Oyadomari and Mori, 2004). In particular, CHOP has been found to affect expression and localization of bcl2 family members and influence the cellular redox status (McCullough et al., 2001; Marciniak et al., 2004). To determine which of these CHOP targets were affected in Tsc-deficient neurons, we compared the mRNA levels of survival and oxidative stress regulated genes by qRT-PCR (Fig. 4A). Tsc2 knockdown did not change the expression of the prosurvival factor bcl2 or of the cellular antioxidant defense gene thioredoxin 2 (Trx-2). Instead, we observed a significant mTOR-dependent increase in the expression (3.5-fold) of the antioxidant enzyme heme oxygenase-1 (HO-1) and of the ER oxidoreductase enzyme ERO1α. HO-1 protein levels were also increased in Tsc2-deficient neurons, and, during rapamycin treatment, this increase was blocked (Fig. 4B, C). However, under the same conditions, rapamycin treatment was not sufficient to prevent cell death.

HO-1 is a member of the heat shock family (Hsp32), and its expression is induced when cells experience oxidative stress (Takahashi et al., 2004), whereas the ERO1α is a glycosylated flavoenzyme implicated in oxidative protein folding and ROS production in the ER by promoting disulphide bond formation (Harding et al., 2003; Sevier and Kaiser, 2008). Therefore, we asked whether Tsc deficiency would induce increased production of ROS. Control and Tsc2-deficient rat hippocampal neurons were treated in culture with MT-Red (Fig. 4D, E). MT-Red generates fluorescence only during oxidation by superoxide produced by mitochondria (Kim et al., 2002). Quantification of MT-Red-labeled cells under an epifluorescent microscope was performed from three independent experiments and revealed a 2.6-fold increase in the percentage of Tsc2-deficient-positive neurons compared with control-infected cells (control, 14.3 ± 2.5% vs Tsc2-deficient neurons, 37.4 ± 1.4%; n = 250 cells per experiment; *p < 0.001 by t test). MT-Red accumulation, as as-

Figure 2. Regulation of UPR genes in Tsc-deficient neurons at baseline and after ER stress induction. A, Real-time qRT-PCR of total RNA from rat hippocampal neurons infected with GL3-Sh (control) and Tsc2-Sh RNAi lentivirus. The ER stress regulated genes CHOP, ATF4, and GRP78 are increased in the Tsc2-deficient neurons in an mTOR-dependent manner as shown by a direct comparison of the effect of rapamycin (Rap.; 20 nM for 24 h) in each genotype. Significant p values are as follows: *p < 0.01 and **p < 0.05. Values normalized against GAPDH represent means of at least three independent experiments, and error bars represent SE. B, IF analysis of control and Tsc2-Sh infected neurons stained with CHOP antibody (red). GFP fluorescence (green) was used to identify infected neurons. Scale bar, 50 μm. C, Quantification of IF analysis shows a significant increase in CHOP nuclear and cytosolic staining in Tsc2-deficient neurons (*p < 0.05). Data represent means of three different experiments, and error bars represent SE. At least 300 cells were counted in each experiment, and statistical analysis was performed by the Student’s t test. D, Representative IF images of control GL3-Sh and Tsc2-deficient neurons treated with DMSO (vehicle) or Tg for 3, 6, and 24 h and stained with CHOP antibody. In the merged panels, infected neurons are in green, CHOP antibody in red, and Hoechst staining in blue. Scale bar, 50 μm. E, Quantification of nuclear expression from at least three different experiments per time point. Values are expressed as means, and error bars represent SE. p values determined by Student’s t test are as follows: *p < 0.05, **p < 0.01.
Lack of Tsc activity correlates with increased CHOP expression. Representative Western blots of protein lysates from GL3-Sh- and Tsc2-Sh-infected hippocampal neurons after ER stress induction. Neurons were left untreated (−), treated with vehicle (DMSO), with 0.5 μM Tg (A), or with 4 μg/ml Tn (B) for 3, 6, and 24 h. At baseline, Tsc2-deficient neurons have increased cleaved caspase 3. The densitometric quantification of CHOP protein induction represents means of three independent experiments, and error bars represent SE. Values were normalized to total Akt level, and statistical analysis was performed by a two-tailed t test with an adjusted significant *p value < 0.0125 after Bonferroni’s correction for multiple pairwise comparisons. C–F, Quantification of cell death after Tg (C, D) and Tn (E, F) treatments of control and Tsc2-Sh neurons by Hoechst staining (C, E) and trypan blue exclusion test (D, F). For each panel, data are expressed as means ± SE from three independent sets of experiments per time point. Statistical analysis was performed with the Student’s t test (*p < 0.005, **p < 0.005 in C, p < 0.05 in D; *p < 0.05, **p < 0.05 in E; *p < 0.05 in F). G, Neuronal fractionation of control and Tsc2-deficient neurons shows increased cytochrome c release into the cytosolic fraction of Tsc2-Sh neurons, indicating activation of the mitochondrial death pathway. Cytochrome c oxidase IV (COX IV) and total Akt were used as mitochondrial (Mito.) and cytosolic (Cyt.) fraction markers, respectively.

Figure 3. Lack of Tsc activity correlates with increased CHOP expression. Representative Western blots of protein lysates from GL3-Sh- and Tsc2-Sh-infected hippocampal neurons after ER stress induction. Neurons were left untreated (−), treated with vehicle (DMSO), with 0.5 μM Tg (A), or with 4 μg/ml Tn (B) for 3, 6, and 24 h. At baseline, Tsc2-deficient neurons have increased cleaved caspase 3. The densitometric quantification of CHOP protein induction represents means of three independent experiments, and error bars represent SE. Values were normalized to total Akt level, and statistical analysis was performed by a two-tailed t test with an adjusted significant *p value < 0.0125 after Bonferroni’s correction for multiple pairwise comparisons. C–F, Quantification of cell death after Tg (C, D) and Tn (E, F) treatments of control and Tsc2-Sh neurons by Hoechst staining (C, E) and trypan blue exclusion test (D, F). For each panel, data are expressed as means ± SE from three independent sets of experiments per time point. Statistical analysis was performed with the Student’s t test (*p < 0.005, **p < 0.005 in C, p < 0.05 in D; *p < 0.05, **p < 0.05 in E; *p < 0.05 in F). G, Neuronal fractionation of control and Tsc2-deficient neurons shows increased cytochrome c release into the cytosolic fraction of Tsc2-Sh neurons, indicating activation of the mitochondrial death pathway. Cytochrome c oxidase IV (COX IV) and total Akt were used as mitochondrial (Mito.) and cytosolic (Cyt.) fraction markers, respectively.

Previous studies have shown that loss of TSC1/TSC2 complex activity correlates with an mTOR-dependent negative feedback on the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which in turn results in reduced Akt activation (Zhang et al., 2006). To investigate whether a similar inhibition was occurring in Tsc-deficient neurons, we analyzed Akt at the phospho-Ser473 activation site using Western blot. When compared with control
In vivo reduce both cell death and oxidative stress. deficient neurons, knockdown of CHOP alone is sufficient to regulated Akt might contribute to increased apoptosis in Tsc-deficient neurons is CHOP independent, and, although down-expression in neurons and display neurological decline with median survival are markedly improved when mutant mice are absence of rapamycin and in the presence of rapamycin (Meikle et al., 2008). To investigate ER stress responses of Akt activation that did not change after CHOP-Sh RNAi (Fig. 5B). These data indicate that reduced Akt activity in Tsc-deficient neurons is CHOP independent, and, although down-regulated Akt might contribute to increased apoptosis in Tsc-deficient neurons, knockdown of CHOP alone is sufficient to reduce both cell death and oxidative stress.

In vivo identification of stress response in brains from Tsc1Δ−/−SynCre− mice and in the tuber of a TSC patient

Our data demonstrated increased oxidative stress in Tsc2-deficient hippocampal cultures in vitro. To determine whether a similar stress response occurs in vivo, we assessed levels of expression for CHOP and HO-1 in total brain lysates from Tsc1Δ−/−SynCre− mice (neuronal Tsc1 knock-out) (Meikle et al., 2007). Tsc1Δ−/−SynCre− mice experience near-complete loss of Tsc1 expression in neurons and display neurological decline with median survival of 35 d. Both the neurological abnormalities and the median survival are markedly improved when mutant mice are treated with rapamycin from P7 to P33 (Meikle et al., 2008). To investigate ER stress responses in vivo, we used Tsc1Δ−/−SynCre+ mice either untreated or treated with rapamycin. Both CHOP and HO-1 protein levels were increased in Tsc1Δ−/−SynCre+ brain lysates and were reduced in mice treated with rapamycin (Fig. 6A,B). Interestingly, Tsc1Δ−/−SynCre+ mice taken off rapamycin after 3 weeks of treatment showed recurrence of HO-1 expression (supplemental Fig. 6, available at www.jneurosci.org as supplemental material).

To extend these findings at the cellular level, immunohistochemical analysis for HO-1 was performed on control and Tsc1Δ−/−SynCre+ brains. Costaining with phospho-S6 antibody was used to identify neurons with increased mTOR activity. Phospho-S6-positive dysplastic cells identified in the hippocampus and the red nucleus of Tsc1Δ−/−SynCre+ brains were also positive for HO-1 (Fig. 6C,D). Furthermore, rapamycin treatment decreased phospho-S6 staining and HO-1 expression in Tsc1Δ−/−SynCre+ brains to levels comparable with controls (Fig. 6D).

To determine whether our findings could be extended to human TSC disease, we performed immunohistochemical analysis on sections from a tuber of a 4-year-old TSC patient. Giant cells with increased mTOR activity were identified in the human tuber by phospho-S6 antibody staining (Fig. 6E). CHOP and HO-1 colabeling was observed in 44% (80 of 115 counted) and 57% (70 of 123 counted) of the phospho-S6-positive cells, respectively. No CHOP or HO-1 staining was observed in the perituber brain regions (Fig. 6F) or in the brain of a non-TSC patient with focal dysplasia (Fig. 6G). In agreement with previous reports, balloon cells that are typically found in focal dysplasias showed some phospho-S6 and SMI-311 staining (Lurton et al., 2002; Baybis et al., 2004). Together, these findings strongly suggest that the ER
and oxidative stress responses identified in vitro in the Tsc2 silenced hippocampal neurons are also present in vivo in both the Tsc knock-out mouse model and the human TSC brain.

**Discussion**

Despite recent progress identifying the genetic mutations and the signaling pathways associated with TSC pathology, the pathogenesis of the diverse neurological symptoms present in this disease remain poorly understood, and treatments are elusive. Here, we demonstrate that Tsc deficiency correlates with the upregulation of specific stress-related cellular responses both in vitro and in vivo (summarized in Fig. 6F). First, we detected ER overload and oxidative damage in Tsc2-deficient hippocampal neurons, in brains from Tsc1−/− SynCre mice and in human TSC tissue. Second, we demonstrated that these cellular abnormalities are the consequence of constitutive mTOR activation because rapamycin treatment abolished stress responses both in vitro and in vivo. Third, we showed that neuronal stress responses in vitro increased vulnerability to cell death via activation of the mitochondrial death pathway and that silencing CHOP reduced apoptosis. The identification of similar stress responses in primary rodent hippocampal neurons with nearly complete Tsc2 gene silencing and in the human TSC brain highlight the damaging neuronal responses that result from mTOR hyperactivity.

We have shown recently that components of the TSC/mTOR pathway are differentially localized during the development of neuronal polarity, as defined by the elaboration of a single neuron and multiple dendrites (Choi et al., 2008). This fine regulation of TSC activity in neurons during the neuronal polarization process, together with the identification of multiple axon formation in neurons lacking Tsc, indicates a critical role for the Tsc/mTOR pathway in axonal specification and connectivity. These findings, along with the identification of a critical role for TSC pathway and that silencing CHOP reduced apoptosis. The identification of similar stress responses in primary rodent hippocampal neurons with nearly complete Tsc2 gene silencing and in the human TSC brain highlight the damaging neuronal responses that result from mTOR hyperactivity.

In previous reports, loss of TSC1/TSC2 complex has been implicated in increased ER stress in MEFs from Tsc1 and Tsc2 knock-out mice (Ozcan et al., 2008). Al-
Immunofluorescent detection of HO-1 expression in ectopic neurons of the hippocampal region from Tsc1c−/−; SynCre+ mice treated with rapamycin at 6 mg/kg every other day from P9 to P33. Occurrence of stress response is evident by positive SMI-311 staining. Scale bar, 50 μm.

Figure 6. Upregulation of stress responses in the brain of Tsc-deficient mice. A, Western blot of brain lysates from Tsc1c−/−; SynCre− (control), Tsc1c−/−; SynCre+ and Tsc1c−/−; SynCre+/− mice treated with rapamycin at 6 mg/kg every other day from P9 to P33. Tsc1c−/−; SynCre− mice show reduced Tsc1 protein levels confirming inactivation of the Tsc1 gene. Increased phospho-S6 (Ser235/236) confirms the presence of active mTOR in the brain lysates from the Tsc1c−/−; SynCre− mice. Occurrence of stress response is shown by increased expression of CHOP and HO-1. Inhibition of mTOR by rapamycin effectively reduces both CHOP and HO-1 levels. Total S6 was used as a loading control. B, Quantification of protein levels performed from the following: Tsc1c−/−; SynCre− mice, n = 5; Tsc1c−/−; SynCre+, n = 7; and rapamycin-treated Tsc1c−/−; SynCre+, n = 6. Statistical analysis was performed by a two-tailed t test with an adjusted significant *p value < 0.017 after Bonferroni’s correction for multiple pairwise comparisons. C–G, Immunohistochemical detection of stress response in vivo. C, Immunofluorescent detection of HO-1 expression in phospho-S6-positive cells in the red nuclei. Equivalent sections were compared between control Tsc1c−/−; SynCre (top) and mutant Tsc1c−/−; SynCre+ mice (middle). Scale bar, 50 μm. Higher magnification of the phospho-S6- and HO-1-positive cells (white box) is shown in the bottom panel. Scale bar, 10 μm. D, Immunofluorescent detection of HO-1 expression in the hippocampal region in mutant Tsc1c−/−; SynCre+ mice with or without rapamycin treatment. Treatment with rapamycin decreases both phospho-S6 and HO-1 expression. Scale bar, 50 μm. E–G, Immunohistochemical analysis on a cortical tuber resected from a 4-year-old TSC patient (E), from the perituber region of a TSC patient (F), and from a non-TSC patient with focal dysplasia (G). Increased level of phospho-S6 staining indicates mTOR activation in the tuber. Compared with the sections in F and G, elevated HO-1 and HO-1 expression are specifically observed only in the giant cells from the tuber region of the TSC brain (E). Enlarged dysmorphic neurons were also identified by positive SMI-311 staining. Scale bar, 50 μm. H, Model for mTOR-dependent neuronal dysfunction after TSC1/TSC2 loss. After loss of TSC function, uncontrolled mTOR hyperactivation results in an increase in protein synthesis and ROS production. Such overloaded cellular machinery results in ER and oxidative stress responses, which induce CHOP and HO-1, respectively, and can be reversed by rapamycin. These cellular stress pathways together with downregulated Akt activity likely contribute to increased vulnerability to neuronal death and dysfunction.
In vivo occurrence of oxidative stress in tubers indicates a concomitant increase in mTOR activity and treatment of our study addressing the molecular targets affected by rapamycin (Meikle et al., 2008; Zeng et al., 2008). These results together with specific TSC mouse models and shown to efficiently improve mitochondrial dysfunction is overall considered to be beneficial (Schipper, 2004b). However, excessive HO-1 can also contribute to the increase of carbon monoxide (CO) and/or free iron levels, which can have toxic effects on mitochondrial function (Ryter and Tyrrell, 2000; Barañano and Snyder, 2001; Schipper, 2004a). Expression of HO-1 in phospho-S6-positive eiptoc neurons of Tsc1c−/− SynCre+ mice and in the dysplastic cells of human tubers indicates a concomitant increase in mTOR activity and occurrence of oxidative stress in vivo. Importantly, mTOR inhibition in vivo reduced CHOP and HO-1 expression. mTOR inhibitors have already been successfully used in several brain-specific TSC mouse models and shown to efficiently improve survival and neurological phenotypes (Ehninger et al., 2008; Meikle et al., 2008; Zeng et al., 2008). These results together with our study addressing the molecular targets affected by rapamycin treatment in vivo provide new insights into the cellular basis of the neuronal dysfunction in TSC.

In the future, it will be important to investigate the damaging effects that could arise from HO-1 overproduction in the TSC brain, such as iron deposition or CO accumulation (Patel et al., 1996). For instance, a highly detrimental effect of iron deposition is the accumulation of free iron in the mitochondria as a result of increased oxidative stress. This would eventually cause mitochondrial dysfunction and energy production failure, affecting several ATP-dependent processes such as the uptake of excitotoxic neurotransmitters (Beal, 1998; Trushina and McMurray, 2007). Such neuronal insults can contribute to glutamate excitotoxicity, which has been implicated in the etiology of seizure-related disorders (Patel, 2002; Schipper, 2004b). In addition, excess CO production could cause dysfunction in synaptic plasticity and consequently lead to defects in cognitive development (Stevens and Wang, 1993; Zhuo et al., 1993). Similarly, the increased ERO1α expression identified in Tsc-deficient neurons could potentially exacerbate neuronal function by enhancing ROS production (Harding et al., 2003; Marcinik et al., 2004). Therefore, the combination of ER and oxidative stress detected in Tsc-deficient neurons may contribute not only to epilepsy but also to neurodevelopmental disabilities in TSC patients.

**Implications of oxidative damage in Tsc mutant brains**

Accumulating evidence has revealed a crosstalk between ER and oxidative stress responses, such that excessive ROS production can contribute to UPR induction and vice versa (Yokouchi et al., 2008). For instance, UPR-regulated genes can create an imbalance in the cellular redox status and release free radicals such as superoxide anions, leading to damage of ER-resident proteins (Verkhratsky and Petersen, 2002). The combined cellular insult that may arise from ER and oxidative stress has been proposed to further contribute to cell death by increasing the accumulation of ROS (Haynes et al., 2004). Our identification of an altered redox balance in Tsc-deficient neurons is consistent with previous reports of increased basal and growth factor-stimulated ROS in Tsc2−/− MEFs (Finlay et al., 2005). In Tsc-deficient neurons, we found that silencing of CHOP was sufficient to reduce the oxidative stress response, thus indicating a tight-linked connection between these two cellular stress pathways.

The brain is highly sensitive to oxidative stress, which has been correlated with the pathogenesis of several neurological disorders (Reynolds et al., 2007). In particular, the cellular toxicities resulting from oxidative stress, such as massive calcium overload, energy depletion, and ROS production, are thought to affect neuronal function by lowering the cellular capacity to respond to stress. A number of studies have suggested that, although the response to stress offers homeostatic control of cellular function, a prolonged stress response itself can be toxic (Kaufman, 2002). For example, the expression of HO-1, the rate-limiting enzyme for the degradation of the heme, is overall considered to be beneficial (Schipper, 2004b). However, excessive HO-1 can also contribute to the increase of carbon monoxide (CO) and/or free iron levels, which can have toxic effects on mitochondrial function (Ryter and Tyrrell, 2000; Barañano and Snyder, 2001; Schipper, 2004a). Expression of HO-1 in phospho-S6-positive eiptoc neurons of Tsc1c−/− SynCre+ mice and in the dysplastic cells of human tubers indicates a concomitant increase in mTOR activity and occurrence of oxidative stress in vivo. Importantly, mTOR inhibition in vivo reduced CHOP and HO-1 expression. mTOR inhibitors have already been successfully used in several brain-specific TSC mouse models and shown to efficiently improve survival and neurological phenotypes (Ehninger et al., 2008; Meikle et al., 2008; Zeng et al., 2008). These results together with our study addressing the molecular targets affected by rapamycin treatment in vivo provide new insights into the cellular basis of the neuronal dysfunction in TSC.

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**References**


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