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Review

Importance of EEG in validating the chronic effects of drugs: Suggestions from animal models of epilepsy treated with rapamycin

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ABSTRACT

Purpose: The development of new drugs for the treatment of epilepsy is a major challenge for modern neurology and its first steps demand basic research. Preclinical studies on animal models of epilepsy are mainly based on the analysis of brain electrical activity to detect seizures, when they are not just limited to behavioral tests like the Racine scale.
Methods: In the present review, we discuss the importance of using time-locked video and EEG recordings (Video-EEG) coupled with behavioral tests as tools to monitor and analyze the effects of anti-epileptic drugs in pre-clinical research. Particularly, we focus on the utility of a multimodal approach based on EEG/behavioral analysis to study the beneficial effects of chronic rapamycin treatment as a potential anti-epileptogenic therapy for a broad spectrum of epilepsy, including both genetic (as in tuberous sclerosis complex) and acquired diseases.
Results: Changes and synchronization of neuronal activity of different areas have been correlated with specific behavior in both physiological and pathological conditions. In the epileptic brain, during a seizure there is an abnormal activation of many cells all at once, altering different networks.
Conclusion: A multimodal approach based on video, EEG analysis and behavioral tests would be the best option in preclinical studies of epilepsy.

1. Introduction

Epilepsy is one of the most important central nervous system disorder affecting between 0.5% and 1% of the population worldwide. Although in same cases there are different effective options for the treatment of epilepsy (i.e. surgery or vagal nerve stimulation), people suffering from this disorder usually require a chronic drug treatment and nearly one third of patients with epilepsy become medically intractable with current available drugs [1,2]. In addition, pharmacoresistance is a major issue with about 30–40% of patients not responding to any kind of antiepileptic drugs [3]. As a consequence, the number of untreatable patients is fast-growing. Moreover, most current treatments are primarily symptomatic therapies that suppress seizures but do not correct the underlying brain abnormalities actually causing epilepsy. In addition to that, epilepsy is often related to mood/anxiety disorder and cognitive impairment [4,5]. Thus, there is a great need for new "antiepileptogenic therapies", also effective on behavior. To develop such treatments, a better understanding of the underlying mechanism of action of new substances is needed. During the development of new therapies, the 'Assay developments' and 'Lead generation' are necessary steps occurring before clinical testing. Both these processes involve animal models-based test systems to identify the effects of therapeutic agents. However, the search for new treatments for seizures, epilepsies and their comorbidities faces considerable challenges. This is due in part to gaps in our understanding of the etiology and pathophysiology of most forms of epilepsy. An additional challenge is the difficulty in predicting the efficacy, tolerability and impact of potential new treatments on epilepsies and comorbidities in humans, using the available resources. In fact, in 2012, out of 30,000 compounds screened by

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the Anticonvulsant Screening Program (ASP) of the National Institute of Neurological Disorders and Stroke (NINDS), only 9 have acquired an indication for seizures treatment [6].

Herein, we focus our attention on the advantages of using a combination of time-locked video and EEG recording (usually referred as video-EEG monitoring) and different behavioral tests when studying chronic effects of antiepileptic drugs. In particular, we take as a relevant example the case of rapamycin in the treatment of epilepsy in tuberous sclerosis complex (TSC), a rare multi-system genetic disease resulting in a combination of symptoms including seizures [7].

2. From neural activity to behavior and back

The brain can be considered as a dynamic network of interacting elements connected each other and with the external environment. The synchronous activation among different brain areas is essential in determining appropriate behavior. During seizures some of the network connections fail, possibly leading to a paroxysmal outcome. In many cases, network failure affects also the interictal period and it could be linked to behavioral disorders [8,9].

Thus, to characterize neural functioning in epilepsy, it may be necessary to examine the brain activation at different levels/times rather than at the level of the single event (i.e. seizure).

2.1. Local field potential (LFP)

Extracellular recording of the electrical signal from animals brain results in a signal that can be roughly segregated by frequency-band separation into a high-frequency part (above ~200–500 Hz), mainly reflecting multiple-unit spiking activity (MUA) and a lower part (below ~200–500 Hz), the so-called 'local field potential' (LFP) [10]. While the MUA most likely represents extracellular action potentials of all neurons around the electrode within a sphere of about 140–300 μm radius [11,12] LFP is primarily generated by transmembrane current passing through cellular membranes/synapses in the proximity of the electrode, ranging from several hundred micrometers to few millimeters, according to the recording conditions [13–16] although the spatial extent of neurons that generate LFP is still a matter of debate [17]. In other words, LFPs reflect primarily a weighted average of synchronized dendro-somatic components of the synaptic signals of a neural population, around the electrode tips. In the last few decades, the interest in LFP has grown up, thanks to both technological improvements in electrodes availabilities (i.e., simultaneous recording of a large number of different sites) and the insight that LFP may provide information about sensory or cognitive processes, deriving from the integration of a certain neuronal population that cannot be measured by studying spiking [18,19].

Commonly, cortical or deep brain recording in animal models of epilepsy are used as a diagnostic tool to detect seizure or other electrical abnormalities (i.e. interictal spiking). However, LFP and simultaneous video recording (namely video-EEG monitoring) and simple LFP analysis could be of great help if coupled with behavioral analysis, when studying the effect of chronic drug treatments in preclinical animal models.

2.2. Video-EEG recording

The study of epilepsy in animal models has greatly taken advantage of simultaneous video and EEG recording. In video-EEG monitoring, a video camera captures the behaviors and movements of a subject/animal concurrently with EEG trace recording, allowing a precise correlation between a given event and the EEG trace (see Figs. 2 and 3 in [20]. Reviewing simultaneous EEG and video data can be also useful for confirming that an observed behavior is an epileptic seizure, instead of a different episode (e.g. scratching, movement disorder). In addition to that, video-EEG monitoring is helpful in seizure classification (e.g. with or without convulsion, absence or partial versus generalized) and to determine its temporal progression (e.g. Racine scale). During interictal periods, the video recording is essential for assessing the animal activity (e.g. rest, active behavior, running, grooming), to better correlate it with EEG. Video monitoring during dark phase is made possible by the use of special video camera or infrared highlighting.

Electrode implantation is carried out under general anesthesia, using inhalation (isoflurane, or sevoflurane) or intravenous (mixture of ketamine, xylazine, and acepromazine, or tiletamine and zolazepam) anesthetics. Montages with 2–4 recording electrodes are generally used to record EEG from mice or rats, after drilling precise holes in the skull with a high-speed micro drill in a stereotactic frame. Usually, four recording electrodes are used for rats whereas only one set of bilateral recording electrodes may be sufficient to monitor EEG activity in mice, according to the smaller head size. Epidural recording screw/wire electrodes are placed symmetrically over central or parietal areas and additional electrodes are positioned over frontal areas [21–27]. In some cases, deep electrodes are used, in particular for hippocampal activity recording. In this case, stainless steel or tungsten insulated wires are used [22,28,29]. The reference and ground electrodes may be placed over posterior (occipital, cerebellum) and frontal regions. Electrodes are soldered to pins/multielectrode connectors, secured to the skull with cranioplastic or dental cement. The connection to the amplifier is ensured by flexible cables, with or without electric swivels, the second allowing easier and stress-free movements of the animal. More sophisticated wireless systems such as New-Behavior (Zurich, Switzerland) [30] or Data Science International (St. Paul, MN) [29] can be successfully adopted. For research purposes it is possible to adapt commercial video-EEG monitoring systems for clinical use, although using a minimal amount of available electrodes. However, it is possible to develop custom systems by assembling different components, connected to a personal computer. In this case, the video camera and biomedical amplifiers should be connected by an analog to digital converter to the computer, running appropriate software for video-EEG monitoring. For recording, individual customized cages are usually adopted. A Plexiglas room with a wire mesh coating (i.e. copper) used as a Faraday cage is usually a convenient and useful solution. According to the design and to the aim of the study, video-EEG monitoring can be performed continuously [23,29] or in a staggered way, i.e. recording selected time-defined EEG periods, ranging from 1 to 48 h, daily or at fixed time points, throughout the entire duration of the experiment. Although a continuous 24/7 recording represents the gold standard, several economic and/or practical issues have to be considered in the design of the study. A continuous recording implies that the acquiring unit has to be full-time dedicated to a single animal until the end of the experiment, and that a huge amount of video data has to be stored, analyzed, and managed. On the other hand, staggered recordings could lead to false negatives, as missed seizures, or more generally to high variability in seizure count up [6].

Seizures are the most important feature to be analyzed within EEG recordings in animal models of epilepsy. Electrographic seizures (Fig. 1A) are generally self-limited (having an unambiguous start and end, standing out from the background activity) epochs of repetitive spike discharges, often starting as low amplitude high frequency activity (tonic phase) and evolving into higher amplitude and slower frequency bursts (clonic phase), lasting at least 10–15 s [22,23,28,31]. A period of voltage suppression is often observed at the end of the seizure (postictal

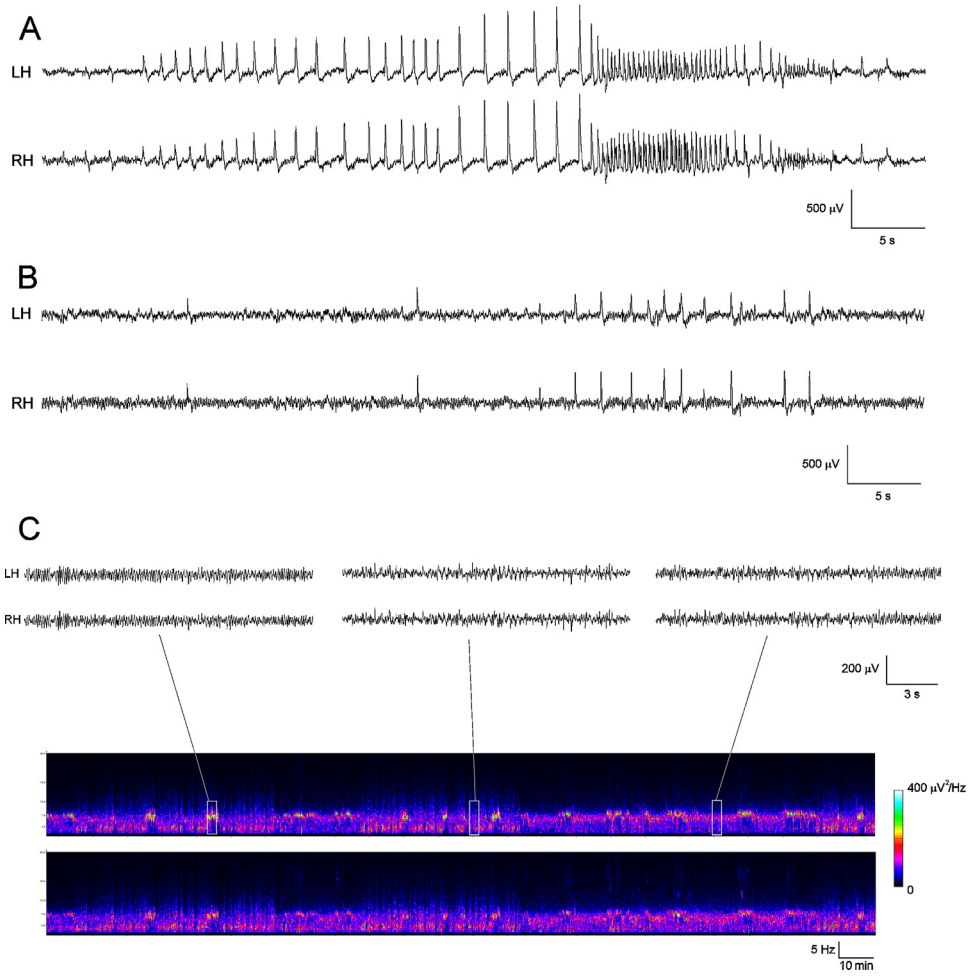

Fig. 1. Video-EEG monitoring in a mouse model of epilepsy: (A) A seizure detected by EEG, recorded from two epidural-screw electrodes, over left (LH) and right (RH) parietal areas, referred to a posterior electrode (cerebellar screw). (B) Background interictal activity characterized by bilateral synchronous sharp waves. (C) DSA relative to 4.5 h of normal background video-EEG monitoring. Different behaving states may be observed and analyzed offline by video inspection associated with EEG traces: REM sleep phase, characterized by a pseudo-sinusoidal activity at around 7.5 Hz (left) and immobility; NREM sleep phase, defined by a slower EEG activity (center) and immobility, and awake phase, where the EEG shows an activity mostly centered around 5–7 Hz frequencies (right) and active locomotion.

phase). Simultaneous video recordings are useful to confirm behavioral correlates of electrographic seizures and to rule out artifacts or other false-positives, mainly attributable to grooming. In addition, in different studies it was found that nonconvulsive electrographic seizures usually occur before ictal EEG events correlated with behavioral seizures [32,33].

The voluminous amount of video-EEG data collected in case of prolonged monitoring studies may require many hours of manual review and analysis. Automated seizure detection algorithms could help in decreasing the manual workload and may sometimes be more reliable than visual analysis [34]. Different automated seizure detection algorithms have been described, for both off-line [35,36] and real-time applications [37].

EEG traces can also be scored according to other epilepsy related features such as interictal spikes [23], repetitive spike trains, or long runs of repetitive spikes [24,26]. Interictal spikes (Fig. 1B) are defined as fast (<200 ms) and sharp waves, with amplitude at least twice the amplitude of the background activity [21]. The interictal background activity can be scored using a four-grade scale [38]: grade 1 (normal) – normal background theta rhythm with no epileptiform spikes, grade 2 (mildly abnormal) – mostly normal background with some epileptiform spikes, grade 3 (moderately abnormal) – mostly abnormal background with frequent epileptiform spikes, grade 4 (severely abnormal) – burst–suppression pattern. The average score for spike frequency (spikes/minute) and background activity (grade 1–4) are the most commonly considered parameters to describe the interictal activity.

In addition to qualitative analysis, quantitative analysis can be performed on EEG data (quantitative EEG or qEEG). Spectral analysis of interictal background activity can easily provide useful information to assess drug efficacy [27,39,40]. EEG power spectra can be obtained using the periodogram method, consisting in the average of Fast Fourier Transformed set of selected homogeneous artifact-free EEG epochs, usually lasting 2 s and possibly tapered with a Hanning window. Absolute band power values are easily extracted from power spectra, as sum of power bins corresponding to the frequencies included within the band of interest. The normalization of individual absolute band powers to the total absolute power results into relative values, permitting comparisons among different animals. Several spectral parameters can be deduced from EEG power spectra, among them theta/delta ratio and mean dominant frequency (MDF) are often utilized. Theta/delta ratio is useful in sleep staging [41]; in general higher values of the ratio correspond to the awake state, while lower values are mostly observed during NREM sleep. MDF is defined as the "center of mass" of a frequency band, and therefore it is calculated as the weighted mean of the frequencies within the band of interest, with their magnitudes as the weights. MDF indicates the shift of the

central frequency within a band toward higher (acceleration) rather than lower (slowing) values, independently of the total power of the band.

Time–frequency analysis can be useful to detect spectral changes throughout long-lasting EEG monitoring periods (Fig. 1C). A compressed representation of the power spectrum (spectral array) can be obtained putting successive spectra on a stack, so that changes in frequency distribution over time can be readily apparent. Two types of spectral array displays are available in commercial instruments: the compressed spectral arrays (CSA) and the density spectral arrays (DSA). The CSA presents the array of power versus frequency versus time data as a pseudo three dimensional perspective plot, whereas the DSA presents the same data as a grayscale-shaded or colored two dimensional contour plot [20]. Although both convey the same information, the DSA is more compact, whereas the CSA permits better resolution of the power data.

2.3. EEG and behavior

As widely demonstrated in humans, even in rodents there is a clear association between regional EEG activity and behavioral states during both physiological and pathological conditions [42–45]. Oscillations provide indices of ongoing neural processing among neurons surrounding the recording site [46,47]. For example, hippocampal theta (4–12 Hz) and gamma (30–120 Hz) oscillations in the hippocampus and prefrontal cortex (PFC) have been correlated with short-term memory processing in rats [47–49], similar to humans [50,51]. On the contrary, pathological disruption of the hippocampus results in altered oscillatory activity, that can impair cognitive ability [47,52–55]. In epilepsy, the relationship between seizures and psychiatric comorbidities is suggested to be bidirectional [56]. According to that, the multidisciplinary approach based on combining video-EEG monitoring analysis and behavioral tests seems to be the best choice to characterize epilepsy-related brain dysfunction, especially when seizures are not yet manifestly evident or during interictal periods. In R6/2 mice it was observed that at the earliest (pre-symptomatic) state of the disease, mice progressively lost their normal diurnal variation in sleep/wake behavior and this was associated with considerable alterations in their sleep EEG [57]. In El mice, a model of multifactorial epilepsy in which seizures are expressed in response to both genetic and environmental antecedent, a power increase within the 1–4 Hz frequency band has been reported during the tail suspension test, suggesting a role for this frequency band in seizure predisposition/induction [58]. An increase in oscillatory activity, within the 5–6 Hz frequency band, has been observed in the knockout mouse for the beta3 subunit of the GABAA receptor treated with diazepam in early life, a mouse model exhibiting spontaneous epilepsy [59]. More recently, high-frequency oscillations (HFOs, 80–500 Hz) have emerged as markers of abnormal neural network activity in both patients and animal models of epilepsy [60–63]. Whereas frequency oscillations in the 100–200 Hz range are believed to represent normal activity [64], several lines of evidence suggest that faster frequency oscillations (200–600 Hz) are generated by action potentials of synchronously bursting principal cells, representing pathological recurrent population spikes [65–67]. However, the relation between HFOs and epileptogenesis has not still been fully elucidated [68].

3. mTOR abnormal activity in the CNS leads to epilepsy

Among the different antiepileptic treatments under investigation, the mammalian target of rapamycin (mTOR) pathway represents a logical candidate, because mTOR regulates multiple cellular functions that may contribute to epileptogenesis, including protein synthesis, cell growth and proliferation, and synaptic plasticity [7]. The importance of the mTOR pathway in epileptogenesis is best illustrated by tuberous sclerosis complex (TSC), one of the most common genetic causes of epilepsy. In mouse models of TSC, mTOR inhibitors prevent the development of epilepsy and underlying brain abnormalities associated with epileptogenesis (Fig. 2). Here we introduce the relevance of video-EEG monitoring associated with behavioral tests as a relevant tool to monitor the effects of rapamycin in TSC mouse models.

3.1. mTOR signaling overview

mTOR is a serine/threonine kinase of the phosphoinositide 3-kinase (PI3K) related kinases and its name is related to its role as the "mammalian Target Of Rapamycin". Rapamycin is a macrolide produced by *Streptomyces hygroscopicus*, discovered in the 18th century on Easter Island, the island later named Rapa Nui by French Polynesian immigrants. mTOR is the catalytic subunit of two distinct complexes called mTORC1 and mTORC2, which are defined by the association of mTOR with unique accessory proteins.

mTORC1 is the best characterized of the two mTOR complexes. An important step forward in understanding mTORC1 regulation came from the discovery that the TSC1 (hamartin)/TSC2 (tuberin) protein complex negatively controls its activity [69]. Various physiological and pathological stimuli converge on this TSC1/TSC2 complex to regulate mTORC1 activity. mTORC1 responds to these upstream signals by modulating multiple downstream pathways, which mediate cellular growth, proliferation, metabolism and survival. As opposed to growth factors and cytokines, many cellular stresses as low energy, hypoxia, and DNA damage act, at least in part, through TSC1/2 to inactivate mTORC1. Once activated, the net effect of mTORC1 signaling is the phosphorylation of downstream proteins that regulate cellular processes involved in protein synthesis and other cellular functions. The best-characterized effectors of mTORC1 are eIF4E binding protein (4E-BP1) and S6 kinase 1 (S6K1) [70].

As compared to mTORC1, little is known about mTORC2 pathway regulation and, in particular, about its upstream activators. In general, mTORC2 promotes cell spreading and F-actin polymerization [71]. In the CNS, mTORC2 controls actin dynamics in neuronal dendritic spine morphology, thus possibly having a role in TSC-related brain manifestation.

3.2. mTOR signaling in the central nervous system

Recent studies between mTOR and neurological disease have demonstrated that mTOR have a significant impact on the nervous system. mTORC1 supports neuronal activity by regulating the translation of specific mRNAs as well as regulation of neurotransmitter receptor expression [72]. In fact, mTOR is required for both (short term) activity-dependent local protein synthesis and (long term) synaptic plasticity [73].

Given the multiple roles of mTOR in the CNS, it is reasonable to think that mTOR pathway could be involved also in epileptogenesis. Particularly, abnormal cell growth due to mTOR activation could affect the excitability of neuronal circuits and promote seizures. In fact, mutations in TSC genes can induce profound alterations in network properties and the imbalance between excitation and inhibition can lead to epilepsy, mental retardation and autism. Finally, mTOR is also a pivotal regulator of the homeostasis of several distinct stem cell pools in which it finely tunes the balance between stem cell self-renewal and differentiation. mTOR hyperactivation in neural stem cells (NSCs) has been etiologically linked to the development of TSC-associated neurological lesions, such as brain hamartomas and benign tumors

[31,74,75]. Thus, mTOR dysregulation in NSCs might contribute to the derangement of their homeostasis, thus leading to tuberous sclerosis complex development.

3.3. "mTORpathies" in the central nervous system

Among the genetic causes of epilepsy, tuberous sclerosis complex (TSC) has provided the strongest link between mTOR and epilepsy. TSC is a common inherited tumor predisposition syndrome, affecting approximately 1 in 7500 individuals [74]. TSC results from mutations of either *TSC1* gene or *TSC2* gene [76]. Individuals with TSC develop benign tumors in multiple organs, including the retina, skin, lung, kidney, and brain. The pathognomonic lesion of TSC is the cortical tuber, a benign growth of immature neuroglial cells in the brain that forms early in development and contain dysmorphic neurons, excessive number of astrocytes and giant cells [77]. The number and location of these tubers correlates with the neurologic complications associated with the disease, including epilepsy, autism, and mental retardation [78]. However, some patients continue to seize after tuberectomy [79].

Individuals with TSC are also predisposed to the development of dysplastic growths called subependymal nodules that arise from cells lining the lateral ventricle [31,75]. While often asymptomatic, subependymal nodules may progress to form subependymal giant cell astrocytomas (SEGAs).

Abnormalities in mTOR signaling that are central to TSC may also be important for epilepsy in other pathologically related tumor syndrome or malformation of cortical development. For instance, patients with *PTEN* mutations have macrocephaly, mental retardation, epilepsy, hamartoma syndrome. Since PTEN is an inhibitor of mTOR, PTEN knockout mice share neurological features that closely resemble the TSC mouse models [80]. Although single gene mutations are rare cause of epilepsy, these genetic disease involving TSC and PTEN mutations demonstrate "proof of principle" that mTOR inhibition may represent an attractive antiepileptogenic strategy and allows the use of the term "mTORopathies" to describe mTOR signaling abnormalities leading to epilepsy and cortical malformations (Fig. 2).

Besides genetic driven epilepsy, mTOR signaling represent a logical mechanism for triggering epileptogenesis in acquired epilepsies. Acquired epilepsy is characterized by an initial insult such as trauma, stroke, or an episode of status epilepticus (SE) that causes subsequent recurrent seizures. The time between the insult and the first seizure is normally referred as the latent period. This latent period is characterized by numerous molecular and cellular abnormalities that could, at least in part, utilize mTOR dependent pathways.

In light of these considerations, experimental evidences for the involvement of mTOR pathway in epileptogenesis and the rationale therapeutic effects of rapamycin have been obtained by different animal models of acquired epilepsy [81]. In the kainate model of acquired epilepsy, a single injection of kainate causes an initial episode of SE, followed by a latent period during which different cellular and molecular mechanisms may contribute to epileptogenesis, as changes in ion channel expression, neuronal death, neurogenesis, synaptic reorganization, axonal sprouting [82]. After this latent period, the animal develops seizures. mTOR is abnormally activated by kainate status epilepticus [22] and in the related pilocarpine model [83]. In the kainate model, there are two phases of transient mTOR activation, an acute phase lasting several hours that occurred during the SE and, after a couple of days, a second longer phase lasting several weeks. Rapamycin administered before SE was able to block both phases of mTOR activation and reduce seizures and consequent neuronal death, mossy fiber sprouting and neurogenesis [22]. Similarly in the in the pilocarpine model, rapamycin can prevent seizures frequency and mossy fiber sprouting [83,84].

The effect of rapamycin in SE-induced models appears to be due to antiepileptogenic and not simply to a seizure-suppressing action. In terms of mechanisms, mossy fiber sprouting, neuronal death, and neurogenesis have all been implied in mediating epileptogenesis and rapamycin may, at least partially, account for the anti-epileptogenic effects [82]. Inhibition of mTOR may also reduce apoptotic pathways potentially contributing to neuroprotective effects, thus protecting the neurons in remaining functional in the face of rising levels of stress in seizures [22,85].

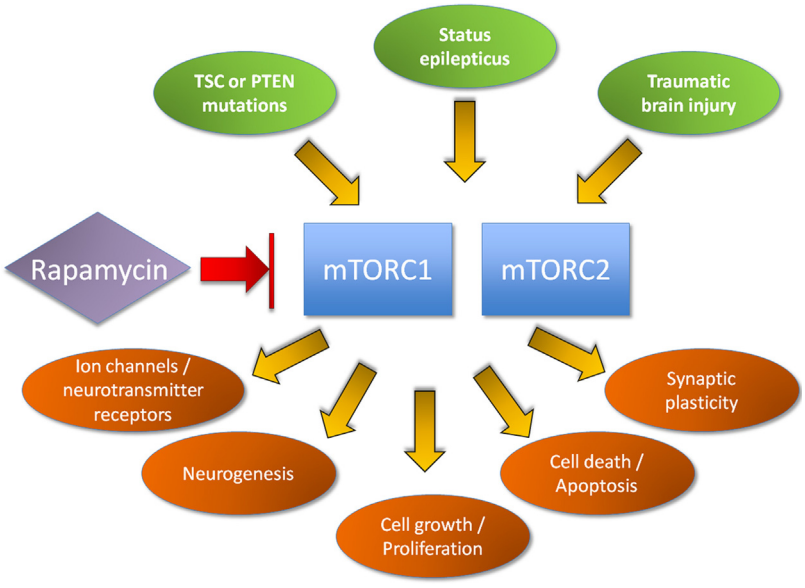

Fig. 2. mTOR and rapamycin: mTOR complexes (mTORC1 and mTORC2) are central signaling hubs for coordinating multiple mechanisms responsible of epileptogenesis. The mTOR signaling pathway may be abnormally activated by a variety of genetic defects or acquired injuries, including upstream TSC or PTEN gene mutations, status epilepticus, or traumatic brain injury. As a consequence, mTOR hyperactivation causes multiple downstream mechanisms causing epileptogenesis through aberrant regulation of ion channels expression, apoptosis, autophagy, axonal sprouting and neurogenesis. Inhibition of mTOR by rapamycin may represent an attractive and effective treatment for preventing epileptogenesis in such a setting.

3.4. Animal models of mTOR signaling abnormalities

Among the genetic causes of epilepsy, TSC remains the prime example of mTOR dysregulation in epileptogenesis. To reproduce temporally and spatially the development of the neurological alterations typical of tuberous sclerosis complex, different CNS-restricted conditional knockout murine models have been generated, by causing loss of either *Tsc1* or *Tsc2* in differentiating or differentiated neuronal cells (Tsc1c/c/Syn-Cre+ and Tsc1c/c/CaMKII-Cre+ mice) [86,87] or in differentiated astrocytes [Tsc1c/c/hGFAP2.2 kb (also known as Tsc1c/c/hGFAP1-Cre+) and Tsc2c/c/hGFAP2.2 kb (also known as Tsc2c/c/hGFAP1-Cre+) mice] [88,89]. Notably, most of these animal models caused by *Tsc1* or *Tsc2* loss show epileptic seizures and neurological abnormalities (Table 1). Given that CTs, SENs and SEGAs are believed to originate from a neural stem cell (NSC) undergoing abnormal differentiation, NSC-targeted mouse models of TSC have also been recently produced by deleting: (1) Tsc2 in embryonic radial glial cells (RGCs) (Tsc2c/−/hGFAP2-Cre+ mice) at embryonic day 13.5 (E13.5) [90] (2) Tsc1 in Emx1-expressing embryonic dorsal telencephalic neuroepithelial progenitors (NEPs) at E9.5 [31,91], (3) Tsc1 in embryonic radial glial cells (RGCs) (Tsc2c/−/hGFAP2-Cre+ mice) at embryonic day 13.5 (E13.5) [75], (4) Tsc1 in embryonic E16.5 progenitors [92] and (5) Tsc1 in postnatal SVZ NSCs [93,94]. Deletion of *Tsc1* or *Tsc2* at different developmental stages results in a gradient of phenotypes, with the most severe phenotypes being associated with mutations in early embryonic neural progenitors [74]. As such, these same CNS restricted TSC mouse models could be exploited to highlight potential genotype-phenotype correlations in TSC.

Among the different models, Tsc2c/−/hGFAP2-Cre [90], in which *Tsc2* mutation occurs in RGCs, recapitulates many aspects of the disease including epilepsy, macrocephaly, cytomegaly, lamination defects and astrogliosis, features that were only partially shown in models induced by mutations in differentiated neuronal and astroglial cells [86–89]. These mice show increased numbers of intermediate progenitors at the expense of post-mitotic neurons, showing that derangement of the homeostasis of neural progenitors due to mTOR activation leads to TSC-associated lesions. However, post-natal SVZ alterations were not documented in this model.

To improve the pathological significance of cell targeting a novel model was generated by deleting *Tsc1* in NEPs [31,91] by using the Emx1Cre donor mice. The *Emx1*-expressing domain gives rise to both the cerebral cortex and the post-natal SVZ [74], which are the two brains regions where tubers and SEN/SEGAs arise. Particularly, Tsc1c/− Emx1-Cre mutant mice recapitulated many aspects of the human disease, including spontaneous epilepsy, cortical lamination defects, hydrocephalus, cytomegaly, macrocephaly, hypomyelination, glia pathology, and, most relevantly, postnatal SVZ abnormalities, which reminded of TSC-associated SENs [31]. Consistent with the role of mTOR in other stem cell compartments, mTOR hyperactivation in NSCs caused enhanced generation of neural stem/progenitors, followed by premature neuronal differentiation and impaired maturation, both during embryonic and postnatal development. Overall, these findings indicate that mTOR is a critical regulator of NSC self-renewal and differentiation of embryonic NEPs/NSCs.

Of note in this model, the functional maturation of the excessively generated mutant neuronal cells was defective, leading to the generation of aberrant neurons and to the development of spontaneous seizures.

3.5. Rapamycin treatment in mouse models of mTOR-dysregulated diseases

The efficacy of mTOR inhibitors in treating epilepsy and neurological lesions associated to TSC has been assessed in several mouse models. Rapamycin treatment reduced the severity of epilepsy, increased survival, reduced cortical cytomegaly and gliosis, improved myelination, increased survival, and restored mTORC1 signaling in several mouse models of TSC [31,75,80,87,95,96]. The effect of blocking the abnormal mTOR activation in the development of epilepsy has been particularly analyzed in knockout mice involving inactivation of the *Tsc1* gene primarily in glial fibrillary acidic protein (GFAP)-positive cells. These mice develop seizures at 4 weeks of age [21]. Early rapamycin treatment started prior to the onset of seizures prevents epilepsy, whereas late treatment reduced seizure frequency in mice that already have epilepsy [96].

However, despite these encouraging results, all these studies clearly indicated the reversibility of rapamycin activity, given that the conditions of mutant mice worsened after rapamycin discontinuation, including the histopathologic abnormalities and epilepsy, leading to death [96]. A study about mTOR inhibition treatment expanded our knowledge. Three different rapamycin treatment regimens – prenatal, postnatal, and pre/postnatal (combined) – were compared in a mouse model of TSC [97]. Combined regimen was the most effective in rescuing brain abnormalities, but surprisingly, it was not beneficial as postnatal regimen in rescuing learning and memory function of mutant animals. Despite the reversibility of rapamycin, its reported effects are of potentially high significance since they appear to be antiepileptogenic and not simply seizures-suppressing. Indeed, in contrast to standard seizure treatments, rapamycin has no a direct effect on neuronal excitability [98] but instead it interrupts gradually the neuropathologic and cellular processes mediating epileptogenesis in these models [95,96].

4. EEG recording in rodents models of epilepsy chronically or sub-chronically treated with rapamycin

Since rapamycin was firstly used for the treatment of epilepsy [96], several chronic or sub-chronic studies have been conducted to test its potential efficacy in different rodent models. However, only few of them went deep into the characterization of both neurophysiological and behavioral analysis. In Table 2, we

Table 1
Phenotypic characterization of most widely used CNS-restricted animal models of mTORopathies showing spontaneous seizures.

Reference	Model	Cell type targeted	Cortical abnormalities	Cellular abnormalities	Lifespan (days)
Uhlmann, 2002, Ann Neurol [88]	Tsc1c/− 2.2Kb hGFAP cre	Astrocytes	No	ND	50
Zeng, 2011, Hum Mol Genet [89]	Tsc1c/c 2.2Kb hGFAP cre	Astrocytes	No	ND	50
Meikle, 2007, J Neurosci [86]	Tsc1c/− Syn cre	Neurons	No	Yes	35
Way, 2009, Hum Mol Genet [90]	Tsc2c/− hGFAP cre	Radial Glia Cells	Yes	Yes	21
Magri, 2011, Cell Stem Cell [31]	Tsc1c/− Emx1Cre mice	Neural Epithelial Progenitors	Yes	Yes	18
Magri, 2013, DM&M [75]	Tsc1c/−/hGFAP2-Cre+ mutant mice	Radial Glia Cells	Yes	Yes	21
Goto, 2011, PNAS [100]	Tsc1c/− NesTA+ TetO–Cre^{+} mutant mice	Radial Glia Cells	No	Yes	21–28
Ljungberg, 2009, DM&M [24]	Ptenc/c Gfap-Cre	Astrocytes	Yes	Yes	70
Zhou, 2009, J Neurosci [101]	Ptenc/c NS-Cre	Neurons	No	Yes	Normal

Table 2
EEG outcomes in animal models of epilepsy sub-/chronically treated with rapamycin.

1st Author year, journal	Animal model	Rapamycin treatment	Neurophysiological and behavioral analysis	Notes
Zeng, 2008, Ann. Neurol [96]	$Tsc1^{GFAP}$CKO mice	3 mg/kg i.p. From 6 weeks (5 days/w)	Video-EEG monitoring 48 h continuous once a week (epidural screws parietal)/ seizures, interictal spike freq, interictal EEG grade (scale of 1–4)	1st paper to use rapamycin in epilepsy. Decreased number of seizures (model characterized in Erbayat-Altay, 2007 Epilepsia)
Zeng, 2009, J Neurosci [22]	Acute kainite induced seizures in rat	6 mg/kg i.p. 24 h after SE daily for 6 days; then every other day for 5 weeks	Video-EEG monitoring (hippocampus/epidural screws frontoparietal and parietal)/ Racine scale	Phenobarbital (30 mg/kg/d i.p. for 6 days) as a control. Rapamycin postponed the onset and decreased the number of seizures.
Ljungberg, 2009, DM&M [24]	NS-Pten mice (cortical dysplasia)	10 mg/kg i.p. From 4 to 5 weeks (5 days/w)	Video-EEG monitoring 2–4 h twice/week from 4 w to 9 w (depidural screws parietal)/ seizure quantification	Rapamycin treatment reduces the severity of electrographic abnormalities. This antiepileptic effect appears to be long lasting, persisting for at least 3w after cessation of the treatment.
Zhou, 2009, J Neurosci [101]	Nse-cre; $Pten^{loxP/loxP}$ (macroceph. autism)	10 mg/kg i.p. From 10–12 weeks for 4–6 weeks (5 days/w)	EEG/EMG (epidural frontal and occipital) from 10 to 12 w for 5 w, 3days/w/OFT and social interaction test	Decreased seizures frequency and duration from 2 w after rapamycin onset. Increased time in the center in the OFT. Increased social interaction (also in presymptomatic group)
Buckmaster, 2011, J Neurosci [28]	Pilocarpine-induced mouse	1.5 or 3 mg/kg i.p. From 24 h after pilocarpine, daily for 2 months	Video-EEG monitoring 9 h/daily for 1 mo, 2 mo after pilocarpine (right, dorsal hippocampus)/ Racine scale	Seizure frequency was similar in vehicle- and rapamycin-treated mice that had experienced status epilepticus. Behavioral seizure severity was similar in vehicle- and 3 mg/kg rapamycin-treated mice
Sunnen, 2011, Epilepsia [26]	NS-Pten mice (cortical dysplasia)	10 mg/kg i.p. (a) w 4–5; (b) w 4–5/10–11/16–17 (5 days/w)	Video-EEG monitoring 4 h at 4, 6 and 9 w (hippocampus/ epidural screws parietal)/ seizure quantification	(a) Decreased epileptiform activity till w10; (b) decreased epileptiform activity till w18 (end of the study); decreased mortality.
Goto, 2011, PNAS [100]	$Tsc1^{cc}$ $Nestin\text{-}rtTA^{+}$ $TetOp\text{-}cre^{+}$/ TSC model mice	PND 8–20 1 mg/kg PND 21 to P40 3 mg/kg i.p. (3 days/w)	Video-EEG monitoring (epidural screw, parietal) PND25 to PND58, over several weeks (16 to 41 h recordings)/ seizure quantification	Increased lifespan (neurological symptoms and death within 2w of wash out). Seizure suppression
Raffo, 2011, Neurobiol Dis [102]	Rat model of infantile spasm (doxorubicin and LPS)	3 mg/kg i.p (a) PND 4–12, 1 mg/kg (b) PND 4–12, 3 mg/kg (c) PND 4 6 mg/kg, P5-6	Video-EEG monitoring (epidural screw, parietal) from PND7/seizure quantification/ reflexes test/Barnes maze	Daily 6 mg/kg resulted in 100% mortality by P12 ($n=4$); dose-related reduction in spasms but no effects on other seizures or interictal abnormalities. Rapamycin improved Barnes maze in Wt.
Sliwa, 2012, Neurosci Lett [103]	Electrical stimulation of the amygdala in rat	6 mg/kg i.p. From 24 h after SE daily for 2 weeks	Video-EEG monitoring 2d-on-2d off (epidural screw, frontal dx and amygdala)	No effects on animals developing epilepsy, latency of the first seizure and frequency.
van Vliet, 2012, Epilepsia [104]	Electrical stimulation of the angular bundle in rat	6 mg/kg i.p. Daily for 7 days after SE; every other day for 5 subsequent weeks	Video-EEG monitoring 24 h/day Seizure scoring/Racine scale	Rapamycin-treated rats developed hardly (9/12) or no (3/12) seizures during the 6-week treatment
Brewster, 2013, PLOS ONE [105]	Pilocarpine-induced rat	6 mg/kg i.p. From 2 weeks after SE every other day for 4 treatments	Video-EEG monitoring (hippocampus/epidural screws parietal)/seizure quantification/MWM/NOR/ Social interaction test	Rapamycin improved memory acquisition in SE rats (no effects on sham). No effects on interictal spike frequency following SE.
Magri, 2013, DM&M [75]	Tsc1c/−/hGFAP2-Cre^{+} mice	6 mg/kg i.p. From P8 to P40 every other day	Video-EEG monitoring from PND12 (depidural screws parietal)/seizure quantification	All mutant mice treated by this regimen stopped developing seizures and were still alive at P40. 10 days after wash out all mutant mice developed seizures
Cambiaghi, 2013, Neuropharm [27]	Tsc1c/− Emx1Cre mice	6 mg/kg i.p. From P8 to P40 every other day	Video EEG monitoring at PND40 (duration 1–2 h). 2 screw parietal electrodes, reference occipital/visual inspection, power spectra, DSA-MDF/behavioral tests: EPM, TST, OFT, FST	Rapamycin-treated mutant mice displayed a reduction in anxiety- and depression-like phenotype, as shown by the EPM/OFT and FST, respectively. These results were inline with EEG power spectra (MDF in the theta-alpha band)

Table 2 (*Continued*)

1st Author year, journal	Animal model	Rapamycin treatment	Neurophysiological and behavioral analysis	Notes
Abs, 2013, Ann Neurol [30]	$Tsc1^{f/2}$::Cag-$CreERT^1$ mice	5–10 mg/kg i.p. At days 1-2-3-4-6-8-10 after seizure onset	Video-EEG monitoring with wireless recording Duration 16 h/Seizures scoring; mean seizure frequency/Interictal spike analysis	Rapamycin treatment fully abolished the seizures

List of abbreviations: EMG, electromyography; EPM, elevated-plus maze; FST, forced-swim test; MWM, Morris water maze; NOR, novel object recognition; OFT, open field test; PND, post-natal day; TST, tail suspension test.

summarized the literature published so far (PubMed 07/2014), according to the following criteria, (i) use of EEG and (ii) chronic/sub-chronic rapamycin treatment in (iii) rodent model of epilepsy). We noticed that EEG or video-EEG monitorings are often limited only to seizure count/duration analysis. In some other cases the video recording is also adopted for better defining Racine scale analysis. In the TSC1-Emx1Cre mice model we analyzed whether chronic rapamycin administration was also associated with changes in behavioral and background EEG features [27]. In this model we observed that rapamycin had a clear effect on reducing depressive-like behavior, also observed in treated wild-type mice but not in heterozygous animals. Similar outcomes were found in an anxiety-like test, although the observed results might be affected by multiple variables, and not exclusively by anxiety. In addition to that, in wild-type animals treated with chronic rapamycin we found a slight but significant decrease in theta-alpha MDF, together with behavioral changes, suggesting a possible mild brain dysfunction associated with drug treatment. Finally, a significant inverse correlation has been found between theta-alpha MDF and the ratio between open arm/total arm entries of the elevated-plus maze (EPM) anxiety-like test. Together these results suggest that both *Tsc1* deletion and chronic rapamycin treatment might have a role in modulating behavioral and brain activity, and point out to the potential usefulness of background EEG analysis in tracking brain dysfunction in parallel with behavioral testing.

However, to our knowledge, we found no evidence for a deeper analysis of the in vivo electrical activity during periods free of seizures in animals treated with rapamycin in other studies. In few of these studies EEG recording and behavioral were conducted, such as depression- or anxiety-like tests (e.g. Forced swimming, Open-field or Sucrose intake), memory (e.g. Novel object recognitio, Morris Water Maze) or social behavior (e.g. Social interaction) tests.

4.1. Local versus surface: clashing or complementary recordings?

Although epidural electrodes are the standard for seizure recording, a more detailed analysis in order to detect the origin and the temporal progression of ictal events should be performed by using intracranial or high-density, multi electrode recordings. According to Table 2, only a minority of studies used deep electrodes in hippocampus, although different areas might be of great interest (e.g. thalamus or deep cortical regions). While scalp (or cortical) recordings might be the best choice in order to detect more global brain effects during chronic treatments (generalized seizures and/or gross alterations), local recordings (e.g. deep electrodes) are essential for spatio-temporal synchronization of distinct brain areas, in particular during inter-ictal periods at various times during chronic therapy. Indeed, different brain areas are usually involved in epilepsy [99] and the possibility to record simultaneously from different areas allows coherence and cross-correlation analysis [47,50,64] for determining the exact cross-talk between them during seizures but, more importantly, in inter-ictal periods and/or during specific behaviors.

5. Conclusions and future direction

Even though EEG monitoring is a technique dating back to nearly one century ago, the possibility to use it in a multidisciplinary approach makes it more attractive for the study of epilepsy. A deeper analysis of LFP recording in animal model of epilepsy chronically treated with antiepileptic drugs (such as rapamycin) should be considered a gold standard in preclinical studies, especially when adopting genetic animal models, for both seizure characterization, background EEG abnormalities and correlation with behavior. Overall outcomes clearly indicate that rapamycin is an ideal drug for chronic tests in animal models of TSC but also in different rodent model of epilepsy. However, results are mixed and some studies reported that rapamycin treatment was not able to ameliorate epilepsy (i.e. seizure frequency). On one hand, these results suggest that a deeper knowledge of rapamycin mechanisms of action in epilepsy is needed. On the other hand, according to the large quantity of positive results, whether not evident in the number/latency of seizure are observed the effect of rapamycin should be tested by background EEG analysis and/or by different behavioral tests, according to the animal model being examined. In addition to that, it would be necessary to develop standard protocols for the analysis of chronic drug treatment in animal models of epilepsy. With regard to chronic rapamycin administration one of the main goals will be to determine well defined time windows and dosage for acute, sub-acute and chronic treatments.

Conflict of interest

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this manuscript is consistent with the Journal's guidelines. All the authors disclose no financial and personal interest.

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