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Rapamycin protects against Aβ-induced synaptotoxicity by increasing presynaptic activity in hippocampal neurons

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ABSTRACT

The mammalian target of rapamycin (mTOR) is involved in the regulation of learning and memory. Recently, rapamycin has been shown to be neuroprotective in models for Alzheimer's disease in an autophagy-dependent manner. Here we show that rapamycin exerts neuroprotection via a novel mechanism that involves presynaptic activation. Rapamycin increases the frequency of miniature excitatory postsynaptic currents and calcium transients of rat hippocampal primary neurons by a mechanism that involves the up regulation of SV2, a presynaptic vesicular protein linked to neurotransmitter release. Under these conditions, rapamycin-treated hippocampal neurons are resistant to the synaptotoxic effect induced by Aβ oligomers, suggesting that enhancers of presynaptic activity can be therapeutic agents for Alzheimer's disease.

1. Introduction

The mammalian target of rapamycin (mTOR), an evolutionarily conserved serine/threonine protein kinase, regulates protein synthesis and macroautophagy, among other processes [1,2]. mTOR is involved in two distinct enzymatic complexes with different functions, a rapamycin-sensitive complex (TORC1) and a rapamycin-insensitive complex (TORC2). mTOR regulates local protein synthesis at the synapses [3] required for the formation, maturation, and function of new spine synapses [4] that can modulate learning and memory [5,6]. Moreover, mTOR-dependent local axonal autophagy can rapidly regulate presynaptic structure and function [7], and autophagy also positively regulates development of the *Drosophila melanogaster* larval neuromuscular junction [8].

Rapamycin is a macrolide antibiotic and immunosuppressant drug used to prevent rejection in organ transplantation produced by the bacterium *Streptomyces hygroscopicus* [9], whose mechanism of action is to inhibit TORC1 activity. It has been shown that long-term treatment with rapamycin increases the lifespan in mice, possibly by delaying aging [10,11], an effect correlated with decreased protein biosynthesis and autophagy activation [12]. In the central nervous system (CNS), rapamycin shows beneficial effects in survival and neuronal plasticity, thus it could contribute to memory improvement in neurodegenerative disorders [4,12–15]. Accordingly, rapamycin was shown to have beneficial effects *in vivo* [16]. The pathological hallmarks of Alzheimer's disease (AD) are the formation of extracellular senile plaques, mainly composed of amyloid-β (Aβ) peptide, and intracellular neurofibrillary tangles containing hyperphosphorylated tau protein [12]. Aβ peptide is generated by sequential proteolysis of amyloid-β precursor protein (APP), with $Aβ_{1-42}$ being the most toxic form of the peptide [12,17]. Recent evidence shows that rapamycin reduces Aβ levels and abolishes AD progression in a transgenic mouse model of the disease by stimulating autophagy [16]. In the same vein, Aβ can activate the mTOR pathway, which is blocked by rapamycin [18,19].

Considering that chronic application of Aβ induces neurotransmission failure [20,21], we examined whether rapamycin can abolish the synaptic deficit induced by Aβ. We investigated the effect of rapamycin on miniature postsynaptic currents, calcium transients and the levels of the presynaptic protein SV2 using patch clamp, quantitative calcium recordings, immunofluorescence and Western blot, respectively. We report that rapamycin, in chronic conditions (48 h), enhances the synaptic transmission affecting mainly the presynaptic site as expressed by upregulation of SV2 levels. In agreement with this finding, rapamycin increased the frequency of calcium transients and miniature postsynaptic

Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; mTOR, mammalian target of rapamycin; CNS, central nervous system

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currents in primary hippocampal neurons that become resistant to the synaptotoxic effects induced by Aβ oligomers.

Taken together, this data indicates that inhibition of the mTOR pathway by rapamycin enhances synaptic transmission making neurons more resistant to the synaptotoxic effects of Aβ oligomers.

2. Materials and methods

2.1. Peptide $A\beta_{1-42}$ preparation

Human synthetic $A\beta_{1-42}$ peptide was purchased from GenicBio (China). $A\beta_{1-42}$ was dissolved in 100% hexafluoroisopropanol (10 mg/mL) and stored in aliquots at −20 °C. The $A\beta_{1-42}$ aggregation and the formation of $A\beta_{1-42}$ oligomers were carried out as previously described [22]. Briefly, for the preparation of $A\beta_{1-42}$ oligomers, aliquots of 5 μL of $A\beta_{1-42}$ (10 mg/mL) were added to 137.5 μL ultrapure water (final $A\beta_{1-42}$ concentration 80 μM) in an Eppendorf tube. After 15 min of incubation at room temperature (~22 °C), the samples were centrifuged at 14,000 g for 15 min, and the supernatant fraction was transferred to a new Eppendorf tube. To form $A\beta_{1-42}$ oligomers, the samples were stirred at 500 rpm using a Teflon-coated micro-stir bar for 24–48 h at room temperature (~22 °C). The formation of oligomers was monitored by turbidity at 400 nm and the structure of oligomers was confirmed by electron microscopy (data not shown) as previously described [22]. $A\beta_{1-42}$ oligomers were used immediately after their preparation or stored at 4 °C (up to 1 h) until used.

2.2. Hippocampal cultures and treatments

Hippocampal neurons were obtained from 18-day-old Sprague–Dawley rat embryos as previously described [23]. The primary hippocampal cultures were maintained in a neuronal feeding medium consisting of 90% minimal essential medium (HyClone, USA), 5% heat-inactivated horse serum (HyClone, USA), 5% fetal bovine serum (Gibco, USA), and a mixture of nutrient supplements. The cultures were treated at 14–15 DIV with 1 μM rapamycin for 48 h at 37 °C and 5% CO_2 alone or in the presence of 500 nM Aβ oligomers [20]. Animal care and protocols were in accordance with the National Institutes of Health recommendations and approved by the Ethics Committee at the University of Concepcion.

2.3. Patch clamp recordings

Patch pipettes (3–5 MΩ) were prepared from filament-containing borosilicate micropipettes. Currents were measured with the whole cell patch clamp technique at a holding potential of −60 mV using an Axopatch 200B amplifier (Axon Instruments). The data were displayed and stored using a 1322A Digidata acquisition board and analyzed with electrophysiological software (Axon Instruments). The external solution contained 150 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4, 330 mOsmol). The standard internal solution in the patch pipette contained 120 mM KCl, 4.0 mM $MgCl_2$, 10 mM BAPTA, and 2.0 mM Na_2-ATP (pH 7.4, 310 mOsmol). Miniature postsynaptic currents were isolated by the addition of TTX (50–100 nM; Alomone Labs, Israel) and were recorded in 2-minute segments. The data were analyzed with the MiniAnalysis 8.0 program (Synaptosoft, Inc., Leonia, NY) to obtain mean averages of peak amplitude and frequency.

2.4. Western blot

Standard Western blotting procedures were followed [24]. Equal amounts of protein were separated on 10% SDS-polyacrylamide gels. Protein bands were transferred onto nitrocellulose membranes, blocked with 5% nonfat milk, and incubated with primary antibodies using the following concentrations: 1:1000 anti-SV2 (Developmental Studies Hybridoma Bank, Iowa City, IA), and 1:5000 anti-α-tubulin (Sigma, USA). Bands were visualized with the ECL Plus Western blotting detection system (PerkinElmer, USA) and the membranes were revealed in the equipment Odyssey (Li-cor Bioscience, USA). Quantification of the signals was made with the Image Studio Version 1.1 program, and α-tubulin was used as an internal control for the experiments.

2.5. Calcium imaging

Neurons were loaded with 5 μM Fluo-4 AM (Molecular Probes, USA) for 20 min at 37 °C. The neurons were then washed twice with DPBS (1X) and incubated for 20 min at 37 °C. The cells were mounted in a perfusion chamber that was placed on the stage of an inverted fluorescent microscope (Eclipse TE; Nikon) equipped with a xenon lamp and a 40× objective (22–24 °C). The cells were briefly illuminated (200 ms) using a computer-controlled Lambda 10–2 filter wheel (Sutter Instruments). Regions of interest (ROI) were simultaneously selected on neuronal soma emitting Fluo-4 fluorescence (excitation 480 nm, emission 510 nm) in an optical field having usually more than 10 cells. Images were collected at 1 s intervals during a continuous 200 second period. The recordings were obtained with an EMCCD iXon + 16 bit camera (Andor, Belfast, Northern Ireland) and both the filter control and image capture were performed by the software Workbench 6.0 (INDEC BioSystems, USA).

2.6. Immunofluorescence

Control and treated hippocampal neurons were fixed for 10 min with methanol and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS). Nonspecific immunoreactivity was blocked with 10% horse serum for 30 min at room temperature (22–24 °C). Monoclonal anti-SV2 antibody (1:200, Developmental Studies Hybridoma Bank, Iowa City, IA) and polyclonal anti MAP2 (1:200, Santa Cruz Biotechnology, CA, USA) antibody was incubated overnight followed by incubation with an anti-mouse secondary antibody conjugated with FITC (1:200; Jackson ImmunoResearch Laboratories) and anti-rabbit secondary antibody conjugated with Cy3 (1:200; Jackson ImmunoResearch Laboratories), respectively, for 1 h at room temperature (22–24 °C). The samples were mounted in fluorescent mounting medium (DAKO, USA) for microscopic analysis.

2.7. Fluorescence measurements and epifluorescence microscopy

FITC and Cy3 immunofluorescence were visualized using a Nikon Inverted Microscope Eclipse TE300 (60X, oil immersion, NA 1.4) with Argon (488 nm) and He–Ne (543 nm) lasers for FITC and Cy3, respectively. After acquisition, images were processed with ImageJ (NIH, Maryland, USA).

2.8. Data analysis

Statistical analyses were performed using GraphPad Prism (GraphPad, San Diego, CA, USA). The values are expressed as mean ± S.E.M. for 3 or more independent experiments. Statistical differences were determined using Student's *t* test or one-way ANOVA with Bonferroni post test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Chronic effects of rapamycin on mEPSCs

Previous studies have shown that rapamycin increases the life span [10,11], as well as the memory parameters in different animal models [4,14]. In agreement with this, acute inhibition of mTOR by rapamycin affects synaptic plasticity and the long-term potentiation in hippocampal neurons by presynaptic and postsynaptic mechanisms, respectively

[6,25]. However, less is known about the chronic effect of rapamycin in cultured primary neurons. Thus, to evaluate the chronic effects of rapamycin on the neuronal network, we used the whole cell patch clamp technique (−60 mV) to determine the chronic effects of rapamycin on miniature postsynaptic currents in primary hippocampal neuron cultures. All recordings were obtained in the presence of 50–100 nM TTX in order to inhibit action potentials [23]. As shown in Fig. 1, we found that the frequency of miniature postsynaptic currents were significantly higher after chronic administration (48 h) of rapamycin (1 μM) as compared to control (Control, 0.633 ± 0.144 Hz, n = 14 vs rapamycin, 1.610 ± 0.250 Hz, n = 10; $p < 0.01$) (Fig. 1A,B). However, no significant differences were observed in the amplitude of the miniature postsynaptic currents (control, 26 ± 1.0 pA, n = 14 vs rapamycin, 28 ± 1.2 pA, n = 10) (Fig. 1C). These results indicate that chronic application of rapamycin enhances neurotransmission in hippocampal neurons by increasing the release of neurotransmitters from presynaptic sites without apparent changes in postsynaptic receptors.

Next, we evaluated the effects of rapamycin on calcium transient currents as defined by their TTX sensitivity [26]. Spontaneous calcium transients were measured with the Fluo4-AM probe allowing us to assess the changes in intracellular calcium as reflected by synaptic and neuronal network activity. As shown in Fig. 2, rapamycin (1 μM; 48 h) significantly increased the frequency of transient calcium currents in hippocampal neurons (control: 100 ± 4% Hz, n = 58 vs rapamycin: 314 ± 7% Hz, n = 73; $p < 0.001$). These results suggest that rapamycin increased the exocytosis of synaptic vesicles at the synaptic site in agreement with the observed changes in the frequency of miniature postsynaptic currents (Fig. 1). To evaluate whether these changes in presynaptic activity correlated with changes in presynaptic or postsynaptic structural components, we examined the effect of rapamycin on the levels of the synaptic vesicle protein SV2 as a presynaptic marker and PSD95 as a postsynaptic marker. The results obtained by Western blot (Fig. 3A) showed an increase in the levels of SV2 in the presence of rapamycin (control, 100 ± 9% vs rapamycin, 140 ± 9%; $p < 0.05$), but no differences were found in the levels of PSD95 (control, 100 ± 17% vs rapamycin, 97 ± 48%), confirming the presynaptic effect of rapamycin observed in the electrophysiological analyses. The increase in SV2 protein levels was corroborated by immunofluorescence that revealed a marked increase in SV2 immunoreactivity in rapamycin-treated neurons as compared to controls (Fig. 3B). Together, these results indicate that chronic application of rapamycin enhances the presynaptic activity of hippocampal neurons and that the upregulation of the presynaptic SV2 protein component may be involved in this effect.

3.2. Rapamycin protects against synaptotoxicity induced by Aβ oligomers

An early feature of AD is the synaptic failure that precedes hippocampal neurodegeneration and neuronal loss [27]. It has been reported that presynaptic proteins such as SV2, SNAP-25, synaptophysin, and synaptotagmin are reduced in AD brains as well as in primary hippocampal neurons exposed to Aβ oligomers [20,28–30]. Recent evidence suggests that the pharmacological inhibition of the mTOR pathway by rapamycin decreases the cognitive deficits in a mouse model of AD and reduces the levels of Aβ peptide [13,16,31,32]. Considering these reports, we quantitatively assessed the effect of Aβ oligomers on SV2 levels in the presence of rapamycin by Western blot (Fig. 4A). Our results demonstrated that rapamycin prevented the reduction of SV2 levels induced by Aβ oligomers (Control 107 ± 6%; Aβ 55 ± 10%; Aβ + rapamycin100 ± 14%; rapamycin 154 ± 5%, $p < 0.05$). These results suggest that the upregulation of SV2 may be involved in the neuroprotective effect of rapamycin against Aβ oligomer-induced synaptotoxicity.

In addition, we determined the effect of rapamycin on the synaptic failure induced by Aβ oligomers on the frequency of miniature postsynaptic currents using whole cell patch clamp (−60 mV; TTX 100 nM). As previously described, chronic application of Aβ oligomers reduced the synaptic activity of hippocampal neurons in culture [20]. By contrast, hippocampal neurons incubated with Aβ oligomers in the presence of rapamycin displayed similar synaptic activity to control neurons (Fig. 4B,C; control 0.36 ± 0.08 Hz; Aβ 0.08 ± 0.02 Hz; Aβ + rapamycin 0.33 ± 0.05 Hz; rapamycin 0.65 ± 0.07 Hz). These results indicate that

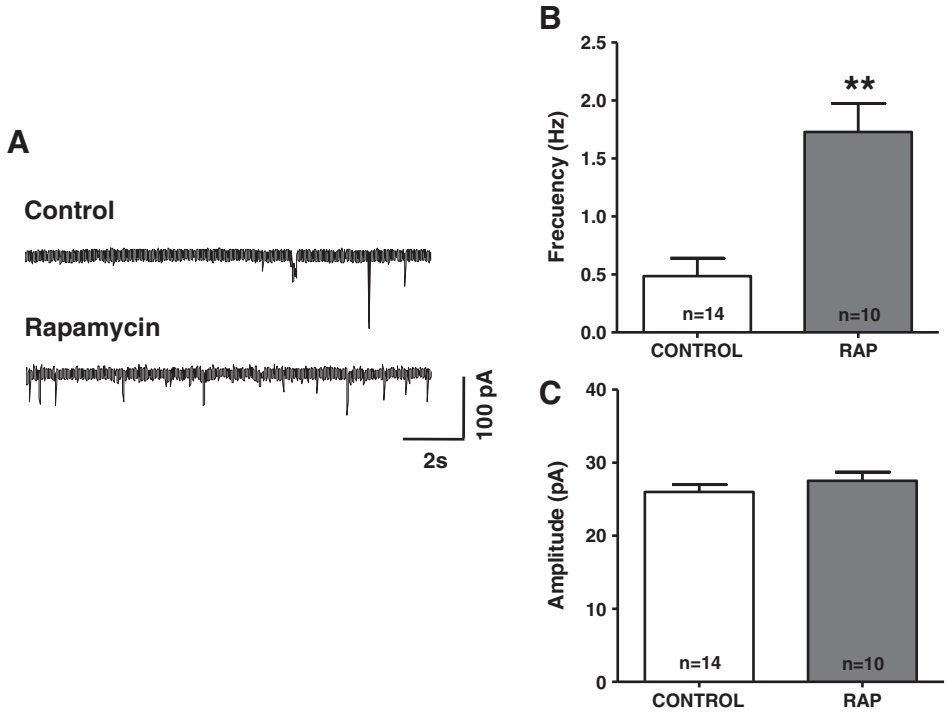

Fig. 1. Effect of rapamycin on synaptic activity of primary hippocampal neurons. (A) Representative traces of miniature spontaneous postsynaptic currents illustrate the enhancing effect of chronic rapamycin treatment (48 h; 1 μM) in hippocampal neurons. (B, C) The bars show the effect of rapamycin (RAP) on the frequency (Hz) and amplitude (pA) of the recordings shown in "A". The values are mean ± SEM obtained from the indicated number of neurons. ** $p < 0.01$ (Student *t*-test).

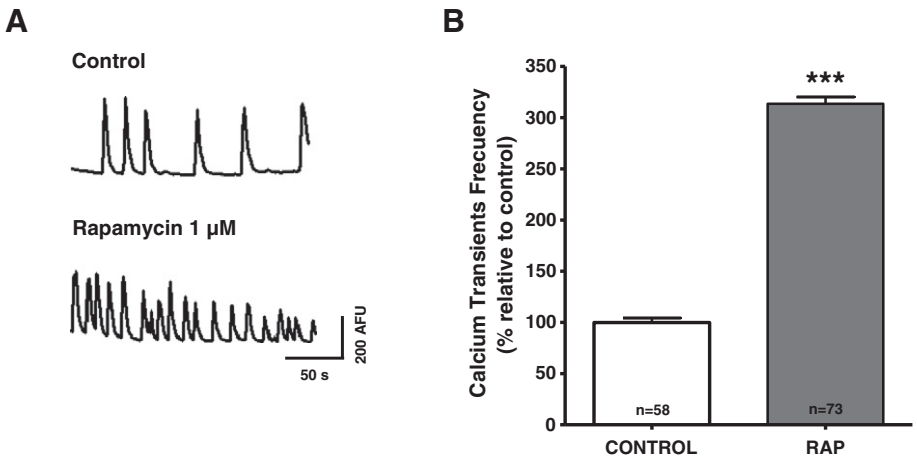

Fig. 2. Chronic treatment with rapamycin increases the frequency of intracellular calcium transients. (A) Representative fluorimetric recordings obtained from control hippocampal neurons and rapamycin-treated hippocampal neurons (48 h; 1 μM). (B) Effect of rapamycin (RAP) on the frequency (Hz) of intracellular Ca^{2+} transients obtained from the indicated number of neurons. Bars are mean ± SEM. *** $p < 0.001$ (Student *t*-test).

rapamycin enhances neuronal network connectivity and protects against synaptic failure induced by Aβ oligomers.

4. Discussion

Rapamycin, a macrolide antibiotic, is known to act by noncompetitive inhibition of the TORC1 complex, a crucial serine–threonine kinase which regulates several cellular processes [33]. Recent evidence shows that rapamycin was effective at increasing the lifespan in mice even though treatment began at 600 days of age [10]. Since there are several pathologies associated with aging and the fact that aging is, by far, the greatest risk factor for most neurodegenerative diseases, it would be interesting to know if the increase in lifespan is correlated with an increased mental health status. The aim of our study was to determine the effect of chronic rapamycin treatments in mature rat hippocampus cultures (14–15 DIV). Our results showed that chronic application of rapamycin increased the frequency of miniature postsynaptic currents, but not the amplitude of the events, indicating a presynaptic site of action. Recent evidence shows that autophagy, induced by the inhibition of mTOR, was strongly activated in the hippocampus of rapamycin-treated mice [16]. In agreement with our observations, previous studies suggest that the effects of autophagy are primarily presynaptic [7,8], and that mTOR-regulated autophagy has a synaptic role, specifically in regulating neurotransmission [7]. These studies support our results, and may suggest that the observed enhancement of presynaptic transmission in mature hippocampal neurons by rapamycin may be a result of the activation of autophagy.

The electrophysiological results were correlated with an increase in the frequency of transient calcium currents. In agreement with these observations, Terashima et al. showed that the activity of a Ca^{2+}-dependent K^+ channel was modulated by rapamycin through its direct association with the FK506 binding protein-12 (FKBP12) which induced a significant increase in the open time of the channel [34]. The increase in transient calcium current frequency induced by rapamycin suggests that rapamycin augmented the calcium-dependent exocytosis of synaptic vesicles. Supporting the electrophysiological and calcium transient observations, we found that rapamycin increased SV2 levels, which is a positive modulator of calcium-dependent exocytosis [35]. In agreement with our results, previous studies showed that rapamycin leads to NMDA-dependent induction of long-term potentiation (LTP) in CA1 neurons of rat hippocampal slices when paired with weak presynaptic stimulation [36], improved memory deficits associated with cannabinoid consumption [37] and rescued memory deficits in a mouse model of tuberous sclerosis in which the mTOR pathway was hyperactivated [14]. It is important to highlight that the effect of rapamycin is not restricted to disease models, but it is also observed in wild type aged animals [10]. Furthermore, recent evidence shows that rapamycin enhances cognitive function in young adult mice and blocks age-associated cognitive decline in older animals [31]. This evidence is consistent with our results, since we observed an improvement in the neuronal network connectivity and function with rapamycin treatment (Figs. 1; 2; 3).

Our results, taken together with previous studies indicate that mTOR activity is required for local protein synthesis at synapses [3], suggest that mTOR may have time-dependent effects on the regulation of synaptic function. Our studies, performed after 48 h of mTOR inhibition, suggest that sustained inhibition of mTOR activity in hippocampal neurons in culture enabled enhanced synaptic transmission [5,6,36]. Because TOR is a conserved Ser/Thr kinase that regulates cell growth and metabolism in response to environmental cues (insulin/IGF, cell energy status, nutrients and stress) via different signaling transduction pathways [33], it is possible that inhibition of mTOR may affect synaptic function in different ways, not only at different times during synaptic activation, but also in a manner dependent on specific culture conditions such as local rapamycin concentrations and age of cultured neurons, among other factors.

An important issue related with aging is the prevalence of age-related diseases like AD. It is not clear how the inhibition of mTOR could have neuroprotective effects. In this regard, it is well known that mTOR is important in the regulation of protein synthesis [2,4], which is critical for synaptic plasticity and memory consolidation [4]. In this context, studies have shown that inhibition of mTOR decreases the late phase of long term potentiation (LTP) [4,6]. Also, there is evidence showing that Aβ decreases the mTOR signaling [38,39], having a toxic effect. Interestingly, Cammalleri et al. showed that there is a time dependence of rapamycin sensitivity since the late long-term potentiation (L-LTP) is sensitive to application of rapamycin only during the induction paradigm, whereas rapamycin application after the establishment of L-LTP was ineffective [40]. On the other hand, several evidence show beneficial effects of rapamycin (i.e. extending the lifespan in various species including mice) [10,41], as well as therapeutic benefits in experimental models of several age-linked neurodegenerative diseases as AD [16,42]. Our results support the neuroprotective effect described for rapamycin, since it prevents the synaptic failure induced by Aβ oligomers (Fig. 4).

There is evidence that shows a relationship between brain seizures and the incidence of Alzheimer's disease [43–45]. Palop et al. showed that Aβ oligomers cause wide fluctuations in the neuronal expression of synaptic activity-regulated genes, as well as epileptiform activity and nonconvulsive seizures [46,47]. They proposed that high levels of Aβ induce aberrant excitatory neuronal activity and this activates

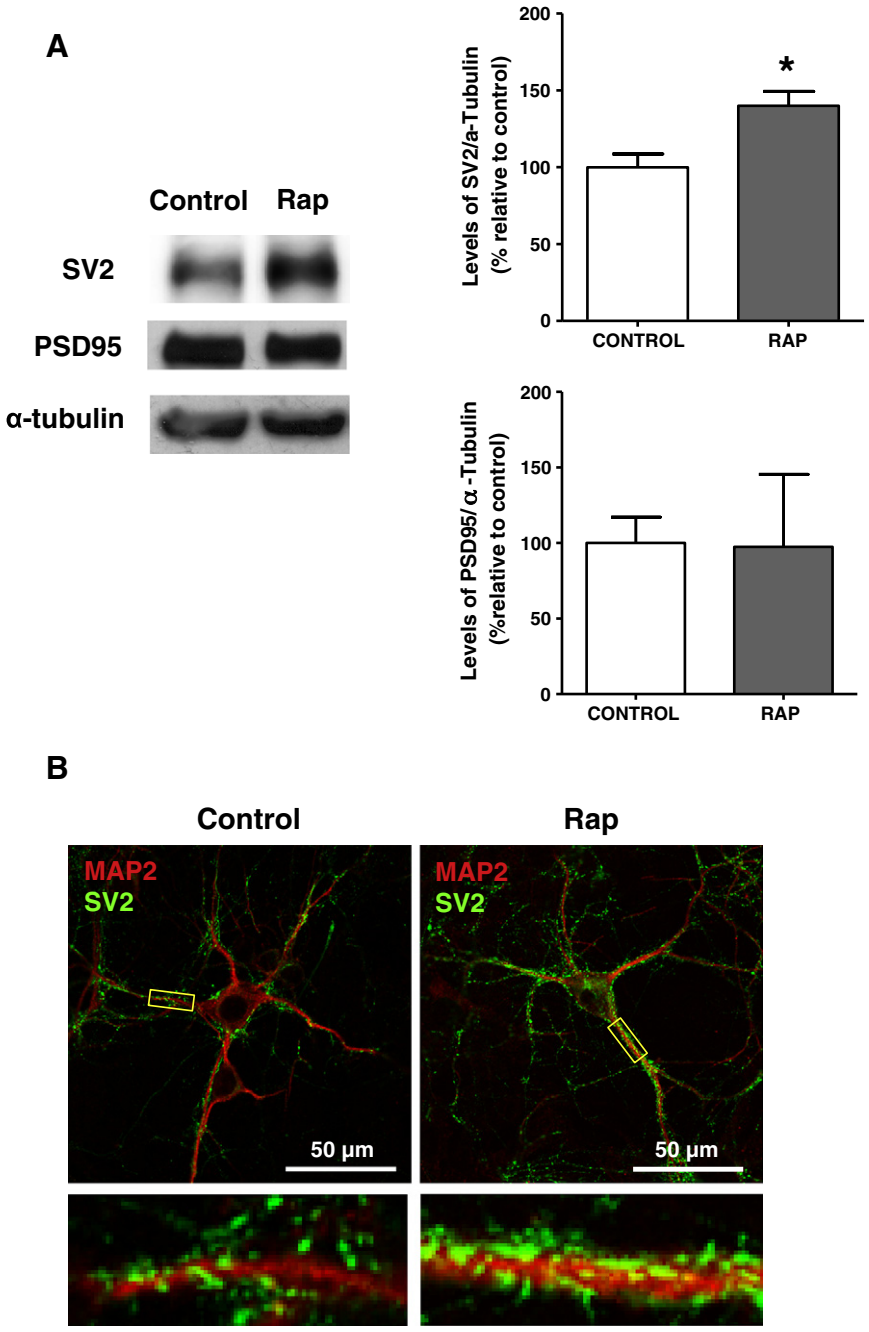

Fig. 3. Chronic treatment with rapamycin increases SV2 levels. (A) Representative Western blots for SV2 and PSD95 levels in hippocampal neurons untreated or treated with rapamycin (RAP) (48 h; 1 μM). α-tubulin levels were used as internal controls. The bars are mean ± SEM obtained from five different experiments. * $p < 0.05$ (Student *t*-test). (B) Epifluorescence images were obtained from control and rapamycin (48 h; 1 μM) treated neurons using antibodies against SV2 (green) and MAP-2 (red). The bars are mean ± SEM obtained from three different experiments.

compensatory inhibitory mechanisms to neutralize this overexcitation, which may contribute to AD-related network dysfunction [46]. Apparently the epileptiform-like effect of Aβ is acute, since it has been shown that Aβ induced a rapid increase in intracellular calcium and miniature currents, indicating an enhancement in vesicular transmitter release, which eventually leads to a blockage in neurotransmission by vesicular depletion [20] leading to a synaptic loss. Related to this, several studies had reported a reduction in presynaptic proteins, such as SNAP25, synaptophysin, synaptotagmin and SV2, in neuronal cultures chronically treated with Aβ oligomers [20,28]. According to this, our results showed a decrease in the levels of presynaptic SV2 proteins and in synaptic activity of hippocampal neurons after chronic treatment with Aβ oligomers (Fig. 4). Although there is evidence showing that the increase in synaptic activity is associated with neurodegenerative effects [46,47], there is also evidence suggesting that synaptic activation protects against Aβ-related synaptic alterations [48]. The intracellular accumulation of Aβ oligomers is associated with early degeneration of distal neurites and synaptic compartments [48,49]. Apparently the synaptic activation by glycine-induced long-term potentiation (g-LTP) and KCl-induced depolarization reduce the levels of intraneuronal Aβ [48]. Also, evidence has shown that moderate levels of NMDAR activity are beneficial for neurons [50]. For example, synaptic NMDAR activation inhibits the production of Aβ [51] and selective enhancement of synaptic activity by low doses of NMDA interrupts Aβ-induced neurotoxicity [52]. On the other hand, recent studies indicate that decreasing mTOR signaling through the administration of rapamycin reduces Aβ levels

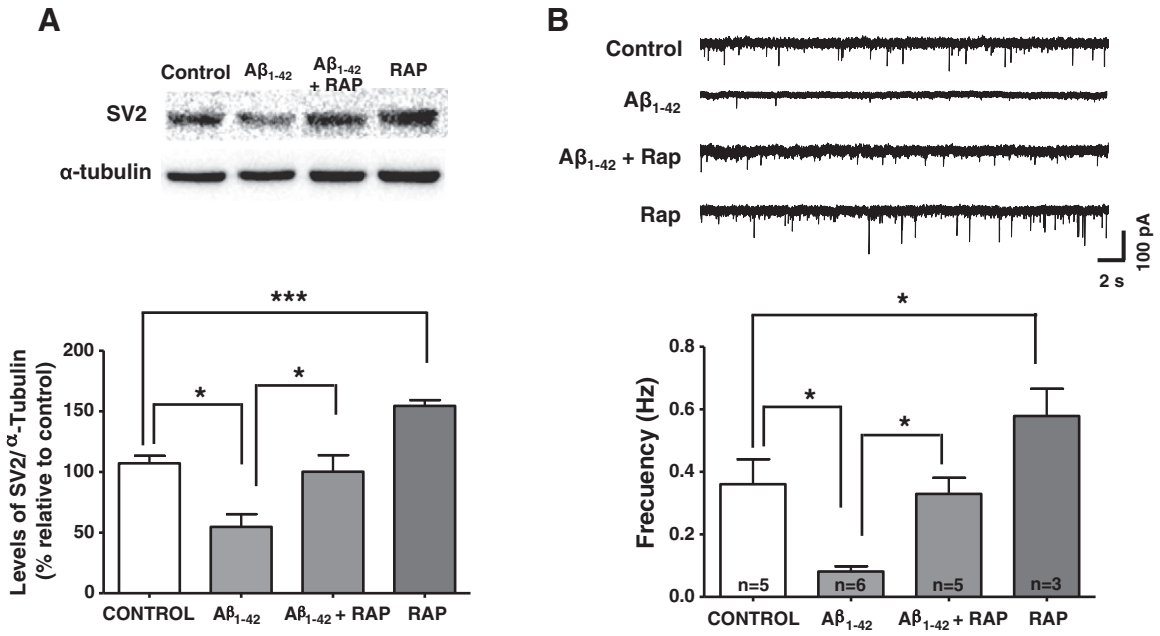

Fig. 4. Chronic treatment with rapamycin protects against toxicity induced by Aβ oligomers. (A) Top, representative Western blots for SV2 levels in hippocampal neurons treated with Aβ oligomers (48 h; 500 nM) in the absence or presence of rapamycin (RAP) (1 μM). Levels of α-tubulin were used as internal controls. Bottom, the graph summarizes the results shown in "A". Bars are mean ± SEM obtained from three different experiments. * $p < 0.05$; *** $p < 0.001$ (one-way ANOVA with Bonferroni post test). (B) Top, representative traces of miniature spontaneous postsynaptic currents illustrate the effect of chronic Aβ oligomer treatment (48 h; 500 nM) in the absence or presence of rapamycin (48 h; 1 μM) in hippocampal neurons. Bottom, the graph summarizes the frequency (Hz) of the recordings shown in "B". The values are mean ± SEM obtained from the indicated number of neurons. *$p < 0.05$ (one-way ANOVA with Bonferroni post test).

[32], and it has been previously shown that an AD mouse model fed with the same rapamycin-supplemented diet that extends lifespan [10], blocked AD-like impairments in spatial learning and memory by autophagy [16]. According to this evidence, our results suggest that the chronic inhibition of mTOR with rapamycin induces an increase in the synaptic activity which might protect against the synaptotoxity of Aβ oligomers by regulating presynaptic components such as SV2 (Fig. 4).

Rapamycin is already used in clinical settings, and it has been previously shown that rapamycin crosses the blood–brain barrier [53], which makes it an interesting drug for treating neurodegenerative diseases. Therefore, our study sheds light on the use of rapamycin or rapamycin-related compounds [54] in clinical trials, supporting the use of "enhancers of presynaptic activity" as drugs for chronic conditions characterized by sustained synaptic failure such as that in AD.

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