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Rapamycin Prevents Epilepsy in a Mouse Model of Tuberous Sclerosis Complex

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Abstract

Objective—Tuberous Sclerosis Complex (TSC) represents one of the most common genetic causes of epilepsy. *TSC* gene inactivation leads to hyperactivation of the mammalian target of rapamycin (mTOR) signaling pathway, raising the intriguing possibility that mTOR inhibitors might be effective in preventing or treating epilepsy in patients with TSC. Mice with conditional inactivation of the *Tsc1* gene primarily in glia (*Tsc1*^{GFAP}CKO mice) develop glial proliferation, progressive epilepsy, and premature death. Here, we tested whether rapamycin could prevent or reverse epilepsy as well as other cellular and molecular brain abnormalities in *Tsc1*^{GFAP}CKO mice.

Methods—*Tsc1*^{GFAP}CKO mice and littermate controls were treated with rapamycin or vehicle starting at postnatal day 14 (early treatment) or six weeks of age (late treatment), corresponding to times before and after onset of neurological abnormalities in *Tsc1*^{GFAP}CKO mice. Mice were monitored for seizures by serial video-EEG and for long-term survival. Brains were examined histologically for astrogliosis and neuronal organization. Expression of phospho-S6 and other molecular markers correlating with epileptogenesis was measured by Western blotting.

Results—Early treatment with rapamycin prevented the development of epilepsy and premature death observed in vehicle-treated *Tsc1*^{GFAP}CKO mice. Late treatment with rapamycin suppressed seizures and prolonged survival in *Tsc1*^{GFAP}CKO mice that had already developed epilepsy. Correspondingly, rapamycin inhibited the abnormal activation of the mTOR pathway, astrogliosis, neuronal disorganization, and increased brain size in *Tsc1*^{GFAP}CKO mice.

Interpretation—Rapamycin has strong efficacy for preventing seizures and prolonging survival in *Tsc1*^{GFAP}CKO mice.

Introduction

Tuberous sclerosis complex (TSC) is caused by mutation of the *TSC1* or *TSC2* gene and characterized by tumor or hamartoma formation in multiple organs.^{1–3} Neurological involvement accounts for the most disabling clinical problems, including seizures, autism, and mental retardation. Epilepsy in TSC is particularly severe, affecting between 60–90% of patients and is often refractory to all available medical and surgical therapies. Thus, more effective treatments with true “anti-epileptogenic” actions are clearly needed for TSC patients with epilepsy.

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Recent excitement has been generated by the finding that the *TSC* genes regulate the mammalian target of rapamycin (mTOR) signaling pathway, which controls cell growth and differentiation.^{7–10} Mutation of either *TSC* gene results in hyperactivation of the mTOR pathway, leading to abnormal cell growth, proliferation and tumorigenesis. Animal and preliminary clinical studies already suggest that mTOR inhibitors, such as rapamycin, decrease growth of TSC-related tumors, such as renal angiomyolipomas and subependymal giant cell astrocytomas.^{11–14} However, the utility of mTOR inhibitors in treating epilepsy in TSC has not been investigated in either clinical trials or animal models. We recently described a mouse model of TSC with conditional inactivation of the *Tsc1* gene in GFAP-positive cells (*Tsc1*^{GFAP}CKO mice), which develops progressive epilepsy, encephalopathy, and premature death,^{15,16} as well as cellular and molecular brain abnormalities likely contributing to epileptogenesis.^{17–19} In the present study, we test whether treatment with rapamycin can prevent or reverse epilepsy and the associated brain abnormalities in *Tsc1*^{GFAP}CKO mice.

Materials and Methods

Animals and Drug Protocols

All experiments were conducted according to an approved Washington University animal protocol. *Tsc1*^{flox/flox}-GFAP-Cre knockout (*Tsc1*^{GFAP}CKO) mice with conditional inactivation of the *Tsc1* gene in glia were generated as described previously.¹⁵ *Tsc1*^{flox/+}-GFAP-Cre and *Tsc1*^{flox/flox} littermates have previously been found to have no abnormal phenotype and were used as controls in these experiments. Rapamycin (LC Labs, Woburn, MA) was initially dissolved in 100% ethanol, stored at –20°C, and diluted in a vehicle solution containing 5% Tween 80, 5% PEG 400 (Sigma, St. Louis, MO) and 4% ethanol immediately before injection. In “early treatment” studies, rapamycin or vehicle treatment was initiated at postnatal day 14, which precedes the onset of seizures and other neurological abnormalities in *Tsc1*^{GFAP}CKO mice. In “late treatment” studies, drug treatment was initiated at six weeks of age, which is typically after onset of seizures in these mice.¹⁶ *Tsc1*^{GFAP}CKO mice and control mice were administered i.p. rapamycin or vehicle 5 days a week, which continued until death or the pre-defined endpoint of the experiment. In one set of studies, mice were monitored daily for survival and weekly for body weight without further interventions. Other studies involved video-EEG monitoring, Western blotting, or histological analysis at defined time points, as described below.

Video-EEG Monitoring

Vehicle- and rapamycin-treated *Tsc1*^{GFAP}CKO mice underwent weekly video-EEG monitoring starting at 4 weeks of age in early treatment studies and 6 weeks of age in late treatment studies. Bilateral epidural electrode placement and continuous digital EEG and video recordings were performed with established methods, as described previously.¹⁶ Forty-eight hour epochs of continuous video-EEG data were obtained once a week from each mouse, until the animal died or the electrodes malfunctioned, and were analyzed for interictal abnormalities and seizures, as reported previously.¹⁶ Briefly, interictal spikes were defined as fast (<200 ms) epileptiform waveforms at least twice the amplitude of the background activity. Electrographic seizures were identified by their characteristic pattern of discrete periods of rhythmic spike discharges that evolved in frequency and amplitude lasting at least 10 seconds, typically ending with repetitive burst discharges and voltage suppression. Seizure frequency (# seizures/48 hr period, based on analysis of the entire EEG record) and interictal spike frequency (# spikes/min) were calculated from each 48 hr epoch. Average interictal grade (scale of 1–4) based on multiple samples from each epoch was scored based on a previously-described scale^{16,20,21}: 1 - normal background activity (+/- 6–8 Hz sinusoidal theta rhythm), no epileptiform spikes; 2 - mostly normal background

activity, few epileptiform spikes; 3 - mostly abnormal background activity, many spikes; 4 - burst-suppression pattern.

Western Blot Analysis

After 5 weeks (1–3 weeks in preliminary studies) of rapamycin or vehicle treatment, Western blotting was performed to assay expression of phospho-S6 and Glt1 by standard methods. Briefly, neocortex and hippocampus were dissected, sonicated, and centrifuged. Equal amounts of total protein extract were separated by SDS-PAGE and transferred to nitrocellulose membrane. After incubating with primary antibody to phospho-S6 (1:1000, Cell Signaling, Beverly, MA), S6 (1:1000, Cell Signaling), actin (1:5000, Sigma) and Glt-1 (1:1000, Alpha Diagnostics, San Antonio, TX), the membranes were reacted with peroxidase-conjugated secondary antibody. Signals were detected by using ECL reagent (Pierce, Rockford IL) and quantitatively analyzed with ImageJ software.

Histology/Immunohistochemistry

After 3 weeks (late treatment) or 5 weeks (early treatment) of treatment, histological analysis was performed to assess glial proliferation and neuronal organization by standard methods. Briefly, brains were perfusion-fixed with 4% paraformaldehyde and cut into 50 μm sections with a vibratome. Some sections were stained with 0.5% cresyl violet. Other sections were labeled with GFAP antibody (anti-rabbit, 1:500, Sigma) and then rhodamine-conjugated anti-rabbit IgG (1:500, Sigma). Images were acquired with a Zeiss LSM PASCAL confocal microscope. GFAP-immunoreactive cells in neocortex and hippocampus were counted by an investigator blinded to the treatment of the mice. In images from coronal sections at ~ 2 mm posterior to bregma and ~ 1 mm from midline, regions of interest were marked in neocortex by a 200 μm wide box spanning from the neocortical surface to the bottom of layer VI and in hippocampus by a 200x200 μm^2 box within the striatum radiatum of CA1 and dentate gyrus. GFAP-immunoreactive cells were quantified in the regions of interest from 2 sections per mouse from a total of 4–5 mice per group. Due to the robust differences in GFAP-positive cells between treatment groups, a more formal stereological approach was not employed. In addition, some sections were counter-stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma), a non-specific nuclear stain for all cells, and quantified for total cell number using similar methods as described above.

Statistics

SigmaStat (Systat Software, San Jose, CA) was used for statistical analysis. For quantitative comparisons (expressed as mean $\pm$ SEM), student's t-test, one-way analysis of variance (ANOVA) with Tukey multiple comparisons post-tests, non-parametric (Kruskal-Wallis) ANOVA, or 2-way repeated measured ANOVA was used, depending on the number of group comparisons and conformity to a normal distribution. Kaplan Meier LogRank test was used for survival analysis. Statistical significance was defined as $P < 0.05$.

Results

Rapamycin blocks activation of the mTOR pathway in a dose-dependent manner

Pilot studies in control mice first documented that rapamycin treatment i.p. for 1 week caused a dose-dependent decrease in S6 phosphorylation, a marker of mTOR pathway activation, with 3 mg/kg almost completely inhibiting S6 phosphorylation (Fig. 1A,B). We next tested the effect of 3 mg/kg rapamycin on mTOR activation in *Tsc1*^{GFAP}CKO mice, with “early treatment” starting at P14 before the onset of seizures in the mice. Vehicle-treated *Tsc1*^{GFAP}CKO mice have elevated phospho-S6 expression compared to controls, and 3 mg/kg rapamycin for 5 weeks blocked S6 phosphorylation in neocortex and hippocampus

of control and *Tsc1*^{GFAP}CKO mice (Fig. 1C, D). Similar results with phospho-S6 expression were also obtained in *Tsc1*^{GFAP}CKO mice after 1 and 3 weeks of rapamycin treatment (86% and 89% decrease in the phospho-S6/total S6 ratio in neocortex after 1 and 3 week treatment, respectively).

Rapamycin prevents progressive astrogliosis, increased brain size and abnormal neuronal organization in *Tsc1*^{GFAP}CKO mice

Tsc1^{GFAP}CKO mice develop progressive increases in astrocyte number (astrogliosis) beginning at about 3 weeks of age diffusely throughout the brain, but most notably in neocortex and hippocampus.¹⁵ To determine whether early rapamycin treatment could prevent astrogliosis, we treated *Tsc1*^{GFAP}CKO and control mice with rapamycin or vehicle for 5 weeks starting at P14. In control mice, rapamycin had no effect on the number of GFAP-immunoreactive cells (Fig. 2A–D). In contrast, while vehicle-treated *Tsc1*^{GFAP}CKO mice showed the expected increase in GFAP-immunoreactive cells compared to control mice, rapamycin treatment prevented astrogliosis in *Tsc1*^{GFAP}CKO mice in both neocortex and hippocampus (Fig. 2A–D). Paralleling the differences in GFAP-positive cells, similar differences were also seen in the number of DAPI-positive cells between different groups (Fig. 2D), indicating that rapamycin inhibited the total cell (astrocyte) number, not simply the level of GFAP-expression by existing astrocytes.

The diffuse increase in astrocyte number in *Tsc1*^{GFAP}CKO mice is also reflected in a generalized increase in brain size. At 7 weeks of age, vehicle-treated *Tsc1*^{GFAP}CKO mice had grossly larger brains compared to control mice (Fig. 2E, top). Rapamycin treatment prevented this enlargement of *Tsc1*^{GFAP}CKO brains, while rapamycin had no obvious effect on brain size of control mice. Quantitative analysis of both brain weight and ratio of brain/body weight (Fig. 2E, bottom) confirmed that rapamycin prevented the abnormal brain enlargement observed in vehicle-treated *Tsc1*^{GFAP}CKO mice.

In addition to the striking abnormalities in astrocyte proliferation, *Tsc1*^{GFAP}CKO mice have previously been shown to have disorganization and dispersion of the pyramidal cell layer of hippocampus.¹⁵ Consistent with previous studies, vehicle-treated *Tsc1*^{GFAP}CKO mice displayed widely-dispersed pyramidal cell layers in all regions of hippocampus (CA1-CA4) compared to control mice (Fig. 2F). Rapamycin partially preserved a more compact organization to the hippocampal pyramidal neurons (Fig. 2F).

Rapamycin reverses the reduction of Glt-1 expression in *Tsc1*^{GFAP}CKO mice

Tsc1^{GFAP}CKO mice have previously been shown to have reduced expression of astrocyte-specific glutamate transporters, Glt-1 and GLAST, which represents a potential glial mechanism of epileptogenesis.¹⁷ Western blotting of forebrain lysates showed that rapamycin increased the expression of Glt-1 in *Tsc1*^{GFAP}CKO mice back toward control levels (Fig. 3). Interestingly, rapamycin also increased Glt-1 expression in control mice.

Early rapamycin treatment prevents the development of epilepsy and premature death in pre-symptomatic *Tsc1*^{GFAP}CKO mice

Next, we asked if early rapamycin treatment, beginning at P14, could prevent the development of seizures, which typically start between 1–2 months of age in *Tsc1*^{GFAP}CKO mice. Vehicle-treated *Tsc1*^{GFAP}CKO mice developed progressive seizures as described previously.^{15,16} In contrast, no seizures were detected in rapamycin-treated mice by weekly video EEG monitoring starting at 4 weeks of age until 17 weeks of age, when the study was terminated (Fig. 4A).

In addition to seizures, untreated *Tsc1*^{GFAP}CKO mice exhibit a progressive encephalopathy and abnormalities in interictal EEG background, also starting between 1–2 months of age.^{15,16} Rapamycin treatment prevented the progressive abnormalities in interictal EEG background compared to vehicle-treated *Tsc1*^{GFAP}CKO mice (Fig. 4B–D). Behavioral correlates to this progressive encephalopathic process are decreasing feeding behavior with age, associated with poor weight gain. Rapamycin-treated *Tsc1*^{GFAP}CKO mice gained weight at a similar rate as rapamycin-treated control mice, in contrast to vehicle-treated *Tsc1*^{GFAP}CKO mice, which gradually lost weight after 7 weeks of age (Fig. 4E). Of note, rapamycin-treated control mice had slightly slower weight gain compared to vehicle-treated control mice, indicating that rapamycin treatment has systemic side effects.

The effect of early rapamycin treatment on long-term survival was also tested. No mortality was observed in littermate controls treated with rapamycin or vehicle. Vehicle-treated *Tsc1*^{GFAP}CKO mice exhibited premature death as described previously,¹⁵ with all mice dying by 4 months. In contrast, 10 of 11 rapamycin-treated mice survived to at least 6 months (Fig. 4F). To determine whether this survival effect might persist after discontinuing rapamycin, at 6 months half of the rapamycin-treated *Tsc1*^{GFAP}CKO mice were switched to vehicle treatment (n = 5), whereas the other half continued to receive rapamycin (n = 5). While all the rapamycin-treated mice continued to survive, all mice switched to vehicle at 6 months died after an average of 62.6 ± 7.8 days. All of these mice were also witnessed to have obvious clinical seizures only after being switched to vehicle. In a separate set of mice, *Tsc1*^{GFAP}CKO mice converted from rapamycin treatment at 6 months to vehicle for 5 weeks had significantly larger brains (brain weight = 0.63 ± 0.01 g in mice converted to vehicle versus 0.43 ± 0.01 g in mice on continued rapamycin treatment, $p < 0.001$ by t-test), increased numbers of GFAP-positive cells, increased phospho-S6 expression, and pyramidal neuron dispersion in the hippocampus, compared to age-matched *Tsc1*^{GFAP}CKO mice with continual rapamycin treatment (Supplemental Figure 1). Thus, the beneficial effects of rapamycin were dependent on continued, long-term treatment and reversed after stopping the drug.

Late rapamycin treatment decreases seizures and improves survival in already-symptomatic *Tsc1*^{GFAP}CKO mice

To determine whether late treatment with rapamycin could reverse the phenotypic abnormalities in already symptomatic *Tsc1*^{GFAP}CKO mice, we treated *Tsc1*^{GFAP}CKO mice with rapamycin or vehicle starting at 6 weeks of age in mice that were already experiencing seizures. No differences in seizure frequency, duration or interictal EEG grade were present between the two groups at 6 weeks immediately before initiation of treatment. However, seizure frequency in the rapamycin group decreased dramatically after the first week of treatment, compared to vehicle-treated mice which had progressively more frequent seizures (Fig. 5A). After 3 weeks of treatment, half (4 of 8) of the rapamycin-treated *Tsc1*^{GFAP}CKO mice became seizure-free for the duration of the study and the remaining mice only had 0.4 ± 0.2 seizures/day, indicating that late rapamycin treatment could suppress or reverse the seizure phenotype in already symptomatic mice. Similarly, late rapamycin treatment stabilized the progressive interictal EEG abnormalities observed in vehicle-treated *Tsc1*^{GFAP}CKO mice (Fig. 5B,C). Furthermore, late-rapamycin treatment dramatically improved survival of *Tsc1*^{GFAP}CKO mice, with no deaths observed during the duration of rapamycin treatment, compared with vehicle-treated *Tsc1*^{GFAP}CKO mice, which all died by 4 months of age (Fig. 5D). Corresponding histological analysis showed that *Tsc1*^{GFAP}CKO mice receiving late rapamycin treatment for three weeks had a lower number of GFAP-positive cells (Fig. 5E) and DAPI-positive cells (data not shown), decreased brain size/weight (Fig. 5F), and more compact hippocampal pyramidal cell organization (Fig. 5G), compared to vehicle-treated *Tsc1*^{GFAP}CKO mice. However, the brain size and number of

GFAP-positive cells from *Tsc1*^{GFAP}CKO mice with late rapamycin treatment was still significantly greater than age-matched vehicle-treated control mice, suggesting that late rapamycin treatment did not completely reverse the histological abnormalities of *Tsc1*^{GFAP}CKO mice.

Discussion

Novel therapies for epilepsy in TSC are clearly needed, given the severity and intractability of seizures in many TSC patients. Currently available medical treatments for epilepsy suppress seizures by directly reducing neuronal excitability, but do not necessarily target the underlying process of epileptogenesis. In TSC, the immediate link between *TSC* gene inactivation and de-regulated mTOR signaling identifies a rational therapeutic strategy that may effectively reverse underlying pathophysiological mechanisms causing the clinical manifestations of TSC. Inhibition of mTOR pathway signaling is already being explored as a possible therapeutic approach for reducing tumor growth in TSC patients.^{11–14} As less is known about mechanisms of epileptogenesis in TSC, the potential role of mTOR inhibitors in treating epilepsy has not been thoroughly investigated. In the present study, we provide novel evidence using a genetically-engineered *Tsc* mouse model that rapamycin may be a very effective treatment for epilepsy in TSC. Early rapamycin treatment before the onset of neurological symptoms prevented the development of epilepsy and associated cellular and molecular brain abnormalities in *Tsc1*^{GFAP}CKO mice, as well as dramatically prolonging survival. Late rapamycin treatment also decreased seizures and prolonged survival in already symptomatic mice, although it only partially improved the underlying histological abnormalities.

The mechanisms of action of rapamycin in treating neurological abnormalities in TSC are incompletely understood, but likely involve effects on cell growth and proliferation. The *TSC1* and *TSC2* gene products, hamartin and tuberlin, constitutively inhibit the protein kinase, mTOR, which regulates cell growth and proliferation via downstream signaling pathways involved in protein translation.^{7–10} Inactivation of either *TSC* gene results in hyperactivation of mTOR and associated downstream effectors (e.g. S6, S6K), accounting for abnormalities in cell size and proliferation observed in multiple organs in TSC.^{22–26} Rapamycin directly inhibits mTOR and thus opposes the hyperactivation of the mTOR pathway and resulting abnormalities in cell growth and proliferation observed in TSC. Somewhat analogous to our present results with *Tsc1*^{GFAP}CKO mice, a rapamycin analog counters abnormal cell growth and brain enlargement, as well as seizures and premature death, in *Pten*-deficient mice.²⁷ Rapamycin may also have similar effects in *Tsc1*^{Synapsin}CKO mice related to neuronal growth and differentiation, but these results have not yet been published in complete form and effects on seizures may be more difficult to monitor due to very early mortality in these mice.²⁸

While the prevailing concept of brain pathogenesis in TSC primarily invokes an early developmental defect in glioneuronal differentiation and proliferation,²⁹ some pathophysiological processes in TSC may remain or become active at later stages. This may be particularly true with astrocyte proliferation in focal TSC brain lesions, such as subependymal giant cell astrocytomas (SEGAs) and, to a lesser degree, tubers. In support of this view, rapamycin has been shown to cause regression of SEGAs in children and adults with TSC, a dramatic effect that could be due to cell loss (e.g. necrosis/apoptosis) or reduced cell size.¹⁴ In the *Tsc1*^{GFAP}CKO mice, while late treatment did not necessarily reverse the increased brain size and astrogliosis back to control levels, early and late treatment with rapamycin was clearly effective in preventing further progression of astrocyte proliferation and brain growth starting from the time rapamycin was initiated. Similarly, rapamycin at least partially prevented progressive neuronal disorganization in the hippocampus of

Tsc1^{GFAP}CKO mice. While the specific mechanisms mediating this neuronal effect are unclear, rapamycin could counter pyramidal cell dispersion by inhibiting intercalating astrocyte proliferation, aberrant glial-mediated neuronal migration, or effects of seizures on neurons.

Although the effects of rapamycin in suppressing cell growth and proliferation are clear both in the *Tsc1*^{GFAP}CKO mice and in TSC-related tumors,^{11–14} less is known about the specific cellular and molecular mechanisms underlying the anti-epileptic action of rapamycin observed in this study. Mechanisms of epileptogenesis in TSC are poorly understood, although numerous abnormalities have been described in human tissue and animal models of TSC that may influence neuronal excitability on the molecular, cellular, and network level. While the most attention is focused on neuronal defects that may cause seizures, studies in *Tsc1*^{GFAP}CKO mice suggest that glia may also contribute to epileptogenesis.^{15–19} On a circuit level, glial proliferation observed in these mice could promote seizures by disrupting normally-balanced excitatory and inhibitory neuronal circuits. In addition, on a molecular level, defects in astrocyte glutamate transport may lead to neuronal excitability due to inadequate buffering of extracellular excitatory glutamate. In the present study, rapamycin prevented or reversed cellular and molecular abnormalities in glial proliferation and astrocyte glutamate transporters in parallel with the inhibition of seizures, suggesting that these changes might account for the beneficial effects of rapamycin on seizures. The rapamycin-induced increase in Glt-1 protein expression in both control and *Tsc1*^{GFAP}CKO mice supports previous work linking the Akt/mTOR pathway to selective transcriptional regulation of Glt-1.³⁰ Alternatively, rapamycin could have anti-epileptic actions that are independent on mTOR inhibition, although to our knowledge rapamycin has not been reported to have anti-epileptic effects in non-mTOR-related epilepsy models. Finally, rapamycin could have other, more direct effects on neuronal physiology, independent of glial-mediated mechanisms.^{31,32} Additional studies are required to more definitively identify the most critical mechanisms underlying epileptogenesis in *Tsc1*^{GFAP}CKO mice and the anti-epileptogenic action of rapamycin.

In addition to the impressive effects of rapamycin in inhibiting seizures in the *Tsc1*^{GFAP}CKO mice, rapamycin had equally dramatic effects on survival of these mice. While all untreated *Tsc1*^{GFAP}CKO mice die by 3–4 months of age, all but one mouse from both the early and late rapamycin treatment groups survived for as long as they continued to receive rapamycin. The mechanisms of death in untreated *Tsc1*^{GFAP}CKO mice likely involve multiple factors, including direct seizure-induced death in some cases (documented during video-EEG monitoring) and a more systemic “wasting” syndrome as reflected in decreased activity and feeding behavior, an encephalopathic EEG, and progressive weight loss. Rapamycin likely improves survival in the *Tsc1*^{GFAP}CKO mice by a combination of direct effects of inhibiting seizures and indirect effects on the general behavior/health of the mice.

While one must be careful in extrapolating animal model data to human disease, the documented anti-epileptogenic efficacy of rapamycin in *Tsc1*^{GFAP}CKO mice with both “early” and “late” treatment paradigms has obvious clinical applications. As some patients are diagnosed with TSC prior to the onset of neurological symptoms due to non-neurological findings or a positive family history, early treatment with rapamycin may be able to prevent the development of epilepsy, especially in patients at high risk (e.g. with an abnormal brain MRI). As other patients are only diagnosed with TSC after the onset of seizures, later rapamycin treatment may be able to improve the symptoms and prognosis of already symptomatic patients. Of important note, however, our data indicate that long-term continued treatment with rapamycin is required in order to maintain a therapeutic effect, as stopping rapamycin treatment resulted in a delayed emergence of the neurological

phenotype in *Tsc1*^{GFAP}CKO mice. Furthermore, the present study did not systematically examine the safety of rapamycin in detail. While both rapamycin-treated *Tsc1*^{GFAP}CKO and control mice did well in terms of survival and gross behavior, rapamycin decreased weight gain slightly in control mice, indicating that rapamycin is not completely without side effects. Rapamycin might also impair synaptic plasticity,^{31,32} which may have detrimental effects on learning and other critical developmental processes of the brain. Thus, additional animal and clinical studies are required to determine the relative risk/benefit ratio of rapamycin treatment for neurological symptoms of TSC. Overall, however, the present study provides exciting pre-clinical data that support the initiation of clinical trials using rapamycin analogs for treating epilepsy in TSC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Rapamycin treatment antagonizes activation of the mTOR pathway in a dose-dependent fashion. (A) Western blotting shows total S6 and phospho-S6 (P-S6) expression in neocortex and hippocampus of control mice administered different daily doses of rapamycin for one week. (B) Quantitative summary demonstrates that rapamycin blocked the activation (phosphorylation) of S6 in a dose-dependent fashion. The ratio of P-S6/total S6 was normalized to the vehicle-treated control group. (C) Western blotting shows total S6 and P-S6 expression in *Tsc1*^{GFAP}CKO mice administered 3 mg/kg rapamycin or vehicle for five weeks starting at P14. (D) Quantitative summary demonstrates that vehicle-treated *Tsc1*^{GFAP}CKO mice have significantly elevated P-S6 levels compared to controls and 3 mg/kg rapamycin inhibits the activation of P-S6 in both *Tsc1*^{GFAP}CKO and control mice. The ratio of P-S6/total S6 was normalized to the vehicle-treated control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by ANOVA ($n = 6$ mice per group).

Figure 2.

Rapamycin treatment prevents histological abnormalities of astrogliosis, increased brain size, and neuronal disorganization in *Tsc1*^{GFAP}CKO mice. (A–D) Consistent with previous studies,¹⁵ vehicle-treated *Tsc1*^{GFAP}CKO mice displayed striking increases in GFAP-positive cells in neocortex (A,C) and CA1 and dentate gyrus (DG) of hippocampus (B,D) compared to control mice. Rapamycin treatment for 5 weeks starting at P14 prevented this increase in GFAP-positive cells in *Tsc1*^{GFAP}CKO mice. Similarly, rapamycin prevented an increase in DAPI-positive cells, indicating that the differences in GFAP-positive cells reflect actual differences in overall cell (astrocyte) number, not merely changes in GFAP expression by existing astrocytes. * $p < 0.001$ by ANOVA, between vehicle-treated *Tsc1*^{GFAP}CKO mice and all other groups ($n = 5$ mice per group). (E) Correlated with glial proliferation, vehicle-treated *Tsc1*^{GFAP}CKO mice developed dramatic, diffuse megencephaly compared to control

mice, which was prevented by rapamycin treatment. Total brain weight and ratio of brain/body weight were significantly increased in vehicle-treated *Tsc1*^{GFAP}CKO mice compared to control mice. Rapamycin treatment prevented this increase in brain weight in *Tsc1*^{GFAP}CKO mice, but had no effect on control mice. * $p < 0.001$ by ANOVA, between vehicle-treated *Tsc1*^{GFAP}CKO mice and all other groups ($n = 10$ mice per group). (F) Consistent with previous studies,¹⁵ cresyl violet staining demonstrated an obvious dispersion of the pyramidal cell layer (marked by arrows) in hippocampus of vehicle-treated *Tsc1*^{GFAP}CKO mice compared to controls, which was partially prevented by rapamycin. Scale bars = 200 μm .

Figure 3.

Rapamycin treatment reverses the reduction of Glt-1 expression in *Tsc1*^{GFAP}CKO mice. (A) Western blotting of cortical homogenates shows Glt-1 expression in vehicle and rapamycin-treated *Tsc1*^{GFAP}CKO and control mice. (B) Consistent with previous studies,¹⁷ quantitative summary demonstrates that vehicle-treated *Tsc1*^{GFAP}CKO mice have significantly lower Glt-1 expression compared to control mice. Rapamycin treatment for 5 weeks starting at P14 increased the expression of Glt-1 in *Tsc1*^{GFAP}CKO mice, as well as control mice. Glt-1 was normalized to actin. * $p < 0.05$, ** $p < 0.01$ by ANOVA ($n = 6$ mice per group).

Figure 4.

Early rapamycin treatment prevents the development of epilepsy and premature death in pre-symptomatic *Tsc1*^{GFAP}CKO mice. (A) Seizures started to develop in vehicle-treated *Tsc1*^{GFAP}CKO mice between 1–2 months of age and became progressively more frequent with age (Note that premature death precluded analysis of seizure frequency beyond 10 weeks in vehicle-treated *Tsc1*^{GFAP}CKO mice). Early rapamycin treatment starting at P14 completely prevented the development of seizures in *Tsc1*^{GFAP}CKO mice (n = 8 mice per group). ** p<0.01, *** p<0.001 by ANOVA. (B) Interictal spike frequency progressively increased with age in vehicle-treated *Tsc1*^{GFAP}CKO mice, but was stabilized by rapamycin.

* $p < 0.05$, ** $p < 0.01$ by ANOVA, comparing vehicle and rapamycin groups ($n = 8$ mice per group). (C,D) Interictal EEG background activity, graded by a documented rating scale,^{16,20,21} became progressively more abnormal with age in vehicle-treated $Tsc1^{GFAP}CKO$ mice, but did not worsen in rapamycin-treated $Tsc1^{GFAP}CKO$ mice. * $p < 0.05$, *** $p < 0.001$ by ANOVA, comparing vehicle and rapamycin groups ($n = 8$ mice per group). (E) Reflective of a progressive encephalopathic process involving decreased feeding behavior, vehicle-treated $Tsc1^{GFAP}CKO$ mice start to exhibit weight loss after 7 weeks of age. Rapamycin-treated $Tsc1^{GFAP}CKO$ mice did not demonstrate such weight loss, but gained weight at a rate comparable to rapamycin-treated control mice. Rapamycin-treated control mice exhibited slightly slower weight gain compared to vehicle-treated control mice. * $p < 0.001$ by ANOVA, involving all group comparisons except KO-Rap versus Cont-Rap ($n = 10$ – 14 mice per group). (F) Survival analysis showed that 50% of vehicle-treated $Tsc1^{GFAP}CKO$ mice died by 9 weeks of age, with all dead by 4 months ($n = 10$). In contrast, almost all rapamycin-treated $Tsc1^{GFAP}CKO$ mice ($n = 10$ of 11) survived during the entire treatment period, starting at P14 and extending until 6 months of age ($p < 0.001$ by Kaplan-Meier LogRank test).

Figure 5.

Late rapamycin treatment decreases seizures and improves survival in already-symptomatic *Tsc1^{GFAP}CKO* mice. (A) Late rapamycin treatment starting at 6 weeks of age, when many mice have already developed seizures, reduced seizure frequency in *Tsc1^{GFAP}CKO* mice. The arrow indicates that rapamycin or vehicle treatment was started immediately following the initial video-EEG session at 6 weeks. ** $p < 0.01$, *** $p < 0.001$ by ANOVA comparing vehicle and rapamycin groups ($n = 8$ mice per group). (B,C) Late treatment with rapamycin prevented the progressive worsening of interictal spike frequency and background EEG grade. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ ($n = 8$ mice per group). (D) Late rapamycin treatment increased survival of *Tsc1^{GFAP}CKO* mice ($p < 0.001$ by Kaplan-Meier LogRank test, $n = 10$ mice per group). (E,F) Late treatment with rapamycin for 3 weeks decreased GFAP-positive cells in neocortex, brain weight, and brain/body weight ratio compared to vehicle-treated *Tsc1^{GFAP}CKO* mice, but not back to the levels of vehicle-treated control mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ ($n = 4$ mice per group). (G) *Tsc1^{GFAP}CKO* mice receiving rapamycin exhibited decreased dispersion of the pyramidal neuron layer (marked by arrows) in hippocampus compared to age-matched vehicle-treated *Tsc1^{GFAP}CKO* mice.