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

Barbara K. Robens a, Alexander Grote b, Julika Pitsch a, Susanne Schoch a, Carlos Cardoso c,d,1, Albert J. Becker a,e,1

a Section for Translational Epilepsy Research, Dept. of Neuropathology, Germany
b Neurosurgery, Bonn Univ. Clinic, Germany
c INSERM, Institut de Neurobiologie de la Méditerranée, Marseille, France
d Aix-Marseille University, UMR 901 Marseille, France
1 These authors contributed equally to the work.

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 FCDIIb, however, lack additional stigmata of TSC. Mutations and allelic variants of the TSC1 gene have been observed in patients with tubers as well as FCDIIb. Those include hamartinR692X and hamartinR786X, stop mutants frequent in TSC patients and hamartinR786Y frequent in FCDIIb. 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 Tsc1fl/fl mice. Expression of only hamartinR692X as well as hamartinR786X 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.

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1. Introduction

Tuberous sclerosis (TSC) is an autosomal-dominant inherited multi organic disorder affecting approximately 1/6000 individuals (Hengstschlager 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 (FCDIIb (Blümcke et al., 2010)), are histomorphologically virtually indistinguishable from cortical tubers, although the patients generally lack additional features of a neurocuteaneous 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; Scheidemihal and Gutmann, 2004; Uhmann 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 (Tavaoile et al., 2005).

We have recently shown that the expression of hamartinR692X as well as hamartinR786X, two stop mutants frequently found in TSC patients...
(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 FCDIII 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 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-Tsc1flox/flox) mouse 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-Tsc1flox/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 Tsc1flox mouse strain used in the present study has a mixed genetic background. We will refer to it as ‘CD1-Tsc1flox/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-Tsc1flox/flox females were crossed with CD1-Tsc1flox/flox males for validation of the new strain or with C57BL/6-Tsc1flox/flox males for all other experiments after increased IUE efficiency compared to CD1 × CD1 breeding. The IUE was performed as described recently (Valve Corporation, USA) into one lateral ventricle. By administering electric pulses charged to 45 V electroporation was conducted. A 7 mm needle was inserted through the skull and Langendorff perfused with warm saline and the interhemispheric fossa was opened. A glass micropipette loaded with the plasmid was inserted into the lateral ventricle at a rate of 0.25 mm intervals and the tissues were electrotonically perturbed with four square-wave pulses of 250 μs, separated by 200 ms. By administering pulses in the lateral ventricle the plasmid DNA (with a concentration of 3.0 μg/μl) was deposited into the ventricle and cells were exposed to the plasmid. The injection site was marked with a 0.9 mm diameter glass micropipette filled with a concentrated solution of India ink. The Ink was expressed into the lateral ventricle at a rate of 0.25 mm intervals and the tissues were electrotonically perturbed with four square-wave pulses of 250 μs, separated by 200 ms. After electroporation, the mouse was returned to its mother for gestation and then exsanguinated through cardiac 4% PFA perfusion for further analysis.

Table 1

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2.3. 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 (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 vechasfield (Vector Laboratories, CA).

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 vechasfield (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).
2.6. Statistical analyses

Different statistical tests were used: Kolmogorov-Smirnov test was used for Sholl analysis. Chi-square test and Two-way ANOVA with Bonferroni post-correction were used for assessing the amount of neurons in different cortical layers. In addition, to verify this approach the neuronal distance-distributions within the cortex were assessed with Mann-Whitney U test. One-way ANOVA with Bonferroni post-correction was carried out for cell size and pS6 fluorescence intensity quantification. For all analyses, neurons from all animals in each group were aggregated for statistical comparison, except for the Chi-square test; here the mean values from each group were compared. All error bars are indicated as the ‘standard error of the mean’ (SEM).

3. Results

3.1. Single cell Cre expression in CD1-Tsc1fl/fl mice leads to increased mTOR pathway activation and dysplastic brain lesions

To knockout Tsc1 in a restricted focal cortical area in a subset of precursor cells during mid-corticogenesis and to investigate the resulting lesion, we in utero electrooporated Tsc1fl/fl mice with a Cre-recombinase (Cre) expressing plasmid at E14. Litter survival after IUE is substantially higher in CD1 mice in comparison to the commonly used laboratory C57BL/6 strain due to very poor mothering instincts of the latter (Baumgart and Grebe, 2015; Cobolli Gigli et al., 2016; and our observations). In an attempt to address this problem, we generated Tsc1fl mice in the CD1 background (CD1-Tsc1fl/fl mice). A successful allele-of-interest transfer from the C57BL/6 background to CD1 was recently published and used for an improved Rett syndrome mouse model for similar reasons (Cobolli Gigli et al., 2016). We first validated that also in this genetic background Cre-mediated deletion of Tsc1 results in an activation of the mTOR pathway by analyzing the level of pS6 immunoreactivity (IR) and cell volume/soma size at P24-30. An increase in these parameters has been reported after Tsc1 knockout in the C57BL/6 background (Feliciano et al., 2011). In accordance, we observed strong pS6 immunolabelling only in the ipsilateral cortex of Tsc1fl/fl mice in utero electrooporated with CAG-Cre-GFP together with mCherry as a volume dye (Fig. 1A, C, right panel) but not in the contralateral side (Fig. 1B, right panel) or after electrooporation of mCherry alone (Fig. 1D, right panel) (mCherry/Tsc1fl/fl 483.2 ± 10.6 µm; mCherry/Cre+/Tsc1−/− 1339.5 ± 39.1; One-way ANOVA with Bonferroni post-correction ***p < 0.001, N = 7–8 mice with 23–30 cells each). Analysis of individual neurons also revealed an increase in soma size in Cre-positive Tsc1fl knockout neurons (Fig. 1C) compared to neurons only electrooporated with mCherry (Fig. 1D) (mCherry/Tsc1fl/fl 163.7 ± 5.3 µm; mCherry/Cre+/Tsc1−/− 391.9 µm; One-way ANOVA with Bonferroni post-correction ***p < 0.001, N = 7–8 mice with 41–60 neurons each). Previous studies showed that hyperactivation of the mTOR pathway in neurons leads to an excessive branching and a thickening of the apical dendrite (Crino et al., 2010; Feliciano et al., 2013). Sholl analysis of sin-

rons leads to an excessive branching and a thickening of the apical

Tsc1

and have strong pS6 immunoreactivity (C). In comparison the control (mCherry) electrooporated ipsilateral cortex shows mCherry expressing neurons positive for NeuN with low pS6 fluorescence intensity (D). Scale bar = 100 µm. Sholl analysis illustrates the number of intersections of dendrites with circles with increasing distance from the soma (E). Tsc1 knockout neurons show a significantly increased number of intersections in comparison to control neurons (E), Kolmogorov-Smirnov test: ***p < 0.001, N = 5 control neurons, n = 8 Tsc1 knockout neurons). Representative images of single cortical neurons electrooporated with CAG-Cre-GFP (F) or mCherry as control (G) used for Sholl analysis. Scale bar = 100 µm.

Electrooporation of CAG-Cre-GFP and mCherry and analyzed Tsc1fl mice with 41–60 neurons each. After IUE at E16 nearly all electroporated control neurons clustered at the upper third of layer II/III (93.4% ± 2.6), Tsc1 knockout neurons on the other hand were observed to be abnormally dispersed throughout layers II/III, only 35.2% ± 7.2 neurons were found in the upper half of layer II/III while 50.4% ± 6.9 of the Cre-knockout cells were localized in similar amounts in layer II/III (39.7% ± 8.0) and IV (54.4% ± 6.9) and also a few in even lower layers (5.9% ± 2.3) (Fig. 3A, B, G). After IUE at E16 nearly all electroporated control neurons clustered at the upper third of layer II/III (93.4% ± 2.6), Tsc1 knockout neurons on the other hand were observed to be abnormally dispersed throughout layers II/III, only 35.2% ± 7.2 neurons were found in the upper half of layer II/III while 50.4% ± 6.9 of the Cre-electroporated neurons were located in the lower layer II/III and 14.4% ± 6.9 in layer IV (Fig. 3C, D, G). Intriguingly, IUE of CAG-Cre-GFP into Tsc1fl mice at E14 revealed a more widely dispersed size increased dysplastic neuronal elements mainly from the middle to the external border of layer II/III, 37.6% ± 8.0 neurons were located in the upper part of layer II/III while 56.7% ± 6.8 neurons were found in lower layer II/III and 5.7% ± 3.5 in layer IV. This Cre-mediated ablation of Tsc1 in the CD1 background causes phenotypically identical changes as previously observed in the C57Bl/6 background indicating the functionality of this mouse line for further studies.

3.2. Tsc1 knockout at different points of embryonic development results in similar neuropathological alterations

Next we examined if the time point of hamartin ablation during embryonic development impacts the observed neuropathological alterations. To this end we performed IUE of CAG-Cre-GFP/mCherry or mCherry alone at E16 to E18. As knockout of Tsc1 at E14 had resulted in a substantially increased soma size, we quantified this parameter as an indicator for mTOR pathway up-regulation due to Tsc1 loss. This quantification revealed an approx. 2-fold enlargement of soma size at P24-30 after Cre-induced Tsc1 knockout both at E16 and at E17 (Fig. 2A-C) (E16: mCherry/Tsc1fl/fl 153.2 ± 4.2 vs. mCherry/Cre+/Tsc1−/− 409.6 ± 9.4; E17: mCherry/Tsc1fl/fl 157.3 ± 3.0 vs. mCherry/Cre+/Tsc1−/− 424.6 ± 10.2; One-way ANOVA with Bonferroni post-correction ***p < 0.001, N = 4–6 mice with 9–33 neurons analyzed per brain slice). There were no morphological differences between neurons of animals IUE at E16 or E17 (see Supplementary Table 1). Electrooporation at E18 did not yield any GFP-expressing neurons marking the end of corticogenesis (data not shown). These data indicate that there is a time window spanning several days during embryogenesis rather than a very restricted time point of vulnerability for lack of hamartin to induce dysplastic neurons.

3.3. Embryonic loss of Tsc1 leads to abnormal positioning of cortical neurons

Aberrant neuronal morphology or polarity have been suggested to alter migration characteristics of developing, migrating cortical neurons (Falace et al., 2014; LoTurco and Bai, 2006). Our experiments demonstrated altered neuronal morphology upon Tsc1 knockout in form of substantially increased soma size, which may potentially interfere with neuronal migration or affect their final position within the cortex. In the following set of experiments we ablated Tsc1 at cortical developmental stages between E14-E18 by in utero electrooporation of CAG-Cre-GFP and mCherry and analyzed final cortical positioning of electrooporated cells at P24-30. Neurons of Tsc1fl mice in utero electrooporated at E14 with control plasmids were mainly located in layer II/III (66.7% ± 8.3) and also in part in upper layer IV (33.3% ± 8.3) whereas Tsc1 knockout cells were localized in similar amounts in layer II/III (39.7% ± 8.0) and IV (54.4% ± 6.9) and also a few in even lower layers (5.9% ± 2.3) (Fig. 3A, B, G). After IUE at E16 nearly all electrooporated control neurons clustered at the upper third of layer II/III (93.4% ± 2.6), Tsc1 knockout neurons on the other hand were observed to be aberrantly dispersed throughout layers II/III, only 35.2% ± 7.2 neurons were found in the upper half of layer II/III while 50.4% ± 6.9 of the Cre-electroporated neurons were located in the lower layer II/III and 14.4% ± 6.9 in layer IV (Fig. 3C, D, G). Intriguingly, IUE of CAG-Cre-GFP into Tsc1fl mice at E14 revealed a more widely dispersed size increased dysplastic neuronal elements mainly from the middle to the external border of layer II/III, 37.6% ± 8.0 neurons were located in the upper part of layer II/III while 56.7% ± 6.8 neurons were found in lower layer II/III and 5.7% ± 3.5 in layer IV. This
was in strong contrast to the normal distribution of neurons electrooporated at respective time points with the fluorescent marker only, which formed a very delicate, homogenous band at the external border of layer II (91.6% ± 3.4), as expected when electroporating at such relatively late time points of embryonic development (Fig. 3E, F, G). Again, we did not observe electrooporated cells in the cortex, when IUE was carried out at E18 (data not shown).

To further substantiate this analysis we quantified the mean distribution of neurons in the cortex by measuring their distance to layer I (E14: mCherry/Tsc1flox/fox 286.2 μm ± 5.8 vs. mCherry/Cre+/Tsc1−/− 413.0 μm ± 4.9; E16: mCherry/Tsc1flox/fox 79.5 μm ± 4.0 vs. mCherry/Cre+/Tsc1−/− 243.5 μm ± 5.8; E17: mCherry/Tsc1flox/fox 53.5 μm ± 5.8 vs. mCherry/Cre+/Tsc1−/− 116.9 μm ± 6.3; Mann-Whitney U test ***p < 0.001, N = 4–11 mice, all neurons on a brain slice were analyzed n = 11–301 cells). This set of experiments indicates that deletion of Tsc1 during embryonic development dramatically impacts final positioning of cortical neurons and that substantially different neuropathological patterns emerge from the manifestation of Tsc1 knockout at different time points of embryonic brain development. A comparison of the different phenotypic causes by control plasmid expression or Tsc1 knockout at E14, E16 and E17 can be found in Supplementary Table 1.

3.4. Differential phenotype rescue requirements for distinct Tsc1 allelic variants

We next analyzed, whether the individual Tsc1 variants that were previously observed in cortical tubers as well as in FCDmib were able to rescue a Tsc1 knockout in vivo. To this end, we co-electroporated CAG-Cre-GFP together with either Tsc1-WT-2A-mCherry, Tsc1-H732Y-2A-mCherry, Tsc1-R692X-2A-mCherry or Tsc1-R786X-2A-mCherry at E14 and performed the analysis at P24–30 (Fig. 4A–D). Due to the presence of the 2A peptide sequence the Tsc1 variants and the fluorescent marker will be expressed as independent proteins. Intriguingly, our results suggest that the Tsc1-WT and the Tsc1-H732Y constructs have the capacity to rescue the Tsc1−/− phenotype, at least based on key aspects such as increased soma size and mTOR pathway activation. Neurons expressing Cre and Tsc1-WT or –H732Y (identified by mCherry labeling) exhibited normal soma size and low pS6 immunoreactivity. In the few neurons that appeared enlarged and also showed increased pS6 immunoreactivity we did not detect Tsc1-mCherry or Cre-GFP fluorescence (Fig. 4A, B). This may be due to low expression levels of the proteins, which would still result in Cre-mediated recombination and Tsc1 ablation but no rescue. Neurons could also be solely electrooporated with the Cre-GFP plasmid. In contrast, both truncated Tsc1 mutants, i.e. Tsc1-R692X and Tsc1-R786X, did not rescue the tuber-like phenotype (Fig. 4C, D). Quantification of the pS6 fluorescence intensity clearly demonstrated a substantially increased pS6 signal (and thus enhanced mTOR activation) in neurons that expressed Cre together with Tsc1-R692X or Tsc1-R786X in comparison to neurons that expressed Cre together with Tsc1-WT or Tsc1-H732Y. With respect to S6 phosphorylation, these experiments indicate a full rescue capacity of Tsc1-WT as well as Tsc1-H732Y since pS6 fluorescence intensity was comparable to cells electrooporated with only mCherry (Fig. 4C, quantification Fig. 4E). On the other hand electrooporation of Tsc1-R692X or Tsc1-R786X led to an increased pS6 fluorescence intensity, comparable to Tsc1−/− neurons electrooporated with Cre-GFP/mCherry (Fig. 1A, D, quantification Fig. 4E).
were co-expressing either TSC1 deficient neurons that were co-expressing either TSC1-R692X or TSC1-R786X or mCherry alone (Tsc1−/−) compared to those expressing TSC1-WT or TSC1-H732Y or neurons without Cre expression (mCherry only) (Fig. 4F) (mCherry/Tsc1lox/lox 163.7 μm² ± 3.0; mCherry/Cre−/−/Tsc1−/−; 391.9 μm² ± 5.3; mCherry/Cre−/−/TSC1-WT 154.8 μm² ± 3.0, mCherry/Cre−/−/TSC1-R786X 173.0 μm² ± 2.7, mCherry/Cre−/−/TSC1-R692X 431.6 μm² ± 4.7, mCherry/Cre−/−/TSC1-R692X 339.5 μm² ± 5.9; One-way ANOVA with Bonferroni post-correction *p < 0.001, N = 6 mice with 50–65 cells each). These results show that the disease-associated truncated TSC1 variants even if strongly abundant cannot restore normal functionality to Tsc1-deficient neurons, whereas the H732Y point mutation in the present setting is as effective as WT-TSC1.

Second hit effects are still rather enigmatic in cortical tubers. Given our present data, we next aimed at elucidating, whether and in which amount of mamartin starting at E14. Furthermore, also in the cultured tumor cellular background that completely loses the expression of WT mamartin starting at E14. Fourth, also in the cultured tumor cells the binding of mamartinH732Y to tuberin and their co-localization was not completely abolished but only reduced to 40% and 20%, respectively (Lugnier et al., 2009). In this context, the full rescue potential of TSC1-H732Y indicates that the remaining interaction of hamartinH732Y and tuberin in neurons in vivo is sufficient for the mutant protein to remain functionally active. Future studies need to be conducted to address this hypothesis and exploit the intriguing possibility that only low levels of TSC1 are sufficient to retain a normal neuronal phenotype which may not be the case for TSC2, possibly providing an explanation for the more severe clinical phenotype observed in TSC patients harboring TSC2 mutations (Curatolo et al., 2015).

On the other hand, expression of the truncation mutants hamartinR692X as well as hamartinR786X did not rescue the Tsc1-knockout phenotype and neurons expressing these hamartin mutants are morphologically similar to Tsc1 knockout neurons. These findings underline the pathogenic potential of these TSC1 mutants. Despite the morphological alterations induced by in vitro expression of hamartinR692X we neither observed spontaneous generalized seizures nor differences in Pentylentetrazol-elicted seizures compared to controls in respectively transgenic mice with a C57BL/6 background (data not shown). The absence of spontaneous seizures was also reported in another TSC mouse model of focal Tsc1 knockout in Tsc1fl/fl mice by in utero electroporation of CAG-Cre (Feliciano et al., 2011). In fact, various parameters and their complex interplay were demonstrated to profoundly influence the excitability of the lesion: the size and the exact cerebral location of the lesion, the composition of compromised precursor cells and finally, also the age of the individual mouse was recently shown to be critical for seizure occurrence, since mice heterozygous for Tsc1 lacking tuber-like lesions had spontaneous seizures only until 9–18 days of age (Lozovaya et al., 2014; Jansen et al., 2005; Meikle et al., 2007; Feliciano et al., 2011). Future studies with more sophisticated EEG-analysis set ups

Fig. 4. Distinct phenotype rescue requirements for TSC1 allelic variants. Brain slices of P24-30 Tsc1fl/fl mice co-in utero electroporated (IUE) with CAG-Cre-GFP and either TSC1-WT-2A-mCherry, TSC1-H732Y-2A-mCherry, TSC1-R692X-2A-mCherry or TSC1-R786X-2A-mCherry at E14 were stained against pS6. Representative images show single cells expressing Cre and TSC1-WT (A) or TSC1-H732Y (B) that appear normal and negative for pS6 and neighboring cells with strong pS6 IR but without TSC1-WT expression. Neurons co-electroporated with CAG-Cre-GFP and TSC1-R692X (C) or TSC1-R786X (D) appear enlarged with high pS6 fluorescence intensity, even in cells without apparent Cre-expression. Quantification shows significantly increased pS6 fluorescence intensity in TSC1-R692X or TSC1-R786X electroporated neurons in comparison to TSC1-WT or TSC1-H732Y electroporated neurons (E). Soma size is significantly elevated in Cre+/TSC1-R692X and Cre+/TSC1-R786X electroporated neurons in comparison to Cre−/TSC1-WT or Cre−/TSC1-H732Y neurons (F), N = 6 mice with 50–65 cells each; One-way ANOVA with Bonferroni post-correction: ***p < 0.001; Scale bar = 100 μm.
allowing the detection of focal seizures with onset in the lesioned area will be mandatory in order to identify the factors contributing to hyperexcitability in the dysplastic lesions here under study.

In a next set of experiments, we co-electroporated this truncated TSC1-R692X mutant with varying amounts of wildtype TSC1. Our data revealed, that already small amounts of hamartin-WT were sufficient
for phenotype rescue. This result strongly stresses mechanisms for substantial silencing of the second allele to be extremely critical for the manifestation of cortical tubers. Peripheral TSC-associated tumors such as angiomyolipoma or rhabdomyoma frequently fulfill the so-called ‘2-hit hypothesis’ for inactivation of tumor suppressor genes, i.e. mutation of the first and associated LOH of the second allele (Henske et al., 1996; Knudson, 1991, 1996). In contrast, LOH at the TSC1/TSC2 genomic loci is only occasionally present in cortical tubers (Carbonara et al., 1995; Henske et al., 1996).

Recent data using state-of-the-art deep sequencing in tubers did not reveal a high frequency of second hit mutations of TSC1 and/or TSC2 or KRAS, an upstream component of the mitogen-activated protein kinase (MAPK) pathway often mutated in cancer (Qin et al., 2010). Obviously, heterozygous germline mutations of TSC1/TSC2 affect also histologically normal appearing cortex. Circumscribed tubers may therefore require additional yet enigmatic pathogenetic factors for their emergence. Mutations in other components of the mTOR pathway as it was recently shown in hemimegalencephaly (Rivière et al., 2012) as well as in FCD IIb, especially with respect to mTOR may be relevant even only in a subset of cells (Lim et al., 2015). On the other hand, hard-to-find intrinsic mutations, genetic mosaicism of cortical tubers or technology limits may likely account for the cases of TSC with no identified mutations (Curatolo et al., 2015; Tyburchy et al., 2015).

The non-static semiology and episodic emergence of seizures, may argue for molecular alterations affecting hamartin/tuberin to occur on dynamic, i.e. epigenetic or protein levels. Recent data demonstrated in smooth muscle-like cells isolated from an angiomyolipoma of a TSC-patient that promoter methylation induces a substantial reduction of tuberin and abnormal developmental effects in respective cells can be successfully antagonized by use of chromatin-remodeling agents (Han et al., 2004; Lesma et al., 2009). It is not resolved, whether parallel mechanisms operate in tubers. Furthermore, post-translational inactivation of tuberin, by non-physiological phosphorylation specific to TSC-associated brain lesions including tubers was attributed to abnormal activation of the Akt and MAPK pathways (Han et al., 2004; Lesma et al., 2009). However, the underlying causes of Akt/MAPK pathway activation remain unclear and may be epigenetically driven. Respective alterations cannot be mimicked or controlled for in the present experimental setup.

Our animal model of TSC partially reproduced the focal abnormal cortical positioning seen in brain biopsies of TSC patients, the so-called cortical tubers as hallmark of TSC. Hamartin loss initiated at E14 led to an increased amount of electroporated, Tsc1 knockout neurons in layer IV; a layer that can be described as a cortical hub for intracolumnar information processing since it converges the majority of the sensory information (Beierlein et al., 2002; Feldmeyer, 2012). This highly interconnected layer amplifies and redistributes thalamo-cortical inputs and an increased number of layer IV neurons should therefore increase integration and recurrent inputs (Lozoya et al., 2014). This in turn will foster increased network excitability and hence, contribute to the epileptogenic potential of TSC.

As consequence, the very low levels of wildtype hamartin expression required to rescue the tuber phenotype may provide a promising basis for future gene therapies aiming at reconstituting hamartin expression in tubers. Everolimus has recently proven to be able to reduce the volume and clinical symptoms of TSC-associated subependymal giant cell astrocytomas (Franz et al., 2014), however, this drug may be limited for long-term or even life-long treatment due to substantial side effects. Experimental gene therapy has recently been shown successful in already manifest developmental brain lesions, i.e. subcortical band heterotopia and seizures (Manent et al., 2008). Given the relatively minute amounts of wildtype hamartin that are required to rescue the tuber phenotype in our present experiments, gene therapeutic reconstitution of hamartin expression in tubers may be a promising locally restricted strategy that should have substantially less side effects than systemic chronic pharmacotreatment – if any at all. New molecular tools are required for the experimental testing of respective experimental therapies in the future.

5. Conclusion

In the present study we used a very recent transgenic mouse model for tuberous sclerosis induced at E14 by in utero electroporation reflecting tuber-like neuropathological features such as abnormal positioning of cortical neurons and dramatically enlarged neuronal cell bodies with strong mTOR pathway activation (Carson et al., 2012; Mizuguchi and Takashima, 2001). Cortical tubers are proposed to be the origin of recurrent seizures, however underlying pathogenetic mechanisms leading to their formation remain controversial (Feliciano et al., 2013). With this model we demonstrated that already minute amounts of TSC1 wildtype were necessary to retain a normal cellular phenotype, arguing in general favor of Knudson’s ‘2-hit hypothesis’ as pathogenetic cause for cortical tuber formation. The strong rescuing capacity of functional hamartin should be further analyzed since it indicates a promising basis for gene therapies. With our CD1-Tsc1floxed/flox mouse model, modified from a recently published mouse model (Feliciano et al., 2011), we obtained substantially increased pup survival, thus facilitating the opportunity to study pathological mechanisms underlying Tsc1 dysregulation.

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Conflict of interest statement

The authors declared no conflict of interest.

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References


