Title: mTOR pathway inhibition as a new therapeutic strategy in epilepsy and epileptogenesis.

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Abstract: Several preclinical and some clinical studies have revealed that the mammalian target of rapamycin (mTOR) signaling pathway is involved in both genetic and acquired epilepsy syndromes. Excessive activation of mTOR signaling, as a consequence of loss-of-function of genes encoding for tuberous sclerosis complex (TSC) 1 and 2, is linked to the development of cortical malformations and epilepsy. This mTOR hyperactivation is associated with different epileptogenic conditions under the term of 'mTORopathies' such as tuberous sclerosis, focal cortical dysplasia, hemimegalencephaly and ganglioglioma. mTOR overactivation produces brain abnormalities that include dysplastic neurons, abnormal cortical organization and astrogliosis. mTOR inhibitors (e.g. rapamycin) have consistent protective effects in various genetic (e.g. TSC models and WAG/Rij rats) and acquired (e.g. kainate or pilocarpine post-status epilepticus) epilepsy animal models. Furthermore, clinical studies in patients with TSC and cortical dysplasia (CD) have confirmed the effectiveness of mTOR inhibitors also in epileptic patients. Therefore, mTOR is currently a very good candidate as a target for epilepsy and epileptogenesis. This review describes the relevance of the mTOR pathway to epileptogenesis and its potential as a therapeutic target in epilepsy treatment by presenting the most recent findings on mTOR inhibitors.
mTOR pathway inhibition as a new therapeutic strategy in epilepsy and epileptogenesis

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Running Title: mTOR in epilepsy and epileptogenesis

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Abstract
Several preclinical and some clinical studies have revealed that the mammalian target of rapamycin (mTOR) signaling pathway is involved in both genetic and acquired epilepsy syndromes. Excessive activation of mTOR signaling, as a consequence of loss-of-function of genes encoding for tuberous sclerosis complex (TSC) 1 and 2, is linked to the development of cortical malformations and epilepsy. This mTOR hyperactivation is associated with different epileptogenic conditions under the term of 'mTORopathies' such as tuberous sclerosis, focal cortical dysplasia, hemimegalencephaly and ganglioglioma. mTOR overactivation produces brain abnormalities that include dysplastic neurons, abnormal cortical organization and astrogliosis. mTOR inhibitors (e.g. rapamycin) have consistent protective effects in various genetic (e.g. TSC models and WAG/Rij rats) and acquired (e.g. kainate or pilocarpine post-status epilepticus) epilepsy animal models. Furthermore, clinical studies in patients with TSC and cortical dysplasia (CD) have confirmed the effectiveness of mTOR inhibitors also in epileptic patients. Therefore, mTOR is currently a very good candidate as a target for epilepsy and epileptogenesis. This review describes the relevance of the mTOR pathway to epileptogenesis and its potential as a therapeutic target in epilepsy treatment by presenting the most recent findings on mTOR inhibitors.

Keywords: mTOR; mTOR inhibitors; Epilepsy; Epileptogenesis; Tuberous Sclerosis Complex; Animal Epilepsy Models;
1. Introduction

Epilepsy is a chronic neurological disorder characterized by recurrent seizures and caused by a large variety of genetic and acquired etiologies. Although many epilepsy patients are seizure-free when treated with drugs, about a third of patients remain still drug-resistant [1-3]. In addition, even when seizures are well controlled with antiepileptic drugs (AEDs), currently available drugs are only a symptomatic therapy in suppressing seizures (antiseizure or anticonvulsant) but do not have disease-modifying properties for preventing or reducing the development of epilepsy (antiepileptogenic) [4-6]. Therefore, novel treatments need to be searched to address both the problem of drugs-resistant epilepsy and the lack of disease modifying therapies.

The mammalian target of rapamycin (mTOR) pathway regulates a number of important physiological functions and in the brain it is clearly involved in cell proliferation, growth and survival, protein synthesis, neuronal morphology and cortical development [7,8]; more recently, it has also been involved in the pathophysiology of several neurological diseases with particular attention to the epileptogenic process being indicated as a potential novel target for epilepsy treatments [9-12]. Dysregulation of the mTOR pathway has been involved in the development of different brain disorders that include focal cortical dysplasia (FCD), tuberous sclerosis complex (TSC), ganglioglioma and hemimegalencephaly, all potentially or certainly leading to epilepsy [9,13].

Hyperactivated mTOR seem to play a pivotal role in the pathogenesis of different animal models of acquired epilepsy, such as infantile spasms (IS), temporal lobe epilepsy (TLE), status epilepticus (SE), absence epilepsy, traumatic brain injury (TBI) and neonatal hypoxia–ischemia [11,14]. Accordingly, different studies have demonstrated that mTOR inhibitors, such as rapamycin and its analogues, decrease the development of seizures preventing epileptogenesis related mechanisms in many animal models and in some cases also some anticonvulsant activity has been evidenced [11,15,16]. From a clinical point of view, small trials have already indicated some kind of activity while larger controlled studies are ongoing using mTOR inhibitors (e.g. everolimus) in patients with
tuberous sclerosis complex and intractable epilepsy [17,18]. The importance of the mTOR pathway in epileptogenesis associated with tuberous sclerosis has been well demonstrated [19], while its role in epileptogenesis occurring in other forms of epilepsy remains to be better clarified and its potential as a target to be confirmed [20-22].

Here, we review the most recent advances concerning the possible role of the mTOR signaling pathway in epilepsy and epileptogenesis, the preclinical studies of mTOR inhibitors treatment in different models of epilepsy, and the available clinical studies in patients with epilepsy.

2. mTOR Pathways in Neurological Diseases

The physiological regulation of the mTOR pathway is essential for normal cellular function; while, its dysregulation may promote the development/progression of disease under pathological conditions such as type 2 diabetes, inflammation, cancer, and cardiovascular disease [8,23,24]. Furthermore, abnormal mTOR signaling has been implicated in a variety of neurological disease [12,25].

In the brain, mTOR mediates several processes involved in CNS development including neurogenesis, cell survival and migration, but it is involved in some other specific processes such as axonal sprouting, axonal regeneration and myelination, dendritic development and microtubule dynamics. A direct role of mTOR in the modulation of glial functions has also been demonstrated [12,26,27]. The mTOR pathway is a key regulator during brain development, in fact, it participates in the control of protein expression and other cellular mechanisms including neuronal and glial differentiation, axon growth, navigation and synaptogenesis, all playing a role in neuronal excitability [28-30]. The mTOR pathway can influence neuronal excitability indirectly through mechanisms controlling synaptic structure and plasticity. In fact, Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways play an important role in the regulation of dendrite arborisation and spine formation, which are critical for the functioning of neurons and neuronal networks [28,29,31].
Accordingly, mTOR pathway also affects neuronal excitability by modulating the expression of ion channels and receptors [32-35].

Considering the relevant role of this pathway in the brain, its dysregulation, such as loss-of-function gene mutations encoding for mTOR inhibitor proteins (e.g. TSC1, TSC2, PTEN), has been involved in neurological diseases such as epilepsy, Parkinson’s disease (PD), Alzheimer’s disease (AD), Huntington’s disease (HD) and brain traumatisms [11,12,36] but also psychiatric diseases such as depression, mental retardation, schizophrenia and cognitive impairment [37].

Recently, particular attention has been given to the role of mTORC1 in major depressive disorder (MDD) [38]. A post-mortem analysis of the prefrontal cortex (PFC) of subjects with MDD revealed deficits in mTOR signaling [39]. Furthermore, Chandran, et al. [40] showed that a chronic unpredictable stress (CUS) exposure produces deficits in mTOR signaling pathway in the amygdala. In agreement, chronic stress associated with depression induced by long-term corticosterone treatment causes an inhibition of the PI3K-Akt-TORC1 pathway [41]. These studies show an association between deficits in synaptic proteins and dysregulation of mTOR signaling in MDD and annulment of these abnormalities may underlie antidepressant activity. For example fast antidepressant response to ketamine, a NMDA receptor antagonist, seems be to mediate by activation of the mTOR pathway in the PFC of rats [42] and depressed patients [43].

Interestingly, abnormal mTOR signaling has also been implicated in diseases as fragile X syndrome [44], Down syndrome, [45] and Rett syndrome [46]. The potential involvement of dysregulated mTOR signaling in these neurological disorders characterized by cognitive deficits is likely linked to its role in physiological mechanisms of learning and memory [47]. In particular, mTOR plays an important role in the consolidation of memory through long-term potentiation (LTP) [33,48].

Furthermore, changes in dendritic morphology may represent a structural substrate for memory persistence and can be regulated by the mTOR pathway [28,29] whereas abnormalities in dendritic morphology have been demonstrated in many neurogenetic syndromes, including TSC, fragile X, Down and Rett [44,49].
In contrast, other studies reported a relationship between enhanced mTORC1 activity and memory improvement; rapamycin seems to disrupt this process in several behavioral models including auditory fear conditioning and Morris water maze task [50,51]. Moreover, rapamycin administration decreased both spatial hippocampus memory and consolidation memory in many brain regions such as amygdala and hippocampus [50,52-54]. However, mTORC1 hyperactivity has been associated with memory deficits in human patients and experimental models of TSC [55]. In a mouse model of TSC, rapamycin rescued memory performance, which could be, at least in part, related to an abnormal mTOR activity [56]. The importance of mTOR signaling in CNS physiology is underscored by the several disorders in which mTOR pathway disruption is implicated, such as tumors, autism, mood disorders, neurodegenerative diseases as well as epilepsy [36,57,58].

Finally, another link between mTOR and neurodegenerative diseases could be autophagy, which represents a catabolic process. It has been demonstrated how mTOR is a crucial regulator of autophagy [59]. A typical hallmark of neurodegenerative diseases, such as Alzheimer’s, Parkinson’s and Huntington’s diseases, is the aberrant accumulation of protein aggregates in the brain [60,61] and associated neuronal death. The clearance of these proteins would seem to be increased by mTOR inhibition [62,63]. Pharmacological manipulation of mTOR signaling is thus proving to be a promising therapeutic branch for the treatment of several neurological disorders [57].

3. Role of mTOR pathway in epilepsy and epileptogenesis

Considering mTOR involvement in cellular functions influencing neuronal excitability, it is not surprising that this signaling pathway can be responsible for or participate to the development of spontaneous seizures, and that this pathway could represent an important target for both epileptogenesis and seizure pharmacotherapy [11,20,64]. Since early 2000, many preclinical and some clinical data have underscored the importance of mTOR pathway in both genetic and acquired
epilepsy syndromes [65]. Excessive activation of mTOR signaling, as a consequence of loss-of-
function mutations of genes encoding for natural mTOR inhibitors such as TSC1 and TSC2 (coding
for the proteins hamartin and tuberin, respectively), phosphatase and tensin homolog (PTEN) and
STE20-related kinase adaptor alpha (STRADalpha), are linked both to the development of cortical
malformations and epilepsy. These malformations or "mTORopathies"-related epilepsies include:
hemimegalencephaly, ganglioglioma, focal cortical dysplasia (FCD), and tuberous sclerosis
complex (TSC) [13,66].
The term "mTORpathies" describes neurological disorders characterized by altered cortical
architecture, abnormal neuronal morphology and intractable epilepsy as a consequence of excessive
mTOR signaling, providing a likely histopathological substrate for epileptogenesis [14,67]. On the
other hand, seizures themselves, in the absence of any other associated pathology, may directly
cause activation of mTORC1 activity [20]. Many experimental models of genetic and acquired
epilepsy, in which mTOR hyperactivation was present, are responsive to mTOR inhibitors [15,16].
Rapamycin and other mTOR inhibitors decrease seizures, delay seizure development, or prevent
epileptogenesis in many experimental models [65]. This evidence supports the hypothesis that
dysregulation of the mTOR pathway seems to be a key condition for the development of
epileptogenesis and epilepsy. To date, the mechanisms by which mTOR inhibition gives rise to the
inhibition of seizure activity in several experimental models is still unclear. Nevertheless, the use of
selective mTOR inhibitors can represent an important new therapeutic strategy for managing or
eventually preventing epilepsy due to these disorders [9].

3.1 Preclinical Studies
Dysfunction of mTOR signaling pathway is involved in the pathophysiology of Tuberous sclerosis
complex (TSC) [68]. However, this dysfunction also plays an important role during the latent phase
of epileptogenesis of some acquired forms of epilepsy, such as temporal lobe epilepsy (TLE),
traumatic brain injury (TBI) [69], infantile spasms (IS) [70] and neonatal hypoxia–ischemia [71].
3.1.1 Tuberous sclerosis complex models

Among the genetic epilepsy syndromes, TSC has drawn particular attention, since it is strongly linked with the dysregulation of the mTOR pathway [10]. TSC is an inherited autosomal disorder resulting from a mutation of one of two tumor suppressor genes: TSC1 and TSC2 [72]. In TSC, benign tumors may develop in multiple organs such as skin, liver, heart, kidney, lung and the brain, in which it is often associated with the development of subependymal giant cell astrocytoma (SEGA) among other tumors [73]. Unfortunately, many TSC patients suffer from drug-resistant epilepsy [74-76]. mTOR dysregulation in TSC directly affects many downstream mechanisms, including alteration of neurotransmitter receptors and ion channel expression, and synaptic and neuronal organization, leading to epileptogenesis process [15,77]. In the context of TSC-associated epilepsy, aberrant mTOR signaling has been repeatedly demonstrated, and conditional knockout of TSC1 or TSC2 in various brain cell populations has been associated with elevated levels of mTOR signaling and seizures in several transgenic mouse models [78,79]. Animal models of TSC are crucial to study the link between mTOR, TSC and epilepsy [80,81]. Mice with TSC1 or TSC2 deleted, in specific neural populations (astrocytes or neurons), show neuropathological phenotypes (i.e. astrogliosis, neuronal autophagy, macrocephaly, seizures and premature death) similar to those found in human TSC [78,82].

Clinical and pre-clinical studies, using mTOR inhibitors (i.e. rapamycin and everolimus) demonstrated the role of mTOR in TSC-associated epilepsy [83,84]. Inhibition of mTOR signaling by rapamycin in a mouse model of TSC with conditional inactivation of the Tsc1 gene primarily in glia (Tsc1GFAPCKO mice) can prevent astrogliosis, neuronal disorganization and seizures early in the course of the disease, suggesting that the aberrant mTOR activation interferes with normal brain function and leads to epilepsy [85]. Similarly, using a knock-out mouse model of TSC in which Tsc1 was ablated in most neurons during cortical development, rapamycin treatment and its derivate everolimus were able to reverse the animal phenotype, rescuing the mutants from epilepsy [86].
Early treatment with rapamycin in Tsc2GFAP1 CKO mice, also rescued the mutants from epilepsy and increased their survival [87,88]. Rapamycin seems effective not only reducing seizures once they start but also in preventing seizures from ever developing as well as many of the pathological and molecular changes (as progressive astrogliosis, inflammatory mechanisms, hippocampal neurodegeneration, brain hypercellularity) in the brain that likely promote epileptogenesis in these mice; this indicates that mTOR may have an anti-epileptogenic effect in these genetic models [85,86,88]. However, following discontinuation of rapamycin therapy, these phenotypes at least partially return and are accompanied by progressive development of severe seizures and early death. Inflammatory signaling mechanisms, particularly the cytokine IL-1β and chemokine CXCL10, are abnormally activated in Tsc1GFAPCKO mice; these inflammatory mediators were reversed by rapamycin treatment, indicating that cytokine and chemokine signaling is downstream from mTORC1 and occurred in astrocyte culture in vitro and before epilepsy onset in vivo [89].

Recently, it has been reported a primary role for TORC1 signaling in epileptogenesis, using mice with biallelic Tsc1 deletion; this latter resulted in activation of TORC1, enhanced neuronal excitability and epilepsy development without any obvious histological changes. Increased TORC1 activation appears sufficient for the development of epilepsy, even in the absence of changes in brain pathology. Rapamycin treatment reduced TORC1 activity, seizure frequency and increased survival indicating an important role of mTOR for managing seizures not only in the presence of major brain pathology but also in other type of epilepsies that result from increased mTOR hyperactivation [90]. Among current AEDs, vigabatrin (VGB) has been shown to have unique efficacy in partial seizures related to TSC and infantile spasms [91] and early treatment, even before seizure onset, can improve the long-term outcome of epilepsy in patients with TSC [92,93]. To date, the exact mechanism by which VGB is effective in TSC remains unclear. In addition to its already proven mechanism of action to increase brain γ-aminobutyric acid (GABA) levels by inhibition of γ-aminobutyrate transaminase (GABA-T) [94], VGB also seems to inhibit mTOR pathway in the neocortex and hippocampus of Tsc1GFAP conditional knockout mice, providing a possible
explanation for the unique effectiveness of this drug in TSC [95]. Therefore, VGB seems to also
directly act on the mTOR pathway; however, an indirect action cannot yet be excluded.
Furthermore, it was reported that a prophylactic antiepileptic treatment of TSC patients (and at
high risk of epilepsy) with VGB, but also levetiracetam, valproic acid and topiramate, markedly
improved their risk of developing mental retardation and reduced the incidence of drug-resistant
seizures [92,93] (Table 1 and 2).

3.1.2 Cortical dysplasia models

Similar to TSC, several other, relatively rare genetic disorders entangle a dysregulation of the
mTOR pathway and an increased risk for tumors and epilepsy. Brain-specific deletion of the mouse
homolog PTEN, an upstream activator of the mTOR pathway [96], mimics several features of
human cortical dysplasia (CD), including neuronal hypertrophy, cortical and hippocampal
disorganization, aberrant mossy fiber sprouting and epilepsy [19]. CD (also known as malformation
of cortical development) is another recognized type of "mTORpathies" characterized by intractable
epilepsy in which mTOR dysregulation plays a key role in determining epilepsy phenotype [97].
Since CD has been linked to mutations of genes encoding for mTOR regulators [68], mTOR
inhibitors through their antiepileptogenic mechanisms might be useful for the treatment of the CD-
related epilepsy [98,99]. Recently, a link in CD between the up-regulated miRNAs (i.e.hsa-miR-21
and hsa-miR-155) and mTOR pathway has been evidenced [100]. Furthermore, PTEN deficiency is
linked with the excessive growth, migration and proliferation of dysplastic cells in CD [101]. To
better understand the role of mTOR in CD and epilepsy, Ljungberg, et al. [102] have characterized
neuron subset-specific Pten knockout (NS-Pten KO) mice as an experimental model of CD. Pten
knock-out mice exhibit neuronal hypertrophy, megalencephaly and seizures as a consequence of
enhanced mTOR activity, and both early and later treatment with rapamycin decreases pathological
abnormalities, suppresses the development of seizures and reduces established late-stage epilepsy
[102]. Rapamycin treatment, at late stages of the pathology, decreased mTORC signaling,
astrogliosis and microgliosis that were found in NS-Pten KO mice of CD [103]. Treatment with
mTOR inhibitors also reverses the neuronal hypertrophy and megalencephaly in PTEN knock-out mice [104,105]. Similar to the TSC models, seizures return with the cessation of rapamycin treatment, although intermittent rapamycin treatment is able to maintain a long-term antiseizure effect [104] (Table 2).

### 3.1.3 Temporal lobe epilepsy models

PTEN inactivation, in human and animals hippocampal dentate granule cells, induces the development of abnormal granule cells and spontaneous seizures similar to temporal lobe epilepsy (TLE) [31]. During epileptogenesis, adult-generated dentate granule cells (DGCs) form aberrant neuronal connections with neighboring DGCs increase neuronal excitability in the hippocampus. Sutula and Dudek [106] demonstrated that PTEN deletion among hippocampal granule cells was sufficient to develop spontaneous seizures in a few weeks, and that mTOR signaling played a fundamental role in this process. Therefore, hyperactivation of the mTOR pathway as a result of PTEN deletion is a possible mechanism of epileptogenesis also in TLE. Moreover, rapamycin administration was effective in inhibiting epileptogenesis and presence of abnormal granule cells in this PTEN animal model [31]. mTOR inhibitors can decrease pathological abnormalities that are associated with epileptogenesis, in particular mossy fiber sprouting [20,107,108]. Accordingly, it was demonstrated that mTOR inhibitors rescued fiber sprouting by promoting the survival of the somatostatin/green fluorescent protein (GFP)-positive interneurons after pilocarpine-induced status epilepticus (SE) in mice [109]. Some studies report that rapamycin is also able to decrease both epileptiform activity and mossy fiber sprouting in mouse models of TLE such as pilocarpine and kainate-post SE spontaneous seizures [107]. Furthermore, rapamycin acts on axonal sprouting and is able to revert abnormal cell growth [19]. However, other studies have shown that rapamycin treatment induced reduction of mossy fiber sprouting but not the frequency of pilocarpine-induced
spontaneous seizures in mice [107,110]. In addition to mossy fiber sprouting, rapamycin treatment reversed neuronal death and neurogenesis that contribute to epileptogenesis, but these data are controversial [20]. Different studies demonstrated that the mTOR pathway is markedly enhanced, in a biphasic manner, after kainate-induced SE in both hippocampus and cortex. The exact mechanism by which kainate induces this mTOR enhancement is unclear. However, it was hypothesized that excessive release of glutamate could be involved in this process [111,112]. Administration of rapamycin, prior or after kainate-induced SE, blocked cell death, neurogenesis, mossy fiber sprouting, and the reduced spontaneous epilepsy in this mouse model of TLE [20,108]. Similar results were obtained in the pilocarpine-SE model [113]. Accordingly, the authors claimed that the mTOR pathway mediates mechanisms of epileptogenesis in kainate and pilocarpine rat models and rapamycin could have anti-epileptogenic effects in these models [20,106,114,115]. However, paradoxical effects of mTOR inhibition have also been reported. In fact, mTOR activation can have both pro-apoptotic and anti-apoptotic effects, depending on different phases of the cell cycle [116]. Rapamycin administration within 1 hour of kainate injection in rats, induced enhancement of the mTOR pathway, higher than with kainate alone, whereas when rapamycin was administered after this time period, the expected inhibition was observed [117]. Therefore, mTOR would seem to act as a master switch that regulates, under different situations, neuronal death and epileptogenesis. It was reported that post-treatment with rapamycin after amygdala electrical stimulation-induced SE, did not stop the epileptogenic process and did not decrease disease severity. These data suggest that the antiepileptogenic effects of mTOR inhibition are not universal within animal models and may depend on several variables [22,110]. In agreement, rapamycin treatment started after electrically induced SE, reduced the development of recurrent spontaneous seizures. Likewise, rapamycin reduced other potential features related to epilepsy and epileptogenesis, such SE-induced neuronal cell loss, mossy fiber sprouting, and blood–brain barrier (BBB) albumin leakage; however, it did not reduce hippocampal microglia or astrocyte activation indicating only partial effects [118].
Very recently, it has been reported that rapamycin treatment after kainic acid–induced SE influences BBB leakage. Moreover, rapamycin is not able to reduce the seizure onset through an improvement of the BBB during the early phase of epileptogenesis. At odds, it is able to reduce BBB leakage during the chronic phase decreasing: gliosis, brain inflammation and angiogenesis. These effects could be related to the inhibitory properties of rapamycin on the development of epilepsy [119,120] (Table 1 and 2). Finally, mTOR activation in astrocytes contributes to TLE and may be targeted to suppress astrogliosis and spontaneous seizure [121].

3.1.4 Other models of epileptogenesis

Recently, mTOR overactivation has been also shown in an animal model of absence epilepsy (the WAG/Rij rat), suggesting that this mechanism might be a very common pathological component for epileptogenesis in different models of epilepsy [122]. It was also established that WAG/Rij rats, a well-validated genetic model of absence epilepsy, epileptogenesis and mild-depression comorbidity [123-125], have higher levels of total mTOR in several brain areas, including the cortex, hippocampus and thalamus in comparison to Wistar rats, [126]. Inhibition of mTOR by rapamycin (started before seizure onset; i.e. at postnatal day 45) permanently reduces the development of spontaneous absence seizures in this model. In addition, WAG/Rij rats in comparison to Wistar rats also showed an age-related decline in hippocampal neural progenitor cell proliferation rate, suggesting that mTORC1 overexpression might be one of the triggers of epileptogenesis [122,126]. Rapamycin effects in this model have been linked to a modulation of inflammatory responses/alterations following its administration; rapamycin would block the lipopolysaccharide dependent increase in pro-inflammatory cytokines in the brain [127].

The mTOR pathway is also activated by hypoxia or toxin–related insults that acutely induce neonatal seizures or infantile spasms [70,128]. In hypoxia-induced neonatal seizures in rodents, activation of mTORC1 pathway was associated with the induction of seizures in the immature rat brain; rapamycin treatment immediately before and after seizures reversed early increases in
glutamate neurotransmission and seizure susceptibility attenuating later life epilepsy and autistic-like behavior [128]. Rapamycin treatment suppresses infantile spasms (IS) permanently and improve cognitive outcome; the suppression of spasms was correlated with the ability of rapamycin to normalize TORC1 activity in perilesional cortical neurons [70].

In a rat model of cryptogenic infantile spasms, in which seizures were triggered by N-methyl-D-aspartate (NMDA), pretreatment with VGB, but not rapamycin in low doses, suppressed IS [129]. While genetic epilepsies affecting the mTOR pathway are relatively rare, there is increasing interest as to whether the mTOR pathway may be involved in other, more common types of epilepsy, such as following acquired brain injury. Traumatic brain injury (TBI) is a major cause of death, mental diseases and disability. Among the consequences, TBI post-traumatic epilepsy is very common and is a cause of significant morbidity and mortality in TBI patients [130]. Akt and mTORC1 activation have been highlighted in a number of traumatic brain injury (TBI) models [131,132]. Studies have reported that mTOR inhibitors might have antiepileptogenic effects in the development of post-traumatic epilepsy in an animal model of TBI [69]. Rapamycin injection 4h following closed head injury significantly improved functional recovery. In rodent models of TBI, mTOR inhibition reduced neuronal death and mossy fibers sprouting and, as a result, improved cognitive outcome [133]. Rapamycin was tested in an experimental model of controlled cortical impact (CCI) injury, a well-validated model of TBI, in which has also been proven that an aberrant activation of mTORC1 occurs. Rapamycin administration, started after CCI, had no effect on acute symptomatic seizures, but significantly prevented the development of chronic post-traumatic epilepsy [134]. mTOR involvement in TBI has also been demonstrated in a rat hippocampal organotypic culture model of post-traumatic epilepsy. Ictal activity was measured both by lactate production and by multiple electrode array (MEA) recordings (Table 1 and 2).

| Table 1. Preclinical evidence on the role of mTOR inhibitors in preventing epileptogenesis |
|----------------------------------|-------------------|------------------------------|-----------------------------|-----------------|-----------------|-----------------|
| Epilepsy Type/Animal Model       | mTOR inhibitor and | Protocol of Administration   | Effect of mTOR inhibitor on epilepsy | Hypothesized Mechanism(s) of | References |
|----------------------------------|-------------------|------------------------------|-----------------------------|-----------------|-----------------|-----------------|

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<table>
<thead>
<tr>
<th>Genetic epilepsy (Tuberous sclerosis)</th>
<th>Tsc1GFAP CKO mice</th>
<th>Rapamycin 3 mg/Kg i.p.</th>
<th>Postnatal day 14 (~2 weeks presymptomatic)</th>
<th>Inhibition of epilepsy development; prolonged survival during treatment</th>
<th>Inhibited abnormal activation of mTOR, astrogliosis, neuronal disorganization and increased brain size. [85]</th>
</tr>
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<tr>
<td>Genetic epilepsy (Tuberous sclerosis)</td>
<td>Tsc2GFAP CKO mice</td>
<td>Rapamycin 3 mg/kg i.p.</td>
<td>Treatment started at P14, 5 days/week, up to the end of experiments</td>
<td>Rescued the animals from epilepsy and increased their survival</td>
<td>Inhibited astrogliosis, neuronal disorganization and increased brain size. Reduced levels of phospho-S6 [88]</td>
</tr>
<tr>
<td>Genetic epilepsy (Tuberous sclerosis)</td>
<td>Tsc1null neuron mice</td>
<td>Rapamycin and Everolimus 6 mg/kg i.p.</td>
<td>Treatment started at P7-P9, every other day, up to 92 days (P100)</td>
<td>Prolonged survival, prevention of spontaneous seizures during treatment</td>
<td>Improved neofilament abnormalities, myelination, and cell enlargement. Reduced levels of phospho-S6 [86]</td>
</tr>
<tr>
<td>Genetic epilepsy (cortical dysplasia)</td>
<td>Pten GFAP KO</td>
<td>Rapamycin 10 mg/kg i.p.</td>
<td>Treatment (2 weeks) started at the 4th and 5th weeks; postsymptoms</td>
<td>Reduced the severity and the duration of the seizure activity, persisted following discontinuation of treatment</td>
<td>Hypothesized changes in subcellular structures and, possibly, in processes that are involved in synaptic plasticity and membrane excitability [135]</td>
</tr>
<tr>
<td>Genetic epilepsy (Cortical dysplasia)</td>
<td>NSE-Pten CKO mice</td>
<td>Rapamycin 10 mg/kg i.p.</td>
<td>Treatment started in mice of 5–6 weeks old (presymptomatic phase)</td>
<td>Decreased seizure duration and frequency</td>
<td>Inhibition of anatomical, cellular and behavioural abnormalities related with mTOR pathway hyperactivation [105]</td>
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<tr>
<td>Genetic epilepsy</td>
<td>Pten KO mice</td>
<td>Rapamycin 6mg/kg/day i.p.</td>
<td>Treatment started 2-5 days post tamoxifen injection</td>
<td>Reduced seizures</td>
<td>Inhibited mossy fibre sprouting [31]</td>
</tr>
<tr>
<td>Acquired epilepsy (TLE)</td>
<td>Kainic-acid induced SE</td>
<td>Rapamycin 6mg/kg/day i.p.</td>
<td>Treatment started 3 days before SE or 24 h after SE, up to 7 weeks.</td>
<td>Suppressed the development of seizures</td>
<td>Blocked cell death, neurogenesis, mossy fibre sprouting [20]</td>
</tr>
<tr>
<td>Acquired epilepsy (TLE)</td>
<td>Pilocarpine-induced SE</td>
<td>Rapamycin 3mg/kg/day i.p.</td>
<td>Treatment started 24 h after SE for 2 months</td>
<td>Blocked mossy fibre sprouting. No effects on seizure frequency</td>
<td>Inhibition of mossy fiber sprouting [107,109,110]</td>
</tr>
<tr>
<td>Acquired epilepsy (TLE)</td>
<td>Amygdala stimulation-induced</td>
<td>Rapamycin 6 mg/kg daily</td>
<td>Treatment started 24 h after SE for 2 weeks</td>
<td>Did not stop the epileptogenic process and no decrease in disease severity</td>
<td>No effect on mossy fiber sprouting [22]</td>
</tr>
<tr>
<td>Epilepsy Type/Model</td>
<td>mTOR inhibitor and dose</td>
<td>Protocol of Administration</td>
<td>Effect of mTOR inhibitor on Seizures</td>
<td>Hypothesized Mechanism(s) of Action</td>
<td>References</td>
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<tr>
<td>Acquired epilepsy (TLE) Pilocarpine induced SE in mice</td>
<td>Rapamycin 6 mg/kg daily</td>
<td>Treatment started 24 h after the onset of SE for 6 consecutive days</td>
<td>Suppressed epileptiform activity</td>
<td>inhibited mTOR pathway and repressing mossy fiber sprouting</td>
<td>[114]</td>
</tr>
<tr>
<td>Acquired epilepsy (TLE) Electrical stimulation of the angular bundle (SE)</td>
<td>Rapamycin 6 mg/kg/day i.p.</td>
<td>Treatment for 7 days, started 4 hours after the induction of SE, and continued until rats were sacrificed, 6 weeks after SE.</td>
<td>Reduced the development of epilepsy</td>
<td>Inhibition of mossy fiber sprouting, reduction in neuronal death, decreased BBB leakage; no reduction of inflammatory response after SE induction</td>
<td>[118]</td>
</tr>
<tr>
<td>Acquired epilepsy (TLE) kainic acid–induced SE</td>
<td>Rapamycin 6 mg/kg day i.p.</td>
<td>Treatment started 4 h after SE, once daily for 7 days, and continued until rats were killed 7 weeks post-SE.</td>
<td>reduction or prevention of recurrent seizures at a later stage.</td>
<td>Reduced BBB leakage during the chronic phase, via reduction of gliosis, brain inflammation and angiogenesis.</td>
<td>[119,120]</td>
</tr>
<tr>
<td>Acquired epilepsy TBI</td>
<td>Rapamycin 6 mg/kg/day i.p.</td>
<td>Treatment started 1 hour after injury and continued for 1 month</td>
<td>Prevented the development of post-traumatic epilepsy</td>
<td>Decreased neuronal degeneration and mossy fibre sprouting, although this effect did not directly correlate with inhibition of epileptogenesis</td>
<td>[134,136]</td>
</tr>
<tr>
<td>Acquired epilepsy Hypoxia-induced seizures in rats</td>
<td>Rapamycin 3 mg/kg i.p.</td>
<td>Treatment started 24 h before and 1 h after exposure to hypoxia</td>
<td>No effect on acute seizures; decreased chronic seizures</td>
<td>inhibited mTORC1 pathway, and subsequent increased glutamatergic neurotransmission.</td>
<td>[128]</td>
</tr>
<tr>
<td>Absence epilepsy model WAG/Rij rats</td>
<td>Rapamycin 1 mg/kg os</td>
<td>Treatment started at P45 and continued for 17 weeks</td>
<td>Decreased the development of absence seizures</td>
<td>inhibition of the release of inflammatory cytokines</td>
<td>[122,126]</td>
</tr>
</tbody>
</table>

SE = Status Epilepticus; TLE = Temporal Lobe Epilepsy; BBB = Blood Brain Barrier; CKO = Conditional Knockout; GFAP = Glial Fibrillary Acid Protein; i.p. = intraperitoneally; KO = Knockout; NS-Pten KO = Neurone Subset-Specific Pten knockout; NSE = Neuron-Specific Enolase; P = Postnatal day; PTEN = Phosphatase and Tensin homolog; TBI = Traumatic Brain Injury; TSC 1 = Tuberous Sclerosis Complex 1; TSC 2 = Tuberous Sclerosis Complex 2; WAG/Rij rat = Wistar Albino Glaxo/Rij-rat.

### Table 2. Preclinical evidence on the role of mTOR inhibitors in preventing seizures

<table>
<thead>
<tr>
<th>Epilepsy Type/Model</th>
<th>mTOR inhibitor and dose</th>
<th>Protocol of Administration</th>
<th>Effect of mTOR inhibitor on Seizures</th>
<th>Hypothesized Mechanism(s) of Action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic epilepsy NS-Pten KO (cortical dysplasia)</td>
<td>Rapamycin 10 mg/kg i.p.</td>
<td>Intermittent treatments (over a period of 5 months)</td>
<td>Decreased seizures and improved survival after additional intermittent treatments</td>
<td>Changes in subcellular structures and, possibly, in processes involved in synaptic plasticity and membrane excitability</td>
<td>[102,104]</td>
</tr>
<tr>
<td>Genetic epilepsy NS-Pten KO mice (cortical dysplasia)</td>
<td>Rapamycin 10 mg/kg/day i.p.</td>
<td>Treatment started at P9</td>
<td>Attenuated epileptiform activity</td>
<td>Suppressed mTOR hyperactivation; Reduced astrogliosis and microgliosis</td>
<td>[103]</td>
</tr>
<tr>
<td>Genetic epilepsy Pten GFAP KO</td>
<td>Tensilimus 7.5 mg/kg</td>
<td>Treatment started from 6 to 16 weeks, when mutant mice were symptomatic</td>
<td>Decreased seizures and mortality</td>
<td>Decreased megalencephaly, cell size</td>
<td>[101]</td>
</tr>
<tr>
<td>Genetic epilepsy Acute biallelic deletion of Tsc1 in adult mice</td>
<td>Rapamycin 5 and 10 mg/kg/day i.p.</td>
<td>Treatment started 2–4 months postnatal</td>
<td>Prolonged survival; No seizures developed during treatment</td>
<td>Effectively reduced pS6 levels</td>
<td>[90]</td>
</tr>
<tr>
<td>Epileptic encephalopathy Multiple-hit rat model of infantile spasms</td>
<td>Rapamycin different doses</td>
<td>After the onset of spasms.</td>
<td>Reduction of acutely-induced spasms in a dose-related way</td>
<td>Unclear</td>
<td>[70]</td>
</tr>
<tr>
<td>TLE following Pilocarpine-induced SE in adult rats</td>
<td>Rapamycin 5 mg/kg/day i.p.</td>
<td>Pre-treatment for 3 days before the induction of seizures with pilocarpine</td>
<td>Reduction seizure activity during treatment, gradually returning after discontinuing treatment</td>
<td>Suppressed mossy fiber sprouting</td>
<td>[108]</td>
</tr>
<tr>
<td>Acquired epilepsy Acute seizure models in Sprague-Dawley rats</td>
<td>Rapamycin 5 mg/kg i.p.</td>
<td>Treatment started, in immature and mature rats, prior to induction of seizures by PTZ, pilocarpine or kainate</td>
<td>Increased severity of seizures; decreased seizure threshold after 3 daily doses of rapamycin in 3–4 weeks old, but not adult, rats</td>
<td>Treatment down-regulates KCC2 expression in CNS, which could increase susceptibility to pilocarpine-induce seizures in immature rats</td>
<td>[32]</td>
</tr>
<tr>
<td>Epileptic encephalopathy Infantile spasms in rats prenatally treated with betamethasone, triggered with NMDA</td>
<td>Rapamycin 3 mg/kg i.p.</td>
<td>Pre-treatment 24 h prior to induction of spasms</td>
<td>No effects in this model</td>
<td></td>
<td>[129]</td>
</tr>
<tr>
<td>Acquired epilepsy Acute seizure models in Sprague-Dawley rats acute seizure tests</td>
<td>Rapamycin 3 or 6 mg/kg i.p.</td>
<td>Used different treatment paradigm in rat pups (P15) and juvenile (P55–60) rats</td>
<td>Variable efficacy on acute seizures which are age, time, treatment paradigm and model dependent.</td>
<td>The lack of effects, above all in immature rats, has been correlated with decreased NPY expression in the cortex and hippocampus</td>
<td>[115]</td>
</tr>
<tr>
<td>Acquired epilepsy Multiple Acute seizure (6Hz, PTZ or kainate) tests in NIH Swiss mice</td>
<td>Rapamycin 4.5 mg/kg i.p.</td>
<td>Short-term treatment (single dose) 3 h before seizure onset and long-term treatment (3 daily doses) before seizure onset</td>
<td>Variable efficacy on acute seizures, which are age, time, treatment paradigm and model-dependent</td>
<td>Reduction in neuronal excitability and/or neurotransmitter release may occur with rapamycin</td>
<td>[137]</td>
</tr>
</tbody>
</table>

**CNS** = Central Nervous System; GFAP = Glial Fibrillary Acid Protein; i.p. = intraperitoneally; KCC2 = Potassium Chloride Cotransporter 2; KO = Knockout; mTOR = mammalian Target Of Rapamycin; NPY = Neuropeptide Y; NS-Pten KO = Neurone Subset-Specific Pten knockout; P = Postnatal day; pS6 = Phospho-S6; PTEN = Phosphatase and Tensin homolog; PTZ = Pentylenetetrazole; TSC 1 = Tuberous Sclerosis Complex 1; TSC 2 = Tuberous Sclerosis Complex 2.

### 3.2 Clinical studies

Despite the current pharmacological and non-pharmacological treatment options, about a third of the epileptic patients remain drug-resistant [138,139]. Indeed, TSC is also characterized by
pharmacologically uncontrolled seizures. The United States Food and Drug Administration (FDA) approved everolimus, a rapamycin analogue, for the treatment of patients with TSC associated with inoperable SEGAs [17,140]. Beneficial effects of mTOR inhibition with everolimus have been reported previously in patients with TSC and epilepsy [74,141-143]. Therefore mTOR inhibitors provide a potential therapy based on the pathophysiology of TSC [77].

In 2009, it has been described for the first time that rapamycin treatment (10 months) induced a reduction in seizure frequency and severity in a 10-year-old girl with difficult-to-treat seizures in TSC, although pre- and post-treatment magnetic resonance imaging did not reveal any change in the cortical tubers [144]. In another study, a child treated with everolimus for a regrowing SEGA, a complete cessation of previously intractable seizures was reported at 12 months follow-up [18]. Everolimus treatment improved seizure control in prospective phase I/II studies in patients with TSC [141] and in patients with TSC and associated SEGA [74]. In one of these studies (prospective open label phase I/II study), Krueger, et al. [74] showed that everolimus used in TSC to limit SEGA (associated with TSC) (primary end point), decreases seizure frequency in approximately 60% of patients studied (secondary end point), but 1 patient experienced increased seizure frequency with the drug [74,145]. In particular, 9 out of 16 patients with TSC showed a decrease in seizure frequency, 6 did not show significant reduction, whereas in 1 an increased seizure frequency was noted. The effect of everolimus, after 12 weeks of treatment in the management of pharmaco-resistant epilepsy in patients > 2 years affected by SEGAs, was also investigated in a prospective, multicentre, open-label, phase I/II clinical trial [141]. The study compared seizure data during the last 4 weeks of everolimus treatment (weeks 13–16) with the 4-weeks period before everolimus initiation (baseline, weeks 1–4).

Everolimus treatment (12 weeks) in pediatric patients with TSC and refractory epilepsy reduced seizure frequency by a median reduction of 73% in 17 of 20 patients examined and a median 70% decrease in cumulative seizure duration. Four of these patients were seizure-free at 12 weeks, and seven had a 90% reduction in seizure frequency [141]. Everolimus was well tolerated; all adverse
events were mostly grade 1 or 2 in severity and usually transient, never requiring everolimus withdrawal. Upper respiratory infections, stomatitis and mucositis were the most common adverse events [141]. Therefore, these findings strongly suggest that everolimus might be an effective treatment for SEGA-related epilepsy. The different animal studies and limited clinical data have led to further current clinical trials for inhibition of mTOR in genetic epilepsies. A compassionate use trial for seven TSC patients with drug resistant epilepsy has demonstrated the efficacy of everolimus; one patient discontinued treatment because of rash, four of six patients exhibited a reduction of seizure frequency of 25-100%, two of six 6 patients, did not show alteration of seizure frequency. Everolimus treatment seemed to be well-tolerated with adverse effects similar to those reported in previous studies [143].

Recently, an open-label case series in seven patients (median age 6 years), with TSC and refractory epilepsy, described the efficacy mTOR inhibitors (six with rapamycin and one with everolimus), in seizures improvement, which were reported to have only minimal adverse effects. Of the intractable seizure group (7 patients), 1 patient had >90% reduction, 4 had 50%-90% reduction, and 2 had <50% reduction. Moreover, this treatment was reported to improve other characteristics of TSC, such as facial angiofibromas and cognition; three reported subjective improvements in learning [146]. Recently, another single case of seizures aggravation after everolimus treatment for SEGA has been reported [147]. Cessation of seizures or a reduction in seizure frequency was also reported in a small number of pediatric patients with TSC who were treated with long-term everolimus. In 6 out of 8 children, at least a 50% reduction in SEGA volume was observed; everolimus resulted in permanent seizure cessation in one child with severe drug-resistant epilepsy and in at least a 50% reduction in the number of seizures in two others [142].

Moreover, in a prospective study of 5 neonatal patients with TSC, it was demonstrated that EEG changes occurring in TSC patients before clinical seizures reflect the process of epileptogenesis in these patients. Therefore, EEG recording could have predictive value during infancy in patients with TSC [148].
More recently, the case of a 13-year-old girl with TSC-associated with refractory generalized seizures who initiated treatment with everolimus experiencing subsequent improvement in several TSC manifestations, including a reduction in seizure frequency from clusters of two or three daily to one every 2 to 4 weeks after 1.5 years of treatment was described [149]. All these clinical studies confirm the potential benefits of mTOR inhibitors on epilepsy associated with TSC, but the open-label design of the studies as well as the heterogeneity of enrolled patients and the small number of cases still does not allow making definite conclusions.

A randomized, blinded, placebo controlled, phase III trial is currently in progress to determine the efficacy of everolimus on seizures in patients with TSC; patients between the ages of 2 and 65 years, with a clinically definite diagnosis of TSC and uncontrolled partial-onset seizures, are currently being enrolled for this study and results are expected during the next year (clinicaltrials.gov identifier NCT01713946) (Table 3).

Recently, a genomics study of infantile spasms and Lennox-Gastaut Syndrome has found a de novo mutation in mTOR gene without associated brain malformations suggesting a possible role of mTOR in these disorders and mTOR inhibitors could be a possible treatment [150]. Furthermore, de novo somatic mutations of PI3K, AKT3 or mTOR genes in patients with hemimegalencephaly (HME) a condition associated with resistant epilepsy have been described [31,151].

**Table 3. Clinical Studies with mTOR Inhibitors in TSC-Associated Epilepsy**

<table>
<thead>
<tr>
<th>Type of Study</th>
<th>Disease</th>
<th>Drug and Dose</th>
<th>Number and Age of Patient(s)</th>
<th>Duration of Treatment</th>
<th>Clinical Result</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case report</td>
<td>TSC and refractory epilepsy</td>
<td>Rapamycin 0.15 mg/kg/day</td>
<td>1 patient; 9-year-old girl</td>
<td>10 months</td>
<td>Reduction in seizure frequency. 1 to 5 brief seizures (&lt; 2 minutes) continued daily</td>
<td>[144]</td>
</tr>
<tr>
<td>Case report</td>
<td>TSC and refractory epilepsy</td>
<td>Everolimus 4.5 mg/m²/day</td>
<td>1 patient; 10-year-old boy</td>
<td>12 months</td>
<td>Complete cessation of epileptic seizures</td>
<td>[18]</td>
</tr>
<tr>
<td>Prospective, open-label, phase I-II study</td>
<td>TSC and related epilepsy</td>
<td>Everolimus 4.7-5.6 mg/m²/day</td>
<td>16 patients; 3 year-old or older</td>
<td>Median duration 21.5 months (range: 4.7-34.4 months)</td>
<td>Reduction in seizure frequency in 9/16 patients, did not change in 6, and increased in 1</td>
<td>[74]</td>
</tr>
<tr>
<td>Prospective, multicenter, open-label, phase I/II clinical trial</td>
<td>TSC and refractory epilepsy</td>
<td>Everolimus 5 mg/m²/day, then titrated to a serum trough level of 5–15 ng/ml</td>
<td>20 patients; median age: 8 years (age range: 2–21)</td>
<td>12 weeks</td>
<td>Reduction in seizure frequency in 17/20 patients (median reduction of 73%). 4 of these patients were seizure-free at 12 weeks, and 7 had a 90% reduction in seizure frequency</td>
<td>[17,141]</td>
</tr>
<tr>
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</tr>
<tr>
<td>Case study series</td>
<td>TSC and refractory epilepsy</td>
<td>Everolimus 5–7 mg/day</td>
<td>6 patients; median age: 5 years (age range: 2–12).</td>
<td>36 weeks</td>
<td>Reduction in seizure frequency in 4/6 patients (of 25% - 100%). The percentage of seizure-free days increased in 3/4 of these patients. In 2/6 patients, no iteration of seizure frequency</td>
<td>[143]</td>
</tr>
<tr>
<td>Open-label, single-center case series</td>
<td>TSC and refractory epilepsy</td>
<td>Sirolimus 1 mg/m²/d. then adjusted to trough blood levels of 4–10 ng/mL</td>
<td>7 patients; median age: 6 years (age range: 3–17).</td>
<td>median duration: 18 months (range: 6–36 months)</td>
<td>1 patient had &gt;90% reduction, 4 had 50%–90% reduction, and 2 had &lt;50% reduction</td>
<td>[146]</td>
</tr>
<tr>
<td>Case study</td>
<td>SEGAs associated with TSC</td>
<td>Everolimus 5 mg/m²/day</td>
<td>1 patient; 13.5-year-old girl</td>
<td>12 days</td>
<td>Seizure aggravation</td>
<td>[147]</td>
</tr>
<tr>
<td>Prospective, double-blind, parallel-group, placebo-controlled, multicenter phase 3 (EXIST-1)</td>
<td>SEGAs associated with TSC</td>
<td>Everolimus 4.5 mg/m²/day then adjusted to attain a blood concentration of 5–15 ng/mL</td>
<td>8 patients; children under the age of 3</td>
<td>35 months (range: 33–38 months)</td>
<td>Cessation of seizures in 1 patient, significant (at least a 50%) reduction in the number of seizures in 2 patients</td>
<td>[142]</td>
</tr>
<tr>
<td>Case report</td>
<td>TSC-associated epilepsy with refractory generalized seizures</td>
<td>Everolimus 5 mg/day</td>
<td>1 patient; 13-year-old girl</td>
<td>1.5 year</td>
<td>Reduction in seizure frequency from clusters of two or three daily to one every 2 to 4 weeks</td>
<td>[149]</td>
</tr>
<tr>
<td>Randomized, blinded, placebo controlled, phase III trial</td>
<td>TSC associated refractory seizures</td>
<td>Everolimus titrated from 3 to 7 ng/mL and also from 9 to 15 ng/mL and placebo</td>
<td>Male or female between the ages of 2 and 65 years</td>
<td>This study is currently recruiting participants</td>
<td>Novartis Pharmaceuticals</td>
<td></td>
</tr>
</tbody>
</table>

EXIST-1 = EXamining everolimus In a Study of TSC; SEGAs = Subependymal Giant cell Astrocytoma; TSC = Tuberous Sclerosis Complex

### 4. Conclusions

Epilepsy represents one of the oldest and most prevalent neurological disorders. Currently available treatments are effective but provide only symptomatic management of the disease, and an unmet need exists for a rational therapy that targets the etiology of epilepsy [2,139]. Understanding epileptogenesis, the process by which a normal brain becomes epileptic, may help identify...
molecular targets for drugs that could prevent epilepsy. To date, the mTOR pathway represents perhaps, the most promising molecular target for producing a better understanding and treatment of this disease. The first favorable point in this regard, is the availability of a number of already marketed drugs acting on this target; such drugs \textit{(i.e.} rapamycin, everolimus \textit{etc}) are already used clinically for other conditions \textit{(e.g.} as immunosuppressants in kidney transplantation, in treatment of advanced renal carcinoma and breast cancer).

Different preclinical data confirm that mTOR dysregulation may play a pathogenetic role in the epileptogenesis of different forms of epilepsy, and that mTOR inhibition may prevent epilepsy development, especially in genetic \textit{mTORopathies}. mTOR inhibitors show efficacy in treating some genetic forms of epilepsy \textit{(e.g.} TSC and WAG/Rij rats) but also possess antiepileptogenic effects in preclinical models. The potential inhibition of mTOR as a therapeutic strategy for preventing epileptogenesis is therefore promising and may be applicable not only to TSC, but also to other forms of epilepsy. These findings are starting to be translated into the clinical arena; successful anticonvulsant effects of both rapamycin and everolimus have been reported in individuals with focal-onset seizures in the context of TSC. While several studies with TSC mouse models suggest that mTOR inhibitors have antiepileptogenic properties for preventing epilepsy, clinical antiepileptogenic drug trials are difficult to conduct and have not yet been attempted with mTOR inhibitors. Some controversial results with mTOR inhibitors also exist and while it is clear that the mTOR pathway might be a good target candidate, some drawbacks of \textquote{general} mTOR inhibition must still be considered and more studies are warranted (ideally with more selective drugs), before full clinical translation of mTOR targeting can result. In particular, the time-window, duration and dosages to be used in prevention of epileptogenesis are still far from being clearly identified. On the other hand, mTOR inhibitors possess only a very low efficacy in preclinical animal models of seizures/epilepsy and therefore, their effectiveness in stopping seizures is probably very limited and/or in any case, it might require time before some kind of effectiveness can be observed; generally, a few days of treatment are necessary to observe an antiseizure effect if any. As most of
the beneficial effects of mTOR inhibition might cease after drug discontinuation, a life-long treatment might be necessary. The effect of long-term mTOR inhibition on developing brain structures and functions is still not fully understood. Despite several drugs acting on mTOR that are already currently available (both considering marketed and experimental drugs), none of these agents has selectivity for the CNS, which obviously underlies the appearance of peripheral side effects that might limit dosages and therefore efficacy. The search for more selective agonists targeting specific effectors of the mTOR pathway can lead to the discovery of better drugs to treat epilepsy. As mentioned above, clinical trials are currently undergoing for both everolimus and rapamycin in epilepsy. The results of these trials will indeed shed light on the possibility to use such molecules in TSC patients; however, this will only be an initial step, which will need further clinical trials to understand the possible use of mTOR inhibitors in other clinical situations with patients at risk of epilepsy development (e.g. traumatic brain injury).

Interactions between mTOR inhibitors and available antiepileptic drugs are not fully known and will have to be analyzed. The role of mTOR inhibitors in epilepsy treatment still needs further research and an extension of clinical trials to non-TSC epilepsy syndromes. Further clinical studies could help to clarify the clinical efficacy, dosage and safety profile of mTOR inhibitors in epilepsy and epileptogenesis. Despite these limitations, recent developments in mTOR pathway modulation open up new perspectives for future therapy of epilepsy and mTOR inhibitors represent a promising therapeutic option for the treatment of epilepsy.

In conclusion, as in the case of many other diseases, we feel that the mTOR pathway represents a great opportunity for future novel drug development in epilepsy therapeutics; the actual available data with mTOR inhibitors, both clinical and pre-clinical are intriguing and highly support future experiments. Finally, a very important task will be that of developing new mTOR inhibitors with a higher selectivity for the central nervous system (CNS) and a better safety profile.
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Supplemental Material

The mTOR Complexes and Signaling Pathways

The mammalian target of rapamycin (mTOR) is a protein serine-threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family [1,2]. Rapamycin (also known as sirolimus) is a macrolide antibiotic molecule extracted from the mycelium of the Easter Island (Rapa-Nui) soil-dwelling bacterium *Streptomyces hygroscopicus*. This macrolide, through its specific ability to inhibit mTOR, is mainly used as an antifungal, immunosuppressant, and anticancer agent [3]. mTOR is activated by phosphorylation in response to several modulators such as growth factors, mitogens and hormones. The molecular factors and downstream target signaling molecules associated with the mTOR pathway are numerous and complex. The signal transduction mechanisms linked to mTOR have been studied extensively and have been related to a huge spectrum of fundamental biochemical and physiological processes, such as metabolism, cell growth, proliferation, differentiation, longevity, apoptosis, and autophagy [4]. In mammalian cells, the functions of mTOR are mediated through two mTOR heteromeric and functionally distinct protein complexes: mTORC1 and mTORC2. These complexes are formed by mTOR and several proteins, two of which are in common to both mTORC1 and 2: one is the mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL), a positive regulator and the other named DEPTOR (DEP-domain containing mTOR-interacting protein) is a physiological negative regulator. mTORC1 has two other associated proteins, the positive regulator named RAPTOR (regulatory-associated protein of mTOR) and PRAS40 (proline-rich AKT substrate of 40 kDa) or AKTS1 (AKT1 substrate), which shows a suppressive action. mTORC2 shows three components in common with mTORC1 and three specific proteins: RICTOR (rapamycin-insensitive companion of mTOR), which plays an important role also for the interaction between mTORC2 and tuberous sclerosis complex 2 (TSC2), a direct activator of this complex [5]; mSIN-1 (mammalian stress-activated protein kinase interacting protein) fundamental for its ability to phosphorylate AKT [6],
and PROTOR-1 (protein observed with RICTOR-1), which is required for efficient mTORC2-mediated activation of SGK-1. Moreover, PROTOR-1 is able to bind RICTOR [7].

In particular, mTORC1 rapamycin-sensitive complex, the better characterized of the two complexes, is fundamental in controlling a wide variety of cellular processes including protein synthesis, autophagy, cell cycle and microtubule dynamics. Conversely, mTORC2, a rapamycin-insensitive complex, regulates both the development of cytoskeleton and cell survival. Recent findings demonstrated that mTORC2 plays a role in genome stability maintenance under oxidative and replicative stress [8] showing a different sensitivity to rapamycin, which inhibits mTOR through the binding to FKBP12 (FK 506-binding protein of 12 KDa). In particular, it has been demonstrated that although mTORC2 is insensitive to acute rapamycin exposure, it can be modulated by prolonged rapamycin treatment [9].

**Upstream of mTOR Signaling**

mTOR pathway is activated by mitogens, brain-derived neurotrophic factor (like BDNF) or hormones (such as insulin and insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF) and ciliary neurotrophic factor (CNTF), glutamate, and guidance molecules [10-12]. All these input signals that positively or negatively control mTORC1 activity are mediated by the activation of receptor tyrosine kinase (RTKs) and converge on tuberous sclerosis complex, comprised of TSC1 (harmatin) and TSC2 (tuberin). TSC1/2 is a GTPase activating protein (GAP) for the small G protein Rheb (Ras homolog enriched in brain) and Rhes (a form of Rheb mainly expressed in the brain) [13,14]. The phosphorylation of TSC1/2 occurs through activation of mTORC1 by channel receptors, GPCRs, RTKs and cytokine receptors via protein kinases (e.g. Akt and ribosomal S6 kinase (RSK) [15]. As a result, TSC1/2 complex results inactivated and induces an increase of Rheb-GTP, that is an important activator of mTORC1 [16]. Rheb-GTP activates mTORC1 through interacting with mTOR [17]. One of the most important upstream modulators of mTOR activity is the heterodimer TSC1/2, whose activity can inhibit mTOR. Only TSC2 has
GTPase-activating properties; however, both TSC1 and TSC2 are required for the function of this heterodimer. A small G protein Rheb (Ras homolog enriched in brain) is a direct downstream target of TSC1/2 and a positive regulator of mTOR function, which induces a conformational change of mTOR that results in activation and phosphorylation of protein effectors [18]. More recently, a third component (TBC1D7) collaborating with TSC1/2 in the activation of Rheb has been identified [19,20]. Rheb-GTP activates the mTORC1 complex interacting with Raptor. In addition, Rheb regulates 4EBP1 binding to mTORC1 reducing mTOR activity through the control of mTOR association with mTORC1 by FKBP38. The latter is structurally linked to an endogenous inhibitor of mTOR named FKBP12.

The TSC1/2 complex receives inputs from different signal-transduction pathways [21], predictably, many of the molecules that bind or regulate the TSC1/2 complex can also regulate mTOR, among them there is the serine/threonine kinase Akt. This kinase is activated by growth factors such as insulin or epidermal growth factor (EGF), through both phosphorylation and the lipid products of phosphatidylinositol 3-kinase (PI3K). Once activated, Akt then directly phosphorylates and reduces the activity of TSC2 and, therefore, increases signaling through Rheb and mTOR [22-24].

Mutations (including nonsense, missense, insertion, and deletion) of TSC1 or TSC2, causing loss of TSCs function produce an overactivation of mTORC1 and brain diseases. TSC2, and its phosphorylation, induces both activation and inactivation of mTORC1 depending on the phospho-acceptor amino acid residues. Phosphorylation of TSC2 (in particular at Thr^{1462}) by Akt and by MAPK (at Ser^{664}) (akaErk) inhibits TSC1/2 activity thus activating mTORC1, whereas phosphorylation of TSC2 (at Ser^{1345} and Thr^{1227}) by AMP-activated protein kinase (AMPK) enhances TSC1/2 activity leading to the suppression of mTORC1 activity [23,25]. Hence, AMPK and Akt exert opposite regulations of TSC2 activity. AMPK is able to inhibit mTORC1 through the phosphorylation of Raptor [26].

In neurons, mTOR activity is also modulated by some neurotransmitters such as glutamate and dopamine receptors [27,28]. In particular, G-protein coupled receptors (GPCRs) such as,
metabotropic glutamate, μ-opioid and cannabinoid receptors, activate mTORC1 in neurons [29-31]. Because GPCRs are known to transduce signals to Akt and/or MAPK, mTORC1 activation triggered by these ligands seems to inhibit TSC2. In particular, group I mGluRs are coupled to mTOR-p70S6K and ERK1/2-p70S6K pathways in striatal and hippocampal synaptoneurosomes [30] and hippocampal CB1 cannabinoid receptor (CB1R) activation modulated the mammalian target of rapamycin (mTOR)/p70S6K pathway inducing amnesic effects [31]

**Downstream of mTOR Signaling**

Many substrates have been identified as mTOR downstream effectors mediating several cellular responses. p70 ribosomal S6 protein kinases 1 and 2 (p70S6K1/2) and eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BPs) are the best-characterized substrates for mTORC1 regulating translation [16,32].

The binding of 4E-BPs to elongation factor 4E binding protein 1 (eIF4E) inhibits the formation of the eIF4F complex, required for the initiation of translation [33]. mTOR phosphorylates and activates S6K and 4EBPs, suppressing this inhibition and promoting ribosomal biogenesis and protein translation [34,35]. Consequently, the protein synthesis is induced by the inhibition of the elongation factor 4E binding protein 1 (4EBP1) [32].

mTORC1 pathway also controls autophagy as a consequence of the inhibition of ULK1 complex (Unc51-like kinase 1)/Atg13 (autophagy-related genes 13)/FIP200 (focal adhesion kinase family-interacting protein of 200 kDa) [36]. mTOR controls the cellular response to hypoxia regulating the transcription/translation of hypoxia inducible factor 1α (HIF1α) [37]. HIF1α-mediated mechanisms are important for angiogenesis following hypoxia and have been involved in different neurodegenerative disease but also in stroke and neonatal hypoxic-ischemic injury [38]. Among the transcription factors regulated by mTORC1 are the sterol-response binding proteins (SREBPs), which regulate lipogenesis [39,40].
References


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**Graphical Abstract**

- **Rapamycin**
- **Everolimus**

**mTOR Complex**

- **mTORC1**
- **mTORC2**

**Hyperactivation of mTOR**

- Abnormal cell proliferation and differentiation
- Change in expression of ion channels/neurotransmitter receptors
- Abnormal dentrite morphology and axon outgrowth
- Abnormal autophagy
- Abnormal neuronal polarization and connectivity
- Hyperexcitability

**Antiseizure Effects in Animal Models and Clinical Studies**

**Antiepileptogenic Effects in Animal Models**

**Epilepsy**

**Epileptogenesis**