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Iron Overload Impairs Autophagy: Effects of Rapamycin in Ameliorating Iron-Related Memory Deficits

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Abstract

Over the years, iron accumulation in specific brain regions has been observed in normal aging and related to the pathogenesis of neurodegenerative disorders. Many neurodegenerative diseases may involve cognitive dysfunction, and we have previously shown that neonatal iron overload induces permanent cognitive deficits in adult rats and exacerbates age-associated memory decline. Autophagy is a catabolic pathway involved in the removal of toxic protein aggregates, which are a hallmark of neurodegenerative events. In the present study, we investigated whether iron accumulation would interfere with autophagy and also sought to determine the effects of rapamycin-induced stimulation of autophagy in attenuating iron-related cognitive deficits. Male Wistar rats received a single daily oral dose of vehicle or iron carbonyl (30 mg/kg) at postnatal days 12–14. In adulthood, they received daily intraperitoneal injections of vehicle or rapamycin (0.25 mg/kg) for 14 days. Results showed that iron given in the neonatal period impaired inhibitory avoidance memory and induced a decrease in proteins critically involved in the autophagy pathway, Beclin-1 and LC3, in the hippocampus. Rapamycin in the adulthood reversed iron-induced memory deficits, decreased the ratio phospho-mTOR/total mTOR, and recovered LC3 II levels in iron-treated rats. Our results suggest that iron accumulation, as observed in neurodegenerative disorders, hinders autophagy, which might play a role in iron-induced neurotoxicity. Rapamycin, by inducing authophagy, was able to ameliorate iron-induced cognitive impairments. These findings support the use of rapamycin as a potential neuroprotective treatment against the cognitive decline associated to neurodegenerative disorders.

Keywords Iron · Autophagy · Rapamycin · Cognitive deficits · Neurodegenerative disorders

Introduction

Iron is an essential micronutrient required for many biological processes in the human body. During central nervous system (CNS) development, high levels of iron are required for the synthesis of neurotransmitters, acting as a cofactor for many enzymes, generation of ATP, and myelination [1]. The functions of iron are based on its biological capacity of interconversion between the ferrous (Fe^{+2}) and ferric (Fe^{+3}) states [1, 2]. However, although crucial for neuronal metabolism, when in excess, this metal can also lead to cellular damage and oxidative stress via free radical production [2], a well-known hallmark of neurodegenerative diseases [3]. Moreover, consistent evidence suggest a pivotal relationship between high iron levels in specific brain regions, such as the hippocampus, substantia nigra, and cortex, and the pathogenesis of neurodegenerative disorders, such as Alzheimer's (AD) and Parkinson's (PD) diseases [4–6]. Moreover, high iron content in brain regions, associated to aging or neurodegenerative disorders, correlates with poor performance in cognitive tests in healthy elderly individuals [7, 8] or in patients with neurodegenerative disorders such as Alzheimer's disease [9, 10].

Further studies are necessary to determine the exact mechanism of neurotoxicity mediated by iron as well as its role on cognitive impairments. Findings from studies using animal

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models have contributed to elucidate the impact of iron accumulation in neurodegenerative disorders (for a review see [11]). Previous studies from our research group have established an animal model of iron administration during the neonatal period, in which maximum iron absorption by the CNS occurs. Iron treatment in this period induces accumulation in brain regions affected by neurodegenerative disorders [12]. Remarkably, in agreement with studies performed in humans, previous studies have shown that iron overload induces persistent memory impairments in several cognitive tasks, including spatial [12], emotional [13–15], and recognition memories [16–19]. In addition, we have also demonstrated that iron loading leads to oxidative damage [16], decreases BDNF levels [20], increases the expression of apoptotic markers [21–23], affects mitochondrial dynamics [22], and results in the accumulation of ubiquitinated proteins [15] in the hippocampus of adult rats.

Autophagy and ubiquitin-proteasome system are the two main pathways of protein and cellular content degradation in eukaryotic cells. The autophagy-lysosome pathway is a major intracellular catabolic process, in which cytoplasmic contents are engulfed and transported to the lysosome for degradation. In addition, autophagy is a pathway responsible to degrade damaged, misfolded, or aggregated proteins, for the maintenance of cellular homeostasis [24–26]. Defective autophagy has been recently implicated in the pathogenesis of several human diseases, including neurodegenerative disorders. Because neurodegeneration is marked by abnormal long-lived protein aggregation, such as amyloid-beta (Aβ) peptide in AD and alpha-synuclein in PD [27–30], studies have consistently suggested autophagy as a protective strategy for effective clearance of these aggregates [27, 29–34].

Furthermore, recent evidence has suggested that iron plays an important role in autophagy regulation since iron overload collaborates with the increase of the protein aggregates [35–37]. Thereby, both in vivo and in vitro studies have reported that autophagy induction could potentially mitigate progression in neurodegenerative diseases with iron deposition, but the relationship between these mechanisms remains poorly understood [33, for a review see [34]).

Autophagy is an intricate machinery regulated by many signaling pathways and can be pharmacologically induced by inhibiting negative regulators such as the mammalian target of rapamycin (mTOR), a serine/threonine kinase [38–40]. This event is regulated by at least sixteen key proteins named autophagy-related genes (ATGs), which are recruited to the pre-autophagosomes elongation and autophagosome formation. A large protein complex is necessary to form the autophagosome nucleation; Beclin-1 is a protein involved in the isolation of membranes to form autophagosomes [41–44]. During the elongation phase, the conjugation of phosphatidylethanolamine (PE) to the microtubule-associated protein 1 light chain 3 (LC3)-I forms LC3-II, which is transported from cytoplasm to the membrane of the pre-autophagosomes. LC3 II is a marker for the progression of autophagy [40, 45–48]. Once autophagosome formation is completed, this structure fuses with lysosomes for degradation [49].

Rapamycin is a macrolide antibiotic that inhibits mTOR [50]. Rapamycin binds to the cytosolic protein FK-binding protein 12 (FKBP12) and inhibits the mTOR kinase activity by phosphorylation of P70S6K [51, 52]. Thus, inhibition of mTOR by rapamycin treatment induces autophagy [38]. A few studies show that rapamycin enhances the clearance of aggregate-prone proteins and reduces their toxicity in neurodegenerative disorders [53–56]. However, additional information about the precise role of autophagy in neuroprotection and associated signaling pathway(s) is necessary. Thus, in the present study, we aimed to investigate whether iron accumulation plays a causative role in autophagy impairments accompanying neurodegeneration. Moreover, we sought to determine whether the pharmacological activation of autophagy by rapamycin could ameliorate iron-induced memory deficits.

Materials and Methods

Animals

Pregnant Wistar rats (CrlCembe:WI) were obtained from the Centro de Modelos Biológicos Experimentais (CeMBE), Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil. After birth, each litter was adjusted within 48 h to contain eight rat pups including offspring of both genders. Each pup was kept together with its mother in a plastic cage with sawdust bedding in a room temperature of 21 ± 1 °C and a 12/12 h light/dark cycle. At the age of 3 weeks, pups were weaned and the males were selected and maintained in groups of three to five in individually ventilated cages with sawdust bedding. For postnatal treatments, animals were given standardized pellet food and tap water ad libitum.

All behavioral experiments were performed at light phase between 09:00 a.m and 4:30 p.m. All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th Edition 2011) and the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI, Brazil). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted and approved by the Institutional Ethics Committee of the Pontifical Catholic University (permit number: SIPESQ no. 8469). All efforts were made to minimize the number of animals and their suffering.

Experimental Design

In order to investigate a possible interplay between iron overload and autophagy, as well as a potential protective effect of rapamycin against iron-induced memory deficits, litters were randomly assigned (simple randomization) to receive either iron carbonyl or vehicle solution. Only male pups from each litter received the treatment from postnatal days 12th to 14th. All litters (those that received iron and those that received vehicle) were kept in the same room with their respective mothers under the same environmental conditions. Subsequently, at the age of 4 months, the two groups were further randomly divided into four groups, resulting in four experimental groups: Veh-Veh, Veh-rapamycin, Iron-Veh, and Iron-rapamycin. During treatments, all groups of rats were kept in the same room under the same environmental conditions. After 14 days of rapamycin treatment, rats were tested in the inhibitory avoidance memory task. Twenty-four hours after the completion of behavioral task, all rats were euthanized by decapitation, and their hippocampi were quickly removed and stored in − 80 °C freezer for posterior biochemical analysis. Animals from each experimental group were randomly assigned for each one of the proteins analyzed. Experimental design is shown in Fig. 1.

Pharmacological Treatments

Neonatal Iron Treatment

The neonatal iron treatment was performed as previously described [13, 15, 16]. Briefly, 12-day-old rat pups received a single oral daily dose (10 ml/kg solution volume) of vehicle (5% sorbitol in water, control group) or 30 mg/kg of body weight of Fe^{2+} (iron carbonyl, Sigma-Aldrich, São Paulo, Brazil) via a metallic gastric tube, over 3 days (postnatal days 12–14). Rats in each group (vehicle or iron) were derived from 7 to 8 different litters, in order to avoid a possible litter effect.

Rapamycin

Adult (4-month-old, weighing approximately 550 g) rats, treated neonatally with either vehicle or iron as described above, received a daily intraperitoneal injection of vehicle or rapamycin (Sigma Aldrich, São Paulo, Brazil). Rapamycin was dissolved in 0.9 g% saline solution containing 0.5 % DMSO (vehicle), and administered daily for 14 days at the dose of 0.25 mg/kg [57]. Inhibitory avoidance training was performed 24 h after the last rapamycin administration.

Inhibitory Avoidance Task

We used the single-trial, step-down inhibitory avoidance (IA) conditioning as an established model of fear-motivated memory. In IA training, animals learn to associate a location in the training apparatus with an aversive stimulus (footshock). The IA behavioral training and retention test procedures were described in previous reports [12, 13, 15]. The IA apparatus was a 50 × 25 × 25-cm^3 acrylic box (Albarsch, Porto Alegre, Brazil) whose floor consisted of parallel stainless steel bars (1-mm diameter) spaced 1 cm apart. A 7-cm wide, 2.5-cm high platform was placed on the floor of the box against the left wall. On the training trial, rats were placed on the platform, and their latency to step-down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, rats received a mild footshock (0.4 mA) and were removed from the apparatus immediately afterwards. A retention test trial was carried out 24 h after the training trial. The retention test trial was procedurally identical to training, except that no footshock was presented. Step-down latencies (in seconds) on the retention test trial (maximum 180 s) were used as a measure of IA retention. Behavioral procedures were performed by an experimenter blinded to animals' experimental condition.

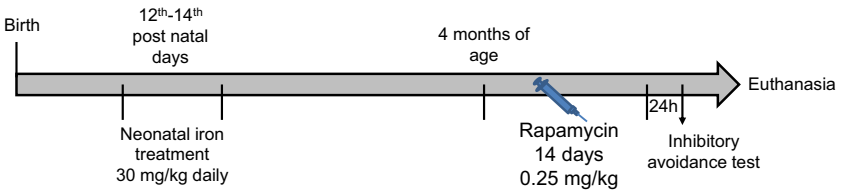

Fig. 1 Experimental design. Groups of rats were treated with vehicle or iron (orally, 30 mg/kg) in the neonatal period at postnatal days 12th to 14th. In adulthood (4 months of age), they received i.p. injections of rapamycin (0.25 mg/kg) or vehicle for 14 days. Twenty-four hours after the last injection, animals were trained in the inhibitory avoidance memory task. Twenty-four hours after the completion of behavioral testing, animals were euthanized by decapitation, and their hippocampi were quickly isolated and stored in − 80 °C for western blot analysis

Western Blot Analysis

Hippocampi obtained from rats treated with iron in the neonatal period and rapamycin in adulthood were homogenized in homogenization buffer containing 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 100 mM NaCl, protease inhibitor cocktail, 0.5%Triton X-100, and 0.1% SDS. After 30 min in ice, samples were centrifuged at 13,500 rpm for 10 min [22, 23]. The supernatant was collected, and the protein content was determined using Bradford assay [58]. Aliquots were stored at − 20 °C.

Twenty-five micrograms of protein was separated on a 10% SDS polyacrylamide gel (or 8% SDS polyacrylamide gel for mTOR and phospho-mTOR analyses) and transferred electrophoretically to a nitrocellulose membrane. Gels for each antibody analyzed included samples from all experimental groups. Membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween 20 and were incubated overnight with the following antibodies: anti-β-actin as loading control (ab8227, Abcam, Cambridge, UK) at 1:1500 and anti-LC3 II (ab48394, Abcam, Cambridge, UK) at 1:1000; or anti-β-actin as loading control (ab8227, Abcam, Cambridge, UK) at 1:1500 and anti-Beclin-1 (ab62557, Abcam, Cambridge, UK) at 1:1000. For Phospho-mTOR and mTOR analysis, we have first probed membranes with Phospho-mTOR antibody (phospho S2448) (ab109268, Abcam, Cambridge, UK) at 1:2000. After that, membranes were stripped and reprobed with mTOR antibody (ab2732, Abcam, Cambridge, UK) at 1:2000, since results are expressed as a ratio phospho-mTOR/total mTOR. For stripping, membranes were washed twice with a mild stripping buffer (containing 15 g glycine, 1 g SDS, 10 ml Tween20, pH 2.2, final volume of 1,0 L) for 30 min and twice with PBS for 10 min at room temperature, before incubating with total mTOR antibody. Goat anti-mouse IgG and goat polyclonal anti-rabbit IgG (both from Abcam, Cambridge, UK) secondary antibodies were used and detected using ECL Western Blotting Substrate Kit (Abcam, Cambridge, UK). Pre-stained molecular weight protein markers (84785, SuperSignal Molecular Weight Protein Ladder, Thermo Scientific, Rockford, USA) were used to determine the detected bands' molecular weight. The densitometric quantification was performed using Chemiluminescent photo finder (Kodak/Carestream, model GL2200) by an experimenter blind to samples experimental condition. Total blotting protein levels of samples were normalized according to each sample's β-actin levels [22, 23].

Statistical Analysis

Latencies to step-down and data from western blot experiments are expressed as mean ± standard error (S.E.). Statistical comparisons were performed using two-way analysis of variance (2-way ANOVA), with neonatal treatment (vehicle or iron) and adult treatment (vehicle or rapamycin) as fixed factors. One-way ANOVA, followed by Tukey's post hoc test, was used to test differences between the experimental groups. In all comparisons, p values less than 0.05 were considered to indicate statistical significance.

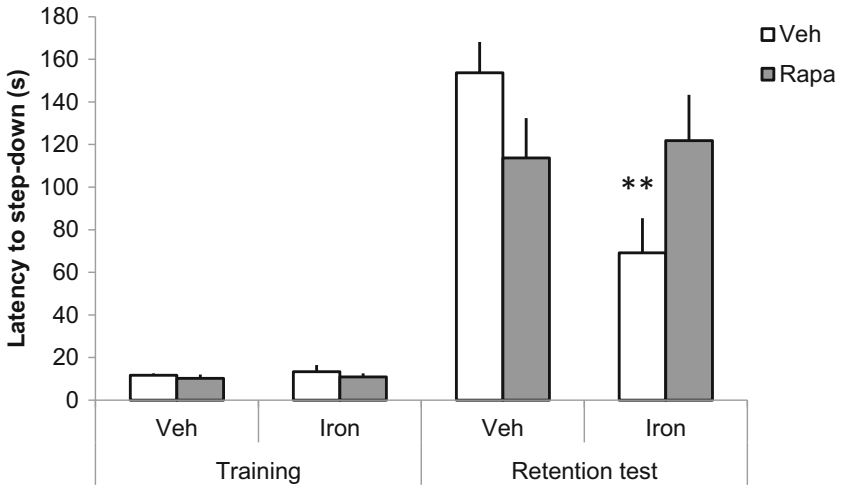

Fig. 2 Subchronic rapamycin in the adulthood rescues iron-induced inhibitory avoidance memory impairment. Inhibitory avoidance task was performed in rats treated neonatally with vehicle (Veh) or iron (30 mg/kg of Fe^{2+}) and given vehicle (Veh) or rapamycin (Rapa, 0.25 mg/kg) in adulthood (4 months of age). Veh-Veh $N = 14$, Veh-Rapa $N = 9$, Iron-Veh $N = 11$, Iron-Rapa $N = 13$. Statistical analysis was performed using two-way ANOVA, with neonatal treatment (vehicle or iron) and adult treatment (vehicle or rapamycin) as fixed factors. Further comparisons were performed using Tukey's post hoc tests. $^{**}p < 0.01$ (Iron-Veh compared to control group Veh-Veh)

Results

Our first goal was to determine if subchronic rapamycin treatment would be able to reverse iron-induced memory deficits. Two-way ANOVA indicated a statistically significant main effect of neonatal iron treatment ($F_{(1,\ 43)} = 4.329$, $p = 0.043$) in latencies to step-down in the retention test session, confirming previous findings that show that iron given in the neonatal period impairs inhibitory avoidance memory. Further comparisons using Tukey's post hoc test indicated that the iron-treated group that received vehicle in adulthood presented a significantly lower latency to step-down in the long-term retention test when compared to the control group ($p = 0.008$). Remarkably, a significant interaction ($F_{(1,\ 43)} = 6.383$, $p = 0.015$) indicates that rapamycin treatment ameliorates memory in iron-treated rats, as latency to step-down in the retention test was higher in the iron-treated group that received rapamycin than in the iron-treated group that received vehicle in the adulthood (Fig. 2). In addition, no significant differences were found when comparing the iron-treated group that received rapamycin in adulthood with the control group (vehicle-vehicle, $p = 0.548$), corroborating rapamycin reversion of iron-induced memory impairment. Also, rapamycin by itself has no significant effect on memory retention since the vehicle-rapamycin group showed no statistical difference from the control group (vehicle-vehicle, $p = 0.440$). No statistically significant effects were found when latencies from the training session were compared.

To confirm the autophagy-stimulating effects of rapamycin, we first analyzed mTOR phosphorylation in the hippocampus of rats that received iron or vehicle in the neonatal period and rapamycin for 14 days in adulthood (Fig. 3). Two-way ANOVA indicated a significant main effect of rapamycin treatment. Besides improving memory retention, rapamycin treatment in the adulthood caused a decrease in the ratio phospho-mTOR/total mTOR ($F_{(1,\ 11)} = 8.00$, $p < 0.016$).

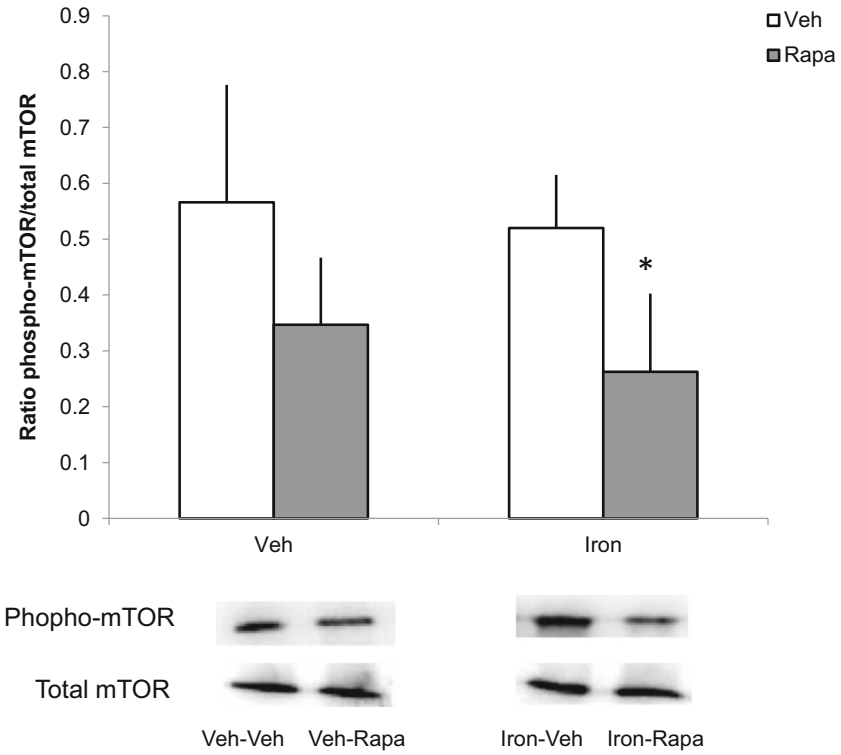

Fig. 3 Rapamycin treatment in the adulthood decreases the ratio phospho-mTOR/total mTOR. **a** Western Blotting of phospho-mTOR and total mTOR in the hippocampus of 4-month-old rats treated with vehicle (Veh) or iron neonatally and treated with vehicle (Veh) or rapamycin (Rapa) in the adulthood. Twenty-five micrograms of protein were separated on SDS-PAGE and probed with specific antibodies. **b** Representative Western blots for phospho-mTOR and total mTOR are shown in the lower panel. Membranes were first probed with Phospho-mTOR antibody and after stripping were reprobed with mTOR antibody, since results are expressed as a ratio phospho-mTOR/total mTOR. Statistical analysis was performed using two-way ANOVA, with neonatal treatment (vehicle or iron) and adult treatment (vehicle or rapamycin) as fixed factors. Veh-Veh $N = 5$, Veh-Rapa $N = 3$, Iron-Veh $N = 3$, Iron-Rapa $N = 4$. Further comparisons were performed using Tukey's post hoc tests. *$p < 0.05$ (Iron-Rapa compared to control group Veh-Veh)

Tukey's post hoc comparison also indicated a significant reduction of the ratio phospho-mTOR/total mTOR induced by rapamycin in the iron-treated group in comparison with the control group (vehicle-vehicle, $p = 0.039$). No other significant effects were found.

In order to examine the effects of iron overload and of the treatment with rapamycin on autophagy in iron-treated rats, we analyzed the expression of proteins critically involved in the autophagic cascade. Beclin-1, a protein involved in autophagy initiation, was quantified. Two-way ANOVA indicated a significant main effect of neonatal iron treatment ($F_{(1,\ 9)} = 6.567$, $p < 0.031$), which induced a decrease in Beclin-1 levels (Fig. 4). No significant effects of rapamycin treatment in the adulthood or interactions were observed. Post hoc comparisons revealed no additional statistical differences.

When LC3 II levels were analyzed, two-way ANOVA revealed significant main effects of iron treatment reducing LC3 II levels ($F_{(1,\ 9)} = 19.27$, $p = 0.002$) and rapamycin in the adulthood rising LC3 II levels ($F_{(1,\ 9)} = 13.175$, $p = 0.005$). Importantly, a significant interaction was observed ($F_{(1,\ 9)} = 10.541$, $p = 0.010$), suggesting that rapamycin in the adulthood was able to restore iron-induced decreases in LC3 II levels (Fig. 5). Further comparisons using Tukey's post hoc tests confirmed these results. Neonatal iron treatment decreased LC3 II levels when compared to control group (vehicle-vehicle $p = 0.002$). Rapamycin was able to restore LC3 II levels, since statistical comparison revealed a significant difference between iron-treated group that received rapamycin and iron-treated group that received vehicle in adulthood ($p = 0.003$). Moreover, LC3 II levels in the iron-rapamycin group were not statistically different from the control group ($p = 0.942$).

Discussion

In the present study, we show that neonatal iron treatment impairs inhibitory avoidance memory, a form of emotionally modulated memory, confirming previous findings from our research group showing that iron overload hinders memory in rodents [12, 15, 16]. Studies with humans have also shown that iron accumulation in brain regions, measured by magnetic resonance imaging, is related to memory deficits in normal aging [8, 59] as well as in neurodegenerative disorders [60, 61]. Using an animal model based on iron administration for 3 days during the neonatal period, a period of high iron uptake by the brain, we have demonstrated that iron overload induces

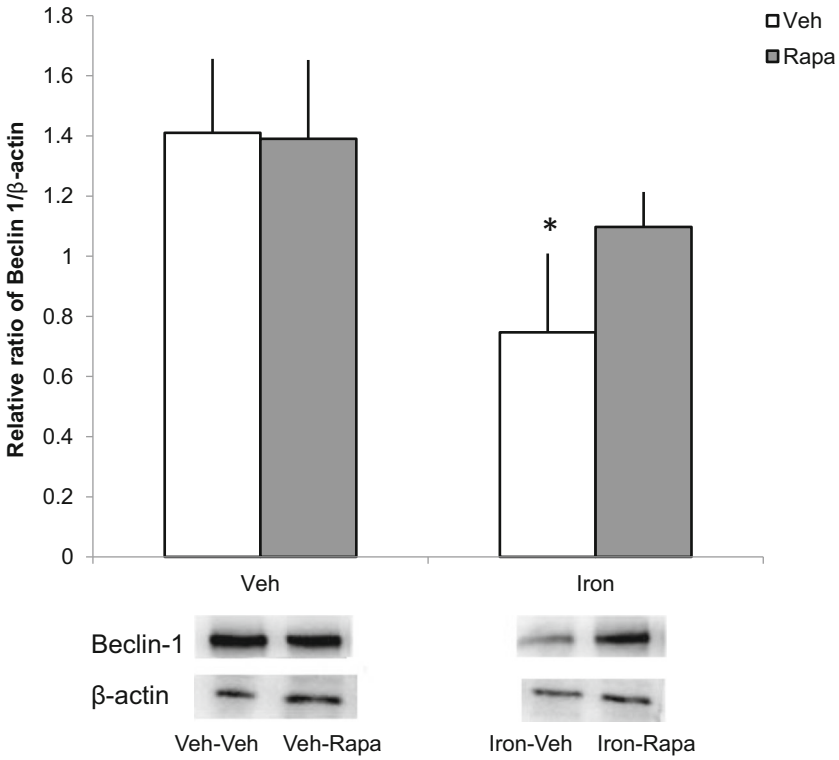

Fig. 4 Neonatal iron treatment decreases Beclin-1 levels. **a** Western Blotting of Beclin-1 in the hippocampus of 4-month-old rats treated with vehicle (Veh) or iron neonatally and treated with vehicle (Veh) or rapamycin (Rapa) in the adulthood. Twenty-five micrograms of protein were separated on SDS-PAGE and probed with Beclin-1 and β-actin antibodies. (**b**) Representative Western blots for Beclin-1 and β-actin are shown in the lower panel. Statistical analysis was performed using two-way ANOVA, with neonatal treatment (vehicle or iron) and adult treatment (vehicle or rapamycin) as fixed factors. Veh-Veh $N = 3$, Veh-Rapa $N = 3$, Iron-Veh $N = 3$, Iron-Rapa $N = 4$. *$p < 0.05$ (significant main effect of iron treatment)

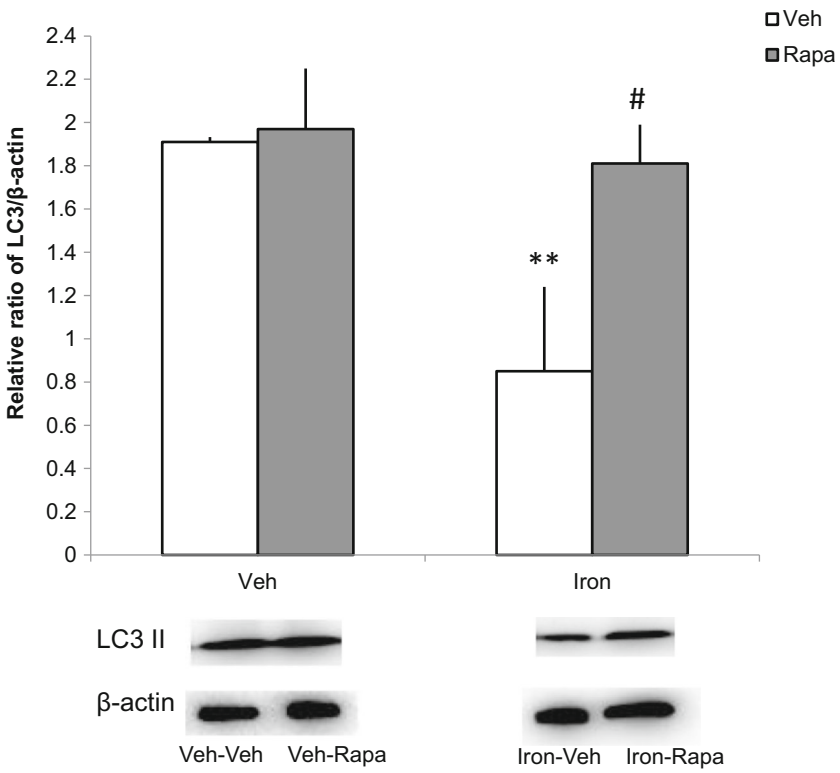

Fig. 5 Neonatal iron treatment induced a decrease in LC3 II levels, which was reversed by rapamycin in the adulthood. **a** Western Blotting of LC3 II in the hippocampus of 4-month-old rats treated with vehicle (Veh) or iron neonatally and treated with vehicle (Veh) or rapamycin (Rapa) in the adulthood. Twenty-five micrograms of protein were separated on SDS-PAGE and probed with LC3 II and β-actin antibodies. Representative Western blots for LC3 II and β-actin are shown in the lower panel. Statistical analysis was performed using two-way ANOVA, with neonatal treatment (vehicle or iron) and adult treatment (vehicle or rapamycin) as fixed factors. Veh-Veh $N = 3$, Veh-Rapa $N = 3$, Iron-Veh $N = 3$ Iron-Rapa $N = 4$ rats per group. Further comparisons were performed using Tukey's post hoc tests. $^{**}p < 0.01$ (Iron-Veh compared to control group Veh-Veh). $\#p < 0.01$ (Iron-Rapa compared to Iron-Veh)

alterations consistently affected in the pathogenesis of neurodegenerative diseases, including increased oxidative stress [16] and altered mitochondrial dynamics [22], induction of apoptosis [21–23] and reactive gliosis [62], and decreased expression of synaptic marker synaptophysin [22] in the hippocampus. Previous findings also indicate that iron overload is associated with impairments in the ubiquitin proteasome system, a major cellular protein degradation pathway [15].

There is increasing evidence indicating the role of misfolded protein degradation mediated by the autophagy system in neurodegenerative diseases, suggesting that failure in this pathway may represent an important contribution to disease progression. This is supported by the presence of aggregates of misfolded proteins, which are substrates for autophagy degradation in most neurodegenerative disorders [27, 63]. However, the exact influence of autophagy is yet to be determined, since the incorrect removal of aggregated protein may have cumulative effects that can be expressed later in life [63]. Although both autophagy impairment and iron deposition seem to be involved in the development and progression of neurodegenerative disorders, if and how they interact remain unknown.

The present findings demonstrate that rapamycin treatment in adult life was able to ameliorate memory retention in the inhibitory avoidance task, suggesting a significant memory improvement in the iron-treated group. In agreement, it was shown that rapamycin improved learning and memory deficits in transgenic mouse models of AD [56, 64], as well as in a rat model of AD-like phenotype, induced by Aβ1-42 peptides injection into the hippocampus [57]. In addition, rapamycin was also able to recover sepsis- [65] as well as anesthesia-induced memory deficits [66]. The mechanisms underlying rapamycin-induced memory recovery are poorly understood. However, a recent study by Glatigny and coworkers [67] demonstrated that fear learning upregulates autophagy in the hippocampus. In agreement, it was found that water maze training increases the number of autophagosomes [68]. Moreover, downregulation of hippocampal autophagy reduces both short- and long-term activity-dependent synaptic plasticity capacities in hippocampal neurons in response to novel neuronal stimuli [67]. Thus, these findings support the view that autophagy may be required for learning and memory.

Our results indicated that rapamycin subchronic treatment in a low dose in adulthood was able to decrease the ratio of phosphorylated mTOR to total mTOR in iron-treated rats. These findings are

corroborated by Liu and coworkers [65], showing that rapamycin decreased mTOR phosphorylation at S2448 and protected against sepsis-induced memory impairments. Based on the observation that autophagy may play a role on memory consolidation, this mechanism may contribute, at least in part, to rapamycin-induced amelioration of iron-related memory deficits. On the other hand, considering that mTOR is an intracellular serine/threonine protein kinase that plays a central role in various cellular processes, we cannot rule out the possibility that inhibition of mTOR by rapamycin might have other effects unrelated to autophagy that could explain its effects on memory.

Aiming to understand the role of iron overload and possible interactions with rapamycin treatment in the autophagy cascade, in this study, we have investigated the effects of iron on key proteins involved in the autophagy pathway in hippocampi of adult rats. Beclin-1 is the mammalian homolog of yeast autophagy-related gene 6 (Atg6), and participates in the autophagosome formation and initiation of autophagy through class III PI3K pathway [28]. It has been reported that Beclin-1 deletion disrupts neuronal autophagy and promotes β-amyloid deposition and neurodegeneration in mice [69]. Here, we demonstrate that the group that received neonatal iron treatment showed a significant decrease in Beclin-1 levels. Also, LC3 II, a protein critically involved in the fusion of autophagosomal membranes was found to be reduced in hippocampi of iron-treated rats, suggesting that autophagy cascade is downregulated by iron overload. Wan and coworkers [37] recently demonstrated using SH-SY5Y cells that iron treatment impaired autophagy, decreasing the levels of autophagy-related proteins including Beclin-1 and LC3, leading to α-synuclein accumulation, and reactive oxygen species (ROS) production. Studies show that iron-loaded lysosomes are sensitive to oxidative stress and to consequent lysosomal disruption [70], while multiple myeloma cell lines exposure to iron chelators led to repression of mTOR signaling, inducing autophagy [71], corroborating that iron availability may modulate autophagy. Iron-induced decreased levels of Beclin-1 and LC3 II suggest that iron overload in vivo compromises downstream autophagy components.

Rapamycin decreased mTOR phosphorylation and also recovered LC3 II levels in iron-treated rats, while no effects were observed on Beclin-1 in iron-treated rats. Although the reasons why rapamycin has not directly recovered Beclin-1 levels, its actions on mTOR phosphorylation and LC3 II suggest stimulatory effects on autophagy. LC3 II association with autophagosomal membranes is closely related with the formation of mature autophagosomes, thus measuring LC3 II levels has been consistently used as a marker of autophagosome formation [63]. Additionally, rapamycin-induced stimulation of autophagy may contribute to neuroprotection against iron-related neurotoxicity, which can ultimately result in memory recovery.

This is a pioneer study, linking for the first time, in vivo iron overload with impaired autophagy, suggesting that iron-induced autophagy deregulation might be related to memory deficits observed in this animal model. From a translational point of view, the present findings alert for the risk imposed by the indiscriminate iron supplementation in infancy, period in which iron access to the brain is maximal. The induction of autophagy by rapamycin alleviated the memory decline induced by iron accumulation, which has been implicated in memory loss in aging and neurodegenerative disorders, giving support for its use as a potential candidate for the treatment of cognitive deficits.

Authors' Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Vanise Hallas Uberti, Betânia Souza de Freitas, Patrícia Molz, and

Elke Bromberg. The first draft of the manuscript was written by Nadja Schröder and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Institutional Ethics Committee of the Pontifical Catholic University, permit number SIPESQ no. 8469).

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