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Research article

Tsc1 haploinsufficiency is sufficient to increase dendritic patterning and Filamin A levels

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HIGHLIGHTS

- *Tsc1* heterozygote neurons display increased dendritic arborization.
- *Tsc1* heterozygosity leads to increased filamin A levels.
- Filamin A and TSC1 levels are correlated.

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ABSTRACT

Most individuals with tuberous sclerosis complex (TSC) are born with a mutant allele of either *TSC1* or *TSC2* and a mosaic of psychological and cognitive defects. *Tsc1* loss of heterozygosity contributes to severe dendritic abnormalities that are rescued by normalizing the levels of the actin-cross linking protein, Filamin A (FLNA). However, it is unclear whether dendrites and FLNA levels are abnormal in an heterozygote *Tsc1* condition. Here, we examined dendritic morphology and FLNA levels in the olfactory bulb of *Tsc1* wild type and heterozygote mice. Using *in vivo* neonatal electroporation to label newborn neurons followed by sholl analysis, we found that *Tsc1* haploinsufficiency is associated with increased dendritic complexity and total dendritic length as well as increased FLNA levels. Since reducing FLNA levels has been shown to decrease $Tsc1^{+/-}$ dendritic complexity, these data suggest that increased FLNA levels in $Tsc1^{+/-}$ mice contribute to abnormal dendritic patterning in the *Tsc1* heterozygote condition of individuals with TSC.

1. Introduction

TSC is an autosomal dominant monogenetic disorder observed in 1/6000 individuals characterized by discrete lesions in diverse tissues, including the skin, heart, kidney, lung, and brain [1]. Most patients are born with at least one detectable mutation in *TSC1* or *TSC2* and are thus heterozygous for one of these genes [2,3]. Very often, there are subsequent inactivating mutations of the other functional allele during development leading to the formation of discrete lesions in several organs, including the brain [4–7].

The cortical lesions, called cortical tubers, are responsible for seizures occurring in >80% of TSC individuals [1]. However, TSC individuals display a mosaic of neuropsychological problems (e.g. autistic traits) and about half of them present with cognitive impairments (i.e. mental retardation). Although epilepsy can lead to both cognitive and psychiatric deficits, it is thought that heterozygosity for *TSC1* and *TSC2* is sufficient to account for some of these deficits. Indeed, $Tsc1^{+/-}$ and $Tsc2^{+/-}$ mice as well as mice with a heterozygote *Tsc2* dominant negative allele ($Tsc2^{\Delta RG}$) display cognitive and social deficits despite the absence of brain lesions or seizures ([8] and for review see [9]). These deficits likely result from a collection of cellular and molecular alterations in heterozygote mice. At the molecular level, mTORC1 and ERK activities have been shown to be increased in $Tsc1^{+/-}$ and $Tsc2^{\Delta RG}$ mice, respectively [10,11]. At the cellular level, retinogeniculate projections have been reported to show abnormalities in targeting in $Tsc1^{+/-}$ mice [12]. Alterations in dendritic patterning have not been observed in hippocampal neurons of $Tsc1^{+/-}$ mice in vivo [13]. This is somehow surprising considering that dendritic defects are often observed in disorders associated with cognitive or psychiatric deficits [14–17]

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and are a classical outcome of increased mTORC1 or ERK activity (see [9,18] for additional references). Because, the defects in axonal patterning were modest, it is possible that a modest defect in dendritic patterning may not be detectable by analyzing the number of first and second dendrites as performed in the published study using *Tsc1*$^{+/-}$ mice [13].

Here, we thus set out to examine the dendritic complexity of neurons in *Tsc1*$^{+/-}$ mice. To efficiently label dendrites, we used mice crossed with a tdTomato reporter line, in which expression of a Cre recombinase (Cre)-encoding plasmid leads to tdTomato expression. We electroporated a Cre plasmid in newborn neurons of the subventricular zone that migrate and become interneurons in the olfactory bulb (OB) [19]. We found that *Tsc1*$^{+/-}$ neurons display a modest but significant increase in dendritic complexity and total length compared to wild type *Tsc1*$^{+/+}$ neurons. Intriguingly, a recent study reported that decreasing the level of an actin-cross-linking protein Filamin A (FLNA) [20] in conditional *Tsc1*$^{+/-}$ neurons of the OB decreased their dendritic complexity [18]. The recent study also reported increased levels of FLNA in conditional *Tsc1*$^{-/-}$ (null) neurons compared to *Tsc1*$^{+/-}$ neurons and in TSC patients, but FLNA levels in *Tsc1*$^{+/-}$ versus wild type *Tsc1* mice were not examined. In light of the data on dendrites in *Tsc1*$^{+/-}$ mice reported here, we examined FLNA levels in these mice and found that there were increased compared to those in *Tsc1*$^{+/+}$ mice. In addition, correlation analysis between TSC1 and FLNA levels in *Tsc1* mice suggest a gene-dosage relationship between *Tsc1* and FLNA levels.

2. Materials and methods

2.1. Animals

Research protocols were approved by the Yale University Institutional Animal Care and Use Committee. Experiments were performed on the following lines of transgenic mice of either gender: *Tsc1*$^{fl/+}$ (fl, floxed, obtained from Jackson Labs), *Tsc1*$^{+/+}$, and *Tsc1*$^{+/-}$ mice (obtained from the National Cancer Institute). All these lines were crossed with Rosa26R-STOP-tdTomato reporter mice (Jackson labs). Tail or toe samples were taken and were subjected to DNA isolation, PCR amplification using previously published primers [21] and amplicons separated by standard electrophoresis methods.

2.2. Neonatal electroporation and vectors

Postnatal electroporation was performed as previously described [19,22,23]. Plasmids (2–3 μg/μl) were diluted in PBS containing 0.1% fast green as a tracer. 0.5–1 μl of plasmid solution was injected into the lateral ventricles of neonatal pups using a pulled glass pipette (diameter < 50 μm). 5 square-pulses of 50 ms-duration with 950 ms-intervals at 100 V were applied using the ECM 830 square wave pulse generator (BTX) and tweezer-type electrodes (model 520, BTX) placed on the heads of P0-P1 pups. The electrodes were positioned to direct current rostrally in the dorso-lateral SVZ. Vectors included pCAG-tdTomato [24] and pCAG-Cre (addgene, C. Cepko).

2.3. Morphometric analysis

Images of tdTomato^{+} basal dendrites were acquired in coronal sections using a Fluoview 1000 confocal microscope and traced with simple neurite tracer software (FIJI, GNU GPL v3, an ImageJ plugin, URL: http://fiji.sc/Simple_Neurite_Tracer). We analyzed the basal dendrites of granule cells in a specific layer for the following technical reason; the basal dendrite can be fully imaged and traced without confusion from a single labeled granule cell. Due to its length, the apical dendrite is often cut or partially cut, and this could lead to erroneous data. In addition, in the external plexiform layer where apical dendrites branch and terminate, it is difficult to distinguish whether the dendrite come from one cell or several cells. Sholl analyses were carried out using the number of intersections in 10 μm-increment concentric circles as a measure of morphological complexity. Z-stacks from 3 different square fields of view were taken from 3 different olfactory bulb (OB) sections. Analysis was performed blindly from at least 3 animals per condition.

2.4. Olfactory bulb lysate and western blot

Mice were anesthesized with pentobarbital (50 mg/kg) prior to olfactory bulb (OB) dissection. Western blotting protocol is detailed in [18]. Samples were homogenized in RIPA buffer, 1× Halt Protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific), and 8 U/ml DNase. Samples were boiled in 2× Laemmli's buffer. 10 or 20 μg protein/sample was loaded into a 4–15% polyacrylamide gel (Bio-Rad Mini Protean TGX gel). Proteins were transferred to PVDF and blocked in 5% milk or 5% BSA. Quantifications were performed using NIH Image J software. Primary antibodies included: FLNA (molecular weight: 281 kDa, Abcam, ab51217, 1:5000), TSC1 (predicted band size: 130 kDa, observed at 150–160 kDa as reported for the company site, Abcam, ab32936, 1:5000), and ERK1/2 (molecular weight of ERK1: 44 kDa, Santa Cruz, sc-94, 1:250,000). We used the Thermo Scientific BenchMark™ Pre-Stained Protein Ladder containing a mixture of 10 proteins spanning 6–180 kDa (Catalog number: 10748010, invitogen).

2.5. Statistics

Analysis was performed on N indicating the number of animals in each condition or n indicating the number of replicates. Data were presented in GraphPad Prism 6. Statistical significance was determined using unpaired Student *t*-test or one way ANOVA with $p < 0.05$ or two way ANOVA with *post hoc* Bonferroni's test for comparison of dendritic crossing. Data are presented as mean ± standard error of the mean (SEM).

3. Results

To investigate whether *Tsc1* haploinsufficiency would lead to alterations in dendritogenesis *in vivo*, we employed neonatal electroporation to fluorescently label developing OB neurons in *Tsc1*$^{+/+}$, *Tsc1*$^{+/-}$, and *Tsc1*$^{fl/+}$ mice crossed with Rosa26R-STOP-tdTomato reporter mice (Fig. 1A). We electroporated pCAG-Cre to excise the floxed *Tsc1* allele and the STOP cassette leading to tdTomato expression at P0 into neural progenitor cells of the subventricular zone. During division, electroporated neural progenitors pass their exogenous plasmids on to neuroblasts, which migrate and integrate into the OB [19]. By 2 weeks, these neuroblasts differentiate into GABAergic interneurons with both apical and basal dendrites. We assessed the complexity and length of the basal dendrites of tdTomato^{+} neurons *in vivo* at 14 days post-electroporation by sholl analysis in coronal sections from littermate mice (Fig. 1B). Sholl analysis revealed that the dendrites of *Tsc1*$^{+/-}$ neurons in both constitutive and conditional *Tsc1*$^{+/-}$ mice displayed a significant increase in their dendritic complexity (2-way ANOVA with *post hoc* Bonferroni's test, n = 27-30 neurons, N = 3 mice each, Fig. 1C) and total dendritic length (One way ANOVA, $P = 0.0184$, $F = 4.194$, Fig. 1D).

Considering that decreasing FLNA levels in conditional *Tsc1*$^{+/-}$ neurons decreased their dendritic complexity and total length [18], we examined whether FLNA levels were increased in *Tsc1*$^{+/-}$ versus *Tsc1*$^{+/+}$ mice at P14. We performed immunoblotting for FLNA, TSC1 and the loading control ERK1/2, which is not altered in *Tsc1* mice