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Minute amounts of hamartin wildtype rescue the emergence of tuber-like lesions in conditional *Tsc1* ablated mice

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ABSTRACT

Tuberous sclerosis (TSC) is a phacomatosis associated with highly differentiated malformations including tubers in the brain. Those are composed of large dysplastic neurons and 'giant cells'. Cortical tubers are frequent causes of chronic seizures and resemble neuropathologically focal cortical dysplasias (FCD) type IIb. Patients with FCD_{IIb}, however, lack additional stigmata of TSC. Mutations and allelic variants of the *TSC1* gene have been observed in patients with tubers as well as FCD_{IIb}. Those include hamartin^{R692X} and hamartin^{R786X}, stop mutants frequent in TSC patients and hamartin^{H732Y} frequent in FCD_{IIb}. Expression of these variants in cell culture led to aberrant distribution of corresponding proteins. We here scrutinized morphological and structural effects of these *TSC1* variants by intraventricular *in utero* electroporation (IUE), genetically mimicking the discrete focal character and a somatic postzygotic mosaicism of the lesion, focusing on the gene dosage required for tuber-like lesions to emerge in *Tsc1*$^{flox/flox}$ mice. Expression of only hamartin^{R692X} as well as hamartin^{R786X} led to a 2-fold enlargement of neurons with high pS6 immunoreactivity, stressing their *in vivo* pathogenic potential. Co-electroporation of the different aberrant alleles and varying amounts of wildtype *TSC1* surprisingly revealed already minimal amounts of functional hamartin to be sufficient for phenotype rescue. This result strongly calls for further studies to unravel new mechanisms for substantial silencing of the second allele in cortical tubers, as proposed by Knudson's '2-hit hypothesis'. The rescuing effects may provide a promising basis for gene therapies aiming at reconstituting hamartin expression in tubers.

1. Introduction

Tuberous sclerosis (TSC) is an autosomal-dominant inherited multi organic disorder affecting approximately 1/6000 individuals (Hengstschläger et al., 2001). This phacomatosis is characterized by benign tumors and dysplastic lesions in many organs including skin, kidney, lungs, heart and brain (Gomez, 1988). In the latter, cortical tubers composed of large, dysmorphic neurons and so-called giant cells are frequently associated with pharmacoresistant epilepsy. Cortical dysplasias, associated with focal epilepsies and composed of dysplastic cytomegalic neurons and so-called balloon cells (FCD_{IIb} (Blümcke et al., 2010)), are histomorphologically virtually indistinguishable from cortical tubers, although the patients generally lack additional features of a neurocutaneous phacomatosis (Taylor et al., 1971).

TSC is commonly caused by mutations in either *TSC1* on chromosome 9q34 (Van Slegtenhorst et al., 1997) or *TSC2* on chromosome 16p13.3 (Carbonara et al., 1994; Sampson and Harris, 1994). Hamartin and tuberin, the corresponding gene products, form a heteromer with a central role as tumor suppressor in the phosphatidylinositol 3-kinase (Pi3K) pathway (Baybis et al., 2004; Van Slegtenhorst et al., 1997, 1998, 1999). Extracerebral neoplasms frequently have a parallel loss of heterozygosity (LOH) (Green et al., 1994). LOH, however, has been rarely detected in TSC brain lesions (Henske et al., 1996). Further evidence suggests that somatic second-hit events in *TSC1* heterozygous individuals may account for cortical tubers (Chan et al., 2004; Crino et al., 2010; Henske et al., 1997), and in fact no tubers have been reported in mice heterozygous for *Tsc1* (Piedimonte et al., 2006; Scheidenhelm and Gutmann, 2004; Uhlmann et al., 2002). However, another study reported second hit mutations to be rare events in cortical tubers (Qin et al., 2010). Furthermore, the TSC pathway in neurons was proposed to be highly sensitive to gene dosage effects as *Tsc1* haploinsufficiency for example impairs neuronal morphology (Tavazoie et al., 2005).

We have recently shown that the expression of hamartin^{R692X} as well as hamartin^{R786X}, two stop mutants frequently found in TSC patients

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(Dabora et al., 2001; Hung et al., 2006; Van Slegtenhorst et al., 1999) results in substantial binding deficits with tuberin and aberrant distribution of the mutated hamartin *in vitro* (Lugnier et al., 2009). In addition, expression of an allelic polymorphism significantly increased in FCD_{IIb} patients, a base exchange from C to G in exon 17 of *TSC1* at nucleotide 2415 resulting in a switch from histidine to tyrosine at codon 732 (hamartin^{H732Y}) (Becker et al., 2002), leads to impaired TSC1-TSC2 heteromer formation and aberrant subcellular distribution of the hamartin^{H732Y} (Lugnier et al., 2009).

Based on these observations, we here aimed to elucidate the morphological and structural effects of respective *TSC1* variants *in vivo* with a particular emphasis on the question which gene dosage is required for a morphological phenotype reflecting tuber-like lesions to emerge.

2. Materials and methods

2.1. Study approval

All experiments were performed in accordance with the guidelines of the European Union and the University of Bonn Medical Center Animal Care Committee.

2.2. Intraventricular in utero electroporation in mice

The C57Bl/6 Tsc1tm1Djk/J (C57BL/6N-*Tsc1*$^{flox/flox}$) mice strain provided by The Jackson Laboratory (JAX) is an established mouse model for Cre mediated *Tsc1* knockout and thus for investigating tuberous sclerosis (Feliciano et al., 2011). For *in utero* electroporation (IUE) we backcrossed C57Bl/6N-*Tsc1*$^{flox/flox}$ mice from the C57Bl/6 background to the CD1 (JAX) background for three generations. For accelerated backcrossing, the genome of the F2 and F3 generation was genotyped for C57Bl/6 specific SNPs. Mice with lowest C57BL/6 SNP-score and homozygous for *Tsc1*-flox were used for further breeding. The *Tsc1*$^{flox/flox}$ mouse strain used in the present study has a mixed genetic background. We will refer to it as 'CD1-*Tsc1*$^{flox/flox}$' mice, since the CD1 background is predominant due to the fact that SNP sequencing after the third generation estimated 90% CD1 and 10% C57Bl/6 genetic content.

CD1-*Tsc1*$^{flox/flox}$ females were crossed with CD1-*Tsc1*$^{flox/flox}$ males for validation of the new strain or with C57BL/6-*Tsc1*$^{flox/flox}$ males for all other experiments because of increased IUE efficiency compared to CD1 × CD1 breedings. The IUE was performed as described recently (Grote et al., 2016). Shortly, pregnant mice (E14, E16, E17 or E18) were anesthetized with isoflurane and injected with Gabrilen (5 mg/kg) and Buprenovet (0.05 mg/kg) 30 min prior to the IUE as analgesia. After exposure of the uterine horns each embryo was injected once with 0.5–1 μl DNA (with a concentration of 3.0–5.0 μg/μl) and fast green (1 mg/ml, Sigma, St. Louis) with a pulled and beveled glass capillary (Drummond Scientific, PA) and a microinjector (Picospritzer III, General Valve Corporation, USA) into one lateral ventricle. By administering five electric pulses charged to 45 V electroporation was conducted. A 7 mm electrode was placed to target cortical ventricular and subventricular progenitors in the somatosensory and motor cortex (Saito, 2006). Successfully electroporated mice were anesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) three to four weeks after birth and sacrificed trough cardiac 4% PFA perfusion for further analysis.

2.3. Generation of constructs

Expression fragments for human *TSC1*-WT, *TSC1*-H732Y, *TSC1*-R786X, *TSC1*-R692X and Cre-recombinase were amplified by Polymerase Chain reaction with specific primers (Table 1) and ligated into the pB-CAG-2A-mCherry or pB-CAG-GFP vector (kindly provided by Joe LoTurco; University of Connecticut) *via* KpnI and AgeI or EcoRI and XmaI restriction sites. Table 1 gives an overview of all used primers and restriction enzyme recognition sequences (small letters).

Table 1
Plasmid generation.

Plasmid		5′–3′ sequence
CAG-Cre-GFP	fw	GCGgaattcATGTCCAATTTACTGACCGTACA
CAG-Cre-GFP	rev	GCGcccgggcATCGCCATCTTCCAGCAGG
TSC1-WT-2A-mCherry	fw	GCGggtaccATGGCCCAACAAGCAAATGTC
TSC1-WT-2A-mCherry	rev	GCGaccggtGCTGTGTTCATGATGAGTCTCA
TSC1-H732Y-2A-mCherry	fw	GCGggtaccATGGCCCAACAAGCAAATGTC
TSC1-H732Y-2A-mCherry	rev	GCGaccggtGCTGTGTTCATGATGAGTCTCA
TSC1-R786X-2A-mCherry	fw	GCGggtaccATGGCCCAACAAGCAAATGTC
TSC1-R786X-2A-mCherry	rev	GCGaccggtGTCATGCTGCAGCTGTCTGA
TSC1-R692X-2A-mCherry	fw	GCGggtaccATGGCCCAACAAGCAAATGTC
TSC1-R692X-2A-mCherry	rev	GCGaccggtGAGGGTGCGGATCTCATCT

2.4. Immunohistochemistry

For co-immunofluorescence stainings, brains from P23-30, electroporated and PFA-perfused mice were collected and cut to 80 μm slices on a Microm HM 650V vibratome (Thermo Scientific, MA). Afterwards, brain slices were washed in 0.1% Triton X-100 PBS solution and blocked with 0.1% Triton X-100, 0.1% Tween 20, 4% bovine serum albumin (BSA) in pH 7.7 TBS for 1 h. This was followed by an overnight incubation with primary antibodies against mouse anti-NeuN (1:400 - Millipore, MA; Antibody Registry: AB_331679 and AB_2298772), Phospho-S6 Ribosomal Protein (Ser235/236) #2211 or (Ser240/244) #2215 (1:500 - Cell Signaling, MA; Antibody Registry: AB_331679 and AB_331682) at 4 °C (both antibodies had similar staining patterns). After three washing steps, brains were incubated with secondary fluorescently labeled antibodies Alexa Fluor® 647 and 405 (1:200 - Life Technologies, NY) for 1 h and mounted on glass plates with vectashield (Vector Laboratories, CA).

2.5. Image analysis and quantification

Z-stack maximum intensity projection confocal images were taken from 80 μm mouse brains after intraventricular *in utero* electroporation with a Nikon Eclipse Ti confocal microscope (Nikon Instruments, Germany) and analyzed for different parameters.

First, neurons form brain slices immunolabeled with antibodies against NeuN and Phospho-S6 Ribosomal Protein Ser240/244 (further referred to as pS6) were analyzed with respect to their pS6 fluorescence intensity and their mean soma size. mCherry-labeled neurons were automatically recognized by the region of interest (ROI) auto-detect function of the NIS elements analysis software (Nikon Instruments, Germany) and parameters such as cell size and fluorescence intensity were generated for each individual ROI. For each brain slice analyzed, all cells that were bright enough to be recognized by the software were included in the analysis.

Final positioning of cortical neurons was analyzed by defining the borders of the six-layered cortex by NeuN staining and the percentage of all electroporated neurons in each layer was calculated. Additionally, the mean distribution of electroporated cells all over the cortex was analyzed by measuring the distance (in μm) of each individual electroporated cell to the upper border of layer I. The mean for each developmental time point was summarized for control and Cre-electroporated *Tsc1* knockout neurons.

Next, confocal maximum intensity projection images of single *in utero* electroporated cortical neurons were subjected to morphometric analyses and quantification using ImageJ software (Wayne Rasband, Maryland). For Sholl analysis each branch from mCherry-labeled *in utero* electroporated neurons was traced from branching point to the tip. On each traced image rings with 10 μm intervals were overlaid and the numbers of neurite intersections were counted for each circle (Ristanović et al., 2006; Sholl, 1953).