GABAergic Interneuron Development and Function Is Modulated by the Tsc1 Gene

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Tuberous sclerosis complex (TSC) is a genetic disease with severe neurologic and psychiatric manifestations including epilepsy, developmental delay, and autism. Despite much progress in defining abnormal signaling pathways including the contribution of increased mTORC1 signaling, specific abnormalities that underlie the severe neurologic features in TSC remain poorly understood. We hypothesized that epilepsy and autism in TSC result from abnormalities of \(^\gamma\)-aminobutyric acidergic (GABAergic) interneurons. To test this hypothesis, we generated conditional knockout mice with selective deletion of the \textit{Tsc1}\textsuperscript{1} gene in GABAergic interneuron progenitor cells. These interneuron-specific \textit{Tsc1}\textsuperscript{1} conditional knockout (CKO) mice have impaired growth and decreased survival. Cortical and hippocampal GABAergic interneurons of CKO mice are enlarged and show increased mTORC1 signaling. Total numbers of GABAergic cells are reduced in the cortex with differential reduction of specific GABAergic subtypes. Ectopic clusters of cells with increased mTORC1 signaling are also seen suggesting impaired interneuron migration. The functional consequences of these cellular changes are evident in the decreased seizure threshold on exposure to the proconvulsant flurothyl. These findings support an important role for the \textit{Tsc1}\textsuperscript{1} gene during GABAergic interneuron development, function, and possibly migration.

Keywords: epilepsy, GABA, hamartin, mTOR, mTORC1

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disease affecting approximately 1 in 6000 people (Osborne et al. 1991). Multiple organ systems are involved including brain, kidneys, lung, and skin (Curatolo et al. 2008); however, brain involvement contributes most significantly to long-term morbidity as epilepsy is seen in up to 90% and autism in up to 50% of individuals with TSC (Hunt and Dennis 1987; Gutierrez et al. 1998; Gomez et al. 1999). Either of these manifestations can be quite debilitating, and many patients are affected by both of these profound neurological complications (Wizinzier 2004). An improved understanding of the pathological mechanisms leading to epilepsy and autism in TSC should enhance current clinical management and catalyze the development of novel therapeutics.

Epilepsy in TSC is quite heterogeneous frequently involving a variety of seizure types, including partial seizures, generalized seizures, and infantile spasms (Holmes and Stafstrom 2007). Infantile spasms affect up to 75% of children with TSC (Thiele 2004) and also occur in the general population as a result of brain dysfunction from a variety of intrinsic or extrinsic causes during prenatal, perinatal, or postnatal brain development (Wong and Trevathan 2001). In contrast to infantile spasms from other causes, TSC-related infantile spasms have a very rapid and sustained response to the seizure medication vigabatrin (Curatolo et al. 2001), an irreversible inhibitor of the \(^\gamma\)-aminobutyric acid (GABA) degrading enzyme GABA transaminase. This response suggests that the mechanisms leading to infantile spasms in TSC include abnormalities of GABAergic neurotransmission.

TSC is caused by loss of function mutations in either the \textit{Tsc1}\textsuperscript{1} or \textit{Tsc2}\textsuperscript{2} genes that encode hamartin and tuberin, respectively. These 2 proteins bind to one another and regulate the mammalian target of rapamycin (mTOR) ser/threonine kinase. mTOR is found within 2 functionally and molecularly distinct complexes, mTORC1 and mTORC2. mTORC1 is defined by the protein raptor (regulatory associated protein of mTOR), mLST8, PRAS40, and DEPTOR and is selectively sensitive to rapamycin (Kim et al. 2002; Liu et al. 2010). mTORC1 regulates cell growth and protein translation through phosphorylation of multiple downstream effectors including ribosomal protein S6 kinase-1 and 4E-BP1 (Fingar et al. 2004; Hay and Sonenberg 2004). mTORC2 in contrast, includes mLST8, mSIN1 as well as rictor (rapamycin insensitive component of TOR). mTORC2 is largely unaffected by rapamycin, however, prolonged exposure may inhibit assembly of the complex (Sarbassov et al. 2006). In contrast to mTORC1, much less is known regarding regulation and function of mTORC2 though it may be involved in cytoskeletal regulation (Sarbassov et al. 2004). Recent studies indicate that hamartin/tuberin differentially regulate the mTOR-containing complexes, inhibiting mTORC1 while activating mTORC2 (Inoki et al. 2003; Huang et al. 2008). Loss of \textit{Tsc1}\textsuperscript{1} or \textit{Tsc2}\textsuperscript{2} then appears to allow increased mTORC1 but decreased mTORC2 signaling (Huang and Manning 2009). Despite considerable progress in our understanding of these signaling pathways, there are still many questions regarding the specific contributions of dysregulated mTORC1 and mTORC2 to the pathogenesis of TSC.

Cortical “tubers” are the pathologic hallmark of TSC and are generally accepted to be the pathologic substrate underlying the observed neurological manifestations (Crino et al. 2006). The severity of epilepsy and autism in TSC tends to correlate with number and location of cortical tubers (Goodman et al. 1997; Wizinzier 2004), and there is clear evidence for dysregulated mTORC1 pathway activity in the “giant” cells present within cortical tuber specimens (Crino 2004). At the neural network level, molecular abnormalities involving inhibitory as well as excitatory neurons in cortical tubers have been suggested to underlie the correlation with epilepsy and autism (White et al. 2001; Valencia et al. 2006; Taki et al. 2009).
The high incidence of both epilepsy and autism in patients with TSC and the dramatic response of infantile spasms to a GABA potentiating agent led us to hypothesize that patients with TSC have altered GABAergic interneuron function. In turn, this may alter the balance of brain excitation and inhibition. To test our hypothesis, we generated mice with conditional inactivation of the Tsc1 gene in ventral neural progenitor cells of the medial, lateral, and caudal ganglionic eminences (MGE, LGE, and CGE), which give rise to neocortical and hippocampal GABAergic interneurons (Wonders and Anderson 2006). These interneuron-specific Tsc1 conditional knockout (Tsc1 interneuron CKO) mice have impaired postnatal growth with approximately 50% dying prematurely. While the brain appears grossly normal, we found decreased numbers of cortical and hippocampal GABAergic interneurons with selective involvement of specific interneuron subtypes. Interneurons from Tsc1 interneuron CKO mice were also enlarged with a marked increase in mTORC1 signaling. Ectopic large cells with increased mTORC1 signaling were also found in layer 1 of the cerebral cortex and the septum. These ectopic cells suggest that Tsc1-deficient GABAergic neurons have impaired migration. Finally, Tsc1 interneuron CKO mice did not exhibit spontaneous seizures but had a reduced seizure threshold when exposed to the proconvulsant agent flurothyl. Overall, these findings support our hypothesis that the Tsc1 gene is involved in GABAergic interneuron development and suggest that inhibitory cortical neurons may contribute to the pathogenesis of epilepsy and possibly autism in patients with TSC.

Materials and Methods

Conditional Knockout Mice

Mice with LoxP sites flanking exons 17 and 18 of the Tsc1 gene ("floxed" allele Tsc1F/F) were generated by Dr David Kwiatkowski (Harvard Medical School, Boston, MA) (Uhlmann et al. 2002). Dlx5/6-Cre-IRE-EGFP transgenic mice express Cre recombinase and EGFP under the control of the Dlx5/6 enhancer and were provided by Dr Kenneth Campbell (University of Cincinnati, Cincinnati, OH) (Zerucha et al. 2000; Stenman et al. 2003). Both of these transgenic lines have been maintained separately in mixed C57/SV129 backgrounds in our colony for the past 4 years. Through interbreeding of Tsc1F/F Tg(Dlx5/6-Cre-IRE-EGFP) and Tsc1F/F Cre-negative mice, Tsc1F/F Tg(Dlx5/6-Cre-IRE-EGFP) (Tsc1 interneuron CKO) mice were generated that have conditional loss of the Tsc1 gene in GABAergic interneuron progenitors (Fig. 1A). Both Tsc1F/F Tg(Dlx5/6-Cre-IRE-EGFP) and Cre-negative littersmates were used as controls; no abnormalities have been seen in any of these genotypes. All experiments using mice were approved by the Vanderbilt University IACUC.

Tsc1 Gene Recombination

Polymerase chain reaction (PCR) of the wild-type and conditional alleles of Tsc1 was performed using primers F4536 (5'-AGGAGGCCCCTCTCTCAGTACCATTGAATG-3') and R4830 (5'-GAAGGAGCATGCGACATGGAAGTGCC-3') with band sizes of 295 bp for the wild type and 480 bp for the floxed allele. Flow cytometry was used to sort cells obtained from EGFP-positive E15.5 embryos. DNA extracted from these cells was then used to confirm recombination and inactivation of the Tsc1 conditional allele using PCR primers F4536 and R6548 (5'-TGGGTTCTCGACCTATCTCTCTGA-3') giving bands of 2012 bp for the un-recombined conditional allele and 308 bp for the recombined null allele.

Immunobistochemistry

P20-P27 Tsc1 interneuron CKO and control mice were anesthetized and perfused with 4% paraformaldehyde in phosphate-buffered saline. Coronal frozen sections (10-20 μm) were then prepared in the coronal plane using standard techniques. The following antibodies were used. Rabbit polyclonal: cleaved caspase-3 (1:200, Cell Signaling), GABA (1:1000, Sigma), phospho-S6 (1:200, Cell Signaling), neuropeptide Y (1:1000, abcam), VIP (1:300, ImmunoStar), and calretinin (1:1000, Millipore). Mouse polyclonal: NeuN (1:1000, Millipore) and GAD67 (1:1000, Millipore). Mouse monoclonal: parvalbumin (1:5000, Sigma) and calretinin (1:1500, Millipore). Rat monoclonal: somatostatin (1:200, Millipore). Primary antibodies were visualized with Alexa Fluor-488 or -568 conjugated secondary antibodies (Molecular Probes). Digital images were acquired with an Olympus BX UCB epifluorescence microscope. For each immunostaining experiment, a minimum of 3 pairs (range, 3-8) of Tsc1 interneuron CKO and littermate control mice were used.

Cell Counting and Size Analyses

Anatomically matched coronal brain sections were selected for analysis using a standard atlas of the mouse brain as a guide (Paxinos and Franklin 2004). Cell counting and size analyses were performed on mice between P20 and P23. In the neocortex, cells immunoreactive for each antibody were counted within 300-μm strips of cortex located 2-3 mm lateral to the midline extending from the pial surface to the gray-white matter junction. Two sections from frontal or parietal cortex were selected for counting of GABA-positive cells. Due to lower total numbers, 3 cortical sections were selected for counting of each interneuron subtype. The counting results of multiple cortical sections from an individual animal were summed for statistical comparisons. Within the hippocampus, immunoreactive cells were counted from defined anatomical zones. Cell size analysis was performed using ImageJ (Version 1.44j, NIH) to calculate area in square microns after manual outlining of cell margins. At least 20 neocortical cells and 10 cells per hippocampal and septal region were measured for each animal. All data collection were performed while blinded to the genotype. An unpaired t-test was used for statistical comparisons.

Video Electroencephalographic Monitoring

Synchronized video-electroencephalography (EEG) monitoring was performed on 2 groups of mice at ages P18 through P40 (n = 6) and P90 through P185 (n = 4). The cranium was exposed and a head mount (V8201, Pinnacle Technologies) was secured using screw subdural electrodes. Each Tsc1 interneuron CKO animal was recorded simultaneously with a littermate control in adjacent recording chambers. Mice were maintained under 12-h dark-light cycles, with free access to food and water. An average of two 24-h epochs of video-EEG were recorded for each mouse over 1-3 weeks with off-line analysis.

Flurothyl Seizure Induction

P18-20 mice were placed into an airight, 2-L acrylic chamber. A 10% flurothyl solution (bis-2,2,2-trifluoroethyl ether, Sigma-Aldrich) dissolved in 95% ethanol was dripped at a rate of 100 μL/min using a precision syringe pump. Latencies to the first myoclonic jerk and subsequent generalized clonic-forebrain seizure were recorded. All flurothyl trials were video recorded for later review. As myoclonic jerks can be subtle, a subset of flurothyl induction experiments was performed with implanted electrodes with synchronized video-EEG to corroborate our visual observations. The experimenter remained blind to animal genotypes throughout all phases of data acquisition. As Tsc1F/F Tg(Dlx5/6-Cre-IRE-EGFP) and Cre-negative animals showed no difference in seizure latencies, the 2 groups were combined as a single control cohort. An unpaired t-test was used for statistical comparisons.

Results

Generation of Tsc1 Interneuron CKO Mice and Verification of Tsc1 Recombination

Hemizygous Dlx5/6-Cre-IRE-EGFP transgenic mice were successively intercrossed with mice homozygous for the Tsc1 floxed allele (Tsc1F/F) to generate Tsc1F/F Tg(Dlx5/6-Cre-IRE-EGFP) mice. Within the hippocampus, immunoreactive cells were counted from defined anatomical zones. Cell size analysis was performed using ImageJ (Version 1.44j, NIH) to calculate area in square microns after manual outlining of cell margins. At least 20 neocortical cells and 10 cells per hippocampal and septal region were measured for each animal. All data collection were performed while blinded to the genotype. An unpaired t-test was used for statistical comparisons.
EGFP) conditional knockout (TscI interneuron CKO) mice. To visualize sites of transgene expression, we utilized EGFP as this fluorescent marker is expressed along with Cre recombinase through an IRES element. At embryonic day (E) 13.5, there was intense EGFP expression in the MGE and LGE (Fig. 1C) consistent with previous reports of this transgene (Zerucha et al. 2000; Stenman et al. 2003). EGFP expression was also observed in cells of the CGE (Fig. 1D) and preoptic area (not shown), additional sources of GABAergic interneurons (Nery et al. 2002; Yozu et al. 2005; Gelman et al. 2009). As previously reported using a similar Dlx5/6 enhancer element (Zerucha et al. 2000; Yozu et al. 2005), expression decreased postnatally with a marked decrease of EGFP in the cortex and hippocampus (data not shown). To confirm selective expression of the transgene in GABAergic interneurons, we used immunofluorescence for the inhibitory neurotransmitter GABA in P0-P2 mice and found greater than 90% of EGFP-positive cells also expressing GABA (data not shown). As currently available antibodies directed against hamartin are not suitable for immunofluorescence, we used flow cytometry to sort dissociated E15.5 neurons from EGFP-positive embryos and extracted genomic DNA to verify that the TscI gene was recombined by Cre recombinase. As expected, Cre-mediated recombination and inactivation of the $Tsc^{1F}$ allele was restricted to the subset of cells expressing EGFP (Fig. 1B).

**Increased Mortality and Impaired Growth of TscI Interneuron CKO Mice**

$TscI$ interneuron CKO mice were born at expected Mendelian proportions, accounting for approximately 25% of all pups. Postnatally, $TscI$ interneuron CKO mice had increased mortality compared with control littermates with approximately 40% dying by P30 (Fig. 2A) with many $TscI$ interneuron CKO pups dying prior to nursing. Additional mortality was evident in older $TscI$ interneuron CKO animals with approximately 40% of mice surviving to 130 days. In addition, $TscI$ interneuron CKO mice were significantly smaller. This size difference became readily apparent by the second week of postnatal life and persisted.
neurons in the cerebral cortex and hippocampus. Cell size was significantly increased in brain sections from Tsc1 interneuron CKO mice (Fig. 4A,B) consistent with increased mTORC1 signaling. This abnormality of cell size was also seen in analyses of specific interneuron subtypes (data not shown). Despite a normal appearing cerebral cortex, we frequently found large pS6 positive cells in isolation or clusters within neocortical layer I of Tsc1 interneuron CKO mice (Fig. 3D). These ectopic pS6 positive cells coexpressed both NeuN and GAD67 (Supplementary Fig. 2) consistent with an interneuron identity. In all Tsc1 interneuron CKO mice, large pS6 positive cells were also seen in the septum (Fig. 3F). The presence of ectopic cells within both the relatively acellular layer I of the neocortex and the ventral septal region suggest that loss of the Tsc1 gene in ventral progenitor cells may result in aberrant interneuron migration.

Reduced Numbers of Cortical and Hippocampal Interneurons

We hypothesized that abnormalities in interneuron development and migration from loss of Tsc1 gene in ventral progenitor cells would result in abnormal numbers of cortical and hippocampal interneurons. Notably, Tsc1 interneuron CKO mice had a significant reduction in GABA-positive neurons within rostral and parietal regions of neocortex (Fig. 5A) as well as the dentate gyrus of the hippocampus (Fig. 5B). A similar trend was observed in the CA1 region of the hippocampus, although this result was not statistically significant. As reduced cortical and hippocampal neurons may result from increased cell death within the neocortex or progenitor regions, we also examined numbers of activated caspase-3 positive cells at several developmental time points (E13.5, E15.5, E17.5, P0, and P20) and saw no significant difference (data not shown). This suggests that the decreased numbers of interneurons were not due to increased rates of apoptosis.

Given the tremendous molecular and functional diversity of interneurons [Markram et al. 2004; Wonders and Anderson 2006; Lodato et al. 2011], we considered the possibility that loss of the Tsc1 gene may have a differential impact within specific GABAergic neuronal subtypes. We counted specific interneuron subtypes within the neocortex examining parvalbumin (PV), somatostatin (SST), vasoactive intestinal peptide (VIP), calretinin (CR), and neuropeptide Y (NPY) expressing interneurons. Intriguingly, we found a 40% reduction of NPY-positive interneurons and 33% reduction of CR-positive interneurons in neocortex of Tsc1 interneuron CKO mice compared with littermate controls while no alterations were seen in the PV, SST, or VIP subtypes (Fig. 5C).

Tsc1 Interneuron CKO Mice Have a Decreased Seizure Threshold

Spontaneous seizures were seen in previous mouse models of TSC generated by the conditional inactivation of the Tsc1 or Tsc2 genes in astrocytes, postmitotic neurons, and neural progenitor cells [Uhlmann et al. 2002; Meikle et al. 2007] suggesting that multiple independent mechanisms may lead to seizures in TSC. Given the reduced numbers of GABAergic interneurons in the forebrain of Tsc1 interneuron CKO mice, we hypothesized a concomitant change in the overall balance of brain excitation and inhibition favoring seizures. To address this possibility in Tsc1 interneuron CKO mice, we

Throughout adulthood for surviving animals (Fig. 2B). Despite clear abnormalities in growth and survival, Tsc1 interneuron CKO brains appeared grossly normal with an intact cortex, hippocampus, and cerebellum. Examination of neocortical lamination using layer-specific markers for nontargeted glutamatergic neurons revealed no obvious differences between Tsc1 interneuron CKO and littermate controls (data not shown). Brains from CKO mice did have a significantly increased brain-to-body weight ratio (Supplementary Fig. 1); however, the absolute brain weight was not increased.

Increased mTORC1 Signaling and Ectopic Cell Clusters in Tsc1 Interneuron CKO Mice

While the brains from Tsc1 interneuron CKO mice appeared grossly normal, many cells within the neocortex and hippocampus had increased levels of phospho-S6 (pS6) indicating increased mTORC1 signaling (Fig. 3D,E). To confirm that the increased mTORC1 signaling was restricted to GABAergic neurons, we measured GAD67/pS6 coexpression in the cortex and found very high concordance (Fig. 3F). As increased cell size is a hallmark of increased mTORC1 signaling [Meikle et al. 2007; Way et al. 2009], we measured the area of GABAergic

Figure 2. Tsc1 interneuron CKO mice are smaller than control littermates and have decreased survival. (A) Approximately, 40% of CKO animals die by P30 with an initial peak in mortality during the first several postnatal days (n = 680 control; 203 Tsc1 interneuron CKO mice). P < 0.0001 by log-rank test. (B) By P18, surviving CKO animals are significantly smaller compared with control littermates. This difference in size persists into adulthood for surviving animals. ** P < 0.01; *** P < 0.001 by 2-way analysis of variance.
implanted electrodes for synchronized video-EEG monitoring. Spontaneous seizures were not detected in Tsc1 interneuron CKO mice despite prolonged video-EEG monitoring from 10 animals (5 Tsc1 interneuron CKO and control littermate pairs) encompassing ages P18–P185. In addition, there were no clear differences in EEG background patterns or epileptiform discharges seen in Tsc1 interneuron CKO mice (data not shown). This suggests loss of the gene in interneurons alone is not sufficient to cause epilepsy. It is possible that spontaneous seizures occurred in the 50% of Tsc1 interneuron CKO mice that died prematurely; however, we have not observed seizures in younger CKO mice during routine handling. To assess more subtle changes in seizure threshold, we treated Tsc1 interneuron CKO and control mice with flurothyl, a volatile chemoconvulsant, and measured latencies to seizure onset (Samoriski and Applegate 1997; Martin et al. 2010). Flurothyl reliably induced myoclonic jerks followed by clonic-forebrain seizures in all Tsc1 interneuron CKO mice; however, there was a significant decreased latency to the initial myoclonic jerk in CKO mice (Fig. 6). Interestingly, the latency to the subsequent clonic-forebrain seizure with continued flurothyl exposure was increased for Tsc1 interneuron CKO mice (Fig. 6).

Figure 3. pS6 expression in control and Tsc1 interneuron CKO brain sections [A and D: neocortex; B and E: hippocampus; C and F: septum] reveals relative increases in pS6 signal and cell size in Tsc1Dlx5/6 CKO mice. (G) Ectopic clusters of pS6 positive cells are frequently seen the normally acellular layer I of cortex from Tsc1 interneuron CKO mice. Double labeling with GAD67 (G) and phospho-S6 (H) demonstrates the majority of GABAergic cells in sections from Tsc1 interneuron CKO mice have increased levels of pS6. (J) Percentage of GAD67+ cells that coexpress pS6 is greatly increased in Tsc1 interneuron CKO brains compared with control littermates. $P < 0.0001$ by chi-square. Scale bar: 100 μm.
Increased mTORC1 Signaling in the Cortex,Septum, and Hippocampus

GABAergic interneurons with increased mTORC1 signaling were found in the cerebral cortex as well as the CA1 and dentate gyrus of hippocampus. The impact of upregulated mTORC1 in these neurons is not known though is expected to alter neuronal morphology as well as function (Meikle et al. 2007; Way et al. 2009; Zeng et al. 2009; Zhou et al. 2009; Sharma et al. 2010). The presence of pS6 positive cells in the septum and layer 1 of the cerebral cortex is particularly intriguing as positioning of these cells may arise if the Tsc1 gene and possibly regulation of mTORC1 signaling is required for the proper migration of GABAergic interneurons.

Pharmacologic experiments testing the impact of increased mTORC1 signaling in this mouse model will be very important. However, GABAergic interneurons are typically born over an interval of many days during embryonic development and extended treatment with mTORC1 inhibitors such as rapamycin could prove challenging given the known toxicity of this drug during early development. Nonetheless, planned experiments will test the impact of transient mTORC1 inhibition during precise windows of interneuron development. In vitro assays will also be employed to directly examine GABAergic interneuron migration using cultured brain slices.

**Discussion**

**Increased mTORC1 Signaling in the Cortex, Septum, and Hippocampus**

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interneuron CKO mice. As GABAergic (mouse indicate that Cre expression is found within ventral Dlx5/6 TSC2 interneuron CKO). *B transgene is evident in our mice (data not 37 control; 12 or 0.0001 by Dlx5/6-Cre-IRES-EGFP deletion. interneuron CKO mice have significantly decreased latency to gene in the septum of model is provided by the finding of additional pS6 cells within and function. Further support for abnormal migration in this have large consequences in GABAergic interneuron location and function. Further support for abnormal migration in this model is provided by the finding of additional pS6 cells within the septum of Tsc1 interneuron CKO mice and layer 1 of the cerebral cortex. These abnormalities may simply reflect a cell autonomous inability of Tsc1-deficient GABAergic neurons to properly move (Bellion et al. 2005; Kappeler et al. 2006; Baudoin et al. 2008). Alternatively our results may be due to an inability to properly interpret the attractive and repulsive cues that normally guide GABAergic interneurons during development (Metin et al. 2006; Hashimoto et al. 2008; Tiveron and Cremer 2008; Nie et al. 2010; Rudolph et al. 2010). A more definitive answer to all of these possibilities will require direct assays of neuronal migration and proliferation, such experiments using live slices of embryonic brains from Tsc1 interneuron CKO mice are underway in our laboratory. **Altered Seizure Threshold** The presence of a decreased threshold to flurothyl induced myoclonic seizures in this model clearly indicates neural network dysfunction resulting from loss of the Tsc1 gene in ventral interneuron progenitors though the underlying mechanisms are not clear. However, it would appear that this network dysfunction is not sufficient to cause spontaneous seizures as is typically seen in patients with TSC. While the development of spontaneous seizures could potentially be an age-dependent process and also dependent on genetic background (Kasugai et al. 2007; Oakley et al. 2009), we speculate that concomitant excitatory neuronal dysfunction is required for spontaneous seizure generation in TSC. The overall balance of excitation and inhibition may then dictate epileptogenesis within regions of the human brain and help explain why only a subset of tubers are generally thought to be responsible for seizure initiation in patients with TSC (Avellino et al. 1997; Kagawa et al. 2005; Chandra et al. 2006; Kassiri et al. 2011). A relative imbalance within specific brain regions may also explain why only about half of all patients with TSC have autism spectrum disorders. The observation that Tsc1 interneuron CKO mice required more time to progress to the generalized clonic-forebrain phase of flurothyl seizure is quite intriguing and suggests the pathway for seizure propagation may be more complex than simple loss of cortical inhibition. Midbrain expression of the Dlx5/6-IRES-EGFP transgene is evident in our mice (data not shown). This subcortical region has been implicated in the modulation and propagation of generalized seizures (Gale 1992; Gale et al. 1993; Sawamura et al. 2001). Specifically, there is evidence that the substantia nigra pars reticulata, a region rich in GABAergic neurons projecting to the superior colliculus and pedunculopontine tegmental nucleus plays a pivotal role in seizure propagation and is involved during the propagation of flurothyl induced seizures (Iadarola and Gale 1982; Gale 1985; Toussi et al. 1987; Xu et al. 1991; Veliskova et al. 2002, 2005). Our results showing decreased survival, increased mTORC1 signaling, increased GABAergic interneuron size, ectopic GABAergic interneurons, and increased seizure susceptibility all contrast a previous report that found Tsc1 was not required for interneuron development (Wang et al. 2007). We speculate that the discrepancy with our findings may be due to differences in the genetic backgrounds of these mouse models or other currently unknown variances. Wang et al. (2007) used a transgenic Dlx5/6-Cre mouse originally generated in theEkker lab (Monory et al. 2006; Kohwi et al. 2007; Yee et al. 2009) while the Dlx5/6-Cre-IRES-EGFP mouse we used was created independently in the Campbell laboratory (Stenman et al. 2003). While published data from the Ekker lab Dlx5/6-Cre mouse indicate that Cre expression is found within ventral progenitor cells, it is conceivable that genomic insertional effects between the 2 different transgenes could cause marked differences in temporal or regional expression patterns altering the timing and pattern of Tsc1 deletion. In conclusion, our findings support GABAergic interneuron dysfunction in the pathogenesis of TSC and suggest novel mechanisms where GABAergic cells mutant for TSC1 or TSC2 can substantially alter the overall function of the cerebral cortex. This may elaborate on emerging concepts of “tubefree” pathology in TSC if relatively few GABAergic interneurons can alter the overall excitation/inhibition balance of the human brain.
Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

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Notes

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