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mTOR inhibition modulates epileptogenesis, seizures and depressive behavior in a genetic rat model of absence epilepsy

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ABSTRACT

Several signaling pathways are believed to be involved in the epileptogenic process that triggers the subsequent changes in the brain causing epilepsy. The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that in the brain, regulates several important physiological functions such as neuronal development and synaptic plasticity, and also seems to be involved in many pathologies, including epilepsy and psychiatric disorders. Previous work in animal models of both genetic and acquired generalized convulsive epilepsies, has suggested that modulators of the mTOR signaling pathway may have beneficial neuroprotective and antiepileptogenic effects. Here, we investigated for the first time, the effect of some treatment schedules (*i.e.* early chronic, sub-chronic and acute) with the specific mTOR inhibitor rapamycin, on the development of absence seizures and seizure parameters as well as depressive-like behavior in WAG/Rij rats, a genetic model of absence epilepsy, epileptogenesis and mild-depression comorbidity. In addition, we studied the possible interaction between rapamycin treatment and the effects of bacterial lipopolysaccharide (LPS) endotoxin administration, which is known to aggravate absence seizures through generation of increased neuroinflammatory responses. We found that rapamycin (early chronic treatment for 17 weeks, starting at P45) exhibited clear antiepileptogenic properties also in this animal epilepsy model; however, this effect was accompanied by unexpected *pro*depressant effects. Both acute and sub-chronic (7 day) treatments also had anti-absence properties, but the sub-chronic treatment produced contrasting *anti*depressant properties in the WAG/Rij rats that were not seen in control Wistar rats. The rapamycin/LPS co-administration studies showed that rapamycin blocked or prevented the LPS-dependent increase in absence seizures, suggesting an anti-inflammatory-like protective action. In conclusion, we have demonstrated a novel antiepileptogenic effect of rapamycin in a well-established animal model of absence epilepsy, and we suggest that this effect may be mediated by the inhibition of inflammatory processes that are developed in the brain of these specific animals during epileptogenesis and during seizures. Our experiments here suggest new insights into this intriguing field, which deserves to be further explored.

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1. Introduction

Epilepsy is one of the most common neurological disorders, affecting approximately 1% (50 million people) of the worldwide population (Ngugi et al., 2011). Most current treatments are symptomatic therapies that suppress seizures without preventing the initial development or progression of epilepsy (Pitkänen and Lukasiuk, 2011). These drugs therefore act primarily as general anticonvulsant or antiseizure agents but do not possess anti-epileptogenic or disease-modifying properties (Temkin, 2001); furthermore, 30% of patients are drug refractory (WHO Epilepsy, 2010). While current antiepileptic drugs (AEDs) mainly target ion channels and/or neurotransmitter receptors that directly contribute to neuronal excitability, more recent research trends have been focused on the identification of primary cell signaling

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pathways that initially trigger various downstream mechanisms mediating epileptogenesis and ultimately a permanent increase in neuronal excitability (Dichter, 2006; Stefan et al., 2006; Pitkänen and Lukasiuk, 2011). Much progress has been made in recent years in identifying potential mechanisms of epileptogenesis, namely the neuropathological changes that occur in nervous system tissue that predispose it to spontaneous seizures. These changes include molecular, cellular, or neuronal network alterations that occur in response to a variety of genetic or environmental insults, and that predispose to seizures via either increased synaptic excitation or decreased inhibition (Dichter, 2006; Stefan et al., 2006; Loscher and Schmidt, 2006; Pitkänen and Lukasiuk, 2011). One signal transduction system that has recently gathered increasing interest as an important regulator of cellular changes involved in epileptogenesis is the mammalian target of rapamycin (mTOR) pathway (McDaniel and Wong, 2011; Russo et al., in press; Zeng et al., 2009a). mTOR is an evolutionarily conserved serine/threonine protein kinase with multiple cellular functions and mechanisms that may influence neuronal excitability and epileptogenesis including protein synthesis, cell growth and proliferation, and synaptic plasticity (Wong, 2010; Sandsmark et al., 2007; Tsang et al., 2007; Huang and Manning, 2008). It is expressed in many tissues, including the brain (Kim et al., 2002), where its activation is controlled by a variety of extracellular stimuli, including glutamate (Cammalleri et al., 2003; Hou and Klann, 2004; Gong et al., 2006) and brain-derived neurotrophic factor (BDNF) (Takei et al., 2004), or intracellular signals, including nutrient and energy status, growth factors and stress (Bolster et al., 2002; Inoki et al., 2002; Kimura et al., 2003). Therefore, mTOR is increasingly recognized to be involved in a large spectrum of physiologic functions under normal conditions and to be dysregulated in a diverse group of human diseases such as cancer, cardiovascular diseases, diabetes, obesity, and neurological disorders (Tsang et al., 2007; Malagelada et al., 2006; Ravikumar et al., 2004; Swiech et al., 2008).

While the most extensively studied roles of mTOR relate to cell growth, proliferation and survival or death, mTOR also appears to be important in numerous other cellular functions. It is also involved in numerous brain functions that can affect neuronal signaling and excitability, such as axonal and dendritic morphology, neurotransmitter receptor expression, synaptic plasticity and memory formation (Garelick and Kennedy, 2011; Russo et al., in press). With regard to epilepsy, mTOR dysregulation has been demonstrated in a variety of genetic and acquired epilepsies, including Tuberous Sclerosis Complex (TSC), focal cortical dysplasias, and animal models of brain injury due to *status epilepticus* or trauma (Zeng et al., 2008; Wong, 2010: for reviews see Cho, 2011; Crino, 2011; Galanopoulou et al., 2012; Russo et al., in press; van Vliet et al., 2012).

Rapamycin (also known as sirolimus) is a lipophilic macrolide antibiotic initially developed as an antifungal agent, but fell out of favor when it was found to have potent immunosuppressive activity. In subsequent decades, the discovery of mTOR led to the elucidation of rapamycin's mechanism of action as a specific mTOR inhibitor (Zhang et al., 2011). Several studies have recently demonstrated mTOR hyperactivation after *status epilepticus* induced by kainate (Zeng et al., 2009b), pilocarpine (Buckmaster et al., 2009; Huang et al., 2010) or electrical stimulation of angular bundle (van Vliet et al., 2012). Rapamycin, administered prior to the onset of spontaneous seizures, retards the development of epilepsy suggesting an antiepileptogenic action in the kainate model (Zeng et al., 2009b). mTOR is also triggered in the pilocarpine model of acquired epilepsy and mediates axonal sprouting, a putative mechanism of epileptogenesis. Accordingly, rapamycin pretreatment is able to prevent mossy fiber sprouting after pilocarpine-induced *status epilepticus*, thereby suppressing spontaneous seizure development (Buckmaster et al., 2009; Huang et al., 2010), similarly, in the study by van Vliet et al. (2012), rapamycin post-SE treatment reduces the development of seizures. It therefore appears from these animal studies of convulsive epilepsy, rapamycin treatment can prevent epileptogenesis (Huang et al., 2010; Zeng et al., 2008); thus, the long-term effects of rapamycin treatment in other seizure models are certainly worthy of further investigation. However, in a recent report (Sliwa et al., 2012), *post*-treatment with rapamycin proved ineffective in preventing epileptogenesis in the amygdala stimulation model of temporal lobe epilepsy and epileptogenesis, suggesting that mTOR might not have a universal role in this phenomenon and might therefore be limited to certain types of epilepsy and epileptogenic processes. Finally, there is also a rapidly growing body of evidence that supports the involvement of brain inflammation and specific inflammatory mediators in both the origin of individual seizures and the epileptogenic process (Vezzani et al., 2011; Vezzani and Friedman, 2011). Since it is known that mTOR is essential for the survival, cytokine production and migration of neutrophils and mast cells (Battaglia et al., 2006; Gomez-Cambronero, 2003; Kim et al., 2008; Weichhart and Säemann, 2009) and that mTOR signaling selectively controls microglial activation and viability in response to pro-inflammatory cytokines, it is conceivable that rapamycin is effective in convulsive epilepsy and epileptogenesis models by primarily controlling neuroinflammation (Dello Russo et al., 2009). The question of whether mTOR inhibition can also prevent other forms of epileptogenesis, specifically the generation of *non*-convulsive absence seizures (Avoli and Gloor, 1982; Castro-Alamancos, 2000: for recent review see Yalçın, 2012), and whether this process could also be mediated by modulation of brain inflammatory responses however, remains completely unanswered.

To address these points, we have examined the effects of various rapamycin treatment protocols (acute, sub-chronic and chronic) on spontaneous slow wave discharge (SWD) seizures and epileptogenesis in WAG/Rij rats, a well-validated model of absence-type epilepsy (Danober et al., 1998; Coenen and Van Luijtelaar, 2003; Shaw et al., 2009). Furthermore, we studied the effects of rapamycin on the increase in absence seizures that can be induced in these animals by the pro-inflammatory endotoxin lipopolysaccharide (LPS) (Kovács et al., 2011). Interestingly, WAG/Rij rats also exhibit low grade depressive-like behavior (dysthymia) (Shaw et al., 2009; Sarkisova and van Luijtelaar, 2011) and it has previously been demonstrated that an early long-term pretreatment with some AEDs, suppresses the onset of absence seizures (anti-epileptogenic effects) and also the development of depressive characteristics (Blumenfeld et al., 2008; Russo et al., 2010, 2011a,b). Since the etiology of depression has recently been linked with neuroinflammation and microglial activation (Hashioka, 2011; Steiner et al., 2011), as well as dysregulation of mTOR signaling (Jernigan et al., 2011; Koike et al., 2011), we also investigated whether rapamycin could influence symptoms of depressive-like behavior in this model. We found that rapamycin possesses anti-absence and anti-inflammatory properties, but variable effects on depressive behavior. Some possible interpretations of these data are discussed.

2. Materials and methods

2.1. Animals

Male WAG/Rij rats and age-matched Wistar rats were used. Rat progenitors were purchased from Charles River Laboratories s.r.l. (Calco, Lecco, Italia) at a body weight of ~60 g (4 weeks old). Following arrival, animals were housed three/four per cage and kept under controlled environmental conditions (60 ± 5% humidity; 22 ± 2 °C; 12/12 h reversed light/dark cycle; lights on at 20.00). Female rats of all strains at 10 weeks of age were placed with same-age group males for mating for 16 h in the ratio 1:1 and examined the next morning for the presence of a vaginal plug, a sign of successful copulation. Dams of all strains were housed 2 per cage,

whereas, all offspring after weaning (P21) were housed three/four per cage. Animals were allowed free access to standard laboratory chow and water until the time of experiments. Procedures involving animals and their care were conducted in conformity with the international and national law and policies (European Communities Council Directive of 24th November 1986, 86/609EEC). All efforts were made to minimize animal suffering and to reduce the number of animal used.

2.2. Experimental summary

The present work involved three different and correlated experiments (see below for details and Scheme 1) to clarify the effects of rapamycin on the epileptogenic process underlying the *development* of absence seizures in WAG/Rij rats (**experiment #1**) and also its acute effects on *established* (chronic) absence seizures in the same model (**experiment #2**). Furthermore, the possible involvement of mTOR signaling on neuroinflammatory processes was evaluated by testing rapamycin treatment *vs.* absence seizures increase induced by LPS (**experiments #2** and **#3**). Finally, we tested the effects of various rapamycin treatment schedules on depressive-like behavior both in WAG/Rij and Wistar rats. In details, in experiment #1, WAG/Rij and Wistar rats were chronically treated as previously described (Russo et al., 2011a,b; see Section 2.4) and at the end of the treatment protocol a group of WAG/Rij rats underwent EEG recordings for the quantification of absence seizures. Recordings were also performed 5 months after the end of treatment to study the temporal duration of the effects; whereas, the remaining rats of both strains were subjected to behavioral tests for the evaluation of depressive-like behavior (forced swimming test and sucrose consumption test; see Section 2.8). Concomitantly, rapamycin blood concentrations were measured throughout the treatment. Experiment #2 measured the effects of rapamycin acutely administered (intraperitoneal administration of various doses) on both chronic absence seizures and depressive-like behavior (forced swimming test only) in WAG/Rij rats at the age of 6 months; furthermore, we have considered the effects of rapamycin on LPS-dependent increase in seizures parameters (Kovács et al., 2011). Experiment #3 aimed to evaluate the effects of a sub-chronic treatment (7 days) on both absence seizures and depressive-like behavior; moreover, it was evaluated the ability of rapamycin sub-chronic pretreatment to prevent LPS-dependent absence seizures increase (Scheme 1).

2.3. Animal surgery and EEG recording

WAG/Rij rats around the age of 6 months were chronically implanted, under anesthesia obtained by administration of a mixture of tiletamine/zolazepam (1:1; Zoletil 100®; 50 mg/kg i.p.; VIRBAC Srl, Milan, Italy), using a Kopf stereotaxic instrument, with five cortical electrodes for EEG recordings (Russo et al., 2004). Stainless-steel screw electrodes were implanted on the dura mater over the cortex: two in the frontal region (AP = 2 mm; $L = \pm 2.5$ mm) and two in the parietal region (AP = −6 mm; $L = \pm 2.5$ mm) according to the atlas coordinates of Paxinos and Watson (1998). The ground electrode was placed over the cerebellum. Rats of the LPS co-administration group were additionally implanted with a guide cannula into the lateral ventricle (AP = −0.8 mm; L = 1.4 mm; H = 3.5 mm) for intracerebroventricular (i.c.v.) administration (Russo et al., 2008). All animals were allowed at least 1 week of recovery and handled twice a day. In order to habituate the animals to the recording conditions, the rats were connected to the recording cables, for at least 3 days before the experiments. Every EEG recording was always performed starting at 9.00 a.m. in order to avoid circadian alterations within groups. The animals were attached to a multichannel amplifier (Stellate Harmonie Electroencephalograph; Montreal, Quebec, Canada) by a flexible recording cable and an electric swivel, fixed above the cages, permitting free movements for the animals. All EEG signals were amplified and conditioned by analog filters (filtering: below 1 Hz and above 30 Hz at 6 dB/octave) and subjected to an analog-to-digital conversion with a sampling rate of 300 Hz. The blinded quantification of absence seizures was based on the number and the duration of EEG SWDs, as previously described (Gareri et al., 2005; Russo et al., 2011a,b).

2.4. Chronic treatment procedure

Rapamycin (LC Laboratories A Division of PKC Pharmaceuticals, Inc. USA) was first dissolved in a minimal amount of ethanol (maximum final dilution <0.1%) and then further diluted to the desired final volume with a solution of tap water: 5% Tween 80: 5% PEG 400 (vehicle). Rats were treated orally (oral-swallowing: o.s.) with rapamycin at a dose of ~1 mg/kg/day. The dose was calculated on the evidence that rats drink on average 12 ml/100 g/day (Russo et al., 2010, 2011a,b); this was further confirmed by checking the volume drunk by rats. Rapamycin treatment was started in rats at 45 days of age (n = 20 WAG/Rij rats and n = 10 Wistar rats) and continued for a further ~17 weeks until the age of ~5 months, when it was stopped. Water bottles were protected from light by wrapping in silver foil, and freshly prepared solutions were replaced twice a week. Thereafter, the animals continued to be normally housed without treatment. Age-matched control rats (n = 20 WAG/Rij rats and n = 10 Wistar rats) were kept under the same housing conditions over the same period of time with vehicle (see above). During this period, animals were weighed weekly every Monday between 9:00 and 11:00 a.m. and every month, a blood sample of ~1 ml was obtained through the tail vein for later analysis of drug blood concentrations from 3 rats of rapamycin-treated groups in a randomized order. Animals were only gently restrained during this process. Furthermore, particular attention was paid to the possible appearance of any obvious drug-induced side-effects such as slower weight gain reported in other studies (Zeng et al., 2008). At the age of ~6 months, after surgery, 10 WAG/Rij rats from each group (treated and untreated) underwent three recording periods, starting at 9.00 a.m., for 3 consecutive days, within 30 and 36 days after treatment discontinuation. Every recording session lasted 3 h without administration of any drug in either group. The same recording schedule was performed at the age of 10 months in the same animals in order to evaluate the duration of the effects, since it was previously demonstrated in other animal models of epilepsy, that after the end of rapamycin

Scheme 1
Experimental Protocol.

Animals	Groups	EEG	SCT + FST
Experiment #1: Chronically treated rats			
WAG/Rij rats	RAP 1 mg/kg/day os for 17 weeks N = 20	N = 10 both at 6 months and 10 months of age	–
N = 40		–	N = 10 both at 6 months and 10 months of age
	Untreated CTRL N = 20	N = 10 both at 6 months and 10 months of age	–
		–	N = 10 both at 6 months and 10 months of age
Wistar rats	RAP 1 mg/kg/day os for 17 weeks N = 10	–	N = 10 both at 6 months and 10 months of age
N = 20	Untreated CTRL N = 10	–	N = 10 both at 6 months and 10 months of age
Experiment #2: Acutely treated rats			
WAG/Rij rats	RAP 0.1, 0.25, 0.5, 1 and 3 mg/kg i.p.	N = 5 for each dose or vehicle	–
N = 50	RAP 0.1, 1 and 3 mg/kg i.p.	–	N = 5 for each dose or vehicle
Wistar rats	RAP 0.1, 1 and 3 mg/kg i.p.	–	N = 5 for each dose or vehicle
N = 20			
WAG/Rij rats	LPS 3 μg/rat in 5 μl saline i.c.v.	N = 5	–
N=25	5 μl saline i.c.v.	N = 5	–
	LPS 3 μg/rat in 5 μl saline i.c.v. + (30 min later) RAP 0.5, 1 and 3 mg/kg i.p.	N = 5 for each dose of RAP	–
Experiment #3: Sub-chronically treated rats			
WAG/Rij rats	RAP 0.5, 1 and 3 mg/k/day os for 7 days	N = 5 for each dose or vehicle;	–
N = 40		EEG at the end of treatment and after 30 days	
	RAP 0.5, 1 and 3 mg/kg/day os for 7 days	–	N = 5 for each dose or vehicle; tests at the end of treatment and after 30 days
Wistar rats	RAP 0.5, 1 and 3 mg/kg/day os for 7 days	–	N = 5 for each dose or vehicle at the end of treatment and after 30 days
N = 20			
WAG/Rij rats	RAP 0.5, 1 and 3 mg/kg/day os for 7 days + on 7th day LPS 3 μg/rat in 5 μl saline i.c.v.	N = 5 for each dose of RAP	–
N = 30	RAP 0.5, 1 and 3 mg/kg/day os for 7 days + on 7th day 5 μl saline i.c.v.	N = 5 for each dose of RAP	–

treatment, both seizure protection and other drug effects disappeared (for a review see Russo et al., in press).

2.5. Acute and sub-chronic rapamycin treatment

WAG/Rij rats ($n = 30$) of ~6 months of age and a body weight of ~280 g were intraperitoneally (i.p.) administered with different doses of rapamycin (0.1–3 mg/kg) in order to determine whether microinjection of this compound might significantly influence the number and duration of SWDs. Every EEG recording session lasted 5 h: 1 h baseline without injection, and 4 h after the i.p. administration of vehicle or rapamycin. Separate groups of rats ($n = 5$ for each dose) were used to determine the effects of vehicle (1% DMSO: 5% Tween 80: 5% PEG 400 in distilled water) and rapamycin. A separate group of rats ($n = 4$) was used to determine the blood levels of rapamycin (1 mg/kg) after acute i.p. administration, a blood sample was obtained as described in Section 2.4 1 h after injection.

To evaluate the effects of a short-term rapamycin treatment (sub-chronic), different groups of rats ($n = 5$ for each dose or vehicle) were orally treated (rapamycin doses 0.5, 1 and 3 mg/kg o.s.; see Section 2.4 for oral administration procedure) for 7 consecutive days, starting treatment one week after surgery for electrode implantation. Starting on the 8th day, rats of the sub-chronic treatment group underwent three recording periods as described for the chronically treated groups in three different days, then treatment was suspended; the same animals were also re-recorded 30 days after the end of treatment.

2.6. Tissue preparation and immunohistochemistry

Six male WAG/Rij rats (6 months old) and 6 male Wistar rats (6 months old) were anesthetized as described in Section 2.3 and sacrificed by transcardiac perfusion with cold PBS, pH 7.4 and subsequently with cold 4% paraformaldehyde (PFA) containing 0.2% saturated picric acid in PBS. Brains were removed, post-fixed overnight at 4 °C in the same fixative solution, then the brain was cut at bregma level according to Paxinos and Watson (1998). Paraffin embedded sections were cut in a coronal plane at a thickness of 4 μm by a microtome. The de-paraffined sections were rinsed in PBS twice and immersed in 0.3% H_2O_2 in PBS for 10 min followed by three rinses in PBS, as previously described (Citraro et al., 2006a). The sections were blocked with 10% goat serum (GIBCO BRL) in PBS for 1 h and incubated overnight at 4 °C with either Anti-mTOR antibody (ab51089) or Anti-mTOR (phosphor S2448) antibody (1:100; Abcam® plc, Cambridge, United Kingdom) followed by peroxidase-conjugated goat anti-rat IgG (1:300; Jackson Immuno Research Laboratory) for 3 h at room temperature. The sections were rinsed in acetate-imidazole buffer (50 mM sodium acetate, 10 mM imidazole, pH 7.2 adjusted with glacial acetate acid) and developed in chromagen solution (0.04% diaminobenzidine; DAB), 2.5% $NiSO_4$ and 0.005% H_2O_2 in acetate-imidazole buffer, pH 7.2 for 10–20 min. Following a series of washes in acetate-imidazole buffer, sections were mounted and coversliped. The immunoreactivity of the sections was analyzed under the microscope.

Immunoreactivity of mTOR was determined by visual inspection of stained sections using light microscopy at 4–40× magnification. A semi-quantitative analysis of the stained sections has also been performed. Three independent specialists in immunohistochemistry, blind to the strain, have performed a microscopical inspection of the selected brain areas (Cortex, hippocampus, thalamus, nucleus reticulari thalami) and have given a value to the different areas according to the following scale: (0) no reactivity; (1) low reactivity; (2) marked staining; (3) very marked staining.

2.7. LPS co-administration protocol

LPS (3 μg/rat in 5 μl saline; Sigma-Aldrich Co. Ltd, Poole, U.K.; E. Coli, serotype O111:B4) was centrally infused as previously described (Kovács et al., 2011) through the guide cannula implanted in the lateral ventricle (see below) in a volume of 5 μl via a Hamilton syringe connected to a CMA/100 infusion pump at a flow rate of 0.5 μl/min (Citraro et al., 2006a,b; Russo et al., 2008). The injection cannula was withdrawn 1 min following infusion. Rapamycin (0.5, 1 and 3 mg/kg; i.p.) was administered 30 min later. Different groups of rats ($n = 5$ for each group) were used for every rapamycin dose.

To evaluate the effects of the sub-chronic rapamycin treatment on the LPS-induced increase in absence seizure parameters (Kovács et al., 2011), different groups of rats ($n = 5$ for each group) were orally treated as above described (see Section 2.5) and were i.c.v. infused with LPS on the 7th day of rapamycin treatment. In the co-administration group, recordings were subdivided as follows: 1 h baseline without injection, i.c.v. LPS injection and 30 min later, rapamycin administration. The sub-chronically treated animals were LPS i.c.v. injected on the 8th day of treatment and recorded 1 h before LPS infusion and for the following 3 h after administration.

2.8. Tests for depressive-like behavior

WAG/Rij rats exhibit many depression-like behavioral symptoms under baseline conditions (in addition to absence epilepsy), and were also recently validated as a useful test model of human low-grade depression (dysthymia), which can be reversed by chronic antidepressant treatment (Sarkisova and van Luijtelaar, 2011). Ten animals of every experimental group were tested, first in the sucrose consumption test and then in the forced swimming test (FST), in order to evaluate the effects of: 1) rapamycin long-term treatment on the development of depressive-like behavior; 2) the acute effects of rapamycin; 3) the effects of the sub-chronic rapamycin treatment. For the purpose of testing the possible behavioral effects of rapamycin in healthy animals, control Wistar rats were also treated with rapamycin following the same protocol used for WAG/Rij rats.

2.8.1. Sucrose consumption test

The sucrose-consumption test is a measure of the '*hedonic*' state of an animal, or the ability to experience pleasure. Its impairment is a fundamental feature of clinical depression (Jones et al., 2008). We have followed the previously used protocol by Sarkisova et al. (2010) for WAG/Rij rats. Briefly, each rat was placed in a test cage, identical to the home cage and the fluid intake (consumption of 20% sucrose solution) was measured by re-weighing preweighed bottles at the end of the test in five consecutive days. Prior to testing, animals were not food- or water-deprived. The values of fluid intake on the 5th day were used for statistical evaluation of differences between groups of rats (Sarkisova et al., 2008, 2010). Since the test was repeated on 5 consecutive days, acute rapamycin effects could not be measured according to repeated treatment (Russo et al., 2013).

2.8.2. Forced swimming test

The FST (Porsolt et al., 1977) is currently the most reliable method for assessing behavioral "despair" and for screening antidepressant drug action in rodents, by measuring the immobility time (IT). We have used a modified version of the test according to Sarkisova et al. (2010) (see also Russo et al., 2011a,b, 2013). The initial 15-min habituation session was excluded and only the 5-min test was carried out in order to avoid possible influences on memory processes by the drug. Animals were individually forced to swim in a clear plastic cylinder (47 cm in height; 38 cm in diameter) containing 38 cm of water (25 ± 1 °C). Test sessions were video-recorded by means of a digital camera (Medi@com Sport Cam Plus; resolution: 640 × 480 – 30 fps) fixed above the cylinders; the obtained files were later analyzed by three independent observers, two of them blind to the treatment protocol. The total duration of immobilization, including passive swimming, was measured. The criterion for passive swimming was floating vertically in the water while making only those movements necessary to keep the head above the water. After the FST, animals were removed and dried with a towel before being placed in their home cages. Every experimental animal group was evaluated in the test starting at 9.00 a.m. and finishing before 11.00 a.m. in order to avoid possible circadian alteration of test results (Russo et al., 2011a,b).

2.9. Determination of drug blood concentrations

Rapamycin blood levels were determined using an Architect i1000sr Immunology Analyzer (Abbott Laboratories, Abbott Park, IL, U.S.A.; Courtais et al., 2010). The Abbott ARCHITECT-i1000 sirolimus assay is a delayed one-step chemioluminescent microparticle immunoassay (CMIA) routinely run in the laboratories of the Clinical Pharmacology Unit at Azienda Ospedaliera "Mater Domini" of Catanzaro. Sample preparation was performed as previously described (Russo et al., 2010, 2011a,b). Analysis of drug blood concentrations was done every month for 4 months (2–5 months of age) of pharmacological treatment on 3 rats randomly selected in the treated groups as above described and on 4 rats i.p. administered with a dose of 1 mg/kg of rapamycin.

2.10. Statistical analysis

All statistical procedures were performed using SPSS 15.0.0 software (SPSS Inc., Chicago, Illinois, USA). EEG recordings were subdivided into 30 min epochs, and the duration and number of SWDs were treated separately for every epoch. Such values were averaged and data obtained were expressed as mean ± S.E.M. for every dose of compound. Long-term treated animals were compared by one-way analysis of variance (ANOVA), the treatment being the only variable, followed by a post-hoc Bonferroni test. The significance of a drug's acute effects in control animals was determined by one-way ANOVA followed by Bonferroni's post-hoc test. Immobility times in the FST were compared by one-way ANOVA followed by Bonferroni's post-hoc test. All tests used were two-sided and $P \leq 0.05$ was considered significant.

3. Results

3.1. Effects of early long-term pretreatment with rapamycin on the development of SWDs

Analysis of EEG recordings from control adult WAG/Rij rats at 6 months of age revealed that the mean number of SWDs (nSWDs) for a 30-min epoch was 8.99 ± 1.72 with a mean total duration (dSWDs) of 38.02 ± 7.01 s and a mean single duration (sSWD) of

4.14 ± 0.48 s (Table 1). Early long-term pretreatment with rapamycin at a dose of ~1 mg/kg/day was able to significantly ($P < 0.05$) reduce the development of absence seizures in adult WAG/Rij rats, compared with untreated controls. nSWDs and dSWDs were respectively decreased by 51.17% ($P = 0.0001$) and 32.14% ($P = 0.005$); however, sSWDs were not significantly modified (−2.41%; $P = 0.11$; Table 1). When the same animals were recorded at 10 months of age (5 months after the end of rapamycin treatment), absence seizure parameters were still consistently reduced similarly to the observed effects at 6 months of age (Table 1), suggesting a persistent drug effect.

Animal growth was very similar within the groups, with no significant differences between control and rapamycin-treated animals over the 17-week treatment period (data not shown). Blood drug concentrations were monitored every month in drug-treated animals. The mean rapamycin blood concentration was very similar every month and no differences were observed between WAG/Rij and Wistar rats; we measured a total mean concentration of 0.88 ± 0.04 ng/ml.

3.2. Effects of acutely administered i.p. rapamycin on absence seizures

The i.p. administration of rapamycin (0.25, 0.5, 1 and 3 mg/kg) in adult WAG/Rij rats ($n = 5$ animal for each dose) was able to induce significant ($P < 0.05$) changes in the number and total duration of SWDs in comparison to control group, whereas the lowest dose tested (0.1 mg/kg) showed no effects (Fig. 1). At the higher doses (0.5, 1 and 3 mg/kg), rapamycin induced a significant ($P < 0.05$) decrease in the number (~−60%) and duration (~−70%) of SWDs compared with control, but these effects were not dose-dependent (Fig. 1). The decrease in the number and duration of SWDs was already evident 30 min after administration of the two highest doses and lasted for the entire time-window of recordings (up to 3 h after injection; Fig. 1). The dose of 0.25 mg/kg also significantly ($P < 0.05$) reduced the number (~−30%) and duration (~−30%) of SWDs, but only during the 3rd hour of recording. The blood concentration of rapamycin (1 mg/kg), 1 h after after injection was 17.66 ± 0.13 ng/ml.

3.3. Effects of a sub-chronic oral rapamycin treatment on absence seizures

EEG recordings from WAG/Rij rats treated sub-chronically with oral rapamycin (o.s.; 0.5, 1 and 3 mg/kg) also showed a significant ($P < 0.05$) reduction in both the number and duration of SWDs; however, the short-term oral treatment appeared more efficacious than acute i.p. rapamycin injection. Once again, the effects rapamycin at these doses, were not dose-dependent and a steady decrease of ~−64% and ~−59% for number and duration of SWDs respectively was observed (Fig. 2). No differences in EEG parameters were noted when statistically comparing the three days of recordings. When the same animals were re-recorded 30 days after the end of treatment, no significant differences were observed between controls and animals previously treated with rapamycin (data not shown).

Table 1
Effects of early long-term pretreatment with rapamycin on SWD parameters recorded in WAG/Rij rats both at 6 months and 10 months of age.

Animal group ($N = 10$)	nSWDs	dSWDs (s)	sSWD (s)
Recordings at 6 months of age (1 month after the end of rapamycin treatment)			
Control group	8.99 ± 0.59	38.02 ± 7.01	4.14 ± 0.48
Rapamycin-treated group (~1 mg/kg/day)	4.39 ± 0.48*	25.8 ± 4.90*	4.04 ± 0.57
Recordings at 10 months of age (5 months after the end of rapamycin treatment)			
Control group	10.42 ± 1.72	48.26 ± 10.01	4.69 ± 0.28
Rapamycin-treated group (~1 mg/kg/day)	5.33 ± 0.46*	24.31 ± 2.48*	4.13 ± 0.2

Rapamycin treatment lasted up to the age of 5 months; therefore for 6 months recordings, there was a 1 month drug-free period, while for 10 months recordings, it was 5 months. nSWDs indicate the mean number of SWDs for every 30-min epoch; dSWDs indicate the mean cumulative duration of SWDs for every 30-min epoch expressed in seconds; sSWD indicates the mean duration of a single SWD expressed in seconds. Data are expressed as mean ± standard error of the mean (SEM) obtained by analyzing three different recordings from every single animal in three consecutive days, with a total recording duration of 3 h. Data marked with (*) are significantly different ($P < 0.05$) from respective controls.

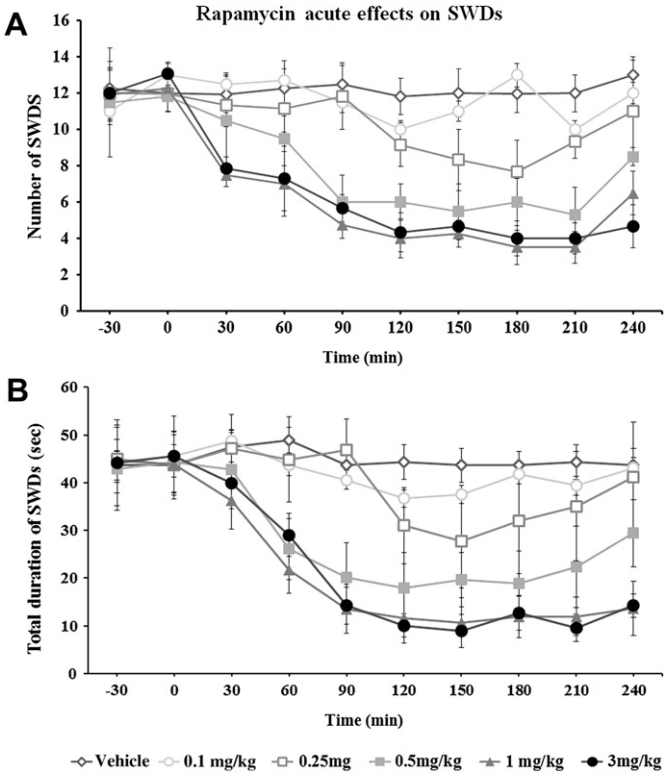

Fig. 1. Effects of acute systemic administration of rapamycin (RAP) in WAG/Rij rats (non-pretreated). Plots show time- and dose-dependent effects of RAP on the number (A) and duration (B) of epileptic SWDs. Data values are means ± SEM ($n = 5$ for each dose).

3.4. mTOR immunostaining

mTOR staining in Wistar rats was marked in the cortex (Score 1.83) and very low (below score 0.6) in the other brain areas considered. WAG/Rij rats, in comparison to control non-epileptic rats, have more mTOR staining in every area, in particular, the cortex and the hippocampus have values higher than 2 on our scale (marked staining) (Fig. 3). Phospho-mTOR was instead more marked only in the cortex with marked levels in WAG/Rij rats (Score 2.27) and low staining in all other areas and no differences between Wistar and WAG/Rij rats (Fig. 3).

3.5. LPS and rapamycin co-administration in acute and sub-chronic paradigms

The i.c.v. administration of LPS in WAG/Rij rats, induced a significant *increase* in both the number and duration of SWDs for up to 210–240 min as previously described (Kovács et al., 2011) (Fig. 4). i.p. administration of rapamycin (0.5, 1 and 3 mg/kg) 30 min after LPS i.c.v. infusion was able to revert the LPS-dependent

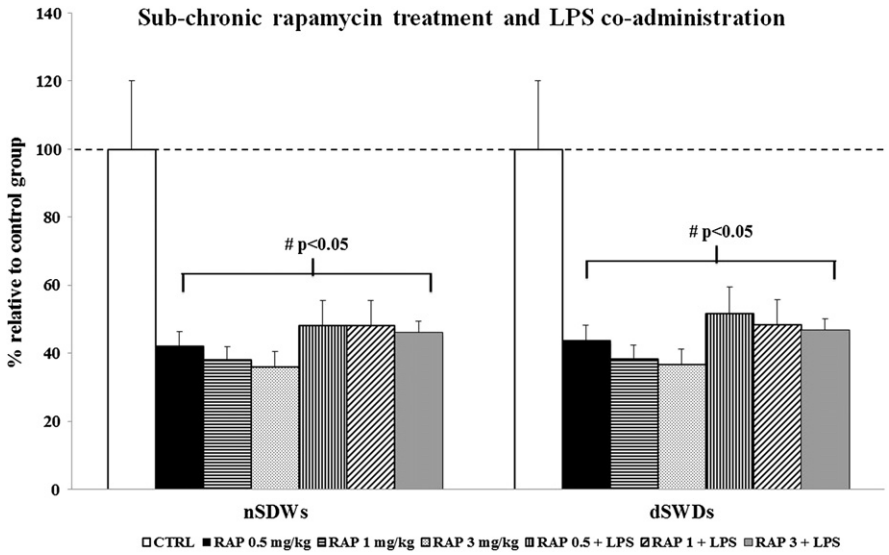

Fig. 2. Effects of a sub-chronic (7 days) treatment with rapamycin or its co-administration with LPS on absence seizures in WAG/Rij rats. Bars indicate seizure parameters (SWDs number and duration for a 30 min epoch). Data are expressed as percentage relatively to control group (first bar on the left of every group). Data values are means ± SEM ($n = 5$ for each dose). # Significantly different ($P < 0.05$) from age-matched control (untreated) WAG/Rij rats. RAP = rapamycin; CTRL = control rats; nSWDs = mean number of SWDs; dSWDs = mean cumulative duration of SWDs, expressed in seconds; LPS = lipopolysaccharide 3 μg/5 μl/rat.

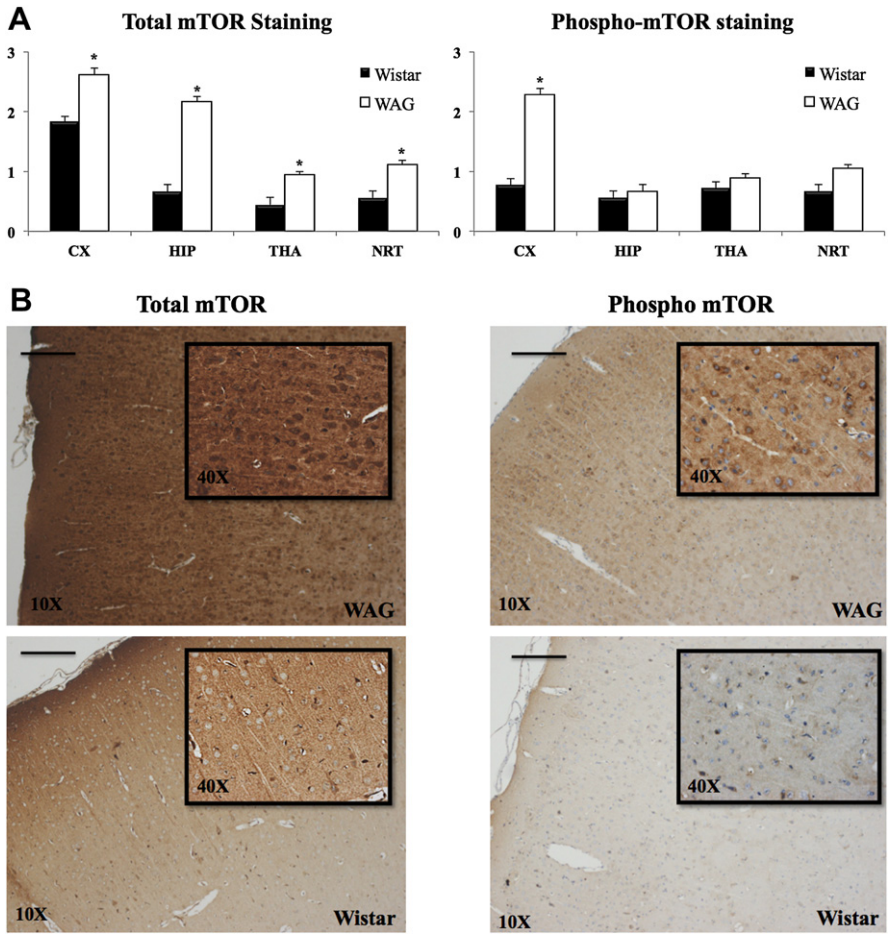

Fig. 3. Immunostaining for mTOR in the brain of WAG/Rij and Wistar rats at the age of 6 months. A) Graph bar for total- (left) and phospho- (right) mTOR values in different brain areas obtained by averaging the scores (see scale on Section 2.6) given to sections by three independent and blinded observers. CX = cortex; HIP = hippocampus; THA = thalamus; NRT = nucleus reticularis thalami; WAG = WAG/Rij rats; *Significantly different ($P < 0.05$) from Wistar rat controls. B) Representative images of immunostained sections of cortices from both WAG/Rij and Wistar rats. Scale bar is 100 μm for 40× magnification images and 250 μm for 10× magnification images. Small boxes are magnifications (40×) of the original 10× images.

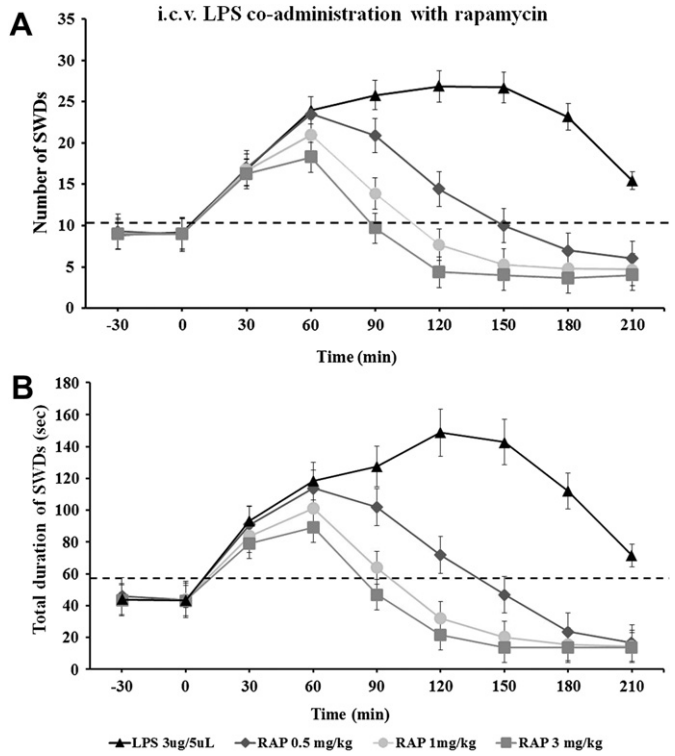

Fig. 4. Effects of acute systemic administration of rapamycin (RAP) 30 min after lipopolisaccharide (LPS) administration in WAG/Rij rats. Plots show time- and dose-dependent effects of LPS alone or in combination with various doses of RAP on the number (A) and duration (B) of epileptic SWDs. Data values are means ± SEM ($n = 5$ for each dose).

increases in nSWDs and dSWDs (Fig. 4). Very surprisingly, this observed reversal effect of rapamycin was dose-dependent, in contrast to the effects of rapamycin administered alone at the same doses (see Fig. 1). When WAG/Rij rats were sub-chronically treated with rapamycin (o.s.; 0.5, 1 and 3 mg/kg), infusion of LPS i.c.v. on the 8th day, failed to significantly modify the number and duration of SWDs at each dose level (Fig. 2), although a slight (non-significant: $P > 0.05$) tendency toward an increase in both parameters was observed. Therefore, rapamycin treatment, whether acute or sub-chronic, was able to prevent the LPS-induced increase in absence seizures in the WAG/Rij rats.

3.6. Rapamycin effects on depressive-like behavior

Finally, it was also of interest to evaluate the effects of rapamycin in the forced swimming test (FST) in both WAG/Rij and normal Wistar rats (for comparison). This produced some interesting and unexpected results. As previously described (Russo et al., 2011a,b), WAG/Rij rats at the age of 6 months showed a significantly longer immobility time (IT) than control Wistar rats (173.69 *vs.* 112.6 s; $P < 0.05$; Fig. 5). Early long-term treatment (1 mg/kg/day; o.s.) with rapamycin in both strains had similar effects, giving a significant increase in IT (+21% and +32% for both WAG/Rij and Wistar rats, respectively; $P < 0.05$). Therefore, after a chronic treatment, even after at least 1 month of drug wash out, rapamycin increased depressive-like behavior in this test (Fig. 5A). However, when the same animals were re-exposed to the same test at 10 months of age (5 months without treatment), no differences were noted between treated and untreated groups for both strains (Fig. 5A); the effect of rapamycin was not therefore sustained. In direct contrast, after a sub-chronic treatment (1 week) with rapamycin (o.s.; 0.5, 1 and 3 mg/kg) at 6 months of age, different results were obtained for the two strains. In WAG/Rij rats, rapamycin dose-dependently *reduced* IT, but this antidepressant effect (−21%) was only significant for the highest dose tested (3 mg/kg ($P < 0.05$; Fig. 5A)), whereas in Wistar rats, rapamycin treatment had no effects at any dose studied (Fig. 5B). Once again, after sub-chronic treatment, when re-testing rats at 30 days after the end of treatment, no differences in IT between groups were observed (Fig. 5). Also, acute treatment, 30 min before the test, did not have any significant effects on IT (data not shown).

Similar results were obtained in the sucrose consumption test (Fig. 6). Rapamycin early long-term treatment (1 mg/kg/day; o.s.) in both strains significantly ($P < 0.05$) reduced sucrose intake on the 5th day of the test, −45% and −54% for Wistar and WAG/Rij rats, respectively. At 10 months of age, 5 months after the end of treatment, sucrose intake values returned to values similar to control. After the sub-chronic treatment, in WAG/Rij rats, the highest dose of rapamycin used (3 mg/kg) significantly increased sucrose intake (+21%; $P < 0.05$), while in Wistar rats no significant differences were observable. Rapamycin therefore produced non-permanent pro- or antidepressive effects in the WAG/Rij rats, depending on the treatment protocol.

4. Discussion

We previously reported that the WAG/Rij rat absence epilepsy model undergoes an epileptogenic process during early life, which stabilizes around the age of 6 months, and that early pretreatment with some AEDs can interfere with this process (Russo et al., 2010, 2011a,b). In the present work, we have demonstrated for the first time that an early long-term treatment with the mTOR inhibitor rapamycin, started prior to the onset of seizures, also reduces the subsequent development of EEG absence epilepsy in adult WAG/Rij rats, thereby displaying antiepileptogenic properties. These results are in line with previous findings in convulsive epilepsy models (for recent review, see Russo et al., in press), where rapamycin was reported to have anti-epileptogenic properties, although data regarding the exact mechanism of action by which it is preventing the development of convulsive seizures, is still lacking. Furthermore, in several studies, mTOR was found to be activated in epilepsy (Russo et al., in press; Sha et al., 2012; Sosunov et al., 2012; Zhang and Wong, 2012); in our study, we found that WAG/Rij rats express more total mTOR than Wistar rats in several brain areas and phospho-mTOR (activated) in the cortex. This result confirms the involvement of mTOR pathway, however, it remains to be elucidated whether, in this animal model, mTOR is activated as a consequence of seizures or is involved in the epileptogenic process and/or seizure generation. The exact mechanism(s) underlying the epileptogenic process in the WAG/Rij rat absence model also remain unclear. EEG recordings were performed 30–40 days after termination of rapamycin treatment, indicating that the observed reduction effects on SWD parameters were not due to the presence of the drug *per se* and that they were persistent up to this age. Since it was previously reported that the protective effects of rapamycin *vs.* convulsive seizures might be transient (Huang et al., 2010), we specifically monitored the same animals at 10 months of age (5 months after treatment suspension) and found that absence seizures where still significantly suppressed. This indicates that up to 5 months after the end of rapamycin treatment, its anti-absence effects were retained. The exact changes induced in the brains of WAG/Rij rats by the treatment, that produced this lasting effect however, will require further investigation. Rapamycin blood levels in our long-term protocol were very low (about 0.9 ng/ml) in comparison to other studies (i.e. van Vliet et al., 2012). This might be explained in several ways (i.e. low rapamycin bioavailability,

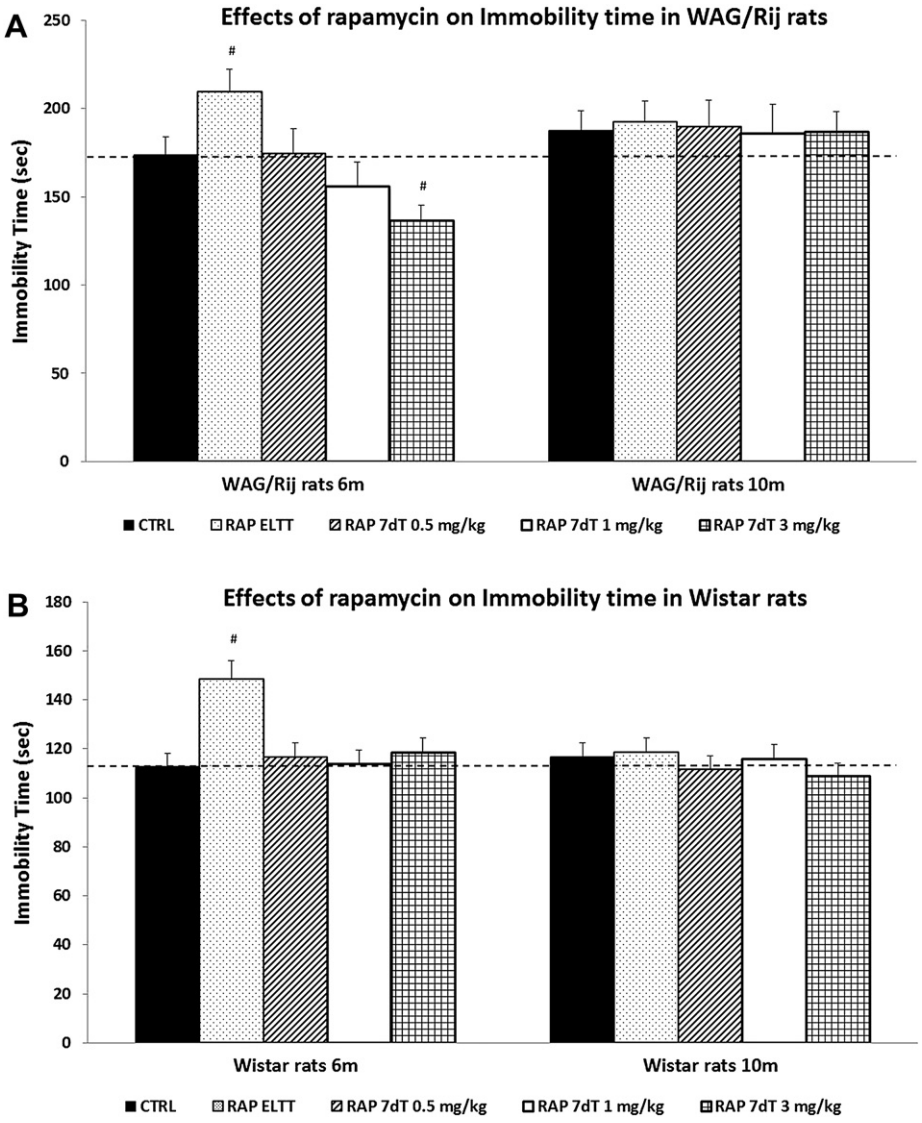

Fig. 5. Effects of rapamycin on immobility time (IT) in the forced swimming test both in WAG/Rij rats (A) and Wistar rats (B) at the age of 6 months (left side) and 10 months (right side). Bars indicate IT expressed in seconds. Data values are means ± SEM ($n = 10$). # Significantly different ($P < 0.05$) from age-matched control (untreated) WAG/Rij rats. RAP = rapamycin; CTRL = control rats; ELTT = early long-term treatment; 7dT = 7 days sub-chronic treatment.

strain differences in drug metabolism), however, further studies might be needed to determine whether lower doses than the one generally used (5–6 mg/kg/day i.p.) might be as much as effective. Actually, Buckmaster and Lew (2011) used two i.p. doses of rapamycin (1.5 and 3 mg/kg/day) in the pilocarpine post-status epilepticus model and reported dose-dependent effects on mossy fiber sprouting but no effects against seizures; unfortunately no blood levels were determined. More experiments are warranted to determine the most appropriate doses to be used and these might be dependent on both animal strain and epilepsy model.

Very surprisingly, we found that rapamycin either acutely or sub-chronically (7 days) administered in WAG/Rij rats at the age of 6 months (with absence seizures already established) was also able to reduce both the number and total duration of SWDs, therefore exhibiting *direct* anti-absence properties. This effect was not however, dose-dependent. Acutely injected rapamycin was effective at doses >0.5 mg/kg, even though at a lower dose of 0.25 mg/kg, it had a significant effect for only a very short time-period. Similarly, the sub-chronic administration of rapamycin for 7 consecutive days had direct anti-absence properties that were not dose-dependent and furthermore, unlike the early long-term treatment, at 30 days after treatment suspension, SWDs parameters returned to baseline levels, indicating only transient effects at this age and with this administration protocol (*c.f.* Huang et al., 2010).

The chronic treatment effects might be explained by the ability of rapamycin, through mTOR inhibition, to affect a variety of cellular and molecular processes, such as neurotransmitter receptor and ion channel expression, synaptic plasticity, neuronal death and apoptosis, and neurogenesis, all which are known to influence neuronal excitability and epileptogenesis (Tang et al., 2002; Kumar et al., 2005; Raab-Graham et al., 2006; Wang et al., 2006). The acute treatment effects might then result from more direct and immediate effects of this compound on neuronal excitability and circuit synchronization. However, rapamycin has been found to have no effect on spontaneous neuronal action potential firing or voltage-dependent Na^+ or K^+ currents underlying action potentials recorded *in vitro* (Victor et al., 1995; Rüegg et al., 2007), therefore, this possibility seems unlikely. Rapamycin alters neuronal expression of both voltage-gated (Raab-Graham et al.,

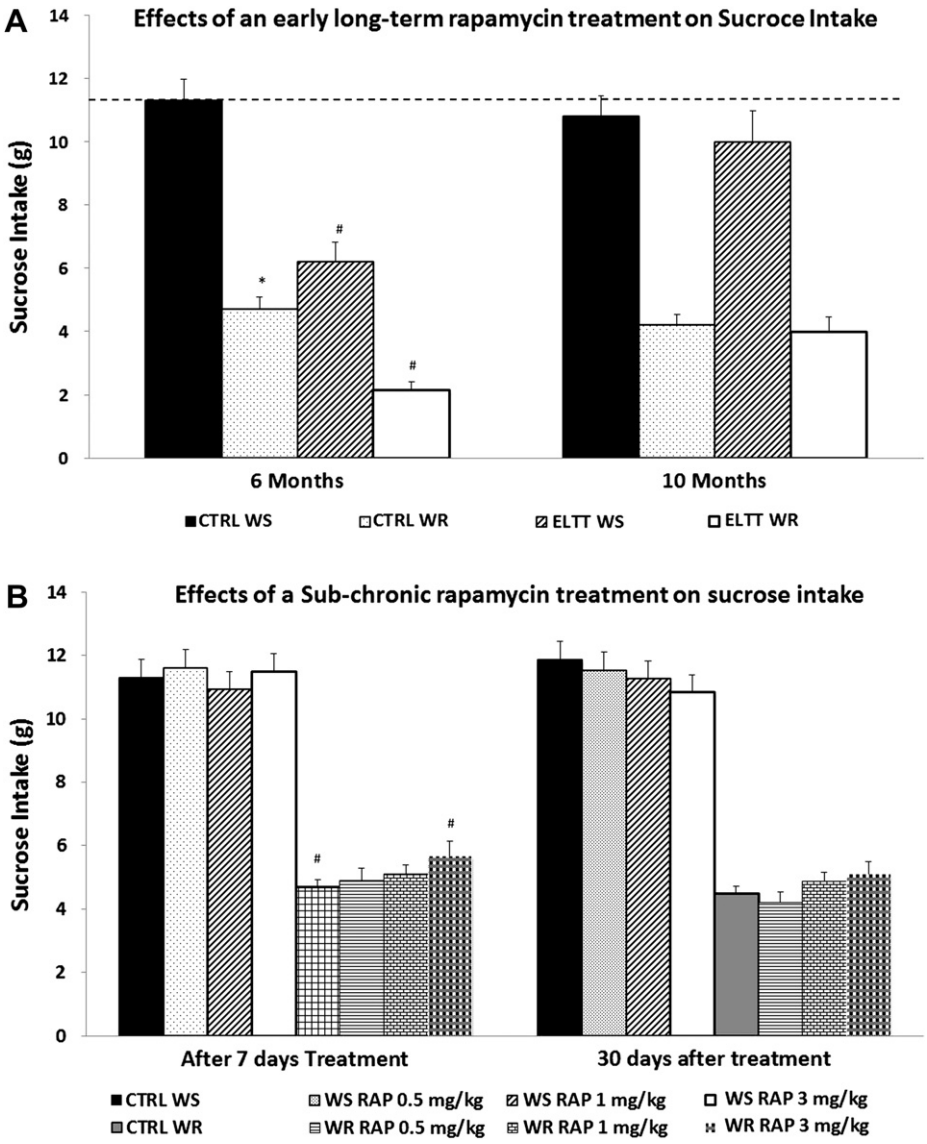

Fig. 6. Effects of rapamycin on sucrose intake in a 15 min test both in WAG/Rij rats (WR) and Wistar rats (WS) at the age of 6 months (left side) and 10 months (right side). Bars indicate sucrose intake expressed in grams (g). Data values are means ± SEM ($n = 10$). A) Effects of an early long term treatment with RAP; B) Effects of a sub-chronic (7 days) treatment with various doses of rapamycin. *Significantly different ($P < 0.05$) from age-matched Wistar rat controls; # significantly different ($P < 0.05$) from age-matched control (untreated) WAG/Rij rats. RAP = rapamycin; WS = Wistar rats; WR = WAG/Rij rats; CTRL = control rats; ELTT = early long-term treatment; 7dT = 7 days sub-chronic treatment.

2006) and ligand-gated ion channels (Sabatini et al., 1999; Wang et al., 2006) and transporters (Li et al., 2006), as well as inhibiting dendritic growth (Jaworski et al., 2005; Kumar et al., 2005; Huang et al., 2007) and changing dendritic spine morphology (Tavazoie et al., 2005). Thus, the involvement of mTOR signaling pathways in epileptogenesis and seizure generation, both convulsive and non-convulsive, may be very complex.

The fact that the observed acute effects of rapamycin (after a certain threshold dose) were not dose-dependent, could indicate that mTOR is involved in the regulation of the release of a 'cascade' of mediators which participate in the generation of SWDs and that inhibition of such release at any stage, produces a very effective 'maximal' anti-absence effect. One possible mechanism that could account for the acute treatment effects of rapamycin on absence seizures is the involvement of cytokines. In recent years, several studies have shown that some epileptic seizures can induce the production of cytokines, *e.g.* interleukins 1β and 6 (IL-1β, IL-6) and tumor necrosis factor-α (TNF-α), which in turn influence the pathogenesis and course of the epilepsies (Szelenyi, 2001; Młodzikowska-Albrecht et al., 2007). Abnormalities in the expression of cytokines and immune cells have been observed in patients with epilepsy and in animal models, indicating cytokines as important mediators of spontaneous seizures (Ravizza and Vezzani, 2006; Lehtimäki et al., 2007; Li et al., 2011). Moreover, the immune system and its associated inflammatory reactions seem to play an important role also in epileptogenesis (Vezzani et al., 2002; Li et al., 2011; Vezzani and Granata, 2005).

Activated astrocytes and microglia are major sources of inflammatory molecules in the brain during epileptic activity in different experimental models of seizures (Vezzani et al., 2011). Glial cells have been suggested to play a role in the mechanisms underlying SWDs in GAERS (Genetic Absence Epilepsy Rats from Strasbourg), another established animal model of absence epilepsy (Melo et al., 2007). Proinflammatory cytokines such as IL-1β and TNF-α are synthesized by the glial cells in the brain (Młodzikowska-Albrecht et al., 2007; Vezzani et al., 2008a,b) and there is also a functional interaction between the cytokines and classical brain neurotransmitters such as glutamate and GABA (De Simoni and

Imeri, 1998; Vezzani et al., 2008a). The mTOR pathway has been shown to be involved in cytokine-dependent microglial activation (Dello Russo et al., 2009), therefore, it is possible that mTOR inhibition by rapamycin blocks this pro-convulsive glial cytokine release resulting in an immediate anti-absence seizure effect. However, van Vliet et al. (2012), demonstrated that rapamycin does not block microglia activation; measures of inflammatory protein plasma levels were unchanged by rapamycin treatment, although, brain levels determination would be more accurate and would better define the pattern of mTOR activity. It is already known that the characteristic thalamo-cortical synchronization which occurs in absence seizures is strongly affected by proinflammatory cytokines (Miller et al., 1991; Vezzani et al., 2002). For example, it has been shown that mature SWDs in GAERS are associated with, and preceded by IL-1β induction in activated astrocytes in the somatosensory cortex, thus suggesting a possible contributing role of this cytokine to SWD generation (Akin et al., 2011). The mechanism here is thought to involve the cytokine-induced release of glutamate from astrocytes, which in turn triggers slow inward currents (mediated by NMDA receptors) and synchronization in adjacent pyramidal cells (Angulo et al., 2004). Furthermore, it has also been demonstrated that in WAG/Rij rats, the absence-like epileptic seizure activity is increased by i.p. administration of LPS, in parallel with increased cytokine levels (IL-1β, IL-6 and TNF-α) (Kovács et al., 2006). Both IL-1β and TNF-α can enhance neuronal hyperexcitability directly via NMDA receptors (Balosso et al., 2008; Viviani et al., 2003; Wang et al., 2000). The cytokine effects on SWDs may also involve IL-1β-mediated inhibition of glial glutamate uptake, leading to a further increase in glutamate extracellular levels (Mascarucci et al., 1998; Hu et al., 2000).

Therefore, considering all the aforementioned evidence favoring a role of inflammatory cytokines in seizure induction, it seemed reasonable to propose that our observed antiepileptogenic and antiepileptic effects of rapamycin could be mediated through inhibition/modulation of brain neuroinflammation in the WAG/Rij rats. To validate this hypothesis, we tested the effects of rapamycin against the increase in SWDs that are known to be produced in WAG/Rij rats by the i.c.v. administration of LPS (Kovács et al., 2011). The pro-absence effect of LPS has previously been demonstrated to be dependent on the release of IL-1β, TNF-α and IL-6 in the WAG/Rij rat brain (Kovács et al., 2006, 2011). We found that administration of rapamycin 30 min after LPS infusion, dose-dependently inhibited the LPS-dependent increase in SWDs. Since the acute effects of rapamycin in WAG/Rij rats were *not* dose-dependent, it is feasible that the antagonism observed was not only functional but involved common mechanisms, *i.e.* inhibition of pro-inflammatory cytokine release by glial cells. Similarly, the sub-chronic rapamycin treatment completely inhibited the LPS-mediated effects. Therefore, our results strongly suggest for the first time, that at least part of the anti-absence action of rapamycin in WAG/Rij rats is mediated by inhibition of brain inflammatory responses; this could also be transferred to other animal models, it would be of great interest to measure the effects of rapamycin on inflammatory components of other epilepsy/epileptogenesis models.

All the above mentioned mechanisms are also in line with our other findings in the behavioral tests. Development of SWDs has previously been associated with depressive-like behavior in this animal model (Sarkisova et al., 2010; Sarkisova and van Luijtelaar, 2011; Russo et al., 2011a,b); however, in further contrast with the hypothesis of Sarkisova et al. (2010) that SWDs are necessary for the development of behavioral alterations (see Russo et al., 2011a,b), we found that early long-term treatment with rapamycin increased immobility time (IT) in the forced swimming test (FST) and reduced sucrose intake in sucrose consumption test (SCT), therefore displaying *pro*depressant properties. This latter effect was not strain specific, since Wistar rats undergoing the same treatment, exhibited identical effects. Thus, mTOR inhibition would appear to be *pro*-depressive, and possibly *vice versa*. In keeping with this, the rapid antidepressant properties of the NMDA receptor antagonist ketamine have been associated with an *increase* in mTOR activity, synaptic protein levels and synaptogenesis in rat prefrontal cortex (Duman et al., 2012) and the antidepressant effects of the mGluR2/3 blocker LY 341495, which also rapidly activates the mTOR pathway, are completely blocked by rapamycin (Dwyer et al., 2011). Furthermore, the hydrophobic dipeptide, leucyl–isoleucine (Leu–Ile), which activates the mTOR pathway, inhibits immobility time in the FST (Furukawa-Hibi et al., 2011). Accordingly, a *deficit* in the mTOR pathway in the prefrontal cortex of human patients is associated with major depressive symptoms (Jernigan et al., 2011), even though mTOR inhibition by rapamycin or other mTOR inhibitors in clinical settings has never been associated with the development of depressive disorders in patients. In fact, in a clinical study in adult maintenance heart transplant recipients, everolimus, a 40-O-(2-hydroxyethyl) derivative of rapamycin, improved memory, concentration and overall psychiatric symptoms when patients were switched over from calcineurin inhibitors (*i.e.* cyclosporin A) (Lang et al., 2009). Overall, we believe that *chronic* inhibition of mTOR, starting treatment early in life (P45: an age where rats can still be considered adolescent; Spear, 2000) might inhibit physiological hippocampal development, leading to pro-depressive behavior (for review on antidepressants and hippocampal neurogenesis see Tang et al., 2012).

At odds with these findings, *sub-chronic* administration of rapamycin (3 mg/kg) showed *anti*depressant properties in the WAG/Rij rats, with no observable effects in healthy controls. This indicated that this action was limited to an already established pathology in the adult brain. The mechanism by which sub-chronic rapamycin might positively influence behavior might also be related to the inhibition of neuroinflammatory mediators released by glial cells. It has previously been demonstrated that LPS injection induces a behavioral syndrome that includes traits of depression (Dantzer et al., 2008; Painsipp et al., 2011). Therefore, like the anti-absence effects, the more immediate antidepressant effects of rapamycin in WAG/Rij rats might depend on a reduction of brain inflammatory responses. This suggests an interesting link between absence seizure generation and expression of depressive-like behavior in this model, the exact mechanism of which needs to be further investigated.

4.1. Conclusions

In conclusion, we have confirmed and extended the knowledge that rapamycin, by inhibiting the activation of mTOR, has anti-epileptogenic/antiseizure properties. We have demonstrated for the first time, that rapamycin has anti-absence effects in an established model of absence epilepsy, and provide strong evidence (through the central administration of LPS), that this effect might be mediated via the inhibition of neuroinflammatory mediators. It is possible that this action might also be largely responsible for the observed antiepileptic effects of rapamycin in other (convulsive) seizure models. In addition, we have shown that rapamycin can have either pro- or antidepressant effects in the WAG/Rij rat model of absence and mild-depression, depending on the treatment protocol (chronic long-term or sub-chronic respectively), which might be related to the age of treatment. Additional studies are warranted to more definitively identify the most critical mechanisms underlying absence epileptogenesis and the antiepileptogenic/antiseizure action of rapamycin, focusing particularly, on neuroinflammatory responses.

Disclosure/conflict of interest

None.

Acknowledgments

None.

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