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The Role of TSC1 in the Formation and Maintenance of Excitatory Synapses

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Tuberous Sclerosis, synapses, spines, neurons, morphology
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INTRODUCTION

Tuberous Sclerosis (TSC) is an autosomal dominant genetic disorder characterized by benign tumors of many organs. The majority of TSC patients are identified as children and most have neurological symptoms including mental retardation and epilepsy. Although it is known that TSC results from mutations in either the TSC1 or TSC2 genes, the pathogenesis of the neurological disorder is unclear. One possibility, inspired by gross pathological findings, is that the presence of benign growths in the brain leads to disorganized and compressed brain tissue and perturbed neural circuits. However, it is equally possible that loss of TSC1 or TSC2 disrupts neuronal function in a cell-autonomous manner. Our hypothesis is that TSC1 is necessary in mature, differentiated neurons for the establishment of proper neuronal morphology and synaptic function. This hypothesis is being testing by examining cell-autonomous defects in TSC1 null neurons located within otherwise normal brain tissue. The approaches used to examine the perturbed cells are immunostaining of activated proteins in the TSC signaling cascade, optical microscopy of neuronal structure, and electrophysiological analysis of electrical properties.

BODY

Task 1. Establish that introduction of Cre recombinase into a TSC1\textsuperscript{C/C} postmitotic neurons results in loss of TSC1 protein.

Task 1 has been completed and the results were presented in Tavazoie et al Figure 1 (see attached appendix).

We exploited a transgenic mouse carrying a conditional Tsc1 allele (Tsc1\textsuperscript{Cre}) in which exons 17 and 18 are flanked by loxP sequences(Uhlmann et al., 2002). Loss of Tsc1 protein in neurons following transfection with a plasmid encoding a Cre recombinase-nuclear localization sequence fusion protein (Cre) was confirmed in dissociated hippocampal cultures prepared from mice homozygous for the conditional allele (Tsc1\textsuperscript{C/C}). Since transfection efficiency of neurons is low, Tsc1 levels were monitored by fluorescence immunohistochemistry (fIHC). In neurons expressing Cre, cytoplasmic Tsc1 levels were significantly reduced at 6 days post transfection (DPT) compared to untransfected neighboring neurons (\(P < 0.05, n = 62-67\)). In several cell types, down-regulation of TSC1/2 upregulates mTOR activity and increases phosphorylation of the ribosomal protein S6 (Inoki et al., 2002; Lai et al., 2005; Potter et al., 2001). Immunostaining against phosphorylated S6 (pS6) revealed a perinuclear cytoplasmic signal in neurons that was abolished by application of rapamycin, a selective pharmacological inhibitor of mTOR (data not shown). PS6 levels were substantially higher in Cre-transfected Tsc1\textsuperscript{C/C} neurons than in neighboring control neurons (\(P < 0.05, n = 68\)). Thus, Cre transfection in post-mitotic, differentiated Tsc1\textsuperscript{C/C} neurons induces recombination of the Tsc1\textsuperscript{C} allele, loss of Tsc1 protein, and upregulation of mTOR.

Task 2. Determine if loss of TSC1 leads to perturbed neuronal morphology
Task 2 has been completed and the results are presented in Tavazoie et al Figure 1 and 2 (see attached appendix). Widespread loss of Tsc1 in the mouse brain, even when limited to astrocytes, leads to pronounced seizures (Uhlmann et al., 2002) which may trigger changes in gene expression and synaptic transmission. To identify cell-autonomous neuronal defects caused directly by loss of Tsc1, we generated genetically mosaic brain tissue in which a small number of neurons lacking Tsc1 were located in otherwise normal brain tissue. This was accomplished by sparse transfection of pyramidal neurons with Cre and GFP in organotypic hippocampal slices prepared from Tsc1 CC mice.

GFP-transfected pyramidal hippocampal neurons were identified by their characteristic morphology (Fig. 1e) and location in a cell-dense band (Fig. 3d). Somas of Cre-transfected Tsc1 CC pyramidal neurons were larger than those of GFP-transfected neurons, reaching a ~2 fold enlargement at 20 DPT (n = 20-31, P < 0.05). Changes in soma size were prevented by cotransfection with a plasmid encoding Tsc1 (n = 8). To control for nonspecific effects of Cre-mediated DNA recombination, similar measurements were made in tissue prepared from B6;129-Gt(Rosa)26Sor tm2Sho/J mice that carry a floxed transcriptional stop upstream of the EGFP coding sequence in the Rosa26 locus (Rosa CC). Cre expression in Rosa CC neurons had no effect on soma size (n = 7-8), confirming that the increased soma size in Tsc1 CC neurons transfected with Cre was due to loss of Tsc1.

In pyramidal neurons, the vast majority of excitatory synapses are made onto the heads of dendritic spines, and the morphology and density of dendritic spines reflect the properties and number of synapses. At 20 DPT, dendritic spines of Tsc1 CC neurons expressing GFP alone displayed roughly spherical spine heads separated from the dendrite by thin necks. In contrast, dendrites of Tsc1 CC neurons coexpressing Cre and GFP possessed elongated spines with greatly enlarged, bulbous heads. Quantification of these changes showed that Tsc1 loss increased spine length and head width and decreased the density of dendritic spines (n = 9-16/2441-4344 cells/spines, P < 0.05). Similar effects on spine size and morphology are seen at 10 DPT (n = 7-8/2577-3338 cells/spines, P < 0.05), a time point at which loss of Tsc1 has no effect on soma size. Changes in spine morphology and density were prevented by expression of Tsc1 (n = 8/2960 cells/spines) and no changes in spine density or morphology were seen with Cre expression in Rosa CC neurons (Sup. Table 1), confirming that the morphological changes seen with Cre expression in Tsc1 CC neurons were due to loss of Tsc1 protein.

Task 3. Determine if loss of TSC1 perturbs synaptic function.

Tasks 3a-d and most of 3e have been completed and the results are presented in Tavazoie et al Figure 3 (see attached appendix). The last portion of task 3e (long-term potentiation) was not assayed we made the unexpected discovery that the glutamate receptor composition of the synapse was altered. Follow-up experiments based on the analysis of evoked synaptic currents in Task 3e demonstrated that the loss of Tsc1 triggered the insertion of calcium permeable AMPA-type glutamate receptors into the synapse. This was demonstrated by analysis of current-voltage rectification curves in the presence of intracellular polyamines and by direct monitoring of synaptically-evoked calcium transients within dendritic spines.
Do the enlarged spine heads seen following loss of Tsc1 contain functionally perturbed synapses? To address this question, whole-cell voltage-clamp recordings were obtained from Tsc1<sup>+/−</sup> pyramidal neurons transfected with Cre and from untransfected neighboring neurons and spontaneous miniature excitatory postsynaptic currents (mEPSCs) were recorded. A red fluorophore was included in the recording pipette solution in order to fill the neuron and confirm its identity. At 10 DPT, mEPSC amplitude was increased by ~20% in Cre-expressing neurons compared to neighboring control neurons (n = 10-12/660-781 cells/events, P < 0.05), indicating an enhanced sensitivity to released neurotransmitter. No changes in mEPSC frequency or in resting membrane resistance (R<sub>m</sub>) and cell capacitance (C<sub>m</sub>) were seen (in controls: R<sub>m</sub> = 197 ± 43 MΩ, C<sub>m</sub> = 256 ± 61 pF; in Cre neurons: R<sub>m</sub> = 200 ± 63 MΩ, C<sub>m</sub> = 182 ± 34 pF). In contrast, at 20 DPT, cells lacking Tsc1 had greatly reduced R<sub>m</sub> (127 ± 15 MΩ vs. 185 ± 20 MΩ in control neurons, P < 0.05) and increased C<sub>m</sub> (234 ± 24 pF vs. 171 ± 17 pF in control neurons) (n = 13 in each condition), making the comparison of mEPSCs between Tsc1-lacking neurons and control neurons difficult.

At 20 DPT, EPSCs evoked by stimulation of Schaffer collaterals were monitored in CA1 pyramidal neurons at a holding potential of −60 mV, at which AMPARs are activated, and at +40 mV, at which the block of NMDA-type glutamate receptors (NMDAR) by Mg<sup>2+</sup> is relieved and the long-lived NMDAR current is revealed. The ratio of evoked AMPAR- to NMDAR-mediated currents increases normally during development as well as following induction of long-term potentiation. In Cre-transfected neurons, the AMPAR/NMDAR current ratio was significantly increased relative to controls (3.7 ± 0.71 vs. 2.0 ± 0.35, in controls, (n = 13 in each condition, P < 0.05) indicating an aberrant relative enhancement of synaptic AMPAR. To examine possible changes in presynaptic function induced retrogradely by postsynaptic loss of Tsc1, responses to a pair of stimuli were recorded and paired-pulse facilitation (PPF) was measured. PPF was similar in untransfected and Cre-transfected neurons (1.73 ± 0.22 and 1.75 ± 0.15, respectively, n = 11-13), suggesting that release probability is not affected by postsynaptic loss of TSC1.

Task 4. Determine if perturbations caused by loss of TSC1 can be rescued by low levels of rapamycin.

Task 4 has been completed and the results are presented in Tavazoie et al (see attached appendix). Rapamycin is a potent and specific inhibitor of mTOR, and is predicted to act downstream of TSC1 and TSC2. To determine if the effects of loss of Tsc1 on neuronal morphology are mediated by increased activity of mTOR, we examined the ability of rapamycin to reverse defects in neuronal morphology. Neurons in hippocampal organotypic slices were transfected and maintained in culture for 14 days, a time point at which the effects of Cre-mediated loss of Tsc1 were apparent. Rapamycin (100 nM) was then added to the culture media, and the cultures were maintained for 6 more days before morphological analysis (total of 20 DPT).

Application of rapamycin to control GFP-expressing rat neurons had no effect on soma size but induced the growth of long, thin spines (n = 6/1702 cells/spines). Surprisingly, the mean length of dendritic spines on control cells in the presence of rapamycin was similar to that of Tsc1 or Tsc2 knockdown cells. However, the rapamycin-induced spine phenotype was distinguished from that resulting from loss of
Tsc1 by the lack of enlarged spine heads. Application of rapamycin to shTsc1-expressing cells reversed the enlargement of the soma and spine heads but further increased spine length. In these cells it was difficult to distinguish long filopodia and spines from nascent dendritic branches, artifactually lowering the measured spine density. Thus, rapamycin-sensitive mTOR activity is epistatic to Tsc1 with respect to the regulation of soma and spine head sizes.

**KEY RESEARCH ACCOMPLISHMENTS**

- Established cell-based, neuronal system for the analysis of morphological and functional perturbations resulting from loss of Tsc1
- Validation of model system, demonstrating that Cre transfection in post-mitotic, differentiated neurons from mice homozygous for conditional Tsc1 alleles leads to loss of Tsc1 and increased pS6.
- Establishment that Tsc1 play a role in regulating neuronal morphology and spine structure in differentiated neurons.
- Establishment that these morphological perturbations are accompanied by function changes in passive membrane properties and synaptic transmission.

**REPORTABLE OUTCOMES**

Tavazoie SF, Alvarez VA, Ridenour DA, Kwiatkowski DJ, Sabatini BL. “Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2.” Nat Neurosci. 2005 Dec;8(12):1727-34.

**CONCLUSIONS**

We have demonstrated that Tsc1 regulates neuronal morphology and function in differentiated, post-mitotic neurons. Loss of Tsc1 leads to changes in cell size (enlarged soma and large spine heads) as well as loss of excitatory synapses (decreased spine density). Furthermore, remaining synapses are functionally perturbed and contain abnormally high levels of AMPA-type glutamate receptors compared to NMDA-type glutamate receptors. Our results indicate that functional perturbations of neurons may contribute to the pathogenesis of TSC. In addition, our experiments with rapamycin suggest that its application is unable to reverse morphological defects resulting from Tsc1 loss and thus may not have therapeutic value in the treatment of the neurological symptoms of TSC.

**REFERENCES**

Tavazoie SF, Alvarez VA, Ridenour DA, Kwiatkowski DJ, Sabatini BL. “Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2.” Nat Neurosci. 2005 Dec;8(12):1727-34.


APPENDICES Reprint attached
Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2

Sohail F Tavazoie1,3,4, Veronica A Alvarez1,4, Dennis A Ridenour1, David J Kwiatkowski2 & Bernardo L Sabatini1

Mutations in the TSC1 or TSC2 tumor suppressor genes lead to tuberous sclerosis complex (TSC), a dominant hamartomatous disorder that often presents with mental retardation, epilepsy and autism. The etiology of these neurological symptoms is unclear and the function of the TSC pathway in neurons is unknown. We found that in post-mitotic, hippocampal pyramidal neurons of mice and rats, loss of Tsc1 or Tsc2 triggered enlargement of somas and dendritic spines and altered the properties of glutamatergic synapses. Furthermore, loss of a single copy of the Tsc1 gene was sufficient to perturb dendritic spine structure. Morphological changes required regulation of the actin-depolymerization factor cofilin at a conserved LIM-kinase phosphorylation site, the phosphorylation of which was increased by loss of Tsc2. Thus, the TSC pathway regulates growth and synapse function in neurons, and perturbations of neuronal structure and function are likely to contribute to the pathogenesis of the neurological symptoms of TSC.

TSC1 and TSC2 are tumor suppressor genes whose protein products, hamartin (TSC1) and tuberin (TSC2), negatively regulate cell growth in a variety of systems. In humans, heterozygous mutations in either TSC1 or TSC2 lead to TSC, an autosomal-dominant hamartomatous disorder characterized by benign tumors in multiple organs including the brain, kidneys, heart and eyes. TSC also typically presents with a constellation of neurological deficits that include epilepsy, mental retardation and autism.

Biochemical and genetic analyses in mammalian systems and Drosophila melanogaster have revealed that TSC1 and TSC2 participate in a conserved growth-regulating pathway involving the mammalian target of rapamycin (mTOR)2–5. In brief, the activation of growth-promoting receptor tyrosine kinases, such as the insulin receptor, stimulates phosphoinositide 3-kinase (PI3K) and the serine/threonine kinase Akt. In vitro, Akt phosphorylates TSC2 at conserved consensus phosphorylation sequences and downregulates its GTPase-activating protein (GAP) activity6–8. Reduced GAP activity allows the buildup of GTP-bound Rheb9,10 and upregulates mTOR, which, through multiple actions, enhances protein translation and cell growth11. Thus, given the loss of heterozygosity of TSC1 or TSC2 found in hamartomas of TSC patients12, the growth of these benign tumors is thought to result from the increased mTOR activity and uncontrolled cell growth that accompanies interruption of the TSC pathway.

The pathogenesis of the neurological symptoms of TSC is unclear, and loss of heterozygosity is not seen within the brains of TSC patients13. The function of TSC1 and TSC2 in mammalian neurons and the defects that arise from hemizygosity of TSC1 or TSC2 are unknown. Many parallels exist, however, between the TSC pathway and those that link extracellular stimuli to synaptic refinement in neurons. For example, activation of the TrkB receptor tyrosine kinase by brain-derived neurotrophic factor (BDNF) stimulates PI3K and Akt to promote dendritic growth14. BDNF also triggers long-term potentiation of synaptic strength in an mTOR-dependent manner15. Similarly, strong activation of metabotropic glutamate receptors depresses synaptic transmission through a PI3K- and mTOR-dependent pathway16,17.

Here we examine the role of the TSC pathway in regulating the growth of post-mitotic, differentiated neurons. We show that the TSC pathway regulates soma size, the density and size of dendritic spines, and the properties of excitatory synapses in hippocampal pyramidal neurons. These morphological effects are independent of regulation of Tsc2 by Akt at conserved phosphorylation sites but do require regulation of cofilin at a conserved LIM-kinase (LIMK) phosphorylation site. Furthermore, the TSC pathway is sensitive to gene-dosage effects, such that loss of a single copy of Tsc1, as is present in all neurons of TSC patients, is sufficient to perturb dendritic spine structure. Our results indicate that the TSC pathway regulates neuronal structure and function and suggest that the neurological symptoms of TSC are, at least in part, due to cell-autonomous perturbations of synapse function.

RESULTS
To uncover defects in neuronal structure and function caused by perturbation of the TSC pathway, we used transgenic mice carrying a conditional Tsc1 allele (Tsc1lox) in which exons 17 and 18 are flanked by loxP sequences18. Loss of Tsc1 protein in neurons following transfection with a plasmid encoding a Cre recombinase–nuclear localization sequence fusion protein (Cre) was confirmed in dissociated hippocampal

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cell-autonomous neuronal defects caused directly by loss of Tsc1, we generated genetically mosaic brain tissue in which a small number of neurons lacking Tsc1 were located in otherwise normal brain tissue. This was accomplished by sparse transfection of pyramidal neurons with Cre and green fluorescent protein (GFP) in organotypic hippocampal slices prepared from Tsc1\textsuperscript{C/C} mice.

GFP-transfected pyramidal hippocampal neurons were identified by their characteristic morphology (Fig. 1e) and location in a cell-dense band. Somas of Cre-transfected Tsc1\textsuperscript{C/C} pyramidal neurons were larger than those of GFP-transfected neurons, reaching a roughly twofold enlargement at 20 DPT (n = 20–31, P < 0.05) (Fig. 1e–g). Changes in soma size were prevented by cotransfection with a plasmid encoding Tsc1 (n = 8). To control for nonspecific effects of Cre-mediated DNA recombination, similar measurements were made in tissue prepared from B6;129-Gr(Rosa\textsuperscript{Sor/PM2Sho})\textsuperscript{J} mice that carry a floxed transcriptional stop upstream of the EGFP coding sequence in the Rosa\textsuperscript{Sor} locus (Rosa\textsuperscript{Sor/C}). Cre expression in Rosa\textsuperscript{Sor/C} neurons had no effect on soma size (n = 7–8) (Fig. 1g), confirming that the increased soma size in Tsc1\textsuperscript{C/C} neurons transfected with Cre was due to loss of Tsc1.

Soma size was also measured in rat neurons in which loss of Tsc2 was induced by RNA interference (RNAi; see Methods and Fig. 1g). Transfection of dissociated rat hippocampal pyramidal neurons with a dual-promoter plasmid encoding cytomegalovirus (CMV) promoter-driven GFP and a U6-driven short-hairpin RNA targeting Tsc2 (shTsc2) reduced Tsc2 and increased pS6 (Supplementary Fig. 1 online). In organotypic hippocampal slice cultures, somas of pyramidal neurons transfected with shTsc2 were enlarged relative to control neurons at 10 DPT (n = 7–13, P < 0.05) (Fig. 1g). This effect was occulted by cotransfection with human Tsc2, which contains nine base pair changes within the region targeted by shTsc2 (n = 9) (Fig. 1g). Thus, soma size of post-mitotic, hippocampal pyramidal neurons is controlled by the TSC pathway. Somatic enlargement occurred with a shorter latency after shTsc2 transfection than after Cre transfection of Tsc1\textsuperscript{C/C} neurons, likely reflecting the rapid degradation of mRNA triggered by RNAi.

**Tsc1/2 regulate density and size of dendritic spines**

In pyramidal neurons, the vast majority of excitatory synapses are made onto the heads of dendritic spines, and the morphology and density of dendritic spines reflect the properties and number of synapses. At 20 DPT, dendritic spines of Tsc1\textsuperscript{C/C} neurons expressing GFP alone displayed roughly spherical spine heads separated from the dendrite by thin necks (Fig. 2a). In contrast, dendrites of Tsc1\textsuperscript{C/C} neurons coexpressing Cre and GFP possessed elongated spines with greatly enlarged, bulbous heads. Quantification of these changes showed that Tsc1 loss increased spine length and head width and decreased the density of dendritic spines (n = 9–16 cells and

cultures prepared from mice homozygous for the conditional allele (Tsc1\textsuperscript{C/C}). Because transfection efficiency of neurons is low, we monitored Tsc1 levels by fluorescence immunohistochemistry (FHC) (Fig. 1a,b). In neurons expressing Cre, cytoplasmic Tsc1 levels were significantly reduced at 6 days post-transfection (6 DPT) compared to untransfected neighboring neurons (P < 0.05, n = 62–67) (Fig. 1b). In several cell types, downregulation of TSC1/TSC2 upregulates mTOR activity and increases phosphorylation of the ribosomal protein S6\textsuperscript{4,5,8}. Immunostaining against phosphorylated S6 (pS6) revealed a perinuclear cytoplasmic signal in neurons (Fig. 1c) that was abolished by application of rapamycin, a selective pharmacological inhibitor of mTOR (data not shown). PS6 levels were substantially higher in Cre-transfected Tsc1\textsuperscript{C/C} neurons than in neighboring control neurons (P < 0.05, n = 68) (Fig. 1c,d). Thus, Cre transfection in post-mitotic, differentiated Tsc1\textsuperscript{C/C} neurons induces recombination of the Tsc1 allele, loss of Tsc1 protein and upregulation of mTOR.

**Tsc1/2 regulate cell growth in differentiated neurons**

Widespread loss of Tsc1 in the mouse brain, even when limited to astrocytes, leads to pronounced seizures\textsuperscript{15}, which may trigger changes in gene expression and synaptic transmission. To identify
2,441–4,344 spines, P < 0.05; Fig. 2b,c). Similar effects on spine size and morphology were seen at 10 DPT (n = 7–8 cells and 2,377–3,338 spines, P < 0.05; Fig. 2), a time point at which loss of Tsc1 has no effect on soma size (Fig. 1g). Changes in spine morphology and density were prevented by expression of Tsc1 (n = 8 cells and 2,960 spines; Fig. 2b,c) and no changes in spine density or morphology were seen with Cre expression in Rosa26 Cre reporter neurons (Supplementary Table 1 online), confirming that the morphological changes seen with Cre expression in Tsc1 Cre neurons were due to loss of Tsc1 protein. The perturbations in spine morphology triggered by loss of Tsc1 were phenocopied by RNAi-mediated knock-down of Tsc2 in rat hippocampal neurons (Supplementary Fig. 2). Thus, at both 10 DPT (n = 7–13 cells and 1,715–5,023 spines) and 20 DPT (5–13 cells and 993–4,262 spines), dendritic spines of shTsc2-expressing neurons were elongated with enlarged spine heads (P < 0.05). These effects were rescued by expression of hTSC2 (n = 9 cells and 2,682 spines; Fig. 2c). Summaries of morphological parameters and numbers of cells and spines analyzed for each experimental condition are given in Supplementary Table 1.

**Functional defects associated with loss of Tsc1**

Do the enlarged spine heads seen after loss of Tsc1 contain functionally perturbed synapses? To address this question, we obtained whole-cell voltage-clamp recordings from Tsc1 Cre neurons transfected with Cre and from untransfected neighboring neurons. Spontaneous miniature excitatory postynaptic currents (mEPSCs) were recorded (Fig. 3a). A red fluorophore was included in the recording pipette solution in order to fill the neuron and confirm its identity. At 10 DPT, mEPSC amplitude was roughly 20% higher in Cre-expressing neurons.
Morphological effects of haploinsufficiency of Tsc1

Individuals afflicted with TSC carry heterozygous mutations in either the TSC1 or TSC2 genes and loss of heterozygosity, although present in hamartomas, is not seen within the brain. Therefore we tested whether loss of a single copy of Tsc1 is sufficient to trigger changes in neuronal morphology (Fig. 4). In tissue prepared from Tsc1+/− mice, Cre-expressing neurons had increased soma size, decreased spine density, increased spine length and increased spine head width relative to GFP-expressing neurons (n = 11 cells and 2,996 spines, P < 0.05). The morphological changes were less pronounced after loss of a single copy than after loss of both copies of Tsc1 (Fig. 4b), suggesting that the TSC pathway is sensitive to gene dosage.

Regulation of neuronal morphology by mTOR

Rapamycin is a potent and specific inhibitor of mTOR, and is predicted to act downstream of TSC1 and TSC2. To determine whether the effects of loss of Tsc1 or Tsc2 on neuronal morphology are mediated by increased activity of mTOR, we examined the ability of rapamycin to reverse defects in neuronal morphology (Fig. 5). Neurons in hippocampal organotypic slices were transfected and maintained in culture for 14 d, a time point at which the effects of Cre-mediated loss of Tsc1 and RNAi-mediated loss of Tsc2 were apparent. Rapamycin (100 nM) was then added to the culture media, and the cultures were maintained for six more days before morphological analysis (total of 20 DPT).

Application of rapamycin to control GFP-expressing rat neurons had no effect on soma size but induced the growth of long, thin spines (n = 6 cells and 1,702 spines; Fig. 5a,c). Surprisingly, the mean length of dendritic spines on control cells in the presence of rapamycin was similar to that of Tsc2 knockdown cells (Fig. 5a,c). Unlike the phenotype resulting from loss of Tsc2, however, the rapamycin-induced spine phenotype did not show enlarged spine heads. Application of rapamycin to shTsc2-expressing cells reversed the enlargement of the soma and spine heads but further increased spine length (n = 8 cells and 1,163 spines, P < 0.05; Fig. 5b,c). In these cells, it was difficult to distinguish long filopodia and spines from nascent dendritic branches, artifically lowering the measured spine density. Similar effects were seen in Cre-expressing Tsc1+/− cells, such that application of rapamycin

Compared to neighboring control neurons (n = 10–12 cells and 660–781 events, P < 0.05; Fig. 3b,c), indicating an enhanced sensitivity to released neurotransmitter. No changes in mEPSC frequency (Fig. 3c) or in resting membrane resistance (Rm) and cell capacitance (Cm) were seen (controls: Rm = 197 ± 43 MΩ, Cm = 256 ± 61 pF; Cre-expressing neurons: Rm = 200 ± 63 MΩ, Cm = 182 ± 34 pF). In contrast, at 20 DPT, cells lacking Tsc1 had greatly reduced Rm (127 ± 15 MΩ vs. 185 ± 20 MΩ in control neurons, P < 0.05) and increased Cm (234 ± 24 pF vs. 171 ± 17 pF in control neurons) (n = 13 in each condition), making the comparison of mEPSCs between Tsc1-lacking neurons and control neurons difficult.

At 20 DPT, EPSCs evoked by stimulation of Schaffer collaterals (Fig. 3d) were monitored in CA1 pyramidal neurons at a holding potential of −60 mV, at which AMPARs are activated, and at +40 mV, at which the block of NMDA-type glutamate receptors (NMDARs) by Mg2+ is relieved and the long-lived NMDAR current is revealed (Fig. 3e). The ratio of evoked AMPAR- to NMDAR-mediated currents normally increases during development as well as after induction of long-term potentiation. In Cre-transfected neurons, the AMPAR/NMDAR current ratio was significantly increased relative to that in controls (3.7 ± 0.71 vs. 2.0 ± 0.35 in controls, n = 13 in each condition, P < 0.05), indicating an aberrant relative enhancement of synaptic AMPARs. To examine possible changes in presynaptic function induced retrogradely by postsynaptic loss of Tsc1, we recorded responses to a pair of stimuli and measured the paired-pulse facilitation (PPF). PPF was similar in untransfected and Cre-transfected neurons (1.73 ± 0.22 and 1.75 ± 0.13, respectively, n = 11–13; Fig. 3f), suggesting that release probability is not affected by postsynaptic loss of TSC1.
First, hTSC2 AA was expressed alone and found to have no effect on phosphorylation of S939 and T1462 (which are mediated by downregulation of Tsc2 by Akt through phosphorylation at two conserved sites in D. melanogaster and mammals7. We investigated whether phosphorylation of Tsc2 and Akt is necessary for regulation of neuronal soma and spine size by Tsc2, and hTSC2 AA is unable to act as a dominant-negative with respect to the neuronal enlargement triggered by upregulation of Akt.

PREVENTED INCREASE IN Soma AND SPINE HEAD SIZES BUT FURTHER INCREASED SPINE LENGTH (Supplementary Table 1). Thus, rapamycin-sensitive mTOR activity is epistatic to Tsc2 with respect to the regulation of soma and spine head sizes.

**Epistatic analysis of Tsc2 and Akt**

It has been proposed that Akt inhibits TSC2 by phosphorylation at two sites (S939 and T1462, numbered by the human sequence) that are conserved in D. melanogaster and mammals7. We investigated whether Akt regulates neuronal morphology and, if so, whether it occurs through phosphorylation of sites S939 and T1462 of Tsc2 (Fig. 6).

Expression in rat hippocampal pyramidal neurons of a constitutively active Akt (AktCA), consisting of a myristoylated Akt lacking its pleckstrin homology domain (myrAktD19), phenocopied loss of Tsc1/Tsc2, resulting in large somas, long spines and increased spine head size (n = 5 cells and 1,052 spines, P < 0.05; Fig. 6a,b). Three experiments were performed to determine if these effects are mediated by downregulation of Tsc2 by Akt through phosphorylation of S939 and T1462 (Fig. 6 and Supplementary Table 1). First, hTSC2 AA was expressed alone and found to have no effect on spine or somatic morphology (n = 5 cells and 1,014 spines). Second, expression of hTSC2 AA did not occlude the changes triggered by overexpression of AktCA (n = 5 cells and 1,149 spines). Third, hTSC2 AA expression in Tsc2 knockdown cells rescued spine and soma morphology with an efficiency equal to that of wild-type hTSC2 (n = 5 cells and 1,824 spines). Thus, phosphorylation of S939 and T1462 is not necessary for regulation of neuronal soma and spine size by Tsc2, and hTSC2 AA is unable to act as a dominant-negative with respect to the neuronal enlargement triggered by upregulation of Akt.

**Morphological changes require signaling through cofilin**

The mechanism by which the TSC pathway regulates neuronal morphology is unknown. Cofilin, a protein that depolymerizes and severs actin filaments and is widely expressed in the mammalian brain, has recently been shown to regulate spine size20,21. Cofilin is negatively regulated by LIMK through phosphorylation at a conserved site (serine 3). We examined whether phosphorylation at this site is regulated by the TSC pathway using IFHC to monitor levels of Ser3-phosphorylated cofilin (p-cofilin) in cultures of dissociated hippocampal neurons (Fig. 7a,b). At 10 DPT, shTsc2-transfected cells had higher p-cofilin levels than control neurons (Fig. 7b; n = 34–42 cells), whereas total cofilin levels were unchanged (n = 26–32 cells). In addition, p-cofilin, but not total cofilin, was increased in Tsc2−/− mouse embryonic fibroblasts (MEFs)22 compared to Tsc2+/+ MEFs (n = 3–5, P < 0.05; Fig. 7c,d). Similar results were obtained with a second commercial p-cofilin-specific antibody (see Methods, data not shown). Furthermore, the intensity of the ~19 kD band recognized by the p-cofilin antibody on western blots reflected levels of phosphorylated cofilin as its intensity was reduced in HEK293T cells transfected with the cofilin Ser3 phosphatase slingshot-1L23. No changes in intensity were noted following LIMK1 transfection, possibly reflecting additional regulation of the kinase activity independently of its expression level.

To determine whether increased cofilin phosphorylation is necessary for the spine enlargement described above, we expressed wild-type cofilin or cofilin with the LIMK phosphorylation site mutated to alanine (cofilin S3A) in shTsc2-transfected neurons in rat organotypic slice cultures (Fig. 7e). At 10 DPT, neurons expressing cofilin and shTsc2 had slightly reduced spine length compared to those expressing shTsc2 alone (n = 9 cells and 2,336 spines, P < 0.05) but similar soma size, spine head width and spine density. In contrast, expression of cofilin S3A in shTsc2-transfected neurons restored soma size, spine head width and spine density to control levels and decreased spine length to levels slightly below those of control neurons (n = 6 cells and 3,200 spines, P < 0.05 compared to shTsc2 neurons; Fig. 7e). Cofilin S3A expression in control neurons had no effect on soma size, spine head width and spine density, but it did reduce spine length (n = 6 cells and 4,243 spines, P < 0.05; Fig. 7f). The morphology of cofilin S3A/shTsc2-expressing cells was identical to that of cofilin S3A/GFP-expressing neurons (Fig. 7g), indicating that cofilin lies downstream of Tsc2 and that phosphorylation at Ser3 is necessary for the morphological changes induced by loss of Tsc2.

**DISCUSSION**

We have shown that the TSC pathway regulates the morphology and function of post-mitotic, hippocampal pyramidal neurons. Loss of Tsc1/Tsc2 triggered multiple changes in neuronal morphology, including increased soma and dendritic spine size as well as decreased dendritic spine density. These effects required both rapamycin-sensitive mTOR activity and regulation of cofilin at a conserved site during phosphorylation of cofilin as its intensity was reduced in HEK293T cells transfected with the cofilin Ser3 phosphatase slingshot-1L23. No changes in intensity were noted following LIMK1 transfection, possibly reflecting additional regulation of the kinase activity independently of its expression level.

**Relationship of Tsc2 and Akt**

We found that expression of constitutively active Akt phenocopies the morphological defects seen with loss of Tsc1 or Tsc2. Increased Akt activity leads to enlarged neuronal size, in agreement with studies using overexpression of Akt or downregulation of PTEN, the lipid kinase inhibitor that blocks mTOR-dependent growth in mammalian cells.
phosphatase that antagonizes PI3K. However, we found that the somatic and dendritic spine enlargement induced by increased Akt activity is independent of phosphorylation of TSC2 at S939 and T1462, the consensus sites conserved across D. melanogaster, rats, mice and humans. This is in contrast to findings in D. melanogaster in which expression of dTsc2S924A/T1518A suppresses the enlargement of ommatidia triggered by overexpression of Akt. Furthermore, we found that expression of hTSC2AA does not perturb spine or soma size and that it reverses the effects of loss of endogenous Tsc2. Again, these results are in contrast with the marked reduction in ommatidia size following dTsc2S924A/T1518A expression but in agreement with recent findings that dTsc2-null phenotype in D. melanogaster development. Lastly, our results are consistent with recent findings that indicate that Akt is regulated by mTOR and thus lies downstream of Tsc1/Tsc2. In summary, our data indicate that, although upregulation of Akt and downregulation of Tsc1/Tsc2 result in similar phenotypes, phosphorylation of Tsc2 at S939 and T1462 is not necessary for the normal control of neuronal size or for the neuronal enlargement induced by upregulation of Akt.

**Molecular mechanisms of morphological perturbations**

In addition to regulating mTOR activity, TSC1 and TSC2 are reported to participate in mTOR-independent signaling cascades that underlie the morphological changes described above. Indeed, we find a link between the TSC pathway and regulation of the cytoskeleton through the sequestration of FK506-binding proteins.

The central role of actin dynamics in determining spine structure strongly suggests that perturbed regulation of the actin cytoskeleton underlies the morphological changes described above. Indeed, we find a link between the TSC pathway and regulation of the cytoskeleton through Akt. Cofilin is an actin depolymerization factor whose activity is downregulated following phosphorylation by LIM kinase at Ser3 (S3) . Haploinsufficiency of LIMK1, a downstream effector of Akt in the TSC pathway, results in the cognitive disorder Williams syndrome. Both cofilin and LIMK1, as well as upstream regulators of the pathway such as PAK and Rho, have been shown to regulate dendritic spine morphology. We found that phosphorylation of cofilin at S3 is necessary for the morphological changes triggered by loss of Tsc2, as they are occluded by expression of cofilin with this site eliminated (S3A). Our results also demonstrate that the balance of active and inactive cofilin is regulated by the TSC pathway in both neurons and MEFs, such that loss of Tsc2 leads to increased

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**Figure 7** Phosphorylation of cofilin is regulated by Tsc2 and is necessary for increased cell growth. (a) Cultured hippocampal neurons transfected with shTsc2 showing GFP fluorescence (green, left), immunostaining for MAP2 (blue, left) and immunostaining for phosphorylated cofilin (p-cofilin) (red, right). Arrows and asterisks highlight shTsc2-transfected neurons and nontransfected neighbors, respectively. Scale bar, 20 μm. (b) Cumulative distribution of p-cofilin (left) and total cofilin (right) immunostaining in shTsc2-transfected and GFP-transfected neurons at 10 DPT. (c) Left, western blots of p-cofilin (left) from Tsc2+/+ MEFs, Tsc2−/− MEFs, control HEK293T cells and HEK293T cells transfected with LIMK1 or slingshot-1L. Right, western blot of total cofilin in Tsc2+/+ and Tsc2−/− MEFs. (d) Summary of band intensities for p-cofilin, cofilin and tubulin in Tsc2+/+ and Tsc2−/− MEFs relative to Tsc2+/+ MEFs (left), and the intensities of p-cofilin bands in HEK cells transfected with LIMK1 or slingshot-1L relative to untransfected cells (right). *P < 0.05. (e) Dendrites of pyramidal neurons in organotypic hippocampal slices transfected with shTsc2 (top), shTsc2+cofilin (middle) or shTsc2+cofilinS3A (bottom). Scale bar, 5 μm. (f) Dendrites of pyramidal neurons transfected with GFP (top) or GFP + cofilin S3A (bottom) imaged at 10 DPT. (g) Summary of the effects of the manipulations shown in e and f on spine density, length, head width and soma area. Shaded areas represent the mean ± 2 s.e.m. of each parameter for control (dark gray) and shTsc2 (light gray) neurons. *P < 0.05 compared to neurons transfected with GFP or shTsc2 alone, as appropriate.
METHODS

Animals. We used mice carrying a conditional Tsc1 allele (Tsc1 

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lox 

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lox 

) consisting ofloxP elements upstream of exon 17 and downstream of exon 18, as well as Sprague-Dawley rats (Charles River Laboratory). Tsc1 

∗ 

lox 

lox 

 mice were generated by crossing Tsc1 

∗ 

lox 

lox 

 mice with C57Bl6 mice (Charles River Laboratories). Rosa26C genotyping was performed using tail genomic DNA and primers F4536 (5′-CTACTTGTCTTGTCCGATGGGCTGTTGCGGATCCNAATGTA AT-3′) and R4641 (5′-CGAGGCACATTCTGACCAGGCTGTTGCGGATCCATGGTGCAA-3′), yielding 295-bp and 480-bp products from wild-type and conditional alleles, respectively.

Plasmids. All enzymes were obtained from New England Biolabs. The following plasmids were gifts: pBS-β-actin-cri (S. Dyacke, Harvard Medical School); hTSC2 and hTSC2AA (E. Henske, Fox Chase Cancer Center, Philadelphia, Pennsylvania); pBS/U6 (Y. Shi, Harvard Medical School); cofilin and cofilin53A (A. Minden, Columbia University); SlingshotII1 and LIM-kinase1 (K. Mizuno, Tohoku University, Japan). pEGFP-N1 (Invitrogen) was used as a GFP control. For production of the dual promoter CMV-EGFP/U6-shRNA vector, pEGFP-N1 was digested with BamHI and BglII to remove the multiple cloning site (MCS) and designated as pEGFP-N1:AMCS. The U6 promoter and its downstream MCS from pBS/U6 were inserted into pEGFP-N1:AMCS at a filled-in AflI site downstream of the SV40 polyadenylation sequence in the forward (pGUF) or reverse (pGUR) orientation relative to EGFP. Four sets of shRNAs directed against Tsc2 were designed from 19–21 bp coding sequences complementary to Tsc2, which were inserted into the MCS of the above plasmid. The shTsc2 clone used in this study was constructed from the following oligonucleotides: GGGTAAAGAGAGCCTGCATCACAAGGTCCTTGGATACGCCGCTTCCTTTACCCCTTCTTTGTT and AATTTACAAAGGGTGAAAGAGGCCGATCCTAACAGGTCCTTGGATACGCCGCTTCCTTTACCCCTTCTTTGTT. For production of shTsc2, pGUF was digested with AflI, klenow-digested with EcoRI and treated with calf intestinal alkaline phosphatase. Oligonucleotides were annealed, phosphorylated with T4 polynucleotide kinase and ligated into the digested pGUF to yield shTsc2.

Cultures and transfection. Dissociated hippocampal cultures were prepared from P3 rats and mice50 and plated at 8 × 10⁴ cells/well on glial monolayers on 12-mm glass coverslips. Cultures were transfected with Lipofectamine 2000 (Invitrogen) after 3–5 days in vitro (d.i.v.) in neuronal media lacking B27, 0.1% Triton X-100/PBS (Sigma) for 10 min, blocked with 1% goat serum/PBS (Jackson Immunolabos) and incubated with the following primary antibodies: anti-phospho-S6 ribosomal protein (1:100, Ser235/236; Cell Signaling), anti-MAP-2 (mouse, 1:500, Sigma), anti-MAP-2 (rabbit, 1:500, Chemicon), anti-Cre recombinease (mouse, 1:1,000, Chemicon), anti-Cre recombinease (rabbit, 1:1,000, Novagen), anti-p-cofilin1 (Ser3) (1:100, Santa Cruz) and anti-cofilin1 (1:200, Cell Signalling). Antibodies specific to tuberin and hamartin (1B2A8 and HF6, respectively) were kindly provided by V. Ramesh (Massachusetts General Hospital). The following secondary antibodies from Jackson Immunolabos were used at a dilution of 1:500: Cy3-conjugated goat anti-rabbit, Cy3-conjugated goat anti-mouse, Cy3-conjugated goat anti-rabbit and Cy5-conjugated goat anti-mouse. Secondary antibody fluorescence was measured in transfected and untransfected neurons using an LSM510 confocal (Zeiss).

Implication for tuberous sclerosis complex

Individuals with TSC show perturbations of cortical architecture including tubers, which are disorganized regions of the brain with disturbed lamination containing increased numbers of astrocytes and sparse neurons1. The correlation between the number of cortical tubers and the severity of seizure symptoms has led to the idea that the neurological deficits in TSC could arise from disruptions of cortical architecture46. Here we show that loss of a single copy of Tsc1 results in defects in neuronal morphogenesis, including increased soma size, decreased spine density and increased spine size. Therefore we propose that cell-autonomous neuronal defects due to haploinsufficiency of TSC1 or TSC2, in addition to the perturbations of brain architecture caused by cortical tubers, subependymal nodules and giant cell astrocytomas, contribute to the pathogenesis of the neurological symptoms of TSC.

Conclusion

S3-phosphorylated coflin without changes in total coflin. Possible mechanisms for this effect include mTOR-dependent translational control of a key regulatory protein as well as direct regulation of the enzymatic activity of Akt, LIMK1, or slingshot by mTOR or a downstream kinase such as Akt29. Alternatively, regulation of Rho through Tsc1-dependent protein-protein interactions30 might mediate downstream regulation of coflin.

Immunofluorescence. Dissociated hippocampal neurons were fixed in 3.7% paraformaldehyde/4% sucrose for 15 min at 20–24 °C, permeabilized with 0.1% Triton X-100/PBS (Sigma) for 10 min, blocked with 1% goat serum/PBS (Jackson Immunolabos) and incubated with the following primary antibodies: anti-phospho-S6 ribosomal protein (1:100, Ser235/236; Cell Signaling), anti-MAP-2 (mouse, 1:500, Sigma), anti-MAP-2 (rabbit, 1:500, Chemicon), anti-Cre recombinease (mouse, 1:1,000, Chemicon), anti-Cre recombinease (rabbit, 1:1,000, Novagen), anti-p-cofilin1 (Ser3) (1:100, Santa Cruz) and anti-cofilin1 (1:200, Cell Signalling). Antibodies specific to tuberin and hamartin (1B2A8 and HF6, respectively) were kindly provided by V. Ramesh (Massachusetts General Hospital). The following secondary antibodies from Jackson Immunolabos were used at a dilution of 1:500: Cy3-conjugated goat anti-rabbit, Cy3-conjugated goat anti-mouse, Cy3-conjugated goat anti-rabbit and Cy5-conjugated goat anti-mouse. Secondary antibody fluorescence was measured in transfected and untransfected neurons using an LSM510 confocal (Zeiss).

Western blotting. Lysates of Tsc2+/− and Tsc2+/− MEFS, and HEK293T cells (control or 24 h after transfection with LIMK1 or 5SH11L), were separated by SDS-PAGE using 8–16% Tris-HCl gels (BioRad). Proteins were transferred onto a polyvinylidene difluoride membrane (PVDF, BioRad) overnight at 4 °C. Membranes were incubated in blocking solution (5% BSA, 0.1% Tween 20 in Tris-buffered saline) for 1 h at 20–24 °C, incubated overnight at 4 °C with primary antibodies (rabbit anti-p-cofilin S3 (1:200, Santa Cruz), rabbit anti-p-cofilin S3 (1:1,000, Cell Signalling), rabbit anti-cofilin S3 (1:1,000, Cell Signalling), rabbit anti-tubulin (1 μg/ml, AbCAM), washed and incubated for 1 h at 20–24 °C with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:2,000, BioRad). Membranes were washed and incubated for 5 min with chemiluminescent substrate (Pierce) before exposure to film. Band densitometry was performed using Quantity One 4.5.0 software (BioRad).

Two-photon laser scanning microscopy and image analysis. Neurons were imaged with custom-built two-photon laser scanning microscopes44 with an excitation wavelength of 910 nm. Images of transfected pyramidal neurons were acquired at 0.8× zoom (image field, 300 × 370 μm), whereas spiny regions of basal and apical dendrites were imaged at 5× magnification (image field, 42 × 42 μm). Optical sections were taken at 1.0-μm spacing. Spine density, length and width, as well as soma size, were measured manually using custom software50 by observers who were blind to genotype. Spine lengths were measured from the junction with the dendritic shaft to the tip. To determine head width and primary dendrite thickness, the fluorescence was measured in a line across each structure and the width of the distribution where fluorescent intensity fell to 30% of maximum was calculated. Measurements performed on 100-nm diameter yellow-green fluorescent microspheres (FluoSpheres, Molecular Probes) indicated that the point-spread function placed a lower limit on measurable widths of 550 nm. The apparent width is the convolution of the fluorescent intensity fell to 30% of maximum was calculated. Measurements performed on 100-nm diameter yellow-green fluorescent microspheres (FluoSpheres, Molecular Probes) indicated that the point-spread function placed a lower limit on measurable widths of 550 nm. Therefore, the summaries of spine head widths are plotted from this lower bound. Soma cross-sectional area was measured in the maximum intensity projection of a low-power image stack by counting the number of pixels within an outline drawn around the soma. Proximal dendrites of Tsc1+/-/− control and Cre-expressing neurons were of similar thickness (control, 1.26 ± 0.1 μm; Cre, 1.49 ± 0.1 μm; n = 9–13 cells).

Electrophysiology. Hippocampal slice cultures from Tsc1−/− mice were placed in a recording chamber perfused with artificial cerebrospinal fluid (ACSF) containing 127 mM NaCl, 25 mM NaHCO3, 1.25 mM Na2HPO4, 2.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 25 mM glucose and saturated with 95% O2, 5% CO2 at 20–24 °C. Whole-cell voltage-clamp recordings were obtained using an Axopatch 200B amplifier (Axon Instruments) from Cre-transfected pyramidal neurons (green fluorescence and visible gold particle in soma) and...
untransfected neighbors at 10–12 DPT and 18–20 DPT. Transfection with GFP alone had no effect on membrane properties (data not shown).

Borosilicate glass pipettes (3–5 MΩ tip resistance; Warner Instruments Inc.) were filled with 120 mM cesium methanesulfonate, 10 mM HEPES, 10 mM EGTA, 4 mM MgCl₂, 0.4 mM NaGTP, 4 mM MgATP, 10 mM phosphocreatine and 0.02 mM Alexa Fluor-594 (Molecular Probes) at a pH of 7.3 (290 MuS). Bicuculline (20 μM, Tocris) was added to the bath to block GABA_A receptors in all experiments and tetrodotoxin (1 μM, Sigma) was included to block sodium channels for mPSC recordings. Series resistance (R_S; >19 MΩ, <20 MΩ for inclusion in data set), input resistance and membrane capacitance were monitored online. mEPSC frequency and amplitude were analyzed in Igor Pro (Wave metrics) using custom software. mEPSCs were evoked at 0.125 Hz with a bipolar electrode placed in the stratum radiatum 250–350 μm from the soma. The AMPAR/NMDAR current ratio was calculated from the ratio of the EPSC peak amplitude at –60 mV to the current at +40 mV 100 ms after the peak. To calculate PPF, paired stimuli at an interpeak interval of 50 ms were delivered and the ratio of peak amplitudes of the EPSCs was calculated.

Statistics. Statistical significance was tested by analysis of variance (ANOVA) with, when appropriate, a Tukey-Kramer correction for multiple pairwise comparisons in Matlab (Mathworks) or Microsoft Excel. Distributions of mEPSC amplitudes and immunostaining intensities were compared using Kolmogorov-Smirnov tests. Band intensities of western blots were compared with paired t-tests. Summary data is presented as mean ± standard error of the mean (s.e.m.).

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

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Supplemental Figure 1

- ○ shTsc2
- ● shGFP

Tsc2 immunostaining (au) vs. pS6 immunostaining (au)
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<th>N cells/spines</th>
<th>SOMA AREA m²</th>
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**Supplemental Table 1**