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A Pulse Rapamycin Therapy for Infantile Spasms and Associated Cognitive Decline

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Abstract

Infantile spasms are seizures manifesting within a spectrum of epileptic encephalopathies of infancy that often lead to cognitive impairment. Their current therapies, including adrenocorticotrophic hormone (ACTH), high dose steroids, or vigabatrin, are not always effective and may be associated with serious side effects. Overactivation of the TORC1 complex of the mTOR pathway is implicated in the pathogenesis of certain genetic and acquired disorders that are linked with infantile spasms, like tuberous sclerosis. Here, we tested the therapeutic potential of rapamycin, a TORC1 inhibitor, as a potential treatment for infantile spasms in the multiple-hit rat model of ACTH-refractory symptomatic infantile spasms, which is not linked to tuberous sclerosis. Rapamycin or vehicle were given after spasms appeared. Their effects on spasms, other seizures, performance in Barnes maze, and expression of the phosphorylated S6 ribosomal protein (pS6: a TORC1 target) in the cortex, using immunofluorescence, were compared. Rapamycin suppressed spasms dose-dependently and improved visuospatial learning, although it did not reduce the frequency of other emerging seizures. High-dose pulse rapamycin effected acute and sustained suppression of spasms and improved cognitive outcome, without significant side effects. Therapeutically effective rapamycin doses normalized the pS6 expression, which was increased in perilesional cortical regions of pups with spasms. These findings support that pathological overactivation of TORC1 may be implicated in the pathogenesis of infantile spasms, including those that are not linked to tuberous sclerosis. Furthermore, a high-dose, pulse rapamycin treatment is a promising, well tolerated and disease-modifying new therapy for infantile spasms, including those refractory to ACTH.

Keywords

seizure; mTOR; learning; rat; cerebral cortex; rapamycin; infantile spasms; Barnes maze; ribosomal S6 protein; seizures

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Introduction

Infantile spasms (IS) appear in a spectrum of epileptic encephalopathies of infancy that often lead to cognitive impairment (Hrachovy and Frost 2003; Pellock et al. 2010). IS have distinct pharmacosensitivity, as they do not respond to most classical antiepileptic therapies. The current therapies for IS include adrenocorticotrophic hormone (ACTH), high dose steroids or vigabatrin, but these are not always effective and can be associated with severe adverse effects (Chiron et al. 1991; Baram et al. 1996; Vigeveno and Cilio 1997; Mackay et al. 2004; Kossoff et al. 2009; Willmore et al. 2009). Early cessation of spasms has been linked with better long-term developmental outcomes (Riikonen 2001; Lux et al. 2004; Darke et al. 2010; Riikonen 2010). However, response to therapy and long-term outcomes can be worse in patients with symptomatic IS (SIS) that are due to underlying structural/metabolic etiologies (Riikonen 1982; Lombroso 1983; Riikonen 2001; Hrachovy and Frost 2003; Mackay et al. 2004; Engel 2006; Berg et al. 2010; Chudomelova et al. 2010). The etiologies of IS are multivariate, with 200 etiologies having been recorded (Hrachovy and Frost 2003), yet an interesting link exists with tuberous sclerosis (TSC), as significant percentage of patients with TSC manifest IS (Curatolo et al. 2001; Chu-Shore et al. 2009; Chudomelova et al. 2010). TSC is a genetic disorder attributed to loss of function mutations in TSC1 or TSC2 genes, which cancel their inhibitory control over mTORC1 (*mammalian target of rapamycin (mTOR) complex 1*) and, as a result, the activity of its downstream targets remains aberrantly increased, leading to excessive cellular proliferation and dysplastic cells (Crino 2010). A commonly used index of overactivated TORC1 pathway is the expression of the phosphorylated form of ribosomal S6 protein (pS6) which regulates the translation of RNAs with a 5' terminal oligopyrimidine sequence (Meyuhas and Drazek 2009).

To enable research into the pathophysiology of IS and the identification of new therapies, seven acute or chronic animal models of IS have been recently developed (Stafstrom 2009; Chudomelova et al. 2010). In this study, we have used the multiple-hit model of SIS (Albert Einstein College of Medicine patent #2080216183), which exhibits the chronicity and evolution of the human condition, including spasms and cognitive deficits (Scantlebury et al. 2010). Spasms in the multiple-hit model of SIS are ACTH-refractory, transiently sensitive to vigabatrin and therefore model the more medically refractory types of IS.

We hypothesized that pathways implicated in the pathogenesis of diseases linked with IS, such as TSC, may be targets for effective therapies. We show that rapamycin, an inhibitor of the TORC1 complex, when administered after the onset of spasms suppresses spasms and improves cognitive outcome in the multiple-hit model, dose-dependently. Although the multiple-hit model is not TSC-linked, we observed a perilesional increase in the expression of pS6-ir, indicative of pathologic overactivation of the TORC1 pathway. Furthermore, the best therapeutic effect of rapamycin occurred with doses that normalized the activity of the TORC1/pS6 pathway at perilesional cortical areas, and did not require continuous administration of rapamycin, avoiding therefore possible side effects associated with long-term rapamycin therapy. In contrast, rapamycin had no effect on the frequency of other types of emerging seizures, until weaning age, suggesting that the networks and pathways controlling spasms are distinct from those involved in other seizures.

Materials and methods

Animals

Male postnatal day 3 (PN3) Sprague Dawley rats (Taconic farms, Inc., Hudson, NY, USA) were used. Postnatal day 0 (PN0) was the day of birth. Rats were kept with their dam in our

American Association for the Accreditation of Laboratory Animal Care accredited animal facility, at constant temperature (21-23°C) and humidity (40-60%), in a 12h dark/12 h light cycle with free access to water and food. All procedures and experiments were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

The multiple-hit model and behavioral testing

Induction of the multiple-hit model, video-monitoring or behavioral testing were as described previously (Scantlebury et al. 2010). Stereotactic infusions of doxorubicin (5µg/2.5µl, right intracerebroventricular) and lipopolysaccharide (2µg/1.5µl, right intraparietal) were done at PN3, under isoflurane anesthesia. The following coordinates were used: doxorubicin: 2.68 mm anterior to lambda; 1.1 mm lateral to sagittal suture; 3.3 mm deep; lipopolysaccharide: 2.55 mm anterior to lambda; 1 mm lateral to sagittal suture; 1.7 mm deep. The skin closure was secured with Vetbond (3M, St Paul, MN). At PN5, p-chlorophenylalanine was injected intraperitoneally (i.p.) in the morning (DLP group) (Scantlebury et al. 2010). The addition of p-chlorophenylalanine increased the frequency of spasms but was not necessary for the initiation of the expression of spasms, as these were already evident at PN4).

Weights and a battery of neurodevelopmental reflexes were done each morning. These included: (a) open field activity, i.e. time to escape from a 12.5cm diameter circular field; (b) negative geotaxis, i.e. time to turn 90 degrees and start climbing up, when placed head downwards over a 45 degrees inclined surface; (c) surface righting time, i.e. time to turn and stand on its four limbs when placed on the supine position; (d) age at eye opening. Barnes maze testing of visuospatial learning and memory was done between PN16-19 (Scantlebury et al. 2010). During Barnes maze testing, each rat was placed individually over the Barnes maze (1m diameter table with 12 holes in the periphery at equal distances, of which only one was open and led to a target dark box). Visual cues were placed in the periphery of the maze and a bright light was placed over the maze. Rats underwent three learning sessions daily for four consecutive days (PN16-PN19: 12 learning trials), so that they could identify and enter the target hole. In the afternoon of PN19 (fourth day of testing) retention was tested in three retention sessions. Each learning or retention session terminated when the rat entered the target hole or, if unsuccessful, at 3min. Controls included age-matched handled and vehicle-injected pups.

Rapamycin

Rapamycin (LC Laboratories, Woburn MA, USA) was given in the afternoon, starting after the onset of spasms, diluted in 4% ethanol, 5% Tween-80, and 5% polyethylene glycol 400 (Fisher Scientific, Pittsburgh PA, USA) (Zeng et al. 2008). Daily administration was done because a single rapamycin dose (1-3 mg/kg i.p.) suppressed cortical pS6-ir in controls for at least 22 hours. Different doses and protocols of rapamycin administration were compared, as listed in Figure 1.

Monitoring

Pups were separated from their dam twice daily for 2-hour sessions of video-monitoring (NVDVR system, AVerMedia, Milpitas, CA) starting at PN4. Only the PN4 afternoon session consisted of one hour pre- and 2 hours post-injection periods. Pre-injection frequencies of spasms (PN4-pre) included the PN4 morning and afternoon pre-injection periods. To minimize the false positive events, “*behavioral spasms*” were considered the sudden, high amplitude and synchronous movements of all four limbs and body presenting as flexion or extension or mixed flexion/extension events. Events that were associated with

flexion or extension in an attempt to change position or with sudden but asynchronous limb movements were not scored as these are common in normal pups.

Epidural EEG/nuchal EMG placement (Pinnacle Technology, Lawrence, KS, USA) was done either at PN6 or at PN9, as described previously (Scantlebury et al. 2010). EEG recordings started the day after implantation. “*Electroclinical spasms*” consisted of spasms with EEG electrodecremental responses (background attenuation) with or without bursts of polyspikes and sharp wave discharges or paroxysmal fast rhythmic activities (Scantlebury et al. 2010). “*Electroclinical seizures*” included behavioral arrests, sudden falls with or without clonus, tonic seizures, wild running seizures, or myoclonic seizures causing sudden whole body jumps and/or falls. These were associated with polyspikes with temporal evolution occasionally followed by electrical attenuation or paroxysmal rhythmic activities, patterns which disrupted the background activities. “*Electrographic seizure-like patterns*” were similar to the ones observed during electroclinical seizures but without any definite clinical correlate.

Histology

Pups were euthanized with pentobarbital (100mg/kg i.p.) and transcardially perfused at PN4 (2 hours post-injection), PN6-7 afternoon, or at the conclusion of the study.

Immunofluorescence for pS6-ir was done on coronal 40µm brain sections using rabbit monoclonal anti-pS6 antibody (1:100; Cell Signaling Technology, Danvers, MA).

Densitometry

400× magnified photographs of cortical layers 2 and 5 were obtained. The lesional areas were those with a needle tract, doxorubicin-positive nuclei or FluoroJade B positive degenerating cells [done as in (Galanopoulou 2008)]. Densitometry of perilesional cortical pS6-ir neurons (in areas adjacent to lesional site but with histological cohesion and morphologically intact neurons) was done as described (Galanopoulou 2008). 2-3 different cortical parietal sections per brain were included in the evaluation, at the level of the anterior dorsal hippocampus. Background-subtracted cellular densities were obtained using the ImageJ software (Wayne Rasband, Research Services Branch, NIMH, Bethesda, MD USA) and were referred as percent of pS6-ir of controls assayed in parallel.

Statistics

Multifactorial ANOVA with Tukey HSD post hoc was used. As the sensitivity of Tukey HSD post hoc decreases with increasing number of comparisons, we also utilized Student's t-test to explore potentially significant intergroup differences (JMP7 software, SAS Institute, Cary NC, USA).

Results

Effects on spasms

DLP pups were treated daily with either rapamycin or vehicle, starting at PN4 after the onset of spasms (groups shown in Figure 1). Because daily rapamycin 6 mg/kg resulted in high mortality (0/4 survivors by PN12), we included the DLP-RAP633 group as a high dose pulse treatment (Figure 1).

There was a dose-related reduction in the frequency of behavioral and electroclinical spasms. Acute suppression of spasms was observed only in the DLP-RAP633 group and not in DLP-RAP1 and DLP-RAP3, suggesting a dose-related effect (Figure 1). Reduction of behavioral spasms was seen also during the subsequent days. Normalization of frequencies of spasms over the pre-injection frequencies indicated that both DLP-RAP3 and DLP-

RAP633 groups had more sustained effect in suppressing spasms compared to DLP-RAP1. Video-EEG analysis, starting at PN7, further documented the dose-related efficacy of rapamycin to suppress electroclinical spasms and the superiority of DLP-RAP633 and DLP-RAP3 groups over DLP-RAP1 in this aspect (Figure 2). For this reason, further behavioral studies were conducted only with the DLP-RAP3 and DLP-RAP633 groups.

Effects on other seizure types and interictal EEG

There was no significant effect of rapamycin on other types of electroclinical seizures or on the interictal epileptic abnormalities (Figure 2). The electroclinical seizures observed in this cohort of pups included most frequently myoclonic seizures manifesting as sudden and intense body myoclonus with or without drop attacks (“myoclonic-astatic seizures”) or body jumps or tonic seizures and a minority of electroclinical seizures consisting of pure tonic seizures or behavioral arrest. The dissociation of rapamycin effect on spasms and other epileptic events suggests different pathogenetic and control mechanisms.

Effects on Barnes maze and neurodevelopmental reflexes

Early cessation of spasms by rapamycin partially improved learning and memory deficits of DLP pups in the Barnes maze (PN16-19), without requiring continuous administration (Figure 3 A). However, rapamycin-treated controls had worse performance in the Barnes maze than vehicle-treated controls. Exploratory behavior of DLP pups improved with rapamycin, and was normalized in DLP-RAP633 group (Figure 2 B). Rapamycin only transiently decreased weight gain, without affecting survival (Figure 2 C-D). There was no significant differences in the daily neurodevelopmental reflexes (surface righting time, negative geotaxis, open field activity, age at eye opening) of rapamycin and vehicle-treated DLP pups.

Expression of pS6-ir in cortical neurons

In cortical layers 2 and 5, pS6-ir colocalized with neuronal markers (Supplementary Material and Supplementary Figure 1). Increased neuronal pS6-ir was noted in both cortical layers 2 and 5 of DLP pups during spasms (PN4, PN6-7) (Figure 4) but not at the age when spasms naturally resolved (PN13) (data not shown).

Daily rapamycin suppressed pS6-ir in an age, dose, and region specific manner (Figure 3). At PN4, DLP pups receiving high rapamycin dose (DLP-RAP6) had normal pS6-ir at layers 2 and 5. In contrast, rapamycin-resistant areas (i.e. with increased pS6-ir) were still present in DLP-RAP3 pups (layer 5). At PN6-7, all rapamycin doses normalized cortical pS6-ir. Although pS6-ir overexpression was highest perilesionally, no interhemispheric differences were seen, due to the bilateral spread of intraventricularly-infused doxorubicin. These suggest that efficient suppression of spasms correlates with normalization of TORC1/pS6 activity at both perilesional cortical layers 2 and 5.

Discussion

In the multiple-hit model of SIS, a 3-day pulse rapamycin treatment, started after the onset of spasms, suppresses spasms and improves cognitive outcome, without significant side effects. Suppression of spasms correlates with the ability of rapamycin to normalize the TORC1/pS6 activity in perilesional cortical neurons. As spasms in the DLP model are ACTH-refractory, high-dose rapamycin pulse treatment may be a potentially more potent and safer therapy for IS than the currently available ones. A dissociation between the effects of rapamycin on spasms vs other seizures was also found, suggesting different pathogenetic and control pathways, in agreement with the clinical experience where infantile spasms and other epileptic seizures have distinct pharmacosensitivity (Mackay et al. 2004).

Acute reduction of spasms by rapamycin was dose-related, occurring only at high doses, which were required to normalize the pathological perilesional overexpression of pS6-ir in cortical regions. Furthermore, both the RAP3 and RAP633 treatment protocols of DLP pups effected more sustained suppression of spasms vs the DLP-RAP1 group, supporting a dose-related effect. The perilesional overactivation of TORC1/pS6 pathway and the therapeutic effect of TORC1/pS6 inhibition on spasms suggest that the dysregulated TORC1 pathway may directly or indirectly precipitate spasms. Indeed, human disorders with dysregulated mTOR activity, like TSC or cortical malformations, can manifest IS (Crino 2007; Chu-Shore et al. 2009). Furthermore, acquired causes of SIS, including hypoxic/ischemic or cytotoxic brain injury, cortical malformations or tumors, or seizures *per se* have been shown experimentally to overactivate TORC1 (Crino 2007; Wong 2010). These support our hypothesis that dysregulation of mTOR may be implicated in the pathogenesis of IS of various etiologies, genetic or acquired. The exact pathways through which rapamycin-mediated normalization of TORC1/pS6 activity leads to suppression of spasms need further investigation. Rapamycin has multiple effects that could directly or indirectly alter neuronal excitability. Rapamycin has anti-inflammatory actions, inhibiting astrocytosis, glial activation and cytokine release, which may promote excitability (Ravizza et al. 2008; Vezzani 2008; Weichhart et al. 2008; Zeng et al. 2008; Maroso et al. 2010). Pathologic overactivation of mTOR pathway may increase the expression of glutamate receptors and excitatory synapses, leading to epilepsy and associated neurodevelopmental deficits, whereas rapamycin may inhibit this process, at least in certain tissues (Wang et al. 2006; Talos et al. 2008; Li et al. 2010; Sharma et al. 2010). Rapamycin has also been reported to upregulate (Zeng et al. 2008) or downregulate (Wu et al. 2010) the expression of the astrocytic glutamate transporter 1, altering therefore the extracellular concentration of glutamate, and hence excitability. However, further studies are needed to determine the exact conditions controlling the direction of this regulation in our model system. Rapamycin may also enhance fasting-induced ketogenesis (Sengupta et al. 2010). Of interest, ketogenic diet has been advocated as an alternative treatment for IS (Eun et al. 2006; Kossoff et al. 2008). A common theme among several of the etiologies of IS are the dysplastic neurons in the epileptogenic focus which are functionally and morphologically altered, promoting epileptogenesis (Cepeda et al. 2010; Orlova and Crino 2010; Blumcke et al. 2011). Dysregulation of the mTOR pathway has been demonstrated in several studies on cortical dysplasias (Iyer et al. 2010; Orlova and Crino 2010) whereas rapamycin reversed the abnormal morphological features induced by mTOR overactivation (Ljungberg et al. 2009; Crino 2010; Orlova and Crino 2010). However the exact timecourse of the effects of rapamycin on the epileptic brain needs to be determined so as to better understand the underlying molecular mechanisms of its acute vs delayed therapeutic effects. Of interest, the existing literature, on other models of epilepsy or seizures or on normal brain tissue, have not provided evidence of a similar acute anticonvulsant effect of rapamycin. Acute rapamycin administration did not change the firing rate or excitatory postsynaptic potentials of normal hippocampal neurons at baseline or after exposure to convulsant stimuli *in vitro* (Daoud et al. 2007; Ruegg et al. 2007). Rapamycin pre-treatment also had no effect upon the severity of kainic acid induced seizures acutely, but prevented the occurrence of spontaneous seizures later on (Zeng et al. 2009). Further studies are needed to determine whether the acute anticonvulsant effect of rapamycin in the multiple-hit model of SIS reflects an inherent sensitivity of the networks generating spasms *per se*, which is absent in the networks generating other types of seizures. Alternatively, the observed differences may be age-related (i.e. higher doses might be required in older animals to observe acute effects), model-specific, or stemming from interactions of rapamycin with other systemic processes (i.e. metabolic state, neuroimmune or neuroendocrine interactions).

We found a dissociation between the effects of rapamycin on spasms vs other electroclinical and electrographic seizure types and interictal spikes, suggesting a specific inhibitory effect

upon the networks generating epileptic spasms, rather than a general anticonvulsant or “anti-excitability” effect. These further emphasize the distinct pathogenetic mechanisms between spasms and other epileptic seizures or interictal events, and is concordant with the clinical experience that IS have distinct pharmacosensitivity from other seizures (Mackay et al. 2004). Rapamycin treatment has been shown to progressively reduce spontaneous seizures in mouse models of TSC or PTEN knockouts as well as in the kainic acid and pilocarpine rat models of epilepsy, with its effects being stronger when its administration preceded the onset of seizures (Zeng et al. 2008; Ljungberg et al. 2009; Zeng et al. 2009; Zhou et al. 2009; Huang et al. 2010). It is therefore possible that rapamycin-mediated suppression of other seizure types or interictal spikes may require longer observation or treatment periods, as was reported in the tuberous sclerosis model (Zeng et al. 2008). However, similar to our study, no significant effect was found in the pilocarpine model of epilepsy, when rapamycin was given after the occurrence of the initial status epilepticus (Buckmaster and Lew 2011). Possible confounders in the comparison of efficacy data across these different studies may be the types of seizures observed, the dosing protocols and different durations of treatment, the age of the rodents and the timing of administration of rapamycin in relevance to the seizure onset. Definition of the exact epileptogenic networks in these models, uniformity of treatment protocols and seizure classification in rodents, as well as larger cohorts will be required for such comparisons.

Early cessation of spasms by high-dose 3-day pulse rapamycin treatment resulted in disease modification. The improvement in learning and memory scores was observed more than 10 days after the last dose of rapamycin in the DLP-RAP633 group. This period is at least 10 times longer than the terminal half life ($T_{1/2}$) of rapamycin in adult rats (approximately 25 hours) (Crowe et al. 1999), and therefore is sufficient to eliminate the drug from the system by the time of Barnes maze testing. The $T_{1/2}$ of rapamycin is expected to be even shorter in developing rats, based on the pharmacokinetic studies in humans: 49-80 hrs in adult human patients vs 10-24 hrs in children younger than 6 years of age (Brattstrom et al. 1997; MacDonald et al. 2000; Schachter et al. 2004). Longer follow up studies will however be useful to determine if this effect persists through adulthood. It is also important to note that the cognitive benefit of DLP pups from rapamycin-mediated suppression of spasms is observed in the absence of any significant reduction in interictal spikes and other-than-spasms seizures. These support that rapamycin-mediated suppression of spasms has disease-modifying effects. This finding also corroborates human studies that advocate the early control of spasms to achieve better neurodevelopmental outcome (Lux et al. 2005; Darke et al. 2010) or pose IS as a risk factor for poorer cognitive outcomes in TSC patients (Chu-Shore et al. 2009). Normalization of pathologically dysregulated mTORC1 activity can improve cognition, even in the absence of seizures, because of its central role in cellular physiology, differentiation and communication (Ehninger et al. 2008; Ehninger et al. 2009). However reduction of TORC1 activity below normal levels, as in the rapamycin-treated controls in our study, worsens cognitive performance, suggesting that homeostatic control of TORC1 activity is paramount in defining good outcome. This may be more feasible with the administration of the high-dose 3-day pulse rapamycin protocol, given only during the acute phase of IS when TORC1 activity is pathologically increased, avoiding therefore the long-term side effects associated with continuous rapamycin use (Bissler et al. 2008; Shor et al. 2009). Indeed, our findings demonstrate good recovery of the normal weight growth, after rapamycin discontinuation, with improved performance in the Barnes maze and good survival. Unlike other models of epilepsies, continuous rapamycin post-treatment was not needed to sustain the therapeutic benefits on spasms and cognition, emphasizing the differences in the pathophysiology of these epileptic syndromes (Zeng et al. 2008; Buckmaster et al. 2009; Zeng et al. 2009; Huang et al. 2010).

Different doses of rapamycin have been used in the studies in epilepsy models, ranging between 1-10 mg/kg daily, i.p. (Zeng et al. 2008; Buckmaster et al. 2009; Ljungberg et al. 2009; Zeng et al. 2009; Zhou et al. 2009; Huang et al. 2010). Most of these studies were performed in adult rodents or pups older than 4 weeks of age, whereas 1-3 week old pups were included here. The age-specific differences in pharmacokinetics and sensitivity to drugs are well established and recognized even in the clinical practice. This may explain the increased sensitivity of chronic high dose rapamycin treatment of the DLP pups (daily administration of 6mg/kg i.p. rapamycin, PN6-12), while similar doses were safely tolerated in more mature rodents. The value of the high dose pulse rapamycin protocol instead (DLP-RAP633) lies on the fact that it preserves the acute efficacy and disease-modifying benefit of high dose rapamycin, without rebound appearance of spasms, avoiding prolonged and potentially toxic treatment protocols. Such high dose pulse treatment protocols have been in practice for other disorders, such as multiple sclerosis, i.e. pulse steroid therapy. Furthermore, it is also relevant that IS are an age-specific syndrome, providing an additional reason to avoid chronic rapamycin therapy, once spasms have resolved.

Rapamycin and its analogs are used for the treatment of TSC-related tumors (Bissler et al. 2008; Shor et al. 2009) and sporadically for the treatment of other types of seizures in TSC patients (Muncy et al. 2009). There is no human experience in patients with IS. Our results support its potential as a therapy of IS, including the ACTH-refractory IS. Although TSC patients with IS who have additional indications for mTOR inhibitors may offer a target population, our results suggest that non-TSC patients with IS may also benefit. Longer follow up studies are required to determine if disease-modification is sustained through adulthood, as well as clarify the long-term outcomes of rapamycin therapy on the EEG and other associated seizures. Identification of the pathways that confer relative resistance to rapamycin may provide new targets for effective, faster-onset disease-modifying therapies of SIS.

Conclusions

We provide preclinical evidence that rapamycin, a TORC1 inhibitor, given after the onset of spasms, may be a new treatment option for the management of infantile spasms, including the ACTH-refractory spasms. The identification of a brief, effective, and safe disease-modifying rapamycin treatment protocol for spasms holds special promise as it will avoid the significant side effects associated with continuous rapamycin administration. Furthermore, the perilesional overactivation of TORC1/pS6 pathway and the therapeutic effect of TORC1/pS6 inhibition on spasms suggest that pathologic overactivation of the TORC1 pathway may contribute to the expression of spasms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ACTH	adrenocorticotrophic hormone
CCX	cerebral cortex
DLP	doxorubicin/lipopolysaccharide/p-chlorophenylalanine treated rats
i.p.	intraperitoneal
ir	immunoreactive
IS	infantile spasms
PN	postnatal day
pS6	phosphorylated ribosomal protein S6
SIS	symptomatic infantile spasms
TSC	tuberous sclerosis complex

Figure 1. Effects of rapamycin on the frequency of spasms in DLP male pups

Rapamycin administration was started after the onset of spasms at PN4 and continued with daily i.p. injections at different doses between PN4-12. Only DLP-RAP633 consisted of a 3 day administration.

Panels A and B: In video-monitored pups, rapamycin administration after the onset of spasms showed dose-related suppression of spasms (rapamycin effect: $F=9.35$, $P<0.0001$; time effect: $F=13.68$, $P<0.0001$; 2-way ANOVA). High doses were required to suppress spasms acutely, within 2 hours of first injection (compare DLP-RAP633 vs DLP-VEH groups at PN4-post). In contrast, the lower rapamycin doses (compare DLP-RAP1 and DLP-RAP3 with DLP-VEH) suppressed spasms with delayed onset (PN6-7) but not acutely. Please note that prior to the onset of spasms comparable frequencies of spasms were seen in groups DLP-VEH, DLP-RAP1, and DLP-RAP633. In the DLP-RAP3 group, despite the higher than the other groups pre-injection frequencies of spasms, rapamycin also demonstrated suppressive effect over spasms, diminishing their frequencies at PN6-7. To further confirm whether the acute suppressive effect on spasms seen only in the high dose pulse treatment group (DLP-RAP633) and not masked due to the higher pre-injection frequencies of spasms in DLP-RAP3 pups, frequencies were normalized against the pre-injection frequencies of spasms (PN4pre timepoint) (*panel B*). A significant increase in spasms between PN4pre and PN4post timepoints was seen in the DLP-VEH group only. DLP-VEH, $n=17$ rats; DLP-RAP1, $n=7$ rats; DLP-RAP3, $n=7$ rats; DLP-RAP633, $n=12$ rats.

Figure 2. Rapamycin decreases electroclinical spasms but has no effect on other electroclinical seizure types

Video-EEG monitoring was done in rapamycin or vehicle treated DLP pups, starting at PN7 (DLP-VEH, n= 6 rats; DLP-RAP1: n=4 rats; DLP-RAP3, n= 9 rats; DLP-RAP633, n= 6 rats). The frequencies of electroclinical spasms (panels A and B) and of other types of electroclinical or electrographic seizures were determined (panels C and D).

Panels A and B: An example of electrographic correlate of a spasm is shown in panel A, appearing as a high amplitude spike with coincident paroxysmal fast activity followed by attenuation of the background activities. A synchronous EMG burst corresponding to the spasm is shown at the cervical EMG channel. Panel B depicts the dose-related effects of rapamycin on the frequency of electroclinical spasms in rapamycin vs vehicle-treated DLP pups. Please note that rapamycin or vehicle treatment started at PN4, i.e. prior to the onset of video-EEG recordings (PN7). Rapamycin had significant effect on frequency of electroclinical spasms [$F=18.03$, $P<0.0001$, 2-way ANOVA]. Age also had a significant effect, as the expression of spasms is age-specific, similar to the human condition [age: $F=9.775$; $P<0.0001$]. Overall DLP-RAP633 and DLP-RAP3 groups manifested less frequent electroclinical spasms compared to DLP-RAP1 (significant reduction of spasms only at PN8) and DLP-VEH groups. Asterisks indicate $P<0.05$.

Panels C - D: Panel C shows an evolving seizure pattern comprised of repetitive slow waves building up to repetitive bursts of high amplitude polyspikes with low amplitude coincident rhythmic activity during the decremental phases, which attenuates towards the end of the event. A myoclonic body jerk is seen at the onset of the EMG activity but no other apparent change in the behavior or movement thereafter. As shown in panel D, video-EEG monitoring till PN19 did not reveal any significant intergroup differences among the 4 treatment groups on regards to hourly frequencies of electrographic and electroclinical seizures (panel D).

Figure 3. Rapamycin effects on learning, memory, exploration, weight and survival

Rapamycin administration was as in Figure 1 till PN12. All rapamycin-treated groups, except DLP-RAP633, received thereafter rapamycin 1 mg/kg i.p. (PN13-PN19). DLP-VEH also received vehicle daily till PN19.

Panel A: Rapamycin administration partially improved the learning and memory impairment of DLP pups in Barnes maze testing at PN16-19. Both DLP-RAP3 and DLP-RAP633 groups partially improved learning and memory. However, effective early cessation of spasms by DLP-RAP633 treatment had lasting improvement in learning and memory, without requiring continuous administration of the drug, suggesting therefore disease-modifying effects. In contrast, rapamycin-treated handled controls (RAP3 group) showed worse performance compared to vehicle-treated handled controls (VEH) ($P < 0.05$, Tukey HSD). The 3-way ANOVA results were: DLP-treatment effect: $F = 464.68$, $P < 0.0001$; DLP * rapamycin interaction effect: $F = 47.34$, $P < 0.0001$; Trial number effect: $F = 3.76$, $P < 0.0001$ (DLP-VEH: $n = 5$ pups; DLP-RAP3: $n = 9$ pups; DLP-RAP633, $n = 5$ pups; VEH: $n = 8$ pups; RAP3: $n = 5$ pups).

Panel B: DLP-RAP633 treatment normalized exploratory performance (measured as “number of holes explored / total number of holes”) compared to other DLP groups. DLP-RAP3 rats explored better than DLP-VEH pups but less than DLP-RAP633 pups ($P < 0.05$, Tukey HSD).

Panel C: Rapamycin administration resulted in significant but only transient decrease in body weight gain in DLP-RAP3 pups ($n = 16$ pups) and DLP-RAP633 pups ($n = 12$ pups) compared to DLP-VEH ($n = 20$ pups) ($P < 0.05$, Student's t-test).

Panel D: Survival till PN20 was similar among groups.

Figure 4. Expression of pS6-ir in parietal cortical neurons of DLP pups

pS6-ir is increased in the parietal cortex of PN4 (panels A-B) and PN6-7 (panels C-D) DLP pups. Panels A and C show representative pS6-ir sections from cortical layer 5 (CCX L5). Panels C-D show the densitometry results from cortical sections at the level of the anterior dorsal hippocampus.

Panel A-B: At PN4, only the high rapamycin dose group (DLP-RAP6) has normalized pS6-ir (AlexaFluor-488 labeled, green) in both layers 2 and 5 (CCX L2, CCX L5). In contrast, DLP-RAP group (all included rats are from DLP-RAP3 group) has normalized pS6-ir in layer 2 but not layer 5 ($n = 4-10$ pups per group). ANOVA results for Layer 2: DLP effect: $F = 14.2$, $P = 0.0007$; RAP3 effect: $F = 15.2$, $P = 0.0005$; RAP6 effect: $F = 9.86$, $P = 0.003$. ANOVA results for Layer 5: DLP effect: $F = 16.9$, $P = 0.0003$; RAP3 effect: $F = 1.43$, $P = 0.2$; RAP6 effect: $F = 9.7$, $P = 0.0005$.

Panels C-D: At PN6-7, however, all tested doses of rapamycin normalize pS6-ir at both layers [CON $n = 8$ rats; DLP-VEH $n = 10$ rats; DLP-RAP $n = 5$ rats; DLP-RAP633 $n = 4$ rats]. DLP-RAP group in panels C-D includes representatives of both DLP-RAP1 and DLP-RAP3, as both these groups had shown efficacy on behavioral spasms at PN6-7 ($n = 2-3$ rats per rapamycin dose group). ANOVA results for Layer 2: DLP effect: $F = 5.5$, $P = 0.0225$; rapamycin effect: $F = 12.6$, $P = 0.0008$. ANOVA results for Layer 5: DLP effect: $F = 11.46$, $P = 0.0014$; rapamycin effect: $F = 22.7$, $P < 0.001$.