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# Pharmacological inhibition of the mammalian target of rapamycin pathway suppresses acquired epilepsy

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ABSTRACT

Inhibition of mTOR by rapamycin has been shown to suppress seizures in TSC/PTEN genetic models. Rapamycin, when applied immediately before or after a neurological insult, also prevents the development of spontaneous recurrent seizures (epileptogenesis) in an acquired model. In the present study, we examined the mTOR pathway in rats that had already developed chronic spontaneous seizures in a pilocarpine model. We found that mTOR is aberrantly activated in brain tissues from rats with chronic seizures. Furthermore, inhibition of mTOR by rapamycin treatment significantly reduces seizure activity. Finally, mTOR inhibition also significantly suppresses mossy fiber sprouting. Our findings suggest the possibility for a much broader window for intervention for some acquired epilepsies by targeting the mTOR pathway.



## Introduction

Epilepsy is a chronic, common and sometimes devastating neurological disorder. Epilepsy is characterized by recurrent seizures that are unpredictable and sometimes progressively severe. It is also associated with significant mortality and morbidities (Rice and DeLorenzo, 1998; Sutula, 2004, 2005). Some forms of epilepsy are caused by an inherited vulnerability to seizures, while other forms are a consequence of neurological insults such as head trauma, stroke, and tumors (Manning et al., 2002; Chang and Lowenstein, 2003; Inoki et al., 2005; Holmes and Stafstrom, 2007).

In epilepsy animal models, an initial brain insult such as status epilepticus induced by electrical stimuli or convulsive agents such as pilocarpine or kainate (KA) triggers widespread neuronal loss followed by neurogenesis, gliosis, and mossy fiber sprouting, along with changes in synaptic transmission in the hippocampus (Gruenthal et al., 1986; Cavazos and Sutula, 1990; Sutula, 1991; Sutula et al., 1992; Sankar et al., 2000; Borges et al., 2003; Borges et al., 2006). The structural changes in these animal models are similar to those observed in human mesial temporal-lobe epilepsy (TLE) (Cavazos and Sutula, 1990; Chang and Lowenstein, 2003). Most animals will subsequently develop spontaneous recurrent seizure within 1–3 months (Williams et al., 2009).

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase and belongs to the phosphatidylinositol kinase-related kinase family (Heitman et al., 1991; Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994). It regulates cell growth, proliferation and survival (Avruch et al., 2006; Reiling and Sabatini, 2006; Um et al., 2006; Wullschleger et al., 2006). In the CNS, the mTOR pathway is regulated by glutamate receptor activation (Lenz and Avruch, 2005; Huang et al., 2007) and is involved in neurite growth, synaptic plasticity and cell survival, presumably by influencing protein translation or Akt activity (Burnett et al., 1998; Tang et al., 2002; Cammalleri et al., 2003; Jaworski et al., 2005; Kumar et al., 2005; Tavazoie et al., 2005; Guertin et al., 2006). The mTOR pathway is closely associated with epilepsy. For example, mutations in the tuberous sclerosis complex (TSC) including TSC1 and TSC2 that act upstream of the mTOR not only lead to a wide spread development of benign tumors and mental retardation, but also a high incidence of epilepsy (Manning et al., 2002; Inoki et al., 2005; Holmes and Stafstrom, 2007). Furthermore, rapamycin treatment that inhibits the mTOR pathway attenuates structural abnormalities and reduces seizures in TSC and PTEN mouse models (Ehninger et al., 2008; Meikle et al., 2008; Zeng et al., 2008; Ljungberg et al., 2009; Zhou et al., 2009), suggesting that the aberrant mTOR activation interferes with normal brain development and leads to epilepsy. Most recent studies also indicate that pharmacological inhibition of the mTOR pathway, either before or immediately following neurological insults, can prevent pathological changes in animal brains and the development of spontaneous recurrent seizure in an acquired epilepsy model (Zeng et al., 2009). Furthermore, chronic hippocampal infusion of the mTOR inhibitor rapamycin reduces mossy fiber sprouting in a pilocarpine

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model (Buckmaster et al., 2009). Therefore, it has been proposed that the mTOR pathway could be a target for preventing epilepsy following neurological insults (Crino, 2008; Ehninger et al., 2008; Meikle et al., 2008; Zeng et al., 2008; Zeng et al., 2009; Zhou et al., 2009).

We report here that mTOR is hyperactivated in rat brains with chronic spontaneous seizures. Inhibition of the mTOR pathway by rapamycin markedly reduces seizure activity, along with inhibition of mossy fiber sprouting. Our data suggest that inhibition of mTOR could be a new therapeutic strategy for managing acquired epilepsy.

## Material and method

### *Animals*

Adult (200–300 g or 8–10 week old) male Sprague–Dawley rats were purchased from Taconic (Taconic, NY). Rats were housed in a room with *ad libitum* access to food and water under a 12-h light/dark cycle (lights on 0700). All experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by our Institutional Animal Care and Use Committees.

### *Drug treatment and seizure monitoring*

Rapamycin (*Tecoland*) was first dissolved in DMSO and further diluted in a vehicle solution containing 5% Tween-20 and 4% ethanol. Rats were pretreated with rapamycin at 5 mg/kg/day i.p. for three consecutive days before the induction of seizures by administration of pilocarpine (300 mg/kg). Pilocarpine administration was performed as described (Huang et al., 2002). Briefly, rats were injected with methylscopolamine and terbutaline (2 mg/kg each i.p. in 0.9% NaCl) 15–30 min prior to pilocarpine (300 mg/kg, i.p.) to minimize peripheral side effects. Seizures were terminated with sodium pentobarbital (25 mg/kg, i.p.) 60 min after administration of pilocarpine. Seizure activity was recorded by a digital camera and graded according to Racine's standard classification (Racine, 1972): stage 1, behavioral arrest with mouth and facial movement; stage 2: head nodding/"wet dog shakes"; stage 3: forepaw clonus; stage 4: rearing and stage 5: rearing and falling. Spontaneous seizures were continuously monitored for 84 h/week (12 h/day, 7 day/week) by a video camera from week 3 to 10 after pilocarpine injection. Seizure events were mainly identified and graded by reviewing video by observers blinded to treatment. To determine the effect of rapamycin on chronic spontaneous seizures, adult rats that had already developed chronic spontaneous seizures were paired based on seizure frequency and seizure scale and assigned into two groups (vehicle vs rapamycin-treated). Rats in the rapamycin group were treated with rapamycin at 5 mg/kg/day i.p. for three consecutive days, followed by treatment on every other day. Spontaneous seizures were monitored via continuous video recording for 12 h/day for three weeks. Total seizure events, total duration and percentage of rats that had at least one seizure during the monitoring period were quantified.

### *Western blot*

Rats were treated with rapamycin (5 mg/kg/day, i.p.) or vehicle for three days followed by pilocarpine (300 mg/kg, i.p.). Pentobarbital (25 mg/Kg, i.p) was given after 1 h to terminate seizures. Rats were sacrificed 30, 60, and 120 min after pilocarpine. Brain tissues were removed immediately and then homogenized in lysis buffer consisting of 50 mM Tris, pH 7.4, 2 mM EDTA and proteinase inhibitor set (Roche). The lysates were then mixed with equal volumes of 2× SDS sample buffer and heated at 95 °C for 5 min. Insoluble cell debris was removed by centrifugation at 10,000 *g* for 10 min, the resulting protein samples were dissolved in 8% Bis-Tris gel and then transferred onto 0.45 μM nitrocellulose membrane. Membranes were first blocked in 5% nonfat dry milk in TBST (25 mM Tris–HCl, pH 7.4; 1.5 M NaCl; 0.05% Tween-20) for 1 h at room temperature and then incubated with rabbit anti-S6 or anti-phospho-S6 antibodies with 1:1000 dilution (Cell Signaling Technology) at 4 °C overnight. After removal of primary antibodies by several washings in TBST, membranes were incubated with HRP-conjugated secondary antibody (1:5000 dilution) in 5% milk in TBST. The signals were visualized with ECL reagent (Pierce). To examine mTOR activity in the epileptic brains, rats with chronic spontaneous seizures were sacrificed and brain tissues were processed accordingly. Rats that were from the same batch, but failed to show chronic spontaneous seizures after status epilepticus induced by pilocarpine were used as controls.

### *Timm staining*

Rats that had already developed chronic spontaneous seizures were treated with rapamycin (5 mg/kg) every other day for three weeks. Mossy fiber sprouting (MFS) was examined by Timm staining as described previously (Chen et al., 2007). Briefly, rats were anesthetized, transcardially perfused with sulfide solution containing 0.9% NaCl, 1.2% $Na_2S \cdot 9H_2O$ and 1.0% $NaH_2PO_4$ and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After further postfixation in 4% paraformaldehyde overnight, the brains were cryprotected in 30% sucrose at 4 °C for 2–3 days. Brain sections were cut in the coronal plane at 40 μm and developed in the dark for 40–45 min in a solution of gum Arabic (50% w/v), hydroquinone (5.67% w/v, protect from light), citric acid/sodium citrate buffer (26% citric acid w/v, 24% sodium citrate w/v), and silver nitrate (17% w/v). After washing, sections were dehydrated in alcohol, cleared in xylene, and mounted on slides with DPX mounting medium. Images were acquired using a light microscope under a low power objective (10×). Supragranular layer (inner molecular layer) optical intensity of Timm staining was measured by drawing a contour along the midline of the granular layer and around the inner third of the molecular layer as indicated in Fig. 6D. Optical intensity in a square box drawn in the middle and outer molecular layers served as background staining. At least three brain sections (1 in every 4 series) from each animal were quantified.

Statistical analysis was performed using GraphPad (GraphPad Software, Inc., La Jolla, CA USA). Groups were compared using Student's *t*-test or Wilcoxon signed rank test.

## Results

### *Activation of mTOR in rat brain by pilocarpine-induced seizures*

Administration of pilocarpine, a muscarinic receptor agonist, is known to cause sustained increases in extracellular glutamate levels in the hippocampus (Liu et al., 1997; Smolders et al., 1997). Previous studies revealed that pretreatment with the NMDA receptor blocker MK801 can antagonize pilocarpine-induced cell death and prevent spontaneous seizures (Rice and DeLorenzo, 1998), suggesting that NMDA receptors are critically involved in this epilepsy model. Our previous studies have shown that excessive activation of NMDA receptors inhibits mTOR activity (Huang et al., 2007), while others have shown that transient activation of NMDA receptors activates mTOR in primary cultured neurons (Lenz and Avruch, 2005). To determine whether pilocarpine treatment influences the mTOR signaling pathway in the rat brain, we used western blots to monitor the phosphorylation of S6, a downstream target of mTOR in rat hippocampus and cortex. We observed that S6 phosphorylation was markedly increased within 30 min in the cortex and hippocampus after pilocarpine injection and peaked around 1 h (Figs. 1A and B). To confirm the involvement of the mTOR pathway in S6 phosphorylation in brains, we first treated rats with the mTOR inhibitor rapamycin (5 mg/kg/day) for three days. We found that phospho-S6 elicited by pilocarpine was almost completely depleted by rapamycin treatment in the cortex and