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| As Published | http://dx.doi.org/10.1523/jneurosci.4796-14.2015 |
| Publisher | Society for Neuroscience |
| Version | Final published version |
| Citable Link | http://hdl.handle.net/1721.1/101096 |
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The Stress-Induced Atf3-Gelsolin Cascade Underlies Dendritic Spine Deficits in Neuronal Models of Tuberous Sclerosis Complex

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Hyperactivation of the mechanistic target of rapamycin (mTOR) kinase, as a result of loss-of-function mutations in tuberous sclerosis complex 1 (TSC1) or TSC2 genes, causes protein synthesis dysregulation, increased cell size, and aberrant neuronal connectivity. Dysregulated synthesis of synaptic proteins has been implicated in the pathophysiology of autism spectrum disorder (ASD) associated with TSC and fragile X syndrome. However, cell type-specific translational profiles in these disease models remain to be investigated. Here, we used high-fidelity and unbiased Translating Ribosome Affinity Purification (TRAP) methodology to purify ribosome-associated mRNAs and identified translational alterations in a rat neuronal culture model of TSC. We find that expression of many stress and/or activity-dependent proteins is highly induced while some synaptic proteins are repressed. Importantly, transcripts for the activating transcription factor-3 (Atf3) and mitochondrial uncoupling protein-2 (Ucp2) are highly induced in Tsc2-deficient neurons, as well as in a neuron-specific Tsc1 conditional knock-out mouse model, and show differential responses to the mTOR inhibitor rapamycin. Gelsolin, a known target of Atf3 transcriptional activity, is also upregulated. shRNA-mediated block of Atf3 induction suppresses expression of gelsolin, an actin-severing protein, and rescues spine deficits found in Tsc2-deficient neurons. Together, our data demonstrate that a cell-autonomous program consisting of a stress-induced Atf3-gelsolin cascade affects the change in dendritic spine morphology following mTOR hyperactivation. This previously unidentified molecular cascade could be a therapeutic target for treating mTORopathies.

Key words: autism; dendritic spines; mTOR; protein synthesis; translation

Introduction

Dysregulation of the mechanistic target of rapamycin (mTOR) kinase is involved in many neurologic disorders, such as tuberous sclerosis complex (TSC), PTEN-hamartoma syndromes, neurofibromatosis 1 (NF-1), and epilepsy (Lipton and Sahin, 2014). As a serine/threonine kinase, mTOR integrates extracellular nutritional stimuli and intracellular energetic status to finely regulate protein synthesis and control cell growth rate. An increasing body of evidence suggests that dysregulated synthesis of certain synaptic proteins is one underlying cellular mechanism of autism spectrum disorder (ASD) (Auerbach et al., 2011; Tsai et al., 2012; Gkogkas et al., 2013; Santini et al., 2013; Ebrahimi-Fakhari and Sahin, 2015). Several specific proteins, such as neuroligin and...
Arc, have been implicated in the synaptic deficits associated with autism (Auerbach et al., 2011; Gkogkas et al., 2013; Ebrahimifakhari and Sahin, 2015); however, systematic translational profiling in these mTOR pathology disease models is lacking.

TSC is an autosomal dominant multisystem disorder caused by loss-of-function mutations in either the TSC1 or TSC2 genes. TSC manifests as a tumor-hamartoma syndrome affecting multiple organs, including the brain, skin, eyes, kidneys, heart, and lungs. The pathognomonic brain lesion in TSC is the cortical tuber, and the most devastating neurologic and psychiatric morbidity in TSC patients include epilepsy, intellectual disability, and autism (Feliciano et al., 2013; DiMario et al., 2015). Approximately 30–50% of patients with TSC are affected by ASD (Asato and Hardan, 2004; Jeste et al., 2008). As a monogenic model for both mTOR pathology and autism, TSC mouse and cellular models have offered valuable opportunities for understanding how mTOR-dependent protein synthesis is involved in the synaptic deficits characteristic of ASD (Tavazoie et al., 2005; Choi et al., 2008; Nie et al., 2010; Nie and Sahin, 2012; Tsai et al., 2012; Bateup et al., 2013). On the other hand, TSC results in epilepsy in ~90% of patients (Chu-Shore et al., 2010); thus, studies of TSC mouse models may also help clarify the role of seizure activity in the etiology and/or progression of autism.

To determine what alterations in mRNA translation occur in response to TSC1/2 loss in neurons, we used Translating Ribosomal Affinity Purification (TRAP) methodology, which provides better temporal resolution and cell type specificity than conventional gene expression profiling (Heiman et al., 2008, 2014). In this study, we introduced the transgene L10a in a hippocampal neuronal culture system using lentiviral vectors, for temporally and spatially specific analysis of translation profiling. We analyzed the differential translational expression profile between Tsc2-deficient and control hippocampal neurons, generating a “TSC translatome.” Although reduced expression of synaptic proteins was anticipated, surprisingly we identified induction of a large number of stress and/or activity-induced proteins as well as a number of cytokines in the Tsc2-deficient neurons. Functionally, our data demonstrate that Atf3-gelsolin cascade downstream of TSC1/2 loss can negatively regulate dendritic spine morphology and impair neuronal connectivity. Our results suggest that hippocampal neurons, when challenged by mTOR hyperactivation, activate a stress-induced prosurvival program at the expense of neuronal connectivity.

Materials and Methods

Antibodies, constructs, and animals. Antibodies to PSD-95 (Pierce), MAP2 (Sigma-Aldrich), GFP (Abcam, Roche, and Rockland), mCherry (Abcam, BioVision), puromycin (KeraFAST), Atf3 (Santa Cruz Biotechnology), Ucp2 (Abcam), p-CAMP response element binding (CREB) (Ser133) (Cell Signaling Technology), tubulin (Cell Signaling Technology), GAPDH (Santa Cruz Biotechnology), and FLAG (Agilent Technologies) were obtained commercially as indicated. Monoclonal anti-GFP antibodies (19C8 and 19F7) for the TRAP assay were purchased from the Memorial Sloan-Kettering Monoclonal Antibody Facility (New York). Gelsolin antibody was a generous gift from Prof. Walter Witke (University of Bonn, Bonn, Germany). For immunocytochemistry, fluorescently labeled secondary antibodies (AlexaFluor-488, -594, or -647, Invitrogen) were used. TTX and rapamycin (both Sigma-Aldrich) and MK801 (Tocris Bioscience) were obtained commercially.

shRNAs against firefly luciferase or rat Tsc2 gene have been described previously (Nie and Sahin, 2012). GFP was replaced with mCherry at cloning sites NheI/EcoR I by using the primer set: 5’-TACAGCCAGCTGTTGCTGATGAGA-3’ and 5’-GCCAGATCTCTACCTGAGATCCTGTC-3’. To clone GFP versus GFP-L10a transgenes into the lentivector pHAGE downstream of the UBC promoter, primer pairs were used as above: 5’-GAAAAAGCGGCGCAGATGGAAA-3’ and 5’-AA GTTATATGATCCGGTCGGGTCGTTGAGAA-3’ for GFP, 5’-GAAAAAGCGGCGCAGATGGAAA-3’ and 5’-AA GTTATATGATCCGGTCGGGTCGTTGAGAA-3’ for GFP-L10a. The cloning sites NotI/BamH1 were used.

shRNA (CAGAGAATACACCCTGCTGATCC) targeting Atf3 was obtained from Sigma-Aldrich, validated and then subcloned into CSGGW vector, which has eGFP inserted downstream of a PGK promoter. Lentivirus particles were packaged and produced by the MGH Viral Core Facility, with a virus titer of 7.596 × 10^8 IU/ml. The pRK-ATF3 plasmid was a gift from Yihong Ye (AddGene, plasmid # 26115) (Wang et al., 2009).

Synl-Cre-Tsc1-wt and Syn-Cre-Tsc1-mice were described previously (Meikle et al., 2007). Hippocampal lysates were generated from brains harvested at postnatal day 21 from both male and female animals. All animal procedures were in accordance with the Guide for the Humane Use and Care of Laboratory Animals, and the study was approved by the Animal Care and Use Committee of Boston Children’s Hospital.

Neuronal culture, drug treatment, and Western blotting. Hippocampal neuronal cultures from rat embryonic day 18.5 or mouse embryonic day 16.5 were obtained as previously described (Nie and Sahin, 2012). Neuron cultures were maintained in Neurobasal medium supplemented with antibiotics, B27 and glutamate. For drug treatments, drugs or vehicle were added to conditioned medium for the time periods indicated. Neurons were then quickly washed with 1X PBS and lysed with 1X RIPA buffer or directly with 1X SDS sample buffer. Lysates were boiled at 95°C for 5 min before SDS-PAGE and Western blotting.

Puromycin incorporation assay. Hippocampal neurons infected with lentiviral sh-Luc versus sh-Tsc2 were treated with anisomycin (20 µM for 1 h), rapamycin (20 nM, overnight), or vehicle before puromycin incubation (1 µM for 30 min). Cells were snap frozen on dry ice, lysed in RIPA buffer (with protease inhibitors), and homogenized. After centrifugation at 13,000 rpm, 4°C, for 10 min, supernatants were collected, mixed with half volumes of 3X sample buffer, and boiled immediately at 95°C for 5 min. Total protein concentrations were determined by Bradford protein assay kit. Equal amounts of proteins were loaded onto 4%-20% gradient SDS-PAGE gels (Bio-Rad). Anti-puromycin antibody (KeraFAST) at 1:5000 dilutions was used for detection of newly synthesized proteins by Western blotting, and postblotting membrane staining by EZBlue reagent (Sigma-Aldrich).

Neuronal transfection, lentivirus infection, and immunocytochemistry. Plasmids and Lipofectamine 2000 were diluted in plain Neurobasal medium at a ratio of 1 µg/ µl for each 13 mm coverslip in a 24-well plate. The plasmid medium was used to add each matching Lipofectamine tube. The mixtures were incubated for 20 min at room temperature. Medium in wells to be transfected was replaced with 500 µl prewarmed plain Neurobasal and the original medium was saved in a 15 ml Falcon tube. The plasmid:Lipofectamine transfection mixture was subsequently added to the top of each well (100 µl per well) and incubated for 2 h. The transfection medium was then washed off and replaced with the saved conditioned medium. In general, transfection was performed on day 7 in vitro (7 DIV) or day 11 (11 DIV) for experiments shown in Figure 7.

Lentiviral particles encoding hU6-driven shRNAs were produced in HEK293T cells as previously described (Nie et al., 2010; Nie and Sahin, 2012). These included GFP or mCherry expressing sh-Luc (against firefly luciferase) versus sh-Tsc2 (against rat Tsc2 gene). Neuronal cultures were infected for 4–6 h on 2 DIV and 9 DIV (for experiments shown in Fig. 7). After washing off the virus-containing medium, cultures were maintained as described above.

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DOI: 10.1523/JNEUROSCI.4796-14.2015

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J. Neurosci., July 29, 2015 • 35(30):10762–10772 • 10763
Transfected or infected neurons were fixed at 16–21 DIV with 4% PFA plus 4% sucrose. Images were captured using Zeiss LSM700 confocal microscope system and analyzed using National Institutes of Health Image.

**Modified TRAP assay.** We followed the TRAP protocol for cell cultures with minor modifications (Heiman et al., 2008, 2014). Before harvesting, neuronal cultures in 10 cm dishes were incubated with 100 μg/ml of CHX for 20 min. After washing twice with PBS containing 100 μg/ml of CHX, cells were lysed with 1 ml ice-cold lysis buffer (20 mM HEPES KOH, pH 7.4, 150 mM KCl, 5 mM MgCl₂, 1% NP-40, EMD Biosciences; 0.5 mM dithiothreitol, 100 μM CHX, protease inhibitors, and RNase inhibitors). The cell lysates were then homogenized on ice by passing through differentially gauged needles. Homogenates were centrifuged for 10 min at 2000 × g at 4°C, to pellet large cell debris, and 1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHPC; Avanti Polar Lipids) was added to the supernatant at a final concentration of 300 μM. After mixture and incubation on ice for 5 min, the lysate was further centrifuged for 10 min at 20,000 × g at 4°C, to pellet unsolubilized material. Monoclonal anti-GFP-coated protein G Dynal magnetic beads (Invitrogen) were added to the supernatant, and the mixture was incubated at 4°C with end-over-end rotation for 30 min. Beads were subsequently collected on a magnetic rack, washed three times with high-salt polysome wash buffer (20 mM HEPES-KOH, pH 7.4, 350 mM KCl, 5 mM MgCl₂, 1% NP-40, 150 mM NaCl, 20 mM Tris-HCl, pH 8.8, 1% sodium orthovanadate, and protease inhibitors). Protein samples were centrifuged at 13,000 rpm for 10 min, and supernatants were collected. Protein concentration was determined by Bradford protein assay. RNA extraction was performed using the RNease miniprep kit (QIAGEN) following the manufacturer’s instructions, and RNA quality was evaluated using NanoDrop spectrometry. The quality of purified RNAs was evaluated based on RNA integrity numbers read by an Agilent Technologies BioAnalyzer 2100.

**Microarray analysis and bioinformatics.** Three biological replicates were performed for each experimental condition (sh-Luc, sh-Tsc2, sh-Tsc2 with rapamycin). Purified RNAs were amplified with the Ovation System (NuGEN) according to the manufacturer’s instructions. Biotin-labeled cDNAs were hybridized onto the Affymetrix rat gene 1.0 ST array. Differential gene expression analysis was conducted between Tsc2 knockdown and control cultures (sh-Luc, sh-Tsc2, sh-Luc with siRNA targeting ATF3). The resulting raw data were analyzed using RMA and the limma package of Bioconductor. Genes with a adjusted p value < 0.05 and a fold change > 2 were selected for further analysis.

**Results**

Global increase in de novo protein synthesis in Tsc2-deficient neurons

mTOR is a master regulator of mRNA translation and protein synthesis (Thoreen et al., 2012). We have previously established a neuronal culture model of TSC that allows us to monitor mTOR activation after knocking down the Tsc2 gene (Nie and Sahin, 2012). To investigate the net effect of Tsc2 knockdown on protein synthesis, we first performed metabolic puromycin incorporation assays (Fig. 1). *De novo* protein synthesis, as quantitated based on Western blotting with an anti-puromycin antibody, was significantly increased in hippocampal neuronal cultures infected with a lentiviral vector carrying Tsc2-shRNA (sh-Tsc2) compared with control neurons infected with shRNA against firefly luciferase (sh-Luc) (1.159 ± 0.054 normalized to sh-Luc). Furthermore, rapamycin treatment (20 nM, overnight) suppressed puromycin incorporation and corrected the difference between Tsc2 knockdown and control cultures (0.797 ± 0.076 vs 0.711 ± 0.075). As a control, anisomycin, a known protein synthesis inhibitor, reduced *de novo* protein synthesis by nearly 90% (0.115 ± 0.024). These data indicate that mTOR activation, as a consequence of Tsc2 knockdown, results in increased *de novo* protein synthesis in hippocampal neuronal cultures.

**Application of a modified TRAP assay for translational profiling**

Loss of neuronal Tsc1 or Tsc2 causes abnormal axon specification, aberrant axon path finding, defects in dendritic morphology, reduced myelination, hyperexcitability, and seizure activity, as well as autistic behaviors in mouse models (Tavazoie et al., 2005; Meikle et al., 2007; Choi et al., 2008; Nie et al., 2010; Tsai et al., 2012; Bateup et al., 2013). Whereas extracellular and intracellular regulators of the TSC-mTOR pathway have been relatively well investigated, many downstream effectors that are regulated by mTOR remain to be explored in detail (Thoreen et al., 2012). We hypothesized that translation of a subset of mRNAs is directly regulated by mTOR in postmitotic neurons when the TSC complex is functionally disrupted. To test this, we used the TRAP assay by introducing a lentivirus-based L10a transgene into neuronal cultures (Heiman et al., 2008). This technique analyzes mRNAs associated with the ribosome rather than steady-state mRNA abundance and thus represents a more direct assessment of ongoing mRNA translation. We found that the GFP-L10a signal expression was enriched in nucleoli and also distributed throughout the soma and within the neurites, even though the latter is much weaker (Fig. 2A). We did not observe a significant increase in cell death or apoptosis in neurons with L10a transgene expression as measured by cleaved caspase-3 staining (data not shown). To perform anti-GFP immunoprecipitation in the Tsc2-

### Table 1. The primers used for qPCR

<table>
<thead>
<tr>
<th>Rat gene</th>
<th>Accession no.</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tsc2</td>
<td>NM_012680.2</td>
<td>GAGTGTGTCACAGAAGATGA</td>
<td>TTGGTAAAGAGACAGAAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>AGAAGACGCACTGTCCTG</td>
<td>CTGGTGGTCCAGTATG</td>
</tr>
<tr>
<td>18S</td>
<td>NR_046237.1</td>
<td>GTAGCTGGTTAGACCCCTAT</td>
<td>CCACTGAACTGTAAGTGC</td>
</tr>
<tr>
<td>Atf3</td>
<td>NM_021922.1</td>
<td>CGAAGAAGGGCCGCTCTA</td>
<td>GACCTGCGGCTGATGTTGATG</td>
</tr>
<tr>
<td>Ucp2</td>
<td>NM_019354.2</td>
<td>ACATTCGACCAAGAGAGG</td>
<td>TCTGGACCACTCAAGGGG</td>
</tr>
<tr>
<td>Snap91</td>
<td>NM_031728</td>
<td>ATAGACCTTTTGGGATAGC</td>
<td>GCAGATTGATGTGACTGGA</td>
</tr>
<tr>
<td>Prep</td>
<td>NM_053385.1</td>
<td>AACACGCTGGGCGATGAGG</td>
<td>AAGAGTGGGCAAGAATGAG</td>
</tr>
<tr>
<td>Hps1</td>
<td>NM_031970.3</td>
<td>AGGCGAGGAGAAAAGG</td>
<td>ATTTGTTGACCTTGGTCAGG</td>
</tr>
</tbody>
</table>

N.B. The primers were designed using Primer3 software.

**Notes:**

The table above lists the primers used for qPCR expression. The primers were designed using Primer3 software. This method allows for the specific amplification of mRNA targets, providing a quantitative measure of gene expression.
defective culture, we infected hippocampal neurons with two different lentiviral vectors: one coexpresses mCherry and shRNA (sh-Luc vs sh-Tsc2) and the other expresses GFP-L10a (Fig. 2B).

The efficiency of Tsc2 knockdown and L10a transgene expression was confirmed by Western blotting. Consistent with previous studies (Tavazoie et al., 2005; Choi et al., 2008; Di Nardo et al., 2009), Tsc2 knockdown in hippocampal neurons resulted in enlarged cell body and phosphorylation of the mTORC1 substrate S6 kinase 1 (S6K1) (Fig. 2D). By using RNA preserving buffer and RNase inhibitors, we performed RNA purification from both immunoprecipitation experiments. With the anti-GFP antibody, we were able to pull down RNAs only from GFP-L10a-expressing cultures. With the anti-GFP antibody, immunoprecipitation experiments yielded RNAs only from GFP-L10a-expressing cultures.

The TRAP genes display differential sensitivity to rapamycin.

We purified ribosome-associated RNAs from three conditions (each in triplicate): (1) sh-Luc (control); (2) sh-Tsc2; and (3) sh-Tsc2 with overnight rapamycin (20 nM) treatment. The amplified cDNAs were subjected to conventional microarray study on Affymetrix rat gene 1.0 chips. The raw data revealed 82 genes differentially expressed at the ribosomal level among these three conditions at false discovery rate (FDR) < 1e-5. (Fig. 3A). The correlation plots from two independent TRAP experiments for each condition confirmed the high reproducibility among replicates (Fig. 3B). Based on the hierarchical clustering, the candidate genes were further classified into three groups. Group 1 contains 17 genes that were downregulated in Tsc2-deficient neurons. Several synapse or synaptogenesis-associated genes, such as Snaph91 and Cbln1, are in this group. Group 2 contains 27 genes that were upregulated by Tsc2 loss, but not rescued by overnight rapamycin treatment. This group includes actin organizing proteins, such as gelsolin (Gsn) and FAM107a. Finally, in Group 3, there were 38 genes that were upregulated in response to Tsc2 loss and were suppressed by overnight rapamycin treatment. Among these, many stress responsive genes, including c-Fos, Ucp2, Hspb1/Hsp27, Hmox1, and Atf3, as well as cytokine genes, such as Ccl2 and Cxcl10, are of particular interest.

We next focused on the annotated genes and used scatter plot analysis to determine which ones are corrected by mTORC1 inhibition by using a twofold change cutoff. Sixteen genes, including Atf3, were differentially expressed in the sh-Tsc2 cultures and corrected by rapamycin treatment (red dots in Fig. 3C; Log2 Fold < 1 when comparing sh-Tsc2 with rapamycin to sh-Luc). In contrast, 14 genes, including Ucp2, remained significantly altered in the Tsc2-deficient neurons despite rapamycin treatment (green dots; Log2 Fold > 1 when comparing sh-Tsc2 with rapamycin to sh-Luc; FDR < 1e-5). Both the activating transcription factor-3 (Atf3) and mitochondrial uncoupling protein-2 (Ucp2) are components of cellular stress response machinery. Together, these findings suggest that Tsc2 loss in neurons induces a potent stress response.

Atf3 is highly upregulated in neuronal TSC models.

To further tease out translational versus transcriptional regulation, we purified total transcripts versus ribosome-associated transcripts after the TRAP procedure. Quantitative real-time PCRs for the candidate genes demonstrated a concordance between transcriptional and translational regulation for the genes we examined, except for the Il1r1 gene, whose translation is enhanced with no significant change in total transcripts. For example, both the total transcripts and the ribosome-associated transcripts of Atf3 and Ucp2 were elevated in Tsc2-deficient neurons. Interestingly, the two genes displayed different responses to rapamycin (20 nM, overnight). Overnight rapamycin treatment reversed the induction of both total transcripts and ribosome-associated transcripts of Atf3. The ribosome-associated transcripts of Ucp2, however, remained high following overnight rapamycin treatment even though its total transcript levels were dramatically reduced (Fig. 4A, B).
We further investigated protein levels of Atf3 in TSC models, as well as the responses to acute versus chronic rapamycin treatment. By Western blotting, we found that hippocampal neurons deficient in Tsc2 had a strong induction of ATF3 (Fig. 4C). This induction of ATF3 was reversed by overnight or prolonged rapamycin treatment (Fig. 4D). Furthermore, we found elevated expression of ATF3 in hippocampal lysates from a neuron-specific Tsc1 knock-out mouse model (Tsc1; Synapsin1-Cre, postnatal day 21; Fig. 4E). By quantification, we found that ATF3 levels in homozygous mutants (CC; Synapsin1-Cre/H11001/Tsc1cc) were 50% higher than that of control littermates (WW; Synapsin1-Cre/H11001/Tsc1ww) (Fig. 4F). Together, these data indicate that ATF3 is induced both in vitro and in vivo in TSC-deficient neurons.

In wild-type neurons, overexcitability due to excessive NMDA-receptor signaling activates the calcium-CREB pathway to eventually enhance the expression of Atf3 (Hashimoto et al., 2002; Zhang et al., 2009, 2011). Atf3 expression is also induced in sensory and motor neuron following axotomy (Tsujino et al., 2000; Seijffers et al., 2007). We thus asked whether the increase in ATF3 protein was due to neuronal hyperexcitability in the Tsc2-deficient cultures. To test whether neuronal hyperexcitability contributed to Atf3 expression in Tsc2-deficient neurons, we treated neurons with TTX (1 µM), which blocks sodium channels and consequently neuronal excitability. As expected, TTX treatment for 24 h reduced activation of CREB, as shown by Western blotting for phospho-CREB (Fig. 5A). However, levels of ATF3 and gelsolin proteins were not significantly altered (Fig. 5A–C). These results indicate that activation of the ATF3/gelsolin pathway is independent of neuronal excitability but likely due to mTOR hyperactivity, given that ATF3 induction is corrected by rapamycin treatment (Fig. 4D).

**Dendritic spine deficits in Tsc2-deficient neuronal culture**

Loss of Tsc1 or Tsc2 in pyramidal neurons of hippocampal organotypic slice cultures results in enlarged soma size, increased spine length and head width, but decreased spine density (Tavazoie et al., 2005). In our dissociated cultures, we found similar morphologic deficits in the Tsc2-deficient hippocampal neurons. The soma size of neurons infected with lentivirus carrying sh-Tsc2 appeared larger than that of sh-Luc expressing neurons (Fig. 6A). The spine densities were significantly reduced in sh-Tsc2 compared with sh-Luc neuronal cultures (52.57 ± 3.204 and 16.85 ± 1.794 per 100 µm in sh-Luc vs sh-Tsc2 cultures at 16–21 DIV, respectively). We did not detect an increase of spine density in response to overnight rapamycin treatment. We also found a significant reduction of spine length in Tsc2-deficient neurons compared with control neurons (2.13 ± 0.079 µm in sh-Luc vs 1.77 ± 0.089 µm in sh-Tsc2 neuronal cultures). Rapamycin did rescue the deficit in spine length (2.03 ± 0.172 µm for sh-Luc vs
2.10 ± 0.175 μm for sh-Tsc2). By contrast, we did not observe differences in the spine width between Tsc2-deficient and control neurons that were treated with either rapamycin or vehicle (Fig. 6B–E).

Given the reduction in spine density in Tsc2-deficient neurons, we asked whether molecular markers of excitatory synapses were affected. In neuronal cultures infected with sh-Tsc2 versus sh-Luc virus, we found a significant reduction in the density of PSD-95 puncta in mutant neurons (18.38 ± 2.120 per 100 μm in sh-Luc vs 9.22 ± 2.274 per 100 μm in sh-Tsc2 neurons; Fig. 6F), consistent with a loss of dendritic spines. Together, these data suggest structural and functional abnormalities of excitatory synapses in cultured Tsc2-deficient hippocampal neurons.

Blockade of the Atf3-gelsolin cascade rescues spine density deficit in Tsc2-deficient neurons

Actin-organizing proteins, such as gelsolin and FAM107a, were also found to be upregulated in our TRAP profiling study. Gelsolin, an actin-severing protein, regulates stability of the actin cytoskeleton and is a direct target of Atf3’s transcriptional activity (Yuan et al., 2013). Therefore, we hypothesized that dysregulation of the Atf3-gelsolin cascade could contribute to the reduced spine density in Tsc2-deficient neurons. To test this hypothesis, we infected hippocampal neurons with mCherry expressing sh-Luc or sh-Tsc2, followed by second infection with lentivirus that bicistronically expresses GFP and sh-Atf3. Western blotting revealed that the gelsolin protein level was significantly increased in Tsc2-deficient neurons. shRNA-mediated Atf3 knockdown in Tsc2-deficient neurons prevented the elevation of gelsolin expression with no effects on mTORC1 activity as measured by phospho-S6 (S240/244) levels (Fig. 7A). The expression of mCherry and GFP by different shRNA lentivectors facilitates the investigation of dendritic morphology. We found a robust rescue of spine density in Tsc2-deficient neurons when Atf3 expression was blocked (Fig. 7B, C). Confirming that this increase in spine density is indeed due to
reduced endogenous Atf3 expression, sequential exogenous overexpression of human FLAG-tagged ATF3 (resistant to Atf3 shRNA) reduced spine density significantly. The spine densities per 100 \mu m were 8.635 ± 2.242 (mean ± SE) in sh-Tsc2, 35.17 ± 7.667 in sh-Atf3, 44.15 ± 5.493 in sh-Tsc2 plus sh-Atf3, and 20.04 ± 1.838 in shTsc2 plus sh-Atf3 and ATF3-FLAG add-back cultures at DIV 16 (Figure 7B, C). A lower spine density obtained from this experiment compared
with Figure 6C is likely due to the age difference of the neuronal cultures (DIV 16 vs DIV 16–21). These data demonstrate a previously unappreciated negative effect of the Atf3-gelsolin cascade on spine number and neuronal connectivity.

Discussion

In this study, we applied a TRAP profiling approach to identify mRNAs that are regulated by TSC/mTOR signaling in hippocampal neurons. Our data in Tsc2-deficient hippocampal neurons uncover several functional groups of mRNAs that encode synapse-related proteins, which are downregulated, and stress-inducible proteins, which are upregulated. Importantly, our data link the activation of the Atf3-gelsolin signaling cascade to dendritic spine deficits of Tsc2-deficient neurons, which has potential therapeutic implications for correcting impaired neuronal connectivity in TSC and related mTORopathies.

Ribosome purification has proven a powerful unbiased tool to elucidate cell-type specific translational profiles (Doyle et al., 2008; Heiman et al., 2008; Sanz et al., 2009; Knight et al., 2012). Expression of eGFP-L10a driven by cell type-specific regulatory elements (TRAP mouse lines) has demonstrated the high sensitivity and robustness of this methodology in various types of neural cell populations (Doyle et al., 2008; Heiman et al., 2008). One limitation of the TRAP methodology as originally published is the low yield of mRNA; however, we were able to overcome this by using lentivector-mediated eGFP-L10a expression for tagging polysomes in large-scale primary hippocampal neuronal cultures (Fig. 2A).

The serine/threonine kinase mTOR is a master regulator of cellular mRNA translation. We found that shRNA-mediated Tsc2 knockdown robustly activated mTOR signaling and increased the overall de novo protein synthesis by ~16% (Fig. 1). To identify the mRNAs or proteins that might contribute to the seizure susceptibility and/or neurocognitive impairment seen in patients with TSC, we applied the comparative TRAP assays in a well-established TSC neuronal culture model (Nie and Sahin, 2012). Expression of the transgene eGFP-L10a does not only assist in cell visualization, but it also allows isolation of ribosomes from the entire cell with no need of dissociation that would otherwise disrupt neuronal processes and their network (Fig. 2A, B).

Figure 6. Acute rapamycin treatment rescues the reduction of dendritic spine length but not of spine density in Tsc2-deficient neurons. A, Hippocampal neurons were transfected with lentivector-based sh-Luc versus sh-Tsc2. Representative images show a dramatic decrease in the dendritic spine density as well as increased soma size in Tsc2-deficient neurons. Scale bars, 20 μm. B, High-magnification images showing the spine density and morphology in sh-Luc versus sh-Tsc2-transfected neurons. MAP2 stains dendrites. Scale bars, 5 μm. C, Quantification of spine densities in nontreated hippocampal neuronal cultures or cultures treated with vehicle versus rapamycin. Acute rapamycin treatment failed to rescue the deficits in spine density in Tsc2-deficient neurons compared with control cultures. ***p < 0.001 (Student’s t test). D, Rapamycin rescued the spine length that decreases in Tsc2-deficient hippocampal neurons. **p < 0.01 (Student’s t test). E, No significant differences in spine width between sh-Luc versus sh-Tsc2 cultures were detected, regardless of treatments with vehicle or rapamycin. F, The densities of PSD-95 puncta per 100 μm in sh-Luc versus sh-Tsc2 cultures are 18.38 ± 2.12 and 9.22 ± 2.27 (mean ± SEM). *p < 0.05 (Student’s t test).
ther enable the study of local translation within axons and dendrites.

**Synaptogenesis-related proteins are downregulated in Tsc2-deficient neurons**

We have identified 13 proteins that are significantly upregulated in Tsc2-deficient neurons despite overall increased protein synthesis (Fig. 1). Of particular interest are presynaptic proteins, such as Snap91 and Chbn1 (Yao et al., 2002; Matsuda and Yuzuki, 2011; Ito-Ishida et al., 2012). Snap91/AIP180 levels and distribution are highly sensitive to neuronal activity (Wu et al., 2010). Knockdown of Snap91/AIP180 impairs axonal development, synaptic vesicle size, and density (Morris et al., 1993; Petralia et al., 2013). Chbn1, also known as precerebellin, induces synaptogenesis in various types of neurons, including hippocampal, cerebellar, and cortical neurons (Matsuda and Yuzuki, 2011). In the cerebellum, trans-synaptic interaction of postsynaptic GluR2 with presynaptic neurexins through Chbn1 mediates parallel fiber synapses onto Purkinje cells. Downregulation of presynaptic proteins may be simply a consequence of impaired neuronal connectivity in TSC, or alternatively, it may reflect a role of the TSC-mTOR pathway in presynaptic differentiation. This might possibly link TSC and another family of autism genes, the neurexins.

**Stress-responsive and anti-inflammatory proteins in TSC and autism**

The vast majority of genes in our profiling dataset are stress and/or activity-related genes, such as c-Fos, Ucp2, Hspb1, Hmox1, and Atf3. Accumulating evidence suggests the presence of oxidative stress, mitochondrial dysfunction, and inflammation in the brain of individuals with autism (Chauhan and Chauhan, 2006; Rossignol and Frye, 2014; Masi et al., 2015). Induced by cellular stress and kainic acid, the mitochondrial uncoupling protein-2 (Ucp2) modulates mitochondrial membrane potential to decrease ATP production, leading to neuroprotection against oxidative stress (Kim-Han and Dugan, 2005; Chuang et al., 2012). Decreased synthesis of ATP has been shown by 31P-magnetic resonance spectroscopy (Minschew et al., 1993), suggesting an involvement of mitochondrial dysfunction in autism. Our previous study has demonstrated an mTOR-dependent induction of Hmox1 (heme oxygenase-1) in Tsc2-deficient neurons, indicating an enhanced oxidative stress response (Di Nardo et al., 2009). Atf3 is a transcription factor in the CREB protein family that is induced by action potential bursting and nuclear calcium signaling (Zhang et al., 2009; Zhang et al., 2011; Hunt et al., 2012). Hmox1 also has anti-inflammatory effects. Other anti-inflammatory proteins we have identified include Ccl2 and Cxcl10. Overall, upregulation of these proteins represents prosurvival or antiapoptotic mechanisms. Given an incidence of ~50% of autism in patients with TSC (Asato and Hardan, 2004; Jeste et al., 2008), the revealed molecular signatures could have diagnostic or prognostic value for the neurocognitive comorbidity in these patients.

**The role of the Atf3-gelsolin cascade in TSC pathophysiology**

Changes in dendritic and synaptic architecture have been described in Tsc1- or Tsc2-deficient neurons. Whereas a number of reports have documented a decrease in spine density following knockdown of Tsc1/2 (Tavazoie et al., 2005; Yasuda et al., 2014; Sugiura et al., 2015), none or only moderate changes were reported in others (Meikle et al., 2007; Bateup et al., 2011). In the present study, we observed morphologic deficits in Tsc2-deficient hippocampal neuronal cultures. These neurons developed enlarged soma size and decreased density of dendritic spines compared with controls. These findings are similar to what has been described previously (Tavazoie et al., 2005). Furthermore, we found that overnight rapamycin treatment did not rescue spine density, even though it did correct the deficit in spine length (Fig. 6B–E). We also detected a reduction in the number of PSD-95-immunoreactive puncta, which is consistent with more sparse dendritic spines in these neurons (Fig. 6F). These data strongly...
indicate an impairment of synaptic connectivity in Tsc2-deficient neurons.

Consistent with our previous in vivo studies (Meikle et al., 2007), we found that acute rapamycin treatment does not rescue spine deficits in cultured Tsc2-deficient rat hippocampal neurons (Fig. 6C), although it did reduce ATF3 to levels that are not different from control neurons (Fig. 4C,D). Different effects of acute rapamycin treatment versus Atf3 knockdown could potentially be explained by a number of factors in our experimental system. It is likely that different mTOR-dependent and mTOR-independent pathways regulate spine density in TSC. Consistent with our results, Yasuda et al. (2014) recently reported that Tsc2−/− neurons show impaired spine synapse formation, which is unaffected by mTORC1 inhibition. The same group went on to show that this is mediated through RhoB-syntenin signaling (Sugiura et al., 2015). Furthermore, rapamycin could affect spine formation, stability, and pruning in ways independent of Atf3. Detailed time-lapse experiments over a greater time period would help to clarify the roles of Atf3, rapamycin, and other players.

Dendritic spines are motile structures that contain high concentrations of filamentous actin. The importance of the actin skeleton for dendritic architecture is exemplified by findings by Zhang et al. (2014), which demonstrated an increased expression of actin-crosslinking protein filamin A (FLNA) responsible for abnormal dendritic morphogenesis in TSC-null mice. Although we did not detect increased FLNA expression in our TRAP screening assay, we found that gelsolin, an actin-severing protein, is one of the genes upregulated in the Tsc2-deficient neurons. Gelsolin was previously reported to be highly expressed in renal cystadenomas in a TSC mouse model (Onda et al., 1999). Gelsolin protein binds to and severs large actin filaments, by which it accelerates filopodia retraction. Interestingly, gelsolin is a direct target gene of Atf3 transcription activity (Yuan et al., 2013). Induction of Atf3 and gelsolin could be neuroprotective in certain contexts (Zhang et al., 2009; Harms et al., 2004; Seijffers et al., 2014). Loss of gelsolin in hippocampal neurons renders vulnerability to glutamate toxicity and exacerbates seizure-induced damage in the brain (Furukawa et al., 1997), suggesting a potent cell-autonomous program that protects neurons from dying of excitotoxicity or seizures.

Our data demonstrate that blocking Atf3 induction prevents gelsolin overexpression and subsequently corrects the loss of dendritic spines in Tsc2-deficient neurons. It is likely that activation of the Atf3-gelsolin cascade, regardless of initial prosurvival benefits, can reduce synaptic connectivity and contribute to long-term neurocognitive impairment seen in patients with TSC. Whether or not gelsolin is connected with mTOR complex-2 (mTORC2) or solely an indirect target of mTORC1 via Atf3 in the hippocampus remains to be investigated. Given that the Atf3-gelsolin pathway is primarily prosurvival, temporal and local manipulation would be imperative for its use in a therapeutic context.

References


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J. Neurosci., July 29, 2015 • 35(30):10762–10772 • 10771


