Full Title

Dysmorphic neuron density is highest in the centre of epileptogenic cortical tubers

Running title

Dysmorphic neuron density in cortical tubers

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Abstract

Cortical tubers are benign, well-circumscribed hamartomas that develop in patients with tuberous sclerosis complex, often resulting in drug-resistant epilepsy. Surgical resection may be required for seizure control, but the minimal extent of resection required for complete seizure control is unclear. Many epilepsy centres perform extensive resections, including perituberal cortex, which may be associated with adverse neurological complications. This approach also reduces the number of patients thought appropriate for epilepsy surgery by excluding those with tubers in eloquent cortical areas.

In vivo and *ex vivo* electrophysiological studies have recently identified the tuber centre as the source of seizures, suggesting smaller resections may be sufficient for effective seizure control. Here we report five epilepsy surgeries performed in four children with TSC and focal motor seizures resulting from solitary epileptogenic tubers in the primary sensorimotor cortex. The resection was limited to the tuber centre, leaving the tuber rim and surrounding perituberal cortex intact. Seizures were eliminated in all cases, and no functional deficits were observed. We hypothesised that this optimal surgical outcome was due to removing dysplastic tissue at the tuber centre, which contained a high density of dysmorphic neurons. Unbiased stereology identified a significantly greater density of dysmorphic neurons within the tuber centre (1951 ± 215) compared to the tuber rim (531 ± 189, $n = 4$, $p = 0.002$).

This study provides the first quantitative evidence of differences in dysmorphic neuron distribution within cortical tubers, with the centre showing the highest density. Taken together with previous electrophysiological studies implicating the tuber centre as the source of epileptic activity, this study supports the hypothesis that dysmorphic neurons are seizure generators within this tissue region. Our results suggest that limiting resection to the tuber centre may provide equivalent reductions in seizure burden to current surgical practice while decreasing the risk of adverse outcomes when tubers are located within eloquent cortical areas such as the motor cortex. Altered surgical practice may result in epilepsy surgery being a viable option for a larger number of patients with TSC and associated malformations of cortical development.

Keywords

Tuberous sclerosis, histopathology, stereology, focal cortical dysplasia, epilepsy surgery

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant, multisystem disorder caused by mutations in the genes encoding hamartin (*TSC1)* or tuberin (*TSC2*) (O'Callaghan, Shiell et al. 1998). Loss of functional hamartin or tuberin results in failure to form a critical negative regulatory protein complex in the mammalian target of rapamycin (mTOR) pathway (van Slegtenhorst, Nellist et al. 1998). mTOR overactivation causes dysregulation of neural stem cell proliferation, differentiation, migration and apoptosis, leading to cortical malformation (Aronica and Crino 2014). TSC is a model for understanding focal epilepsy due to focal cortical dysplasia (FCD) due to clinical, histopathological and neuroimaging similarities, particularly with FCD type IIb (Crino 2007). This association has been strengthened by recent genetic investigations of patients with FCD, which have identified pathogenic variants in genes encoding multiple components of the mTOR pathway (Baulac, Ishida et al. 2015, D'Gama, Geng et al. 2015, Leventer, Scerri et al. 2015, Lim, Kim et al. 2015, Scerri, Riseley et al. 2015, Mirzaa, Campbell et al. 2016, Sim, Scerri et al. 2016, D'Gama, Woodworth et al. 2017, Lim, Gopalappa et al. 2017).

Epileptic seizures occur in up to 90% of patients with TSC, often commencing during infancy, having a focal basis, being drug-resistant, and associated with neurodevelopmental impairments (Chu-Shore, Major et al. 2010). Epilepsy surgery is performed in some patients with TSC and drug-resistant epilepsy, with a variety of approaches being employed to delineate seizure localisation and extent of the resection required, and favourable seizure outcomes are reported in the majority (Harvey 2019). The underlying cellular substrate and mechanisms of epileptogenesis in TSC and FCD are debated (Wong 2008, Harvey 2019), being attributed to either the visible pathological lesion, occult dysplasia in surrounding or remote cortex, molecular mechanisms related to the mTORopathy, or pervasive cerebral network disturbances. Electrophysiological studies in FCD suggest a substructure within the lesion, with specific zones pertaining to seizure onset and propagation (Brazdil, Halamek et al. 2010). The dysplastic tissue that defines FCD and TSC contains abnormal cell types, referred to as dysmorphic neurons and balloon cells (Najm, Sarnat et al. 2018). Single cell electrophysiological studies in FCD type IIb and TSC have provided evidence that dysmorphic neurons are the epileptogenic generators (Cepeda, Andre et al. 2005, Cepeda, Andre et al. 2010).

Our previous MRI and electrocorticography (ECoG) studies in children with TSC undergoing epilepsy surgery suggested that tubers, not perituberal cortex, are the source of focal seizures in TSC (Mohamed, Bailey et al. 2012). Subsequently, we localised seizure generation to the centre, not rim, of the tubers (Kannan, Vogrin et al. 2016). Combined with our histopathological

observation of apparently increased dysmorphic neuron number in the tuber centre compared to the tuber rim, we drew similarities with bottom-of-sulcus FCD (Harvey, Mandelstam et al. 2015) and considered similar, small, targeted, ECoG- and imaging-guided resections in TSC.

Here we demonstrate proof of principle, reporting five surgeries in four children with TSC in whom resection of an epileptogenic tuber in primary motor cortex was limited to the dysplastic tuber centre, eliminating the related seizures and EEG abnormalities and avoiding potential motor deficit. Additionally, we performed unbiased stereology and show that the density of dysmorphic neurons is greatest in the tuber centre. This study provides strong evidence that dysmorphic neurons, concentrated in the centre of epileptogenic cortical tubers, are the cellular substrate of drug-resistant focal epilepsy in TSC. Furthermore, our findings provide impetus to consider targeted procedures to alleviate seizures in TSC, including when tubers are present in areas previously not thought to be amenable to epilepsy surgery.

Materials and methods

Clinical details

The Royal Children's Hospital Human Research Ethics Committee approved this study (HREC 29077) and informed consent was obtained for all participants in accordance with the Declaration of Helsinki. The four children had drug-resistant, focal motor seizures with unilateral tonic or clonic manifestations, beginning in infancy and occurring at multiple daily frequency at the time of surgery (Supplementary Table 1). TSC was clinically diagnosed and confirmed by identification of pathogenic variants in *TSC2* in all patients. Three children developed infantile spasms, promptly and effectively treated with vigabatrin; Patient 3 was treated with vigabatrin before seizure onset and did not develop infantile spasms. Scalp EEG showed focal interictal epileptiform discharges (IEDs) and ictal rhythms in the contralateral central region. One or more candidate tubers were visible on MRI in the central region.

The children were aged 9 months to 2.8 years at the time of surgery, performed between April 2012 and November 2016; Patient 3 underwent separate surgeries for independent right and left-sided focal motor seizures. In all patients, surgery was performed in one stage with intraoperative ECoG, targeting candidate tubers exposed following a stereotactic craniotomy. The precentral and postcentral gyri and adjacent sulci were identified from cross-referencing the cortical surface appearance with the 3D surface-rendered MRI. The candidate tubers, their centres and sulcal boundaries were identified in a similar fashion, supplemented by cortical palpation. The concept and boundaries of tuber centres and rims were previously reported (Kannan, Vogrin et al. 2016). One or two 4-contact depth electrodes (5 mm spaced) were used

to record from the tuber centres, and either subdural strip (Patients 3a, 3b and 4) or strip and grid (Patients 1 and 2) electrodes (10 mm spaced) were used to record from the tuber rims and perituberal cortex. ECoG was recorded from 1-5 candidate tubers in the central and surrounding regions. Runs of rhythmic IEDs or electrographic seizures recorded from the tuber centre indicated their epileptogenicity.

Patient 3 underwent an ineffective resection of a non-epileptogenic left parietal tuber, due to false localisation of her right-sided focal motor seizures, prior to the eventually effective left precentral gyrus tuber-centre resection. At Patient 3's second surgery, the centre of an additional non-epileptogenic tuber in the left precentral gyrus was resected, as is our practice for exposed candidate tubers. Patients 1, 2 and 4 developed independent, non-motor seizures from remote foci that were successfully resected in Patients 1 and 2.

Tuber stereology details

Tissue samples were collected from the centre (resected tissue) and rim (biopsy) of the operated tuber in Patients 2, 3 and 4; no centre biopsy was obtained in Patient 1. These were processed for routine histological examination. Tissue was placed in formalin, embedded in paraffin, stained with H&E and NeuN, and then reviewed by conventional light microscopy. Each specimen showing typical features of TSC with cortical dyslamination, dysmorphic neurons and balloon cells (Blumcke, Thom et al. 2011).

Approximately 2 mm of residual paraffin embedded specimen was later cut at 15 µm and 1:10 serial sections were stained with mouse anti-human neurofilament protein monoclonal antibody (Covance, SMI-311R) to identify dysmorphic neurons (see supplementary methods) and counterstained with haematoxylin. Stereological analysis of dysmorphic neuron number was performed on blind-coded slides to prevent interpreter bias.

Dysmorphic neuron density estimations were performed using the optical fractionator method (West, Slomianka et al. 1991) via stereology software (StereoInvestigator, Version 11.08.1 [64-Bit]; MicroBrightField (MBF) Bioscience, Williston, Vermont, USA) and a 40x oil immersion lens on a light microscope (DMBL2; Leica, Germany). Patient dysmorphic neurons were only included in the count if they met the ILAE's criteria (Blumcke, Thom et al. 2011), which required immunoreactivity for neurofilament, whole cell size measured >20 µm and nuclei measured >15 µm. For each sample, dysmorphic neurons were counted in every 10th section cut and stained, giving a sampling fraction (ssf) of 1/10. A trace was drawn around the boundaries of the tissue on each section at 5x, within which a counting frame grid was overlaid. The size of each counting frame for the optical dissector probe was 150 µm x 150 µm, giving

an area of the counting frame (Aframe) of 22,500 μm^2. The distance between counting frames along the x and y axes of the section plane grid was variable between tissues, an arbitrary 5% of total surface area per section was chosen to be sampled to account for specimen size variability. The height of the optical dissector (h) was 10 µm, with an upper guard zone of 2.5 µm. The actual thickness of the mounted sections (t) was measured at five random points within each section analysed.

The estimated total number (N) of dysmorphic neurons in each sample was determined as the sum of cells counted ($\Sigma Q-$) on all sections, multiplied by the inverse of the ssf (1/10), the inverse of the area sampling fraction, asf (Aframe/Astep), and the inverse of the thickness sampling fraction, tsf (h/t), according to the formula $N = \Sigma Q- \times 1/(ssf \times asf \times tsf)$. The coefficient of error (CE) for each stereological estimate of total dysmorphic neuron number was calculated with the Gunderson-Jensen estimator (Gundersen and Jensen 1987) using a smoothness factor of 0 and a = 1/12 (Slomianka and West 2005). The sampling scheme for estimating total dysmorphic neuron number is summarised in Supplementary Table 2. The numerical density (cells/mm^3; Nv) of dysmorphic neurons was defined as the estimated total number (N) of cells divided by the estimated total volume (Vref) of each sample (given as a default output by StereoInvestigator dictated by the surface area covered by each section trace and the principles of Cavalieri's basic estimator), according to the formula NV = N/Vref.

Statistical analysis

For statistical analysis, the unpaired two-tailed student's t-test method was employed via Graph Pad Prism (V6, San Diego, USA), where a p value less than 0.05 was considered evidence of statistical significant. Unless otherwise stated, results are expressed as mean ± standard error of the mean (SEM).

Results

Tuber centre-only resections

The operated, epileptogenic tubers were in the precentral (Patients 1, 3a, 3b, 4) or postcentral gyrus (Patient 2), abutting the central sulcus. Intraoperative ECoG showed runs (Patient 3a, 3b, 4) or continuous (Patients 1, 2) rhythmic spike-wave activity and electrographic seizures (Patients 1, 3a) in the tuber centre, recorded by depth electrodes. These were confined to the tuber centre (Patients 3a, 3b, 4) and not recorded in the adjacent strip or grid electrodes recording from the tuber rim, or propagated to the tuber rim (Patients 1, 2). ECoG from the surrounding perituberal cortex showed independent spike-wave activity in patient 1 and 2. The

grey matter of the tuber centre was resected in a subpial fashion using an ultrasonic aspirator, to the grey-white junction. The abnormal white matter underlying the cortex of the tuber was not resected. Post-resection ECoG from the tuber rim and perituberal cortex, using subdural strip electrodes, showed no epileptiform activity; independent spiking was recorded in the perituberal cortex of Patients 1 and 2.

No patient experienced postoperative weakness, even transiently, and none has any apparent motor or sensory deficit on examination at follow-up. The focal motor seizures ceased immediately following the five surgeries in the four patients, with no recurrence during the 2.4-7.0 (mean 5.0) years of close clinical monitoring. Routine postoperative scalp EEG recordings three months following surgery, and subsequent video-EEG monitoring for independent seizures, did not reveal IEDs or electrographic seizures in the operated central region, except for Patient 4 who developed ipsilateral centrotemporal spikes without Rolandic epilepsy.

Antiepileptic medications were ceased following these surgeries in Patients 3 and 4. Patient 3 remains off antiepileptic medication. Patient 4 was commenced on lacosamide for infrequent, non-motor seizures of remote origin. Patients 1 and 2 had medications reduced following this and subsequent surgeries, to oxcarbazepine monotherapy. No patient has regular, ongoing seizures. Patients 2, 3 and 4 have normal intellectual abilities, Patients 2 and 4 have mild learning or behavioural difficulties, and all attend mainstream schooling. Patient 1 has an intellectually disability and autism spectrum disorder and attends a special developmental school.

Histopathology and dysmorphic neuron density

Routine pathological examination and diagnosis of cortical tubers revealed considerable variance of the underlying architecture and cellular constituents within biopsy specimens that suggested more severe pathology in the centre of cortical tubers including subjective impression of a higher dysmorphic neuron density (Figure 1). In comparison, the balloon cells appear similarly distributed across the specimens. Collectively, pathological examination of these specimens suggested variance in dysmorphic neuron numbers as a consistent differential between rim and centre specimens. Taken together with our pre-operative, intra-operative and post-operative observations that support epileptogenesis as arising from within the centre of cortical tubers, we hypothesised that a higher density of dysmorphic neurons in the tuber centre is the potential pathological substrate of seizure generation in TSC. Therefore, we performed unbiased quantitative stereology to determine if there were differences in dysmorphic neuron density between the tuber rim and tuber centre.

Stereological estimation of dysmorphic neuron demonstrated a greater density of dysmorphic neurons within the tuber centre compared to the rim (centre: 1951 ± 215 cells/mm^3, mean ± SEM, n=4 Vs rim: 531 ± 189 cells/mm^3, mean ± SEM, n=5, p=0.002, unpaired two-tailed t-test) (Figure 2). The significantly higher density of dysmorphic neurons seen at the centre compared to the rim was observed in tissue from each patient when analysed independently. Patient results for dysmorphic neuron densities ranged from a minimum of 66 cells/mm^3 in rim biopsies to a maximum of 2568 cells/mm^3 in centre biopsies, demonstrating that different regions of cortical tubers can have a considerable variance in dysmorphic neuron density.

Discussion

The surgical approach to epilepsy treatment in TSC varies significantly between epilepsy centres. Workup of such children is complex and involves clinical, imaging and preoperative and intraoperative electrophysiological studies to identify the seizure-generating tubers and determine the best approach for their removal with minimal risk of side effects. Surgery should be considered early in the treatment process to avoid the risks of ongoing seizures and medication side effects to the developing brain. Some children with TSC are not considered to be good candidates for epilepsy surgery due to epileptogenic tubers in eloquent cortical areas and the perceived risks of deficits following epilepsy surgery.

A major determinant for the inclusion of the four children reported here was the localisation of seizures in sensorimotor cortex. Their seizures had clinically apparent and localising focal motor features that aided determination of postoperative outcome (unlike non-motor seizures commonly seen in young children with TSC). Notably, only a single tuber was found to be epileptogenic and resected, and follow-up was lengthy. Resection was limited to the tuber centre, leaving the tuber rim and surrounding perituberal cortex intact, other than a small biopsy of the tuber rim. This approach led to elimination of the related seizures and EEG abnormalities and avoided potential motor deficit.

Much of the literature suggests that epileptogenicity in TSC relates to the "mTORopathy" in perituberal cortex, resulting in occult cortical dysplasia, derangements of cellular functioning and widespread epileptogenic networks. Studies to determine the seizure generating zone of cortical tubers have shown variable results with some suggesting seizure onset in the perituberal cortex (Madhavan, Weiner et al. 2007, Major, Rakowski et al. 2009, Marcotte, Aronica et al. 2012, Chalifoux, Perry et al. 2013, Ruppe, Dilsiz et al. 2014, Sosunov, McGovern et al. 2015)

and others suggesting onset within the tubers (Ma, Elliott et al. 2012, Mohamed, Bailey et al. 2012, Kannan, Vogrin et al. 2016, Despouy, Curot et al. 2019). Many studies have lacked detailed methods to determine the spatial and temporal electrophysiological abnormalities seen within and around tubers. The widely held belief that seizures originate in the perituberal cortex has led to wide resection approaches or inappropriate exclusion of patients from surgery as a treatment option. Our intracranial EEG monitoring studies in children with TSC and uncontrolled epilepsy revealed that focal seizures and interictal epileptiform discharges arise from the centre of epileptogenic tubers and propogate to tuber rim, perituberal cortex and non-epileptogenic tubers (Mohamed, Bailey et al. 2012). These findings are consistent with MRI and histopathology which suggests more marked cortical dysplasia in the tuber centres (Kannan, Vogrin et al. 2016). These findings have led to a change of practice in our centre to smaller, centre only resections in TSC and "bottom of sulcus" FCDs.

Prior to this study, there were limited descriptions of dysmorphic neuron distribution in cortical tubers (Muhlebner, Iyer et al. 2016, Muhlebner, van Scheppingen et al. 2016). On clinical review of routine histopathology, we observed a higher density of dysmorphic neurons at the tuber centre and hypothesised that this could be the cause of increased epileptogenicity at the tuber centre we detected with our electrophysiology studies. Therefore, we performed quantitative analysis using stereological methods to assess the density of dysmorphic neurons in various regions of the cortical tubers of TSC. Our results demonstrate for the first time that the centre contains a higher density of dysmorphic neurons when compared with the rim. We suggest that removal of this nidus of high density of dysmorphic neurons at the tuber centre may be sufficient to control seizures.

It has been suggested that dysmorphic neurons are the essential pathogenetic element driving epileptogenesis in cortical dysplasia, and hence are the source of ictal discharges (Chassoux, Devaux et al. 2000, Battaglia, Colciaghi et al. 2013). This proposed role for dysmorphic neurons as the source of seizure generation in TSC and other FCDs is reasonable given the electrophysiological properties of these cells compared with other cell populations present in dysplastic tissue. Compared to normal pyramidal neurons, dysmorphic neurons possess atypical passive membrane properties. Due to the increased size and hence increased membrane area of these cells, they exhibit a larger cell capacitance, a longer time constant and a lower input resistance (Cepeda, Hurst et al. 2003). In addition, dysmorphic neurons are predisposed to hyperexcitability via larger amplitudes of macroscopic Ca2+ currents and Ca2+ influx. Studies demonstrating high expression of ionotropic and metabotropic glutamate receptor subtypes

and/or subunits in these cells also provide evidence of dysfunctional molecular pathways contributing to hyperexcitability (White, Hua et al. 2001, Wang, Greenwood et al. 2007, Boer, Troost et al. 2008). Conversely, balloon cells, which are the other recognised abnormal cell type in tubers, have a high input resistance, they do not exhibit voltage-gated Na+ or Ca2+ currents, and do not generate spontaneous synaptic currents (Cepeda, Hurst et al. 2003). Further to this, studies demonstrating an insensitivity of balloon cells to excitatory amino acids suggest a low epileptogenic potential (Cepeda, Andre et al. 2012). Interestingly, evidence for increased clearance of glutamate in areas with a high density of balloon cells, thereby leading to a decrease in the spread of epileptogenic activity, may imply a protective or antiepileptic function (Gonzalez-Martinez, Ying et al. 2011). However, BCs also express VGLUT2, suggesting they could participate in seizure propagation by release of intracellular glutamate (Devinsky, Vezzani et al. 2013). Future studies will need to determine balloon cell densities and their spatial relationship to dysmorphic neuron density gradients in both epileptogenic and non-epileptogenic tubers, which could help improve our understanding of the interactions between these two cell types, in addition to the requirements for tuber epileptogenicity.

A recent study using new hybrid electrodes with tetrodes demonstrated the tuber is critically involved in seizure onset, with neuronal spiking activity suggesting a gradient of epileptogenicity from the tuber centre to the perituberal tissue (Despouy, Curot et al. 2019). This study provides additional support to the hypothesis that the tuber centre play a major role in seizure generation in cortical tubers and, by extension, the histopathologically similar FCD type II and larger malformations in the spectrum to hemimegalencephaly. However, the fact that seizure activity is elicited in other cortical malformations that do not feature dysmorphic neurons suggests that additional pathomechanisms contribute to epileptogenicity in these disorders (Blumcke, Thom et al. 2011). For example, FCD type I is characterised by cortical dyslamination and clusters of pyramidal neurons that are largely morphologically normal, but are misoriented, mildly hypertrophic or immature (Blumcke, Aronica et al. 2016), but electrophysiologically normal (Cepeda, Hurst et al. 2003, Ben-Ari, Gaiarsa et al. 2007). These immature neurons have been implicated in the epileptogenesis of FCD type I along with the aberrant connectivity that is associated with cortical dyslamination. Unifying this mode of epileptogenesis with our proposed dysmorphic neuron pathomechanism is a recent study examining spontaneous pacemaker GABA receptor-mediated synaptic activity, thought to arise from a subclass of GABAergic interneurons (Cepeda, Chen et al. 2014). Such activity represents an abnormal signal that serves to induce widespread depolarisation through GABA

synaptic events and hence synchronise a local network of dysmorphic neurons and other cell types, including immature neurons, in FCD type I. This ‘epileptogenic pacemaker’ is thought to be responsible for specific electroencephalographic (EEG) signatures observed in some cortical dysplasia cases (Chassoux, Devaux et al. 2000). Follow up studies are required to determine the spatial relationship of the cells responsible for this activity with dysmorphic neurons. Close associations between these two cell populations could translate into refined seizure onset zone localisation through EEG interpretation.

Our study provides strong evidence for a high density of hyperexcitable dysmorphic neurons concentrated in the centre of cortical tubers as being the pathological substrate of drug-resistant epilepsy in cortical tubers. It is yet to be determined if a similar mechanism underlies other cortical malformations, including other forms of type II FCD. Our findings, taken together with recent electrophysiological data, argue against performing multiple tuberectomies or large cortical resections in TSC and suggest that limited resections, focussed on epileptogenic tuber centre removal, may be more appropriate. This approach will lead to fewer operative complications, fewer post-operative deficits and the consideration of epilepsy surgery as an effective and early treatment of the drug resistant seizures of TSC for a larger number of patients.

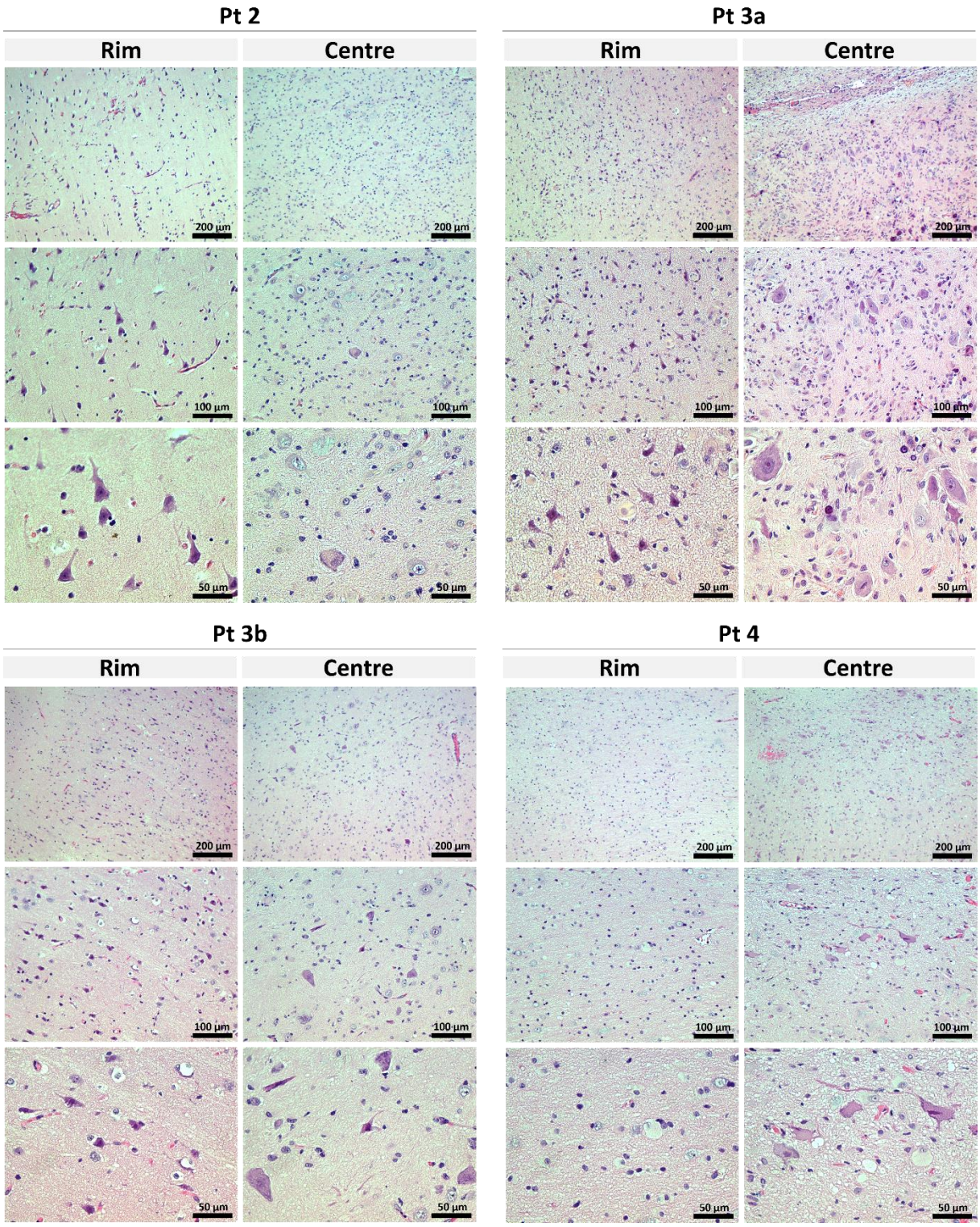

Figure 1: Differences in dysmorphic neurons numbers identified in specimens from the centre of cortical tubers compared to the rim.

Haemotoxylin and Eosin staining from paired biopsies taken from the rim and the centre of cortical tubers demonstrate spatial differences in the laminal architecture and cellular constituents within specimens. Specimens from the rim largely comprised grey matter in

which cortical lamination appeared relatively normal and cytomegalic or dysmorphic neurons were rarely identified. Balloon cells were identified to varying degrees in rim biopsies. Conversely, biopsy resected from the centre of the cortical tuber also was largely comprised of grey matter but demonstrated marked dyslamination with irregular clusters of large, cytomegalic and dysmorphic neurons and balloon cells. Scale bars are indicated.

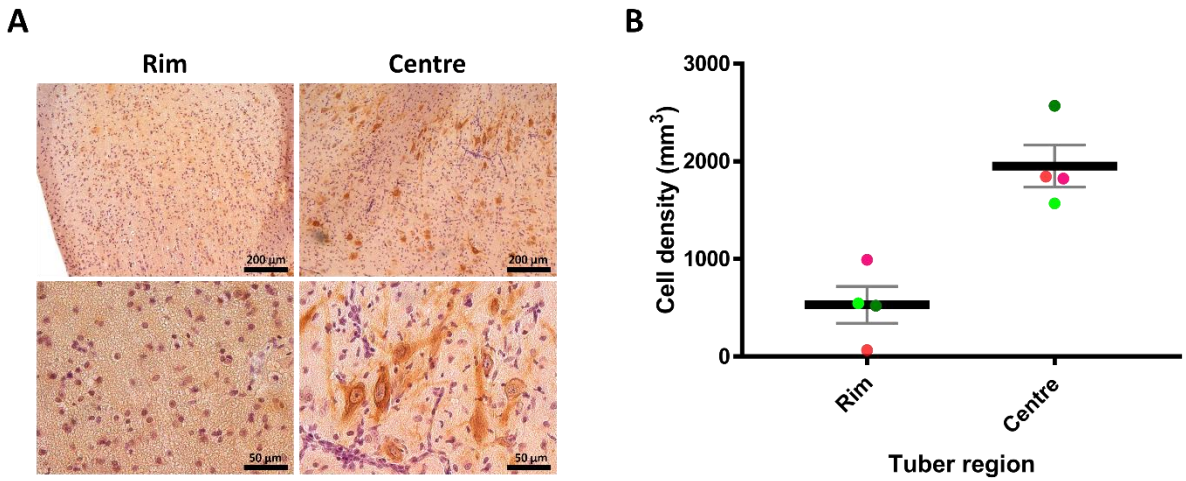

Figure 2: The density of dysmorphic neurons is greatest in the centre of the tuber
(A) Representative images of anti-human neurofilament protein immunohistochemistry staining the dysmorphic neurons in tuber rim and tuber centre demonstrating a typically observed cluster of dysmorphic neurons in the centre biopsy that were absent from the rim. Scale bars are indicated. (B) Unbiased quantitative stereology determined the density of dysmorphic neurons in the tuber centre was ~4 fold greater than the tuber rim. Error bars represent standard error of the mean.

Supplementary material

See attached file

References

Aronica, E. and P. B. Crino (2014). "Epilepsy related to developmental tumors and malformations of cortical development." Neurotherapeutics **11**(2): 251-268.

Battaglia, G., F. Colciaghi, A. Finardi and P. Nobili (2013). "Intrinsic epileptogenicity of dysplastic cortex: converging data from experimental models and human patients." Epilepsia **54 Suppl 6**: 33-36.

Baulac, S., S. Ishida, E. Marsan, C. Miquel, A. Biraben, D. K. Nguyen, D. Nordli, P. Cossette, S. Nguyen, V. Lambrecq, M. Vlaicu, M. Daniau, F. Bielle, E. Andermann, F. Andermann, E. Leguern, F. Chassoux

and F. Picard (2015). "Familial focal epilepsy with focal cortical dysplasia due to DEPDC5 mutations." Ann Neurol **77**(4): 675-683.

Ben-Ari, Y., J. L. Gaiarsa, R. Tyzio and R. Khazipov (2007). "GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations." Physiol Rev **87**(4): 1215-1284.

Blumcke, I., E. Aronica, H. Miyata, H. B. Sarnat, M. Thom, K. Roessler, B. Rydenhag, L. Jehi, P. Krsek, S. Wiebe and R. Spreafico (2016). "International recommendation for a comprehensive neuropathologic workup of epilepsy surgery brain tissue: A consensus Task Force report from the ILAE Commission on Diagnostic Methods." Epilepsia **57**(3): 348-358.

Blumcke, I., M. Thom, E. Aronica, D. D. Armstrong, H. V. Vinters, A. Palmini, T. S. Jacques, G. Avanzini, A. J. Barkovich, G. Battaglia, A. Becker, C. Cepeda, F. Cendes, N. Colombo, P. Crino, J. H. Cross, O. Delalande, F. Dubeau, J. Duncan, R. Guerrini, P. Kahane, G. Mathern, I. Najm, C. Ozkara, C. Raybaud, A. Represa, S. N. Roper, N. Salamon, A. Schulze-Bonhage, L. Tassi, A. Vezzani and R. Spreafico (2011). "The clinicopathologic spectrum of focal cortical dysplasias: a consensus classification proposed by an ad hoc Task Force of the ILAE Diagnostic Methods Commission." Epilepsia **52**(1): 158-174.

Boer, K., D. Troost, W. Timmermans, J. A. Gorter, W. G. Spliet, M. Nellist, F. Jansen and E. Aronica (2008). "Cellular localization of metabotropic glutamate receptors in cortical tubers and subependymal giant cell tumors of tuberous sclerosis complex." Neuroscience **156**(1): 203-215.

Brazdil, M., J. Halamek, P. Jurak, P. Daniel, R. Kuba, J. Chrastina, Z. Novak and I. Rektor (2010). "Interictal high-frequency oscillations indicate seizure onset zone in patients with focal cortical dysplasia." Epilepsy Res **90**(1-2): 28-32.

Cepeda, C., V. M. Andre, J. S. Hauptman, I. Yamazaki, M. N. Huynh, J. W. Chang, J. Y. Chen, R. S. Fisher, H. V. Vinters, M. S. Levine and G. W. Mathern (2012). "Enhanced GABAergic network and receptor function in pediatric cortical dysplasia Type IIB compared with Tuberous Sclerosis Complex." Neurobiol Dis **45**(1): 310-321.

Cepeda, C., V. M. Andre, H. V. Vinters, M. S. Levine and G. W. Mathern (2005). "Are cytomegalic neurons and balloon cells generators of epileptic activity in pediatric cortical dysplasia?" Epilepsia **46 Suppl 5**: 82-88.

Cepeda, C., V. M. Andre, I. Yamazaki, J. S. Hauptman, J. Y. Chen, H. V. Vinters, G. W. Mathern and M. S. Levine (2010). "Comparative study of cellular and synaptic abnormalities in brain tissue samples from pediatric tuberous sclerosis complex and cortical dysplasia type II." Epilepsia **51 Suppl 3**: 160-165.

Cepeda, C., J. Y. Chen, J. Y. Wu, R. S. Fisher, H. V. Vinters, G. W. Mathern and M. S. Levine (2014). "Pacemaker GABA synaptic activity may contribute to network synchronization in pediatric cortical dysplasia." Neurobiol Dis **62**: 208-217.

Cepeda, C., R. S. Hurst, J. Flores-Hernandez, E. Hernandez-Echeagaray, G. J. Klapstein, M. K. Boylan, C. R. Calvert, E. L. Jocoy, O. K. Nguyen, V. M. Andre, H. V. Vinters, M. A. Ariano, M. S. Levine and G. W. Mathern (2003). "Morphological and electrophysiological characterization of abnormal cell types in pediatric cortical dysplasia." J Neurosci Res **72**(4): 472-486.

Chalifoux, J. R., N. Perry, J. S. Katz, G. C. Wiggins, J. Roth, D. Miles, O. Devinsky, H. L. Weiner and S. S. Milla (2013). "The ability of high field strength 7-T magnetic resonance imaging to reveal previously uncharacterized brain lesions in patients with tuberous sclerosis complex." J Neurosurg Pediatr **11**(3): 268-273.

Chassoux, F., B. Devaux, E. Landre, B. Turak, F. Nataf, P. Varlet, J. P. Chodkiewicz and C. Daumas-Duport (2000). "Stereoelectroencephalography in focal cortical dysplasia: a 3D approach to delineating the dysplastic cortex." Brain **123 (Pt 8)**: 1733-1751.

Chu-Shore, C. J., P. Major, S. Camposano, D. Muzykewicz and E. A. Thiele (2010). "The natural history of epilepsy in tuberous sclerosis complex." Epilepsia **51**(7): 1236-1241.

Crino, P. B. (2007). "Focal brain malformations: a spectrum of disorders along the mTOR cascade." Novartis Found Symp **288**: 260-272; discussion 272-281.

D'Gama, A. M., Y. Geng, J. A. Couto, B. Martin, E. A. Boyle, C. M. LaCoursiere, A. Hossain, N. E. Hatem, B. J. Barry, D. J. Kwiatkowski, H. V. Vinters, A. J. Barkovich, J. Shendure, G. W. Mathern, C. A. Walsh and A. Poduri (2015). "Mammalian target of rapamycin pathway mutations cause hemimegalencephaly and focal cortical dysplasia." Ann Neurol **77**(4): 720-725.

D'Gama, A. M., M. B. Woodworth, A. A. Hossain, S. Bizzotto, N. E. Hatem, C. M. LaCoursiere, I. Najm, Z. Ying, E. Yang, A. J. Barkovich, D. J. Kwiatkowski, H. V. Vinters, J. R. Madsen, G. W. Mathern, I. Blumcke, A. Poduri and C. A. Walsh (2017). "Somatic Mutations Activating the mTOR Pathway in Dorsal Telencephalic Progenitors Cause a Continuum of Cortical Dysplasias." Cell Rep **21**(13): 3754-3766.

Despouy, E., J. Curot, M. Denuelle, M. Deudon, J. C. Sol, J. A. Lotterie, L. Reddy, L. G. Nowak, J. Pariente, S. J. Thorpe, L. Valton and E. J. Barbeau (2019). "Neuronal spiking activity highlights a gradient of epileptogenicity in human tuberous sclerosis lesions." Clin Neurophysiol **130**(4): 537-547.

Devinsky, O., A. Vezzani, S. Najjar, N. C. De Lanerolle and M. A. Rogawski (2013). "Glia and epilepsy: excitability and inflammation." Trends Neurosci **36**(3): 174-184.

Gonzalez-Martinez, J. A., Z. Ying, R. Prayson, W. Bingaman and I. Najm (2011). "Glutamate clearance mechanisms in resected cortical dysplasia." J Neurosurg **114**(4): 1195-1202.

Gundersen, H. J. and E. B. Jensen (1987). "The efficiency of systematic sampling in stereology and its prediction." J Microsc **147**(Pt 3): 229-263.

Harvey, A. S. (2019). Invasive EEG in tuberous sclerosis. Invasive Studies of the Human Epileptic Brain: Principles and Practice. K. P. Lhatoo SD, Lüders HO. Oxford, UK Oxford University Press**:** 213-234.

Harvey, A. S., S. A. Mandelstam, W. J. Maixner, R. J. Leventer, M. Semmelroch, D. MacGregor, R. M. Kalnins, Y. Perchyonok, G. J. Fitt, S. Barton, M. J. Kean, G. C. Fabinyi and G. D. Jackson (2015). "The surgically remediable syndrome of epilepsy associated with bottom-of-sulcus dysplasia." Neurology **84**(20): 2021-2028.

Kannan, L., S. Vogrin, C. Bailey, W. Maixner and A. S. Harvey (2016). "Centre of epileptogenic tubers generate and propagate seizures in tuberous sclerosis." Brain **139**(Pt 10): 2653-2667.

Leventer, R. J., T. Scerri, A. P. Marsh, K. Pope, G. Gillies, W. Maixner, D. MacGregor, A. S. Harvey, M. B. Delatycki, D. J. Amor, P. Crino, M. Bahlo and P. J. Lockhart (2015). "Hemispheric cortical dysplasia secondary to a mosaic somatic mutation in MTOR." Neurology **84**(20): 2029-2032.

Lim, J. S., R. Gopalappa, S. H. Kim, S. Ramakrishna, M. Lee, W.-i. Kim, J. Kim, S. M. Park, J. Lee, J.-H. Oh, H. D. Kim, C.-H. Park, J. S. Lee, S. Kim, D. S. Kim, J. M. Han, H.-C. Kang, H. Kim and J. H. Lee (2017). "Somatic Mutations in TSC1 and TSC2 Cause Focal Cortical Dysplasia." The American Journal of Human Genetics.

Lim, J. S., W. I. Kim, H. C. Kang, S. H. Kim, A. H. Park, E. K. Park, Y. W. Cho, S. Kim, H. M. Kim, J. A. Kim, J. Kim, H. Rhee, S. G. Kang, H. D. Kim, D. Kim, D. S. Kim and J. H. Lee (2015). "Brain somatic mutations in MTOR cause focal cortical dysplasia type II leading to intractable epilepsy." Nat Med **21**(4): 395-400.

Ma, T. S., R. E. Elliott, V. Ruppe, O. Devinsky, R. Kuzniecky, H. L. Weiner and C. Carlson (2012). "Electrocorticographic evidence of perituberal cortex epileptogenicity in tuberous sclerosis complex." J Neurosurg Pediatr **10**(5): 376-382.

Madhavan, D., H. L. Weiner, C. Carlson, O. Devinsky and R. Kuzniecky (2007). "Local epileptogenic networks in tuberous sclerosis complex: a case review." Epilepsy Behav **11**(1): 140-146.

Major, P., S. Rakowski, M. V. Simon, M. L. Cheng, E. Eskandar, J. Baron, B. A. Leeman, M. P. Frosch and E. A. Thiele (2009). "Are cortical tubers epileptogenic? Evidence from electrocorticography." Epilepsia **50**(1): 147-154.

Marcotte, L., E. Aronica, M. Baybis and P. B. Crino (2012). "Cytoarchitectural alterations are widespread in cerebral cortex in tuberous sclerosis complex." Acta Neuropathol **123**(5): 685-693.

Mirzaa, G. M., C. D. Campbell, N. Solovieff, C. P. Goold, L. A. Jansen, S. Menon, A. E. Timms, V. Conti, J. D. Biag, C. Olds, E. A. Boyle, S. Collins, G. Ishak, S. L. Poliachik, K. M. Girisha, K. S. Yeung, B. H. Chung, E. Rahikkala, S. A. Gunter, S. S. McDaniel, C. F. Macmurdo, J. A. Bernstein, B. Martin, R. J. Leary, S. Mahan, S. Liu, M. Weaver, M. O. Dorschner, S. Jhangiani, D. M. Muzny, E. Boerwinkle, R. A. Gibbs, J. R. Lupski, J. Shendure, R. P. Saneto, E. J. Novotny, C. J. Wilson, W. R. Sellers, M. P. Morrissey, R. F. Hevner, J. G. Ojemann, R. Guerrini, L. O. Murphy, W. Winckler and W. B. Dobyns (2016). "Association of MTOR Mutations With Developmental Brain Disorders, Including Megalencephaly, Focal Cortical Dysplasia, and Pigmentary Mosaicism." JAMA Neurol **73**(7): 836-845.

Mohamed, A. R., C. A. Bailey, J. L. Freeman, W. Maixner, G. D. Jackson and A. S. Harvey (2012). "Intrinsic epileptogenicity of cortical tubers revealed by intracranial EEG monitoring." Neurology **79**(23): 2249-2257.

Muhlebner, A., A. M. Iyer, J. van Scheppingen, J. J. Anink, F. E. Jansen, T. J. Veersema, K. P. Braun, W. G. Spliet, W. van Hecke, F. Soylemezoglu, M. Feucht, P. Krsek, J. Zamecnik, C. G. Bien, T. Polster, R. Coras, I. Blumcke and E. Aronica (2016). "Specific pattern of maturation and differentiation in the formation of cortical tubers in tuberous sclerosis omplex (TSC): evidence from layer-specific marker expression." J Neurodev Disord **8**: 9.

Muhlebner, A., J. van Scheppingen, H. M. Hulshof, T. Scholl, A. M. Iyer, J. J. Anink, A. M. van den Ouweland, M. D. Nellist, F. E. Jansen, W. G. Spliet, P. Krsek, B. Benova, J. Zamecnik, P. B. Crino, D. Prayer, T. Czech, A. Wohrer, J. Rahimi, R. Hoftberger, J. A. Hainfellner, M. Feucht and E. Aronica (2016). "Novel Histopathological Patterns in Cortical Tubers of Epilepsy Surgery Patients with Tuberous Sclerosis Complex." PLoS One **11**(6): e0157396.

Najm, I., H. B. Sarnat and I. Blumcke (2018). "The international consensus classification of Focal Cortical Dysplasia - a critical update 2018." Neuropathol Appl Neurobiol.

O'Callaghan, F. J., A. W. Shiell, J. P. Osborne and C. N. Martyn (1998). "Prevalence of tuberous sclerosis estimated by capture-recapture analysis." Lancet **351**(9114): 1490.

Ruppe, V., P. Dilsiz, C. S. Reiss, C. Carlson, O. Devinsky, D. Zagzag, H. L. Weiner and D. M. Talos (2014). "Developmental brain abnormalities in tuberous sclerosis complex: a comparative tissue analysis of cortical tubers and perituberal cortex." Epilepsia **55**(4): 539-550.

Scerri, T., J. R. Riseley, G. Gillies, K. Pope, R. Burgess, S. A. Mandelstam, L. Dibbens, C. W. Chow, W. Maixner, A. S. Harvey, G. D. Jackson, D. J. Amor, M. B. Delatycki, P. B. Crino, S. F. Berkovic, I. E. Scheffer, M. Bahlo, P. J. Lockhart and R. J. Leventer (2015). "Familial cortical dysplasia type IIA caused by a germline mutation in DEPDC5." Ann Clin Transl Neurol **2**(5): 575-580.

Sim, J. C., T. Scerri, M. Fanjul-Fernandez, J. R. Riseley, G. Gillies, K. Pope, H. van Roozendaal, J. I. Heng, S. A. Mandelstam, G. McGillivray, D. MacGregor, L. Kannan, W. Maixner, A. S. Harvey, D. J. Amor, M. B. Delatycki, P. B. Crino, M. Bahlo, P. J. Lockhart and R. J. Leventer (2016). "Familial cortical dysplasia caused by mutation in the mammalian target of rapamycin regulator NPRL3." Ann Neurol **79**(1): 132-137.

Slomianka, L. and M. J. West (2005). "Estimators of the precision of stereological estimates: an example based on the CA1 pyramidal cell layer of rats." Neuroscience **136**(3): 757-767.

Sosunov, A. A., R. A. McGovern, C. B. Mikell, X. Wu, D. G. Coughlin, P. B. Crino, H. L. Weiner, S. Ghatan, J. E. Goldman and G. M. McKhann, 2nd (2015). "Epileptogenic but MRI-normal perituberal tissue in Tuberous Sclerosis Complex contains tuber-specific abnormalities." Acta Neuropathol Commun **3**: 17.

van Slegtenhorst, M., M. Nellist, B. Nagelkerken, J. Cheadle, R. Snell, A. van den Ouweland, A. Reuser, J. Sampson, D. Halley and P. van der Sluijs (1998). "Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products." Hum Mol Genet **7**(6): 1053-1057.

Wang, Y., J. S. Greenwood, M. E. Calcagnotto, H. E. Kirsch, N. M. Barbaro and S. C. Baraban (2007). "Neocortical hyperexcitability in a human case of tuberous sclerosis complex and mice lacking neuronal expression of TSC1." Ann Neurol **61**(2): 139-152.

West, M. J., L. Slomianka and H. J. Gundersen (1991). "Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator." Anat Rec **231**(4): 482-497.

White, R., Y. Hua, B. Scheithauer, D. R. Lynch, E. P. Henske and P. B. Crino (2001). "Selective alterations in glutamate and GABA receptor subunit mRNA expression in dysplastic neurons and giant cells of cortical tubers." Ann Neurol **49**(1): 67-78.

Wong, M. (2008). "Mechanisms of epileptogenesis in tuberous sclerosis complex and related malformations of cortical development with abnormal glioneuronal proliferation." Epilepsia **49**(1): 8-21.