Neuroscience 219 (2012) 33–47

RAPAMYCIN DOWN-REGULATES KCC2 EXPRESSION AND INCREASES SEIZURE SUSCEPTIBILITY TO CONVULSANTS IN IMMATURE RATS

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Abstract—Seizure susceptibility to neurological insults, including chemical convulsants, is age-dependent and most likely reflective of overall differences in brain excitability. The molecular and cellular mechanisms underlying development-dependent seizure susceptibility remain to be fully understood. Because the mammalian target of rapamycin (mTOR) pathway regulates neurite outgrowth, synaptic plasticity and cell survival, thereby influencing brain development, we tested if exposure of the immature brain to the mTOR inhibitor rapamycin changes seizure susceptibility to neurological insults. We found that inhibition of mTOR by rapamycin in immature rats (3–4 weeks old) increases the severity of seizures induced by pilocarpine, including lengthening the total seizure duration and reducing the latency to the onset of seizures. Rapamycin also reduces the minimal dose of pentylenetetrazol (PTZ) necessary to induce clonic seizures. However, in mature rats, rapamycin does not significantly change the seizure sensitivity to pilocarpine and PTZ. Likewise, kainate sensitivity was not significantly affected by rapamycin treatment in either mature or immature rats. Additionally, rapamycin treatment down-regulates the expression of potassium-chloride cotransporter 2 (KCC2) in the thalamus and to a lesser degree in the hippocampus. Pharmacological inhibition of thalamic mTOR or KCC2 increases susceptibility to pilocarpine-induced seizure in immature rats. Thus, our study suggests a role for the mTOR pathway in age-dependent seizure susceptibility. **© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: mTOR, epilepsy, development, rapamycin, seizure, KCC2.

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Abbreviations: ANOVA, analysis of variance; DV, doros-ventral; EDTA, ethylenediaminetetraacetic acid; EEG, electroencephalography; GP, globus pallidus; KCC2, potassium-chloride cotransporter 2; mTOR, mammalian target of rapamycin; NMDA, N-methyl-D-aspartate; PTEN, phosphatase and tensin homolog; PTZ, pentylenetetrazol; ROI, regions of interest; RT, room temperature; SE, status epilepticus; SNR, substantia nigra; TSC, tuberous sclerosis complex; VA/VL, ventral anterior/ventral lateral.

INTRODUCTION

The mammalian target of rapamycin (mTOR) is a member of the phosphatidylinositol kinase-related kinase family (Sarbassov et al., 2005). In the CNS, the mTOR pathway is regulated by glutamate receptor activation (Huang et al., 2007; Lenz and Avruch, 2005) and modulates neurite outgrowth (Kumar et al., 2005; Tavazoie et al., 2005), synaptic plasticity (Tang et al., 2002) and cell survival (Dawson et al., 1993), presumably by influencing protein translation or Akt activity (Burnett et al., 1998; Jaworski et al., 2005). The role of the mTOR pathway in epilepsy has recently been intensively interrogated (Wong, 2010). Hyperactivation of mTOR causes widespread benign tumors and mental retardation, along with a high incidence of epilepsy in tuberous sclerosis complex (TSC) patients (Crino et al., 2006; Holmes and Stafstrom, 2007; Thiele, 2010). Pharmacological inhibition of the mTOR pathway successfully attenuates structural abnormalities and reduces seizures in TSC (Meikle et al., 2008; Zeng et al., 2008), cortical dysplasia (Ljungberg et al., 2009), and phosphatase and tensin homolog (PTEN) (Sunnen et al., 2011; Zhou et al., 2009) mouse models of epilepsy. Additionally, rapamycin has been shown to attenuate infantile spasm (Raffo et al., 2011) and to prevent pathological changes in the hippocampus (Buckmaster et al., 2009; Zeng et al., 2009; Huang et al., 2010) and spontaneous recurrent seizures in adult acquired epilepsy models (Zeng et al., 2009; Huang et al., 2010), although not in the mouse pilocarpine model (Buckmaster and Lew, 2011). Moreover, a recent clinical study revealed that inhibition of mTOR by everolimus markedly reduces the volume of subependymal giant-cell astrocytomas and seizure frequency in TSC patients (Krueger et al., 2010). Thus, the mTOR pathway has been increasingly viewed as a favorable therapeutic target against seizures associated with genetic defects such as TSC (Crino, 2008) and perhaps in acquired epilepsy following neurological insults (McDaniel and Wong, 2011).

Despite the positive outcomes from rapamycin treatment in TSC/PTEN genetic animal models and to some extent in acquired epilepsy models, it remains essentially unknown if there are any negative effects from chronic exposure to rapamycin in the developing brain. Given that the mTOR pathway is involved in brain development by regulating neurite outgrowth and synaptic plasticity, the immature brain, which undergoes extensive growth of neurons and modification of neuronal circuitries, could

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http://dx.doi.org/10.1016/j.neuroscience.2012.05.003

be especially sensitive to mTOR inhibition. The present experiments were undertaken to examine if rapamycin influences seizure susceptibility to convulsants in the immature rat.

EXPERIMENTAL PROCEDURES

Animals

Immature (50–75 g or 3–4 weeks old) and mature (200–300 g or 8–10 weeks old) male Sprague–Dawley rats were purchased from Taconic (Taconic, Hudson, NY, USA). Animals were housed with *ad libitum* access to food and water in an animal facility with temperature control and a 12-h light–dark cycle (lights on 0700). All experiments were performed according to the guidelines set by the Animal Care and Use Committee as well as the National Institutes of Health guide for care and use of laboratory animals. Efforts were made to minimize suffering and unnecessary use of animals. Rats used in all experiments were 3–4 weeks old unless otherwise indicated.

Drug treatment

Rapamycin (Tecoland, Edison, NJ, USA) was first dissolved in dimethylsulfoxide (DMSO) and further diluted in a vehicle solution containing 5% tween-20 and 4% ethanol. Rats were pretreated with rapamycin at 5 mg/kg/day i.p. for one to three consecutive days prior to the induction of seizures by administration of pilocarpine (255–300 mg/kg, i.p.), kainate (10–20 mg/kg, i.p.) or PTZ (35–60 mg/kg, i.p.). For the most experiments, animals were treated with pilocarpine at 255 mg/kg or rapamycin at 5 mg/kg unless otherwise indicated. Pilocarpine administration was performed as previously described (Huang et al., 2002). Briefly, rats were injected with methylscopolamine and terbutaline (2 mg/kg each i.p. in 0.9% NaCl) 15–30 min prior to pilocarpine (255–300 mg/kg, i.p.) to minimize peripheral side effects. Seizures were terminated with sodium pentobarbital (25 mg/kg, i.p.) 60 min after administration of pilocarpine. For the experiment determining the seizure threshold to PTZ, rats were first injected with PTZ at 25 mg/kg i.p., followed by repeated administration of 5 mg/kg at 5-min intervals until a bilateral forelimb clonic jerk was induced as described (Bough et al., 2006). All compounds were purchased from Sigma–Aldrich (St. Louis, MO, USA) except where indicated otherwise.

Seizure monitoring

Behavioral seizure activity was continuously recorded by a digital video recorder surveillance system (Advanced Security Inc., Chino Hills, CA, USA) for 60 min immediately following injection of pilocarpine or kainate. Videos were then examined by trained researchers. Total seizure duration was defined as the total time each rat experienced clonic seizures, including both brief episodic seizures typically lasting less than a minute and status epilepticus (SE). Seizure activities were graded according to Racine's standard classification (Racine, 1972): stage 1, behavioral arrest with mouth and facial movement; stage 2: head nodding; stage 3: forelimb clonus; stage 4: rearing; and stage 5: rearing and falling. In the present study only stages 3–5 seizures were counted as they are most easily distinguished from typical rat behavior using video surveillance. An individual behavioral seizure event was defined as continuous seizure activity without full recovery to a Racine scale value of less than 3 for more than 10 s. The highest seizure scale found in each behavioral seizure episode was recorded. The average seizure scale for individual animals was calculated by averaging all seizure scales that occurred during 60 min of recording; animals which did not develop seizures at a scale greater than 3 were counted as 0. The average seizure scales in each group were then calculated.

Seizure activity was also monitored by electroencephalography (EEG) using an 8206 EEG/EMG data conditioning and acquisition system following the manufacturer's instructions (Pinnacle Technology Inc., Lawrence, KS, USA). Briefly, rats at p18–19 were sedated with pentobarbital at 25 mg/kg (i.p.) and anesthetized by inhalation of isoflurane, and placed in a stereotaxic frame. EEG electrodes used for the epidural cortical recording were stainless-steel 1/8'' screws (Pinnacle Technology Inc.) threaded into holes drilled through locations on the skull following the stereotaxic coordinates (AP +2 mm; ML +2 mm) using bregma as the reference. The reference electrode was placed at (AP −5 mm; ML −2 mm). The ground was placed in the skull (AP −5; ML +2). Note: AP+ stands for anterior and AP− for posterior relative to bregma. ML+ stands for lateral to the right and ML− to the left relative to the midline. Rats were allowed 5 days for recovery and then treated with either rapamycin at 5 mg/kg (i.p.) or vehicle daily for three days. On the fourth day posttreatment, rats were treated with either pilocarpine at 255 mg/kg (i.p.) or kaniate at 20 mg/kg (i.p.). Electrographic seizures were recorded for 1.5 h and manually identified as characteristic spikes and waves of high-frequency and high-amplitude (twice the base level) rhythmic activity persisting for more than 15 s. SE was defined as continuous seizure activity that lasted over 15 min without full recovery of amplitude to the basal level more than 10 s. Seizures that started near the end of the 1.5-h recording and lasted for more than 10 min were also counted as SE. Total seizure duration was defined as total time that each rat spent in electrographic seizures, including episodic seizures and SE. Because prolonged seizures can significantly increase mortality, we monitored seizure activity for 1.5 h, which is long enough to allow SE to occur while minimizing mortality. One rat in the rapamycin-treated group died from a severe seizure during the course of recording and was excluded from the study.

Tissue isolation and western blot analysis

Rats (50–75 g or 3–4 weeks old) were treated with either vehicle or rapamycin (5 mg/kg/day, i.p.) for three days prior to being sacrificed. Rat brains were placed into a stainless steel matrix and sectioned coronally into 3-mm thick slices by inserting four razor blades at 4, 7, 10, and 13 mm from the anterior-most point of the cortex. Regions of interest (ROI) were identified as described in the monograph *A Stereotaxic Atlas of the Developing Rat Brain* by Nancy M. Sherwood, and then excised using a 2-mm hole punch. Isolated tissues were subjected to western blot analysis as described (Huang et al., 2010). Briefly, tissues were homogenized in lysis buffer consisting of 50 mM Tris, pH 7.4, 2 mM EDTA and a proteinase inhibitor set (Roche, San Francisco, CA, USA), followed by mixing with an equal volume of 2X LDS sample buffer comprised of 20% β-mercaptoethanol and heated at 95 °C for 5 min. The resulting protein samples were resolved in an 8% Bis-Tris gel in MES buffer and then transferred onto a 0.45-μm nitrocellulose membrane. The membranes were first blocked in 5% nonfat dry milk in tris buffered saline and tween 20 (TBST) (25 mM Tris–HCl, pH 7.4; 1.5 M NaCl; 0.05% Tween-20) for 1 h at room temperature (RT) and then incubated with primary antibodies at 1:1000–2000 dilution at 4 °C overnight. Antibodies were purchased as indicated below: rabbit anti-s6, p-s6, mouse anti-GAPDH (Cell Signaling, Danvers, MA, USA), rabbit anti-M1 and M3 antibodies, rabbit anti-GABA α1 and 3 rabbit anti-KCC2, rabbit anti-NKCC1 (Millipore, Billerica, MA, USA), rabbit anti-NMDA 2B (Abcam, Cambridge, MA, USA) and mouse anti-GluR2 (Zymed, San Francisco, CA, USA). After removal of primary antibodies by washing 3 times with TBST, membranes were incubated with HRP-conjugated secondary antibody (1:10,000 dilution for rabbit antibody; 1:3000 for mouse antibody)

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