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Rapamycin suppresses PTZ-induced seizures at different developmental stages of zebrafish

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ABSTRACT

The mTORC1 complex integrates different inputs from intracellular and extracellular signals to control various cellular processes. Therefore, any disruption in the mTORC1 pathway could promote different neurological disorders. mTORC1 overactivation has been verified in different genetic and acquired epilepsy animal models. Therefore, inhibitors of this complex could have both antiepileptogenic and antiseizure effects. In our study, we investigated the effects of rapamycin pretreatment on pentylenetetrazole (PTZ)-induced seizures in zebrafish. Our results have shown that the latency to reach the tonic–clonic stage (stage III) of progressive behavioral alterations shown during PTZ-induced seizures was prolonged in larval (7 days post fertilization, 7 dpf), juvenile (45 days post fertilization, 45 dpf) and adult (6–8 months) zebrafish after pretreatment with rapamycin. Furthermore, rapamycin pretreatment did not alter the locomotor activity in zebrafish. Therefore, the results obtained in our study indicate that rapamycin pretreatment is an important mechanism to control the progress of seizures in zebrafish throughout different developmental stages (larval, juvenile, and adult). Taken as a whole, our data support that rapamycin has immediate antiseizure effects and could be a potential alternative therapy for seizure control in epilepsy.

1. Introduction

Epilepsy is a common neurological disorder, affecting around 65 million people worldwide. This disorder is characterized by the occurrence of recurrent and unpredictable seizures, which occur due to abnormal activity of neuronal cells (Moshé et al., 2015; Lin and Baines, 2015). The mechanist target of rapamycin (mTOR) protein is a 289-kDa serine-threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and forms two distinct multi-protein complexes: mTORC1 and mTORC2. mTOR complexes integrate intracellular and extracellular signals and act as central regulators of cell activity, metabolism, growth, proliferation, and survival (Laplante and Sabatini, 2012, 2013). Rapamycin is an anti-fungal macrolide compound that acts by inhibiting mTOR signaling and is produced by the soil bacterium *Streptomyces hygroscopicus*, which was isolated from a soil sample obtained in Rapa Nui (Vézina et al., 1975).

mTORC1 is the best characterized of the two mTOR complexes and integrates different inputs from intracellular and extracellular signals: growth factors, stress, energy status, hormones, and amino acids in the control of various cellular processes, including protein and lipid synthesis, differentiation, and autophagy (Laplante and Sabatini, 2013; Maiese et al., 2013). Regarding the central nervous system (CNS), the mTORC1 complex regulates a variety of neuronal functions: cell proliferation, survival, growth, and plasticity. Disruption of the mTORC1 pathway has been implicated in different neurological disorders, and previous studies have shown that the mTORC1 signaling overactivation is related to epilepsy occurrence (Wong, 2010). The mTORC1 overactivation has been verified in genetic and acquired epilepsies: tuberous sclerosis complex, focal cortical dysplasias, and animal models of epilepsy acquired after *status epilepticus* or trauma (Wong, 2010). Therefore, as a mTORC1 inhibitor, rapamycin could be an important alternative in epilepsy treatment (Ryther and Wong, 2012).

Several studies have shown that rapamycin has seizure suppressive and antiepileptogenic effects in animal models of epilepsy. Rapamycin treatment attenuated the development of posttraumatic epilepsy in a mouse model of traumatic brain injury, decreasing the frequency of seizure occurrence (Guo et al., 2013). Furthermore, rapamycin treatment showed protective effect following *status epilepticus* since rats treated with this compound presented less seizure frequency when compared

Abbreviations: AED, antiepileptic drug; CNS, central nervous system; mTOR, mechanist target of rapamycin; PTZ, pentylenetetrazole; VPA, valproate.

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with untreated animals (van Vliet et al., 2012). In addition, rapamycin treatment in epileptic rodents significantly reduced seizure frequency, suggesting an antiseizure effect (Huang et al., 2010). Therefore, different studies suggest a protective effect of rapamycin against epilepsy. However, whereas the antiepileptogenic effects of rapamycin have been shown to be a reality, the acute action of rapamycin in suppressing seizures needs to be further investigated in animal models of seizure. Moreover, the anticonvulsant effects of rapamycin appear to vary depending on the stage of development. In immature rats, rapamycin pretreatment had anticonvulsant effects against PTZ-induced seizures. However, the same treatment was ineffective against PTZ-induced seizures in adult animals (Chachua et al., 2012). Nevertheless, few studies have reported the effects of rapamycin throughout the development and more investigation is necessary to address this issue.

Zebrafish is a small freshwater teleost which has become widely used as a model organism to understand seizure modulation. PTZ-induced seizures both in larval and adult zebrafish caused behavioral, molecular, and electrographic alterations that would be expected from a seizure episode (Baraban et al., 2005; Pineda et al., 2011). In addition, both larval and adult zebrafish show response to classic antiepileptic drugs (AEDs), such as valproate (VPA), carbamazepine, and phenytoin (Berghmans et al., 2007; Lee et al., 2010; Siebel et al., 2013).

Despite the large number of clinically-used anticonvulsant drugs, approximately 30% of epileptic patients are refractory to current pharmacological treatments (Moshé et al., 2015). Therefore, novel therapeutic approaches that prevent or reverse the molecular and cellular mechanisms of epilepsy are necessary. Considering that the mTORC1 pathway is a prominent target in anticonvulsant therapies and zebrafish is an effective model widely used in seizure studies, the investigation of rapamycin effects on PTZ-induced seizures in zebrafish may improve our knowledge on the modulation of mTORC1 on seizure control.

2. Materials and methods

2.1. Animals

Larval (7 days post fertilization, 7 dpf), juvenile (45 days post fertilization, 45 dpf), and adult (6–8 months) wild-type zebrafish (*Danio rerio*) used in this study were obtained from our breeding stock held at Pontifícia Universidade Católica do Rio Grande do Sul, Brazil. Zebrafish embryos were obtained from natural mating of adult zebrafish bred and maintained in an automated re-circulating tank system (Zebtec, Tecniplast Group, Buguggiate, VA, Italy). Fertilized eggs were collected, washed with system water (reverse osmosis water equilibrated with Instant Ocean salts), free of debris and transferred to sterile cell culture plates. Plates were maintained in an incubator at 28.5 °C and monitored daily until 7 dpf. Following this, the animals were housed in thermostated thanks (Zebtec, Tecniplast Group, Buguggiate, VA, Italy) filled with unchlorinated water constantly aerated at a targeted temperature of 26 ± 2 °C. Fish were kept under a 14–10 h light/dark cycle photoperiod and fed daily, three times a day, with fish food that was supplemented with live artemia. The protocol was approved by the Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS) under number 11/00255-CEUA.

2.2. Materials

The drugs rapamycin, pentylenetetrazole (PTZ), tricaine, and valproate (VPA) were purchased from Sigma (St. Louis, MO, USA).

2.3. Rapamycin pretreatment

This study investigated the effects of rapamycin pretreatment on PTZ-induced seizures in larval (7 dpf), juvenile (45 dpf) and adult (6–8 months) zebrafish. All animals received the rapamycin treatment 30 min before the PTZ exposure. In order to verify the zebrafish response to a classic AED in our PTZ-induced seizure model, we treated a group of animals with VPA. Rapamycin and VPA groups were treated and analyzed in an identical manner. Rapamycin and VPA concentrations and doses were selected based on preliminary studies conducted in our laboratory.

Zebrafish larvae were individually placed in 96 well plates (1 larva per well with 100 μL of solution) and submitted to treatment with 0.1% DMSO (control group), rapamycin at 0.12, 0.25, 0.5, 1, and 2.5 μM or VPA at 3 mM for 30 min. In juvenile and adult animals, the 1% DMSO (control group), rapamycin at 0.25, 0.50, 1, 2.5, and 5 mg/kg and VPA at 100 mg/kg were applied by intraperitoneal (i.p.) injection. I.p. injections were conducted using a 3/10-ml U-100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16) × 31G Short Needle (Becton Dickinson and Company, New Jersey, USA) according to the protocol established by Phelps et al. (2009). Anesthesia of juvenile and adult animals prior to the injection was given by its immersion in a 10% tricaine solution until the animal showed lack of motor coordination and reduced respiration rate. After the injection, the animals were placed in a separate tank with aerated unchlorinated tap water (26 ± 2 °C) to facilitate the recovery from anesthesia.

2.4. Locomotor activity

In order to analyze the rapamycin effects on locomotor activity, animal behavior was recorded during the 30 min following rapamycin treatment and prior to PTZ exposure. The same protocol was applied to verify the VPA effects on locomotion. Larvae were individually placed in 96 well plates (1 per well, with 100 μL of solution). Juvenile and adult animals were individually placed in glass tanks (12 cm × 8 cm × 13.5 cm, length × width × height). The animal behavior was registered by a video camera for 30 min and further analyzed using the ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA) to track the distance traveled by the animals.

2.5. PTZ-induced seizures

To induce seizures, zebrafish were individually exposed to 7.5 mM PTZ (Baraban et al., 2005; Wong et al., 2010). Larvae were individually exposed to PTZ in 96-well plates. PTZ solution was applied in the larval medium in order to obtain a final concentration of 7.5 mM in a total volume of 200 μl. Juvenile and adult zebrafish were individually exposed to 7.5 mM PTZ by their immersion in 250 mL beakers. All PTZ treatments were videotaped and evaluated later by trained observers. The seizure-like behavior was classified according to each stage: stage I — dramatically increased swimming activity, stage II — whirlpool swimming behavior, and stage III — clonus-like seizures followed by loss of posture, when the animal falls to one side and remains immobile for 1–3 s, as previously reported for zebrafish (Baraban et al., 2005; Wong et al., 2010). The animals were submitted to the PTZ treatment until they reached stage III, which corresponds to tonic–clonic seizure stage in zebrafish.

2.6. Statistical analysis

The results are expressed as mean ± S.E.M. The locomotion activity and seizure latency were analyzed by one-way ANOVA followed by Dunnett's post-hoc test or Student's *t*-test, as indicated in the legends of the figures. $P < 0.05$ was considered as significant.

3. Results

3.1. Locomotor activity response following classic AED treatment along different developmental stages

Results regarding the locomotion analysis have shown that the treatment with the classic AED VPA before PTZ-induced seizures did

not alter the locomotor activity in larval, juvenile, and adult zebrafish (Fig. 1A).

3.2. Behavioral seizure response following classic AED treatment along different developmental stages

In order to investigate seizure development and zebrafish response to a classic AED along three different life stages (larval, juvenile, and adult), we analyzed the latencies to the first typical behavior characterizing each seizure stage (I, II, and III). Larvae, juveniles, and adults have shown progressive behavioral alterations until they reached the most severe seizure stage, stage III, which corresponds to tonic–clonic seizure in zebrafish. Furthermore, zebrafish have shown response to a classic AED in all life stages analyzed, in view of the fact that VPA pretreatment increased the latency to stages II and III onset in larval, juvenile, and adult zebrafish (Fig. 1B).

There were no significant differences in the latency to reach stage I between larval zebrafish pretreated with VPA and their respective control group (Fig. 1B; $F(1, 12) = 3.83$). Juvenile zebrafish treated with VPA reached stage I with similar latencies when compared to control group (Fig. 1B; $F(1, 18) = 1.90$). Finally, adult zebrafish pretreated with VPA did not show different latencies to reach stage I in comparison to the respective control group (Fig. 1B; $F(1, 18) = 7.50$).

Larvae pretreated with VPA presented increased latency to reach stage II when compared to control group (Fig. 1B; $F(1, 13) = 2.31$; $P < 0.0005$). Juveniles pretreated with VPA showed an increase in the latency to reach stage II in comparison to control group ($F(1, 18) = 1.84$; $P < 0.05$). Finally, adults pretreated with VPA reached stage II with higher latencies in relation to control group (Fig. 1B; $F(1, 18) = 1.61$; $P < 0.0005$).

Larval zebrafish pretreated with VPA showed increased latency to reach stage III in comparison to control group (Fig. 1B; $F(1, 25) = 1.00$; $P < 0.005$). Concerning juvenile animals, the latency to reach stage III was significantly increased with VPA pretreatment (Fig. 1B; $F(1, 25) = 1.06$; $P < 0.0005$). Finally, adult zebrafish pretreated with VPA reached stage III with higher latencies when compared to control group (Fig. 1B; $F(1, 25) = 2.20$; $P < 0.0005$).

3.3. Locomotor activity response following rapamycin treatment along different developmental stages

Results regarding locomotion analysis have shown that the treatment with rapamycin before PTZ-induced seizures did not alter the locomotor activity in all tested concentrations (larvae) and doses (juveniles and adults) (Fig. 2A, B, and C).

3.4. Behavioral seizure response following rapamycin treatment along different developmental stages

In order to investigate seizure development following rapamycin treatment along three different zebrafish developmental stages (larval, juvenile, and adult), we analyzed the latencies to the first typical behavior characterizing each seizure stage (I, II, and III). Rapamycin pretreatment provided significant protection against PTZ-induced seizures in zebrafish, in all life stages studied, in view of the fact that larvae, juveniles, and adults pretreated with rapamycin spent longer time to reach seizure stage III, which corresponds to tonic–clonic seizure in zebrafish.

Considering larval zebrafish, animals reached stage I with similar latencies for all rapamycin concentrations tested (Fig. 3A; $F(5, 110) = 1.75$). Similarly, rapamycin did not alter the latencies to reach stage II in larval zebrafish (Fig. 3A; $F(5, 110) = 2.51$). Finally, rapamycin, at 1 μM, promoted a significant increase in the latencies to stage III onset in larval zebrafish (Fig. 3A; $F(5, 110) = 5.62$; $P < 0.0005$).

Rapamycin pretreatment did not change latencies to reach stage I for all concentrations tested in juvenile zebrafish (Fig. 3B; $F(5, 73) = 2.87$). Stage II onset was also not altered by rapamycin in juvenile zebrafish (Fig. 3B; $F(5, 76) = 2.75$). However, the latency for the first signal of stage III was significantly increased in juvenile zebrafish treated with 0.5 mg/kg rapamycin when compared to the control group (Fig. 3B; $F(5, 110) = 5.32$; $P < 0.0005$).

Considering adult zebrafish, there were no significant alterations in the latencies to reach stage I for all rapamycin concentrations tested (Fig. 3C; $F(5, 56) = 2.88$). The latencies to reach stage II did not show significant differences between rapamycin pretreated and control groups. However, stage III onset was increased after the pretreatment with 0.5 mg/kg and 1 mg/kg rapamycin in adult zebrafish (Fig. 3C; $F(5, 110) = 6.40$; $P < 0.005$).

4. Discussion

The mTOR signaling pathway acts as a molecular system integrator in response to nutrients, neurothrophic factors, and neurotransmitters, controlling protein synthesis and autophagy (Lipton and Sahin, 2014). This mechanism coordinates neural stem cell proliferation, synaptic plasticity, neuronal death, and neurogenesis (Lipton and Sahin, 2014). Concerning the cellular and molecular processes that may be regulated by mTORC1 in the CNS, pharmacological modulation of the mTOR signaling pathway could be a promising alternative in different neurological disease therapies, such as in epilepsy treatment (Lipton and Sahin, 2014). In agreement, hyperactivation of mTOR signaling is evident in models of acute seizure (Zhang and Wong, 2012), *status epilepticus* (Okamoto et al., 2010), and epileptic patients (Berdichevsky et al.,

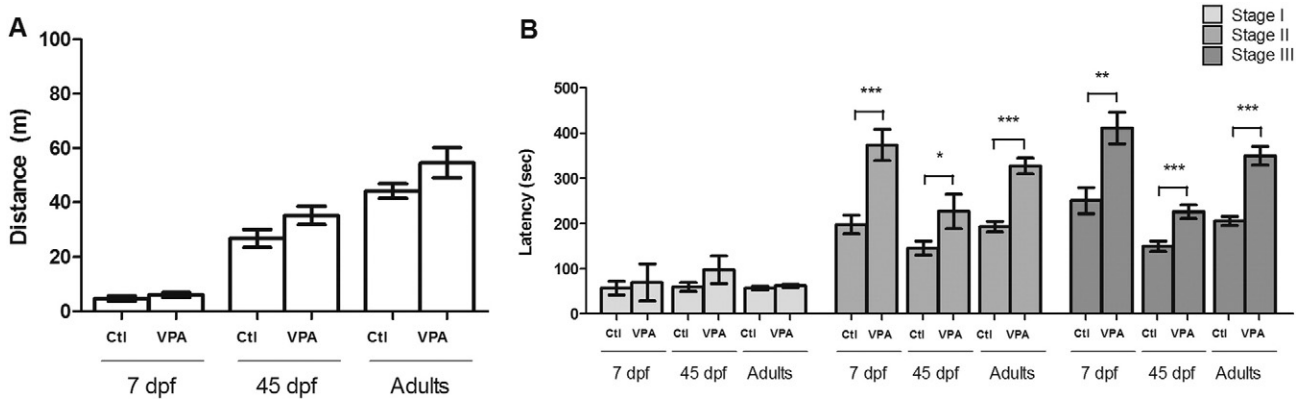

Fig. 1. Effect of VPA on locomotor activity and progression of PTZ-induced seizures in zebrafish at different developmental stages. (A) Locomotor activity measured as distance traveled during the 30 min following VPA pretreatment in larval (7 dpf), juvenile (45 dpf) and adult zebrafish. (B) Latency to the first behavioral manifestation of each seizure stage (I, II, and III) during PTZ exposure. VPA was administered at 3 mM (7 dpf) and 100 mg/kg (45 dpf and adults). Data were expressed as mean ± S.E.M. of at least 10 animals for each group and were analyzed by Student's *t*-test. The symbols represent statistical difference when compared to the respective vehicle control group (Saline). *P < 0.05, **P < 0.005, ***P < 0.0005.

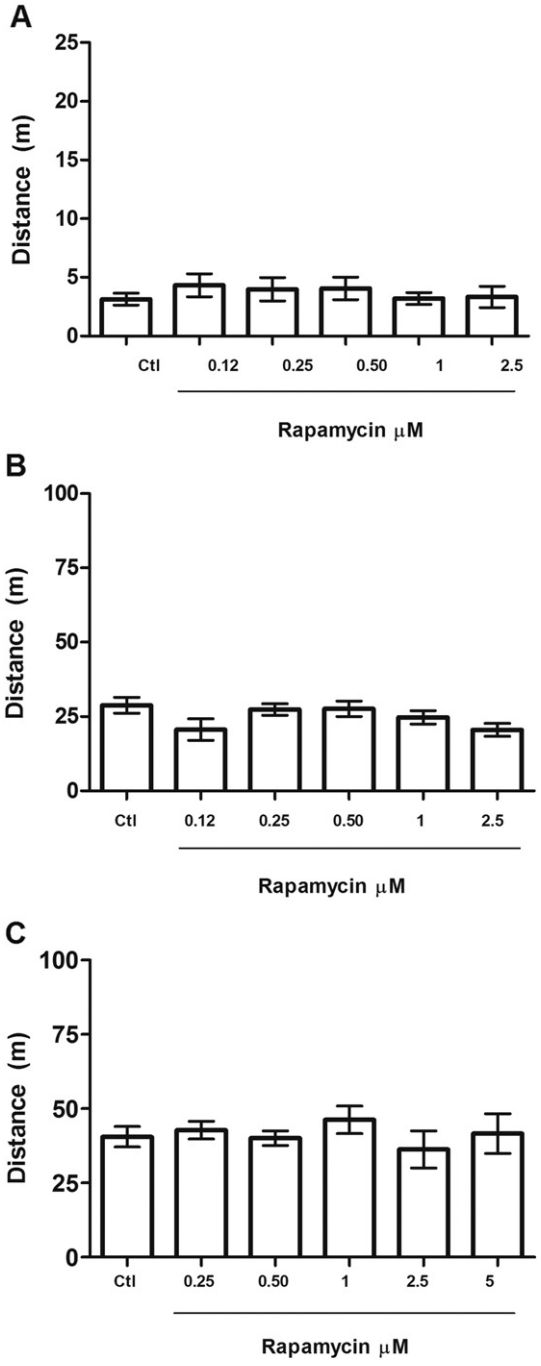

Fig. 2. Effect of rapamycin on locomotor activity in zebrafish at different developmental stages. Locomotor activity measured as distance traveled during the 30 min following rapamycin pretreatment in larval (A), juvenile (B) and adult (C) zebrafish. Rapamycin was administered at 0.12, 0.25, 0.50, 1 and 2.5 μM (7 dpf) and 0.25, 0.50, 1, 2.5 and 5 mg/kg (45 dpf and adults). DMSO was administered at 0.1% (7 dpf) and 3% (45 dpf and adults). The data are expressed as the mean ± S.E.M. of at least 15 animals for each group and were analyzed by one-way ANOVA followed by Dunnett's post-hoc test.

2013; Sun et al., 2013). As a result, inhibitors of this complex could have both antiepileptogenic and antiseizure effects. In our study, we observed that rapamycin pretreatment delayed the progression of seizure behavioral alterations, showing protective effect in a PTZ-induced acute seizure model.

Taking into account the mTOR role along different life stages, we believe it is important to characterize mTOR modulation in larval, juvenile, and adult zebrafish. During development, the mTOR pathway controls neuronal growth by promoting their differentiation, neurite elongation and branching, and synaptic formation (Swiech et al., 2008; Takei and Hiroyuki, 2014). Following the developmental period, mTOR signaling is still essential, participating in the control of several forms of synaptic plasticity that enable learning and memory formation processes (Swiech et al., 2008; Takei and Hiroyuki, 2014). As a result, mTOR is essential along different life stages and its disturbance may cause disorders ranging from abnormal neural development to neuronal degeneration. mTOR hyperactivity during development is clearly associated with tuberous sclerosis, whereas mTOR hypoactivity and hyperactivity in adults have been associated with Alzheimer's disease and epilepsy, respectively (Swiech et al., 2008; Takei and Hiroyuki, 2014). Interestingly, our data have shown that rapamycin pretreatment has protective effect in larval, juvenile, and adult animals. In our study, rapamycin pretreatment increased the latency to the onset of stage III of progressive seizure behavioral alterations observed in zebrafish during PTZ exposure in all studied life stages. Similarly, a recent study tested the potential anticonvulsant properties of rapamycin in immature and adult rats using two different methods to induce acute seizures: blocking inhibitory (flurothyl and PTZ models) and facilitating excitatory (NMDA and kainate models) signaling pathways. Rapamycin treatment by a single dose administration was effective in seizure models induced by the antagonist of GABAergic neurotransmission. In immature rats, one dose of rapamycin (3 mg/kg) 4 or 24 h prior to PTZ exposure had evident anticonvulsant effects. In adult animals, acute rapamycin treatments did not provide anticonvulsant effects against PTZ-induced seizures. Nevertheless, the pretreatment with rapamycin 4 h prior the induction of flurothyl seizure model has shown anticonvulsant properties in both immature and adult rats (Chachua et al., 2012).

Considering the current knowledge, the antiepileptogenic actions of mTORC1 modulation are more evident, in view of the fact that mTORC1 regulates synaptic plasticity, neuronal death, and neurogenesis (Ryther and Wong, 2012). A previous study has shown that the mTOR signaling pathway is activated during kainate-induced seizures and that rapamycin (6 mg/kg) pretreatments suppress this seizure-induced mTOR activation and also inhibit the epilepsy development, showing antiepileptogenic effects (Zeng et al., 2009). Rapamycin treatments were also able to suppress the epileptogenesis process in a rodent model of temporal lobe epilepsy. Daily rapamycin (10 mm) infusion during 1 or 2 months inhibits aberrant mossy fiber sprouting caused by pilocarpine exposure (Buckmaster et al., 2009). Therefore, the long-term effects of rapamycin administration have been shown to be promising in epilepsy treatment. On the other hand, the immediate action of rapamycin in suppressing seizures is less studied until now. However, it is well known that mTORC1 inhibition could regulate protein synthesis of ion channels and neurotransmitter receptors, suppressing seizure occurrence in patients with epilepsy (Ryther and Wong, 2012). Therefore, it is plausible that mTOR inhibitors may have antiseizure effects in addition to antiepileptogenic action (Ryther and Wong, 2012).

In our study, we demonstrated that acute rapamycin treatment has protective effects against seizures in zebrafish. Rapamycin administration 30 min before PTZ exposure delayed seizure progression in zebrafish at different life stages. In agreement with our findings, different studies showed immediate protective effects of rapamycin against seizure occurrence. Huang et al. (2010) demonstrated antiseizure effects of rapamycin in rats with spontaneous recurrent seizures induced by pilocarpine. In this study, the animals were treated with rapamycin or vehicle control and observed for three weeks. Animals that received rapamycin treatment presented reduced seizure frequency and duration in comparison with the vehicle control group (Huang et al., 2010). Another study showed that rapamycin treatment can inhibit the aggravation of absence seizures induced by the intracerebral administration of the pro-inflammatory bacterial endotoxin lipopolysaccharide in a rat model of absence epilepsy (Russo et al., 2013). Lipopolysaccharide administration induced a time-dependent increase in both number and total duration of spike–wave discharges, with a peak

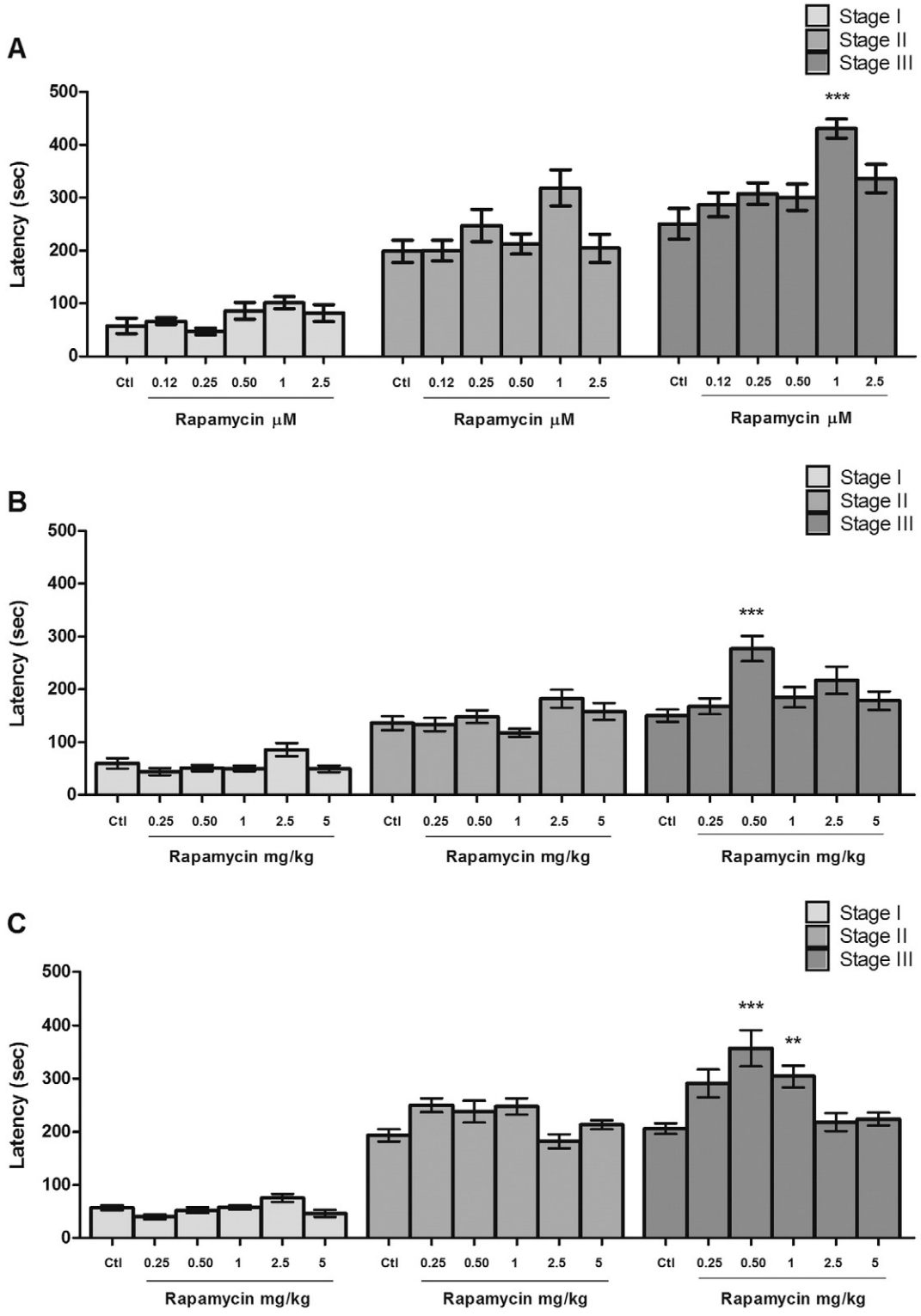

Fig. 3. Effect of rapamycin on progression of the PTZ-induced seizures in zebrafish at different developmental stages. Latency to the first behavioral manifestation of each seizure stage (I, II, and III) during PTZ exposure in larval (A), juvenile (B) and adult (C) zebrafish. Rapamycin was administered at 0.12, 0.25, 0.50, 1 and 2.5 μM (7 dpf) and 0.25, 0.50, 1, 2.5 and 5 mg/kg (45 dpf and adults). DMSO was administered at 0.1% (7 dpf) and 3% (45 dpf and adults). The data are expressed as the mean ± S.E.M. of at least 15 animals for each group and were analyzed by one-way ANOVA followed by Dunnett's post-hoc test. The symbols represent statistical difference when compared to the respective vehicle control group (DMSO). $^{**}P < 0.005$, $^{***}P < 0.0005$.

at around 120 min post-injection. This proconvulsant effect was significantly inhibited by rapamycin (0.5, 1, and 3 mg/kg) treatment given 30 min after lipopolysaccharide administration (Russo et al., 2013). In contrast, rapamycin pretreatment 1 h before kainate exposure promoted an increase in severity and duration of observed seizures and neuronal cell death in rats. The same study showed no difference between animals pretreated with rapamycin 10 h before seizure induction in comparison with animals that did not receive treatment (Chen et al., 2012). Considering clinical studies, the effect of rapamycin on seizure control was evidenced in a phase I/II clinical trial. Patients with

confirmed diagnosis of tuberous sclerosis and medically intractable epilepsy were treated for 12 weeks. The median seizure frequency decreased by 73% and the median cumulative seizure duration decreased by 70% (Krueger et al., 2013).

In summary, rapamycin treatment in view of epileptic seizure control shows a plethora of effects. These effects seem to change according to treatment period, rapamycin dose, life stage, and animal models. The results obtained in our study indicate that rapamycin pretreatment is an important mechanism to control the progress of seizures in zebrafish along different development stages (larval, juvenile, and adult zebrafish). Taken as a whole, our findings support that rapamycin exerts immediate antiseizure effects. Therefore, rapamycin treatment could be a potential alternative therapy for seizure control in epilepsy and might be worthy of further investigation.

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