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ORIGINAL CONTRIBUTION

Effects of lipoic acid supplementation on age- and iron-induced memory impairment, mitochondrial DNA damage and antioxidant responses

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Abstract

Purpose To investigate the effects of lipoic acid (LA) supplementation during adulthood combined with supplementation later in life or LA administration only at old age on age-induced cognitive dysfunction, mitochondrial DNA deletions, caspase 3 and antioxidant response enzymes expression in iron-treated rats.

Methods Male rats were submitted to iron treatment (30 mg/kg body wt of Carbonyl iron) from 12 to 14th post-natal days. Iron-treated rats received LA supplementation (50 mg/kg, daily) in adulthood and old age or at old age only for 21 days. Memory, mitochondrial DNA (mtDNA) complex I deletions, caspase 3 mRNA expression and antioxidant response enzymes mRNA expression were analyzed in the hippocampus.

Results LA administration in adulthood combined with treatment later in life was able to reverse age-induced effects on object recognition and inhibitory avoidance memory, as well as on mtDNA deletions, nuclear factor (erythroid-derived 2)-like 2 (*Nrf2)* expression, and antioxidant enzymes disruption induced by iron in aged rats. LA treatment only at old age reversed iron-induced effects to a lesser extent when compared to the combined treatment.

Conclusion The present findings support the view that LA supplementation may be considered as an adjuvant against mitochondrial damage and cognitive decline related to aging and neurodegenerative disorders.

Keywords Lipoic acid · Aging · Iron · Memory · Mitochondria · Antioxidant

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Introduction

Ageing is a complex biological process, inevitable and multifactorial, characterized by a progressive loss of homeostatic balance by individuals, leading to decline of biochemical and physiological functions [1, 2], including reduction of cognitive function, as well as increased risk for neurodegenerative disorders [3].

Iron is an essential micronutrient for human nutrition, however, impaired iron homeostasis is among the many pathways commonly altered in aging and age-associated neurodegeneration [4, 5]. Evidence suggests that iron accumulation, reactive oxygen species (ROS) production, and mitochondrial dysfunction are features of neurodegenerative diseases, playing key roles in the process of cell death [5]. It has been demonstrated that iron overload during brain development, a period of maximum iron uptake by the mammalian brain, leads to progressive cognitive decline and increased iron content in brain regions, which are

accompanied by oxidative stress in adulthood and old age in rats [6–10]. Iron overload at the post-natal period is also associated with increased apoptotic markers and mitochondrial dysfunctions in the hippocampus of adult rats [11–14].

Dietary supplements may represent an option for preventing, delaying or controlling the progression of age-associated biological changes that may result in neurodegeneration [15, 16]. Over the years, a growing interest has been given to lipoic acid (LA), a naturally occurring dithiol compound synthesized enzymatically in the mitochondrion, as a nutritional supplement and therapeutic agent [17–20]. It is usually present in food sources of animal and vegetable origin, such as muscle meats, liver, heart and to a lesser extent in fruits and vegetables [17, 21]. It has been suggested that LA, might display neuroprotective properties due to its ability to cross the blood–brain barrier, gaining access to the CNS where it can be reduced to dihydrolypoic acid (DHLA) [17, 18, 21] and LA has been found in rat brain tissue after oral administration [22]. Studies have reported that LA improves cognitive functions [19, 20] and prevents oxidative damage related to mitochondrial dysfunction associated with aging [18–20]. Moreover, LA may present powerful antioxidant properties, inhibiting the formation of free radicals, acting as chelating agents of transition metal ions, such as iron, thereby reducing the bioaccumulation of the element in the brain, suggesting that LA can be a potential candidate for the treatment and prevention of neurodegenerative diseases [21, 23].

Although there are some symptomatic treatments, no treatment available is effective in curing or slowing the progressive nature of neurodegenerative diseases and associated cognitive dysfunction. It has been proposed that failure of novel therapeutic agents may be due to the fact that treatments are introduced long after the pathological process in the central nervous system has initiated. Thus, the present study aimed to test whether the administration of LA in the adult period and aged period would have greater beneficial effects on age- and iron-induced cognitive and biochemical dysfunctions than LA supplementation in the aged period only.

Material and methods

Animals

Pregnant Wistar rats (3 months old) (CrlCembe:WI) were obtained from the Centro de Modelos Biológicos Experimentais (CeMBE) of the Pontifical Catholic University in Porto Alegre, RS, Brazil. After birth, each litter was adjusted within 48 h to eight rat pups including offspring of both genders in the same proportion and kept at standard laboratory conditions. At the age of 3 weeks, pups were weaned and the males were selected and raised in groups of three to five in individually ventilated cages with sawdust bedding. For post-natal treatments, the animals received standardized pellet food and tap water ad libitum. This study was approved by the Institutional Ethics Committee for the Use of Animals of the Pontifical Catholic University (CEUA SIPESQ #7510) and all experimental procedures were performed in accordance with the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI, Brazil).

Dietetic treatments

Neonatal iron treatment

Male rats from randomly distributed litters were treated with vehicle solution (Sorb, sorbitol, C6H14O6 to 5%) or iron (30 mg/kg/body wt of Carbonyl iron, Sigma-Aldrich Brazil Ltda, São Paulo, Brazil) in the neonatal period during three post-natal days (12th to 14th post-natal days) [6, 11–14]. In this model, iron supplementation is given during the period of maximal iron absorption by the brain, and it has been demonstrated that iron supplemented during this period increases iron content in brain regions of rodents [9, 24].

Supplementation with lipoic acid

At the age of 19 months, a subset of rats treated orally with sorbitol/iron in the neonatal period (as described above) received LA (50 mg/kg, Sigma-Aldrich Brazil Ltd, São Paulo, Brazil) diluted in water or water only (vehicle), orally by gavage daily for 21 days. Another subset of rats treated as described, received LA or water orally by gavage daily for 21 days at the age of 6 months (adulthood) and later at 19 months of age (combined LA regimen). For more details, please see, Supplementary material—Fig. 1. Drug solutions were freshly prepared immediately prior to administration. The dose of LA used was chosen based on previously published articles [25, 26].

Behavioral procedures

Open-field behavior

To evaluate the possible sensory-motor effects of LA on iron-treated rats, behavior during the exploration of an open field was evaluated in old age. The open-field behavior test was performed as previously described [27] and is presented as Supplementary material—Table 2.

Object recognition task

The object recognition test was performed as previously described [7, 27]. The animals were tested in elderly (19 months old), 14 days after the beginning of LA supplementation. Briefly, on the first day, rats were submitted to a habituation session in which they were placed in a rectangular open field (40 cm × 45 cm × 60 cm), and were left to explore it for 5 min. Twenty-four hours after the habituation session, animals were placed in the open field, where they were exposed to two identical objects (A1 and A2). Animals were left to explore the objects until they had accumulated 30 s of total time exploring the objects or for a maximum of 10 min. For the evaluation of the long-term memory (LTM), 24 h after the training session, rats were allowed to explore the open field for 5 min in the presence of two objects: the familiar object (A) and a novel object (B). All experimental sessions were filmed and subsequently analyzed. The exploration of objects was measured using two stopwatches to register the time spent exploring them during the experimental sessions. The "recognition index" was expressed by the ratio TB/(TA + TB), where: TA = time spent exploring the familiar object (A) and TB = time spent exploring the novel object (B) [7, 27].

Inhibitory avoidance

The general procedures for inhibitory avoidance training and retention tests were described in Uberti et al. [28]. The inhibitory avoidance training apparatus (50 cm × 25 cm × 25 cm) was an acrylic box with a floor composed 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 one wall.

On training trials, performed on the 20th day of LA treatment in old age, rats were placed on the platform and their latency to step down on the grid with all four paws was measured with a digital chronometer. Immediately after stepping down on the grid, rats received 0.4-mA, 3.0-s footshock and then were removed from the apparatus immediately afterward. The retention test trial was performed 24 h after training by placing the rats on the platform and recording their latencies to step down. Step-down latencies on the retention test trial (maximum 180 s) were used as a measure of inhibitory avoidance memory retention.

Twenty-four hours after the completion of inhibitory avoidance testing, rats were euthanized by decapitation. Hippocampi were rapidly dissected and the left hemisphere was placed in a refrigerated RNA-later solution (Sigma-Aldrich, São Paulo, Brazil) for RT-qPCR assays and the right hemisphere was placed in PBS solution for mitochondrial Complex I deletion assays. Samples were stored at – 80 °C for subsequent molecular analyses.

Molecular analyses

Quantitative PCR (qPCR) analysis of mitochondrial Complex I deletion

Total DNA was extracted from frozen hippocampus using the ReliaPrep™ gDNA Tissue Miniprep System (Promega Corporation, Promega Biotecnologia do Brasil, Ltda, São Paulo, Brazil) in accordance to the manufacturer's instructions. The extract was used for qPCR analysis without further purification. Two different regions of the mitochondrial genome were analyzed; one within the *nd1* gene, which rarely undergoes deletions and another within the *nd4* gene, which is often deleted. Primers used to identify *nd1* and *nd4* regions are described in Supplementary material—Table 1. Standard qPCR reactions were performed in quadruplicate using the 7500 Real-Time PCR System (Applied Biosystems). The results were expressed as *nd4 /nd1* ratio [29, 30].

Quantitative reverse transcription PCR (RT-qPCR) analysis of caspase 3 and antioxidant enzymes

RT-qPCR was used to analyze the mRNA levels of caspase 3 (*Casp3*), Nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*), and anti-oxidant enzymes, glutathione peroxidase (*Gpx1*) and NAD (P) H: quinone oxidoreductase 1 (*Nqo1*), as previously described [11, 13]. PCR primer sequences are available in Supplementary material—Table 1. Molecular analysis was performed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines for RT-qPCR experiments [31, 32]. Extraction of total RNA with the TRIzol reagent (Invitrogen, Carlsbad, USA) from hippocampus of aged rats was performed according to the manufacturer's instructions. The purity (Abs 260/280 nm ~ 2.0) and concentration of the RNA were determined by NanoDrop Lite (Thermo Fisher Scientific) and after treated with Deoxyribonuclease I—Amplification Grade (Sigma-Aldrich, São Paulo, Brazil) to eliminate genomic DNA contamination. All samples were tested by 1% agarose gel electrophoresis with Gel Red nucleic acid dye (Biotium, Hayward, USA). The cDNA species were synthesized ImProm-II™ Reverse Transcription System (Promega Corporation, Promega Biotecnologia do Brasil, Ltda, São Paulo, Brazil) from 1 µg of the total RNA, following the manufacturer's instruction.

Quantitative PCR was performed using SYBR® Green I (Thermo Fisher Scientific, Waltham, USA) to detect double-strand cDNA synthesis on the 7500 Real-time PCR System (Applied Biosystems). The PCR cycling conditions were the same as described previously [13]. All real-time assays were carried out in quadruplicate and, in all cases, a reverse transcriptase negative control was included by substituting the templates for DNase/RNase-free distilled water in each

PCR reaction. *Hprt1* was used as reference gene for normalization. The efficiency per sample was calculated using LinRegPCR 2018.0 Software (http://LinRegPCR.nl). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Cq}$ method [11, 13, 32].

Statistical analysis

Data were expressed as mean ± standard error and analyzed using SPSS (Statistical package for the social sciences) 16.0 software. Levene's test of equality of error variances was used to test the assumption of homogeneity of variance. Variances were similar among the experimental groups for all tested variables. The comparisons were performed by means of a two-way analysis of variance (2-way ANOVA), using neonatal treatment (vehicle or iron) and adult supplementation (water or LA) as fixed factors. Values of $p < 0.05$ were used as a level of significance.

Results

Behavioral analysis

We first investigated the effects of iron overload and LA supplementation later in life only or in adulthood combined with administration later in life on memory of aged rats, using two diverse learning and memory paradigms: object recognition and inhibitory avoidance, an aversive type of memory.

When results from object recognition of aged groups that received LA only in old age were analyzed using 2-way ANOVA, we found no significant main effects of neonatal treatment or LA supplementation neither in the training session (Neonatal treatment: $F_{(1,27)} = 0.044$, $p = 0.835$; Old age LA supplementation $F_{(1,27)} = 0.154$, $p = 0.698$; Fig. 1a), nor in the retention test session (Neonatal treatment: $F_{(1,27)} = 0.208$, $p = 0.652$; Old age LA supplementation $F_{(1,27)} = 0.038$, $p = 0.848$; Fig. 1a), suggesting that LA, administered at old age only, was not able to rescue recognition memory deficits associated to aging. Statistical comparison of recognition indexes of aged groups in which LA administration was performed in adulthood combined with treatment later in life, revealed a significant main effect of LA-combined treatment in the retention test session ($F_{(1,31)} = 12.47$, $p = 0.001$, Fig. 1b). No significant main effect of neonatal iron treatment was found ($F_{(1,31)} = 0.077$, $p = 0.784$, Fig. 1b) and no interaction ($F_{(1,31)} = 0.154$, $p = 0.697$, Fig. 1b). In the training session, no significant main effects of neonatal treatment ($F_{(3,31)} = 0.014$, $p = 0.907$, Fig. 1b) or combined LA supplementation (ANOVA, $F_{(3,31)} = 0.068$, $p = 0.796$, Fig. 1b) were found. These findings show that the LA-combined treatment group presented significantly higher recognition indexes in the memory

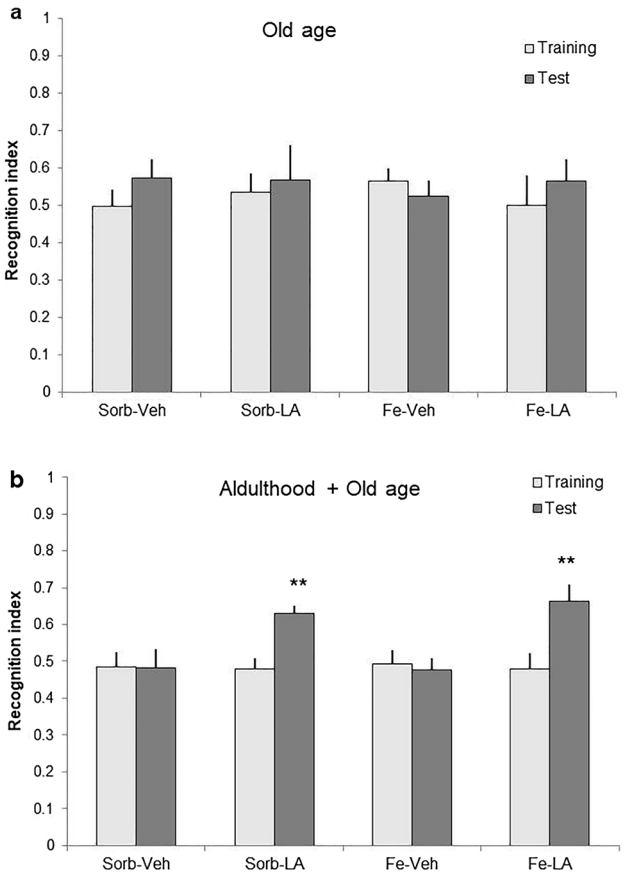

Fig. 1 Lipoic acid combined treatment (adulthood and old age) rescues recognition memory impairment in aged rats. **a** Object recognition was performed in rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) at old age only (19 months of age). Behavioral testing was performed after 14 days of treatment. Sorb-Veh $N = 9$, Sorb-LA $N = 8$, Fe-Veh $N = 8$, Fe-LA $N = 6$. **b** Object recognition task was performed in rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) combined treatment at adulthood (6 months of age) and at old age only (19 months of age). Behavioral testing was performed after 14 days of treatment. Sorb-Veh $N = 9$, Sorb-LA $N = 7$, Fe-Veh $N = 9$, Fe-LA $N = 10$. Data expressed as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA. **Indicates main effect of LA-combined treatment, $p < 0.01$

retention test than the groups that did not receive LA, suggesting that LA-combined treatment reversed age-induced recognition memory deficits.

Comparisons of inhibitory avoidance results from aged groups that received LA only in old age showed no significant difference when analyzing neonatal treatment (2-way ANOVA, $F_{(1,32)} = 3.44$, $p = 0.073$, Fig. 2a), while a significant main effect of LA supplementation (2-way ANOVA, $F_{(1,32)} = 17.92$, $p < 0.0001$, Fig. 2a) was observed, when comparing retention test latencies. Noteworthy, we also found a significant interaction (2-way ANOVA, $F_{(1,32)} = 19.98$, $p < 0.0001$, Fig. 2a). When comparing training latencies,

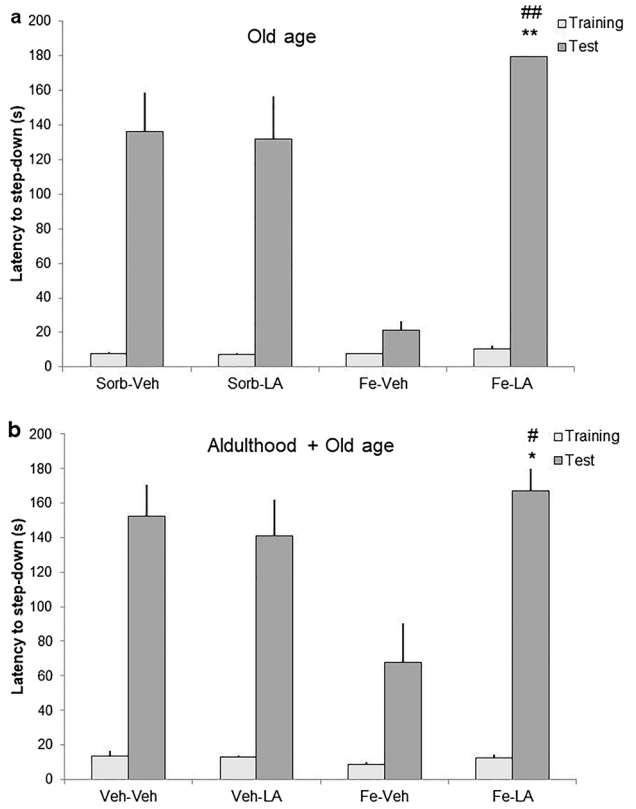

Fig. 2 Lipoic acid treatment at old age only and LA-combined treatment rescues inhibitory avoidance memory impairment in aged rats. **a** Inhibitory avoidance task was performed in rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) at old age only (19 months of age). Sorb-Veh $N=11$, Sorb-LA $N=8$, Fe-Veh $N=10$, Fe-LA $N=7$. **b** Inhibitory avoidance task was performed in rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) combined treatment at adulthood (6 months of age) and at old age only (19 months of age). Sorb-Veh $N=7$, Sorb-LA $N=9$, Fe-Veh $N=11$, Fe-LA $N=12$. Statistical analysis was performed using two-way ANOVA. Data expressed as mean ± S.E.M. **Indicates main effect of LA treatment at old age only, $p<0.0001$; ##Indicates significant interaction $p<0.0001$; *Indicates main effect of combined LA treatment, $p<0.05$; #Indicates significant interaction $p<0.01$

no significant main effects of neonatal treatment (2-way ANOVA, $F_{(1,32)}=2.09$, $p=0.158$, Fig. 2a) or old age LA supplementation (2-way ANOVA, $F_{(1,32)}=0.92$, $p=0.345$, Fig. 2a) were revealed. Interestingly, contrarily to results found in object recognition test, LA supplementation only at old age was able to recover aversive memory deficits. Similar results were found when we compared groups that received LA-combined treatment. A significant main effect of LA-combined treatment when comparing latencies in the memory retention test (2-way ANOVA, $F_{(1,35)}=5.18$, $p=0.029$, Fig. 2b) and a significant interaction (2-way ANOVA, $F_{(1,35)}=8.17$, $p=0.007$, Fig. 2b) were found. When comparing training latencies, no significant main effects of neonatal treatment (2-way ANOVA, $F_{(1,35)}=2.19$, $p=0.148$, Fig. 2b) or LA-combined treatment (2-way ANOVA, $F_{(1,35)}=0.82$, $p=0.372$, Fig. 2b) were found. These results suggest that LA-combined treatment recovered aversive memory deficits in aged rats (Fig. 2b).

To control for possible sensory-motor effects related to iron and/or LA treatment that could interfere with memory acquisition, we evaluated general exploratory activity in the open field. No significant main effects of neonatal treatment or LA supplementation were found when the number of crossings, number of rearings, or number of fecal pellets were compared, suggesting that iron and/or LA administered at old age and in the adulthood and old age did not alter motor activity, motivation or anxiety measured in a 5-min open-field session (For complete description of Results please see Supplementary material, Table 2).

Molecular analysis

We first aimed at examining if iron overload, as a source of oxidative insult, would induce mtDNA damage in the hippocampus of aged rats. We also were interested in analyzing the effects of LA supplementation later in life only, in comparison with LA given in adulthood combined with administration later in life. Statistical comparison using 2-way ANOVA indicated a significant main effect of neonatal iron treatment ($F_{(1,17)}=21.05$, $p<0.0001$, Fig. 3a), while no main effect of LA supplementation at old age only ($F_{(1,17)}=2.42$, $p=0.138$, Fig. 3a), nor significant interactions ($F_{(1,17)}=1.78$, $p=0.199$, Fig. 3a) were found. When analyzing the results of LA-combined treatment in adulthood and later in life, 2-way ANOVA indicated significant main effects of neonatal iron treatment ($F_{(1,19)}=11.23$, $p=0.003$, Fig. 3b) and combined LA supplementation ($F_{(1,19)}=8.12$, $p=0.010$, Fig. 3b) and a significant interaction ($F_{(1,19)}=9.30$, $p=0.007$, Fig. 3b). These results suggest that iron overload increases the levels of mtDNA deletions, and that LA given at old age only was not able to reverse these alterations, while the LA-combined supplementation was able to ameliorate iron-induced mtDNA deletions.

Mitochondria play a key role in regulating apoptosis, and then we next analyzed the effects of iron loading and the two different LA regimens on *caspase 3* gene expression in the hippocampus of aged rats. Two-way ANOVA revealed a significant main effect of iron neonatal treatment ($F_{(1,13)}=15.04$, $p=0.002$, Fig. 4a), but no significant main effect of LA supplementation ($F_{(1,13)}=0.012$, $p=0.915$, Fig. 4a) when comparing groups that received iron in the neonatal period and LA treatment only in old age, indicating that iron in the neonatal period increased *caspase 3* gene expression in aged rats. When groups that received LA-combined treatment were analyzed, *caspase 3* was also increased in iron-treated rats, although this effect fell short of significance (2-way ANOVA, main effect of neonatal

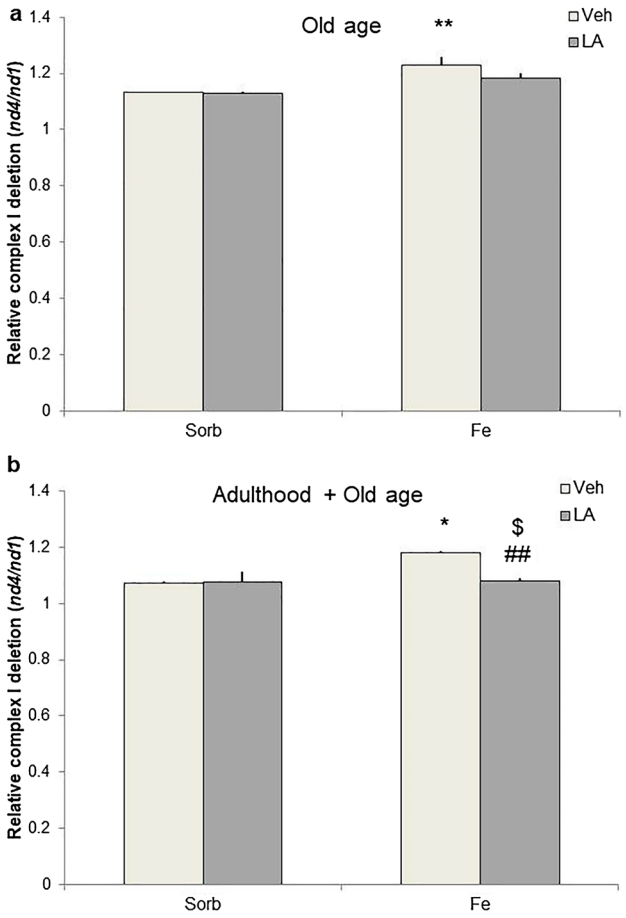

Fig. 3 Iron increases mtDNA deletions in the hippocampus of aged rats and combined LA supplementation reverses this effect. **a** Relative complex I deletion in hippocampal mtDNA of aged rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) at old age only (19 months of age). Sorb-Veh $N=6$, Sorb-LA $N=5$, Fe-Veh $N=5$, Fe-LA $N=5$. **b** Relative complex I deletion in hippocampal mtDNA of aged rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) in the adulthood (6 months of age) and at old age (19 months of age). Sorb-Veh $N=6$, Sorb-LA $N=6$, Fe-Veh $N=5$, Fe-LA $N=6$. Data expressed as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA. **Indicates main effect of neonatal iron treatment, $p<0.0001$; *Indicates main effect of neonatal iron treatment, $p<0.01$; $Indicates main effect of combined LA supplementation, $p<0.05$; ##Indicates significant interaction, $p<0.01$

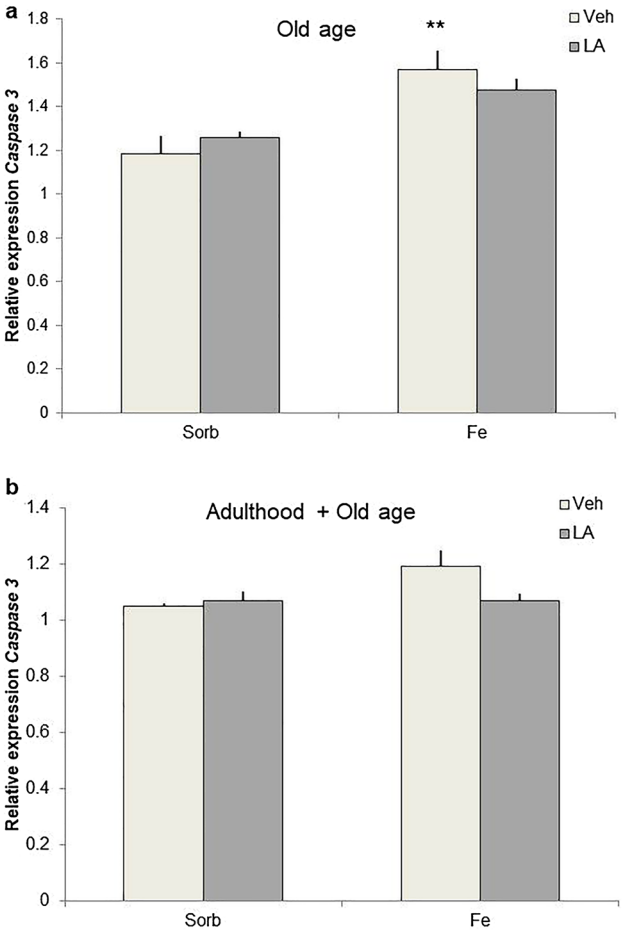

Fig. 4 Iron increased *caspase 3* gene expression in the hippocampus of aged rats. **a** *Caspase 3* expression in the hippocampus of aged rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) at old age only (19 months of age). Sorb-Veh $N=4$, Sorb-LA $N=4$, Fe-Veh $N=5$, Fe-LA $N=4$. **b** *Caspase 3* expression in the hippocampus of aged rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) in the adulthood (6 months of age) and at old age (19 months of age). Sorb-Veh $N=5$, Sorb-LA $N=4$, Fe-Veh $N=4$, Fe-LA $N=3$. Data expressed as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA. **Indicates main effect of neonatal iron treatment, $p<0.01$

treatment; $F_{(1,12)}=3.96$, $p=0.070$, Fig. 4b). No main effect of LA supplementation was found ($F_{(1,12)}=2.34$, $p=0.152$, Fig. 4b).

To substantiate the hypothesis that iron overload in the neonatal period represents an oxidative insult later in life, we next investigated the effects of iron on the expression of *Nrf2*, a transcription factor that regulates antioxidant defenses. We found a significant main effect of neonatal iron treatment ($F_{(1,15)}=7.36$, $p=0.016$, Fig. 5a) when comparing the groups that received iron and LA treatment only later in life, but no significant main effect of LA supplementation was found ($F_{(1,15)}=3.00$, $p=0.104$, Fig. 5a). Interestingly, a significant interaction ($F_{(1,15)}=5.73$, $p=0.030$, Fig. 5a) was revealed, suggesting that LA supplementation later in life differentially affected *Nrf2* expression in iron-treated rats. When comparing the groups that received LA-combined treatment, we found a significant main effect of iron treatment ($F_{(1,16)}=5.23$, $p=0.036$, Fig. 5b). Although a main effect of LA-combined treatment fell short of significance ($F_{(1,16)}=4.50$, $p=0.050$, Fig. 5b), a significant interaction ($F_{(1,16)}=7.31$, $p=0.016$, Fig. 5b) was found, suggesting

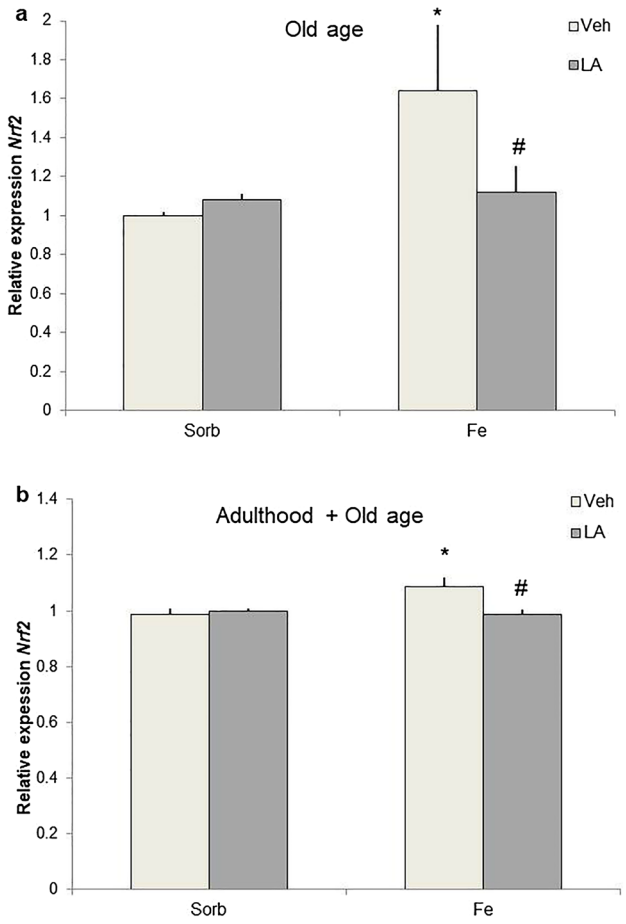

Fig. 5 Iron increased *Nrf2* expression in the hippocampus of aged rats and LA reversed this effect. **a** *Nrf2* expression in the hippocampus of aged rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) at old age only (19 months of age). Sorb-Veh $N=5$, Sorb-LA $N=6$, Fe-Veh $N=3$, Fe-LA $N=5$. **b** *Nrf2* expression in the hippocampus of aged rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) in the adulthood (6 months of age) and at old age (19 months of age). Sorb-Veh $N=4$, Sorb-LA $N=6$, Fe-Veh $N=4$, Fe-LA $N=6$. Data expressed as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA. *Indicates main effect of neonatal iron treatment, $p<0.05$; #Indicates significant interaction, $p<0.05$

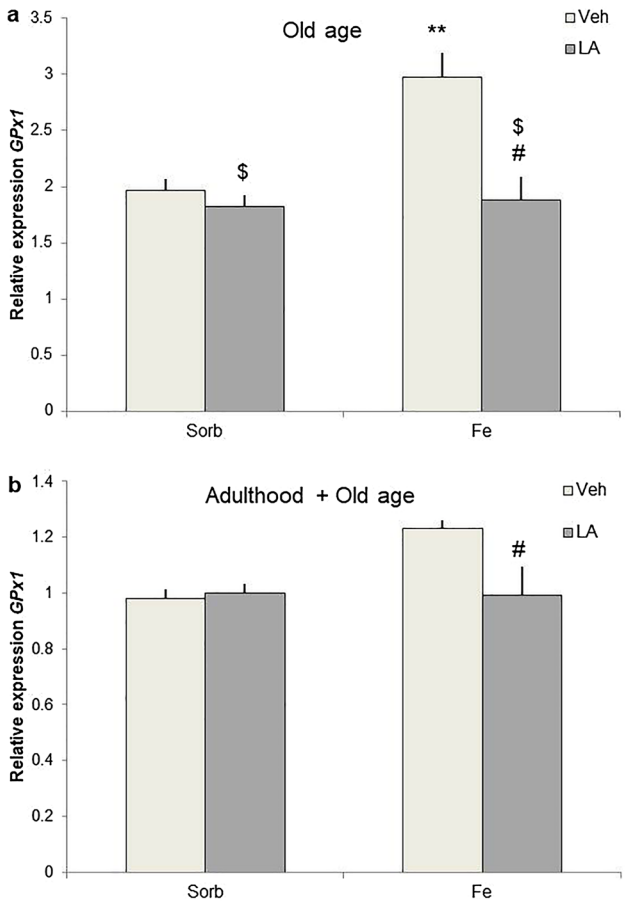

Fig. 6 Iron increased *Gpx1* expression in the hippocampus of aged rats and LA treatment reversed this effect. **a** *Gpx1* expression in the hippocampus of aged rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) at old age only (19 months of age). Sorb-Veh $N=4$, Sorb-LA $N=5$, Fe-Veh $N=4$, Fe-LA $N=4$. **b** *Gpx1* expression in the hippocampus of aged rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) in the adulthood (6 months of age) *and* at old age (19 months of age). Sorb-Veh $N=6$, Sorb-LA $N=5$, Fe-Veh $N=4$, Fe-LA $N=4$. Data expressed as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA. **Indicates main effect of neonatal iron treatment, $p<0.01$; $Indicates main effect of LA supplementation at old age only, $p<0.01$; #Indicates significant interaction, $p<0.05$

that LA-combined treatment was able to reverse iron-induced increases on *Nrf2* expression.

We next further analyzed the transcriptional regulation of antioxidant enzymatic defenses, such as *Gpx1* and *Nqo1*. Statistical comparisons of *Gpx1* expression indicated a significant main effect of neonatal iron treatment ($F_{(1,13)}=11.09$, $p=0.005$, Fig. 6a) and a significant main effect of LA supplementation at old age only ($F_{(1,13)}=15.15$, $p=0.002$, Fig. 6a). In addition, a significant interaction was revealed ($F_{(1,13)}=8.74$, $p=0.011$, Fig. 6a), suggesting that LA was able to reverse iron-induced increases in *Gpx1* expression. When the groups that received iron and LA-combined treatment were compared, iron neonatal treatment also induced an increase in *Gpx1* expression, although this effect failed to reach statistical significance (main effect of neonatal treatment, $F_{(3,15)}=4.15$, $p=0.060$, Fig. 6b). LA-combined treatment also did not reach statistical significance (main effect of LA supplementation, $F_{(3,15)}=3.44$, $p=0.084$, Fig. 6b), although a significant interaction ($F_{(3,15)}=4.68$, $p=0.047$, Fig. 6b) was found, suggesting that LA-combined supplementation affects *Gpx1* expression in iron-treated rats. Next, we analyzed another antioxidant defense regulated by *Nrf2*, *Nqo1*. Two-way ANOVA revealed a significant main effect of neonatal iron treatment ($F_{(1,17)}=15.40$, $p=0.001$,

Fig. 7a), which increased *Nqo1* expression; LA supplementation at old age only was not able to reverse this effect, as no significant main effect of LA supplementation ($F_{(1,17)}=0.89$, $p=0.358$, Fig. 7a), nor significant interactions were found ($F_{(1,17)}=0.99$, $p=0.332$, Fig. 7a). When the groups that received iron and LA-combined treatment were compared, 2-way ANOVA revealed a significant main effect of iron treatment ($F_{(1,15)}=10.56$, $p=0.005$, Fig. 7b), while the effects of LA-combined treatment fell short of significance ($F_{(1,15)}=3.84$, $p=0.069$, Fig. 7b). However, a significant interaction ($F_{(1,15)}=5.00$, $p=0.041$, Fig. 7b) was revealed, suggesting that LA-combined reversed iron-induced increases in *Nqo1* expression.

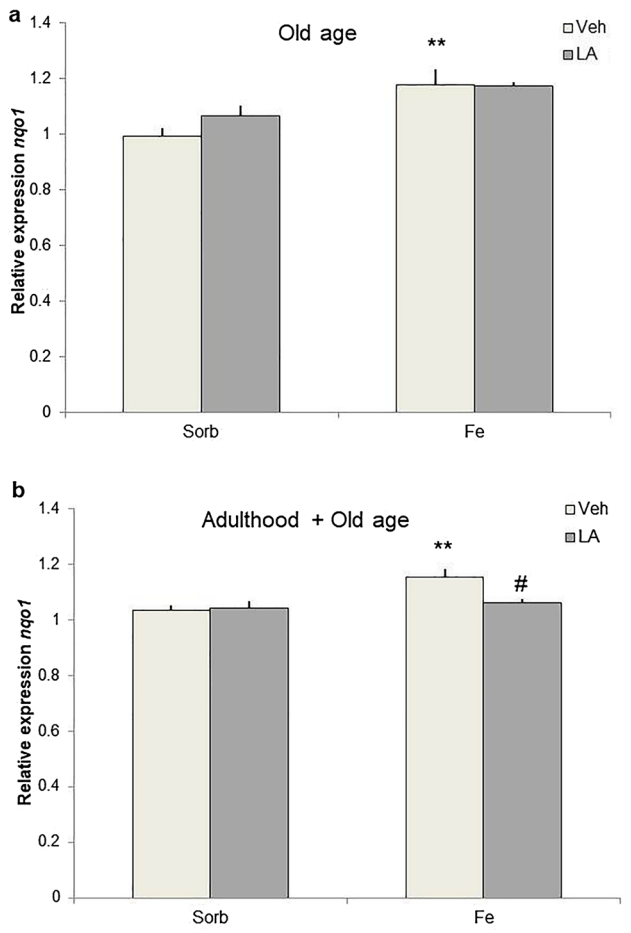

Fig. 7 Iron increased *Nqo1* expression in the hippocampus of aged rats and LA-combined treatment reversed this effect. **a** *Nqo1* expression in the hippocampus of aged rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or LA (50 mg/kg) at old age only (19 months of age). Sorb-Veh $N=5$, Sorb-LA $N=6$, Fe-Veh $N=5$, Fe-LA $N=5$. **b** *Nqo1* expression in the hippocampus of aged rats treated neonatally with vehicle (Sorb) or iron (30 mg/kg of Fe and given vehicle (Veh) or LA (50 mg/kg) in the adulthood (6 months of age) and at old age (19 months of age). Sorb-Veh $N=5$, Sorb-LA $N=5$, Fe-Veh $N=4$, Fe-LA $N=5$. Data expressed as mean ± S.E.M. Statistical analysis was performed using one-way ANOVA **Indicates main effect of neonatal iron treatment, $p<0.01$; #Indicates significant interaction, $p<0.05$

Discussion

Studies performed in human subjects indicate that iron accumulation in brain regions throughout life impacts cognitive performance in a variety of cognitive tests in healthy elderly [33, 34] as well as in patients with neurodegenerative diseases, including Alzheimer's disease (AD) [35–37]. Remarkably, indiscriminate iron supplementation to healthy infants has been considered a major risk factor for the development of neurodegenerative disorders later in life [38]. Using an animal model of iron overload during the neonatal period, we have shown that iron induces persistent memory deficits in a variety of memory tasks, including aversive and recognition memory, in adult rats [6, 9, 12, 28]. We [7] and others [39, 40] have previously demonstrated that long-term object recognition memory is impaired in aged compared to adult rats, and using a different experimental approach, we also demonstrated that iron treatment impairs short-term recognition memory in aged rats, which is not impaired by aging itself [7, 41]. In agreement, the present findings show that aged rats present long-term recognition memory impairments, considering that rats that received vehicle or iron in the neonatal period explored objects at chance level of 50%, showing no preference towards the novel object in the long-term memory retention test. Considering that aging impairs long-term recognition memory, the present experimental conditions do not allow us to determine whether iron overload would lead to further long-term recognition memory deficits in aged rats. Combined LA supplementation was able to reverse age-induced recognition and aversive memory impairments, while LA treatment at old age only reversed iron-induced aversive memory impairments, as a significant interaction was observed. Neither iron nor LA treatments altered general exploratory behavioral parameters analyzed in the open field, suggesting that the effects described here are related to the mnemonic aspects of behavior (please see Supplementary material—Table 2). Previously, it has been showed that dietary LA supplementation improved both learning and memory retention in different paradigms in Tg2576 transgenic mouse model of AD and in senescence-accelerated SAMP8 mice [19, 42]. Studies have also showed that LA supplementation was able to recover spatial and temporal memory in aged rats [43] and ameliorate spatial memory deficits induced by D-galactose in mice [44]. Here we show that LA supplementation in adulthood combined with supplementation in old age was able to reverse emotional memory deficits in aged rats.

Various mechanisms underlying LA memory-improving effects have been proposed. These include improvement of

memory-related signaling pathways, enhancing mitochondrial function, scavenging free radicals to decrease oxidative damage, or increasing the levels of the antioxidants to enhance the antioxidant defenses in the brain [43, 45].

We aimed to investigate the effects of iron loading as an additional risk factor, on mitochondrial and oxidative mechanisms, and the mechanisms associated to the beneficial effects of LA on age-induced memory deficits. Deletions in mtDNA are present in aged brain where dementia is often an accompanying feature implying that mitochondrial respiratory impairment may be causally linked with cognitive decline [46]. Previous studies demonstrated that aging itself increases the levels of mitochondrial Complex I deletions in approximately 40–50%, in comparison to adult rats [29, 30]. The present study demonstrates that iron overload in the neonatal period induced higher mtDNA deletions in the hippocampus of aged rats with a magnitude of 9–10%. It is noteworthy that this effect, although seemingly small, may contribute to amplify age-induced mitochondrial dysfunctions. Combined LA supplementation was able to reverse iron-induced increases in mtDNA deletions. One possible mechanism for the protective effects of LA may be related to its capacity of recycling endogenous antioxidants, such as vitamins C and E, and raise glutathione levels [25, 47]. However, considering that the neonatal iron exposure may lead to increased mitochondrial DNA deletions either by bringing about a burst of deletions early in life or via a protracted increased accumulation rate for mitochondrial DNA deletions throughout life, it is plausible that LA would promote selective removal of damaged mitochondria or stimulate mitochondrial biogenesis. In fact, a study by Fernández-Galilea and coworkers [48] demonstrated that LA increased mitochondrial content in cultured human adipocytes as revealed by electron microscopy and by mitotracker green labeling. Additionally, a recent in vivo study showed that dietary LA significantly increased mRNA expressions of mitochondrial biogenesis and electron transport chain-related genes in liver and muscle of zebrafish [49].

Caspase 3 was identified as a key mediator of neuronal programmed cell death and its induction is a crucial event of neuronal cell death program involved in many chronic neurodegenerative diseases [50]. In previous studies, we demonstrated that iron overload in the neonatal period induces a significant increase in caspase 3 protein levels, which is accompanied by increased *caspase 3* gene expression, as well as increased caspase 3 substrate cleavage in the hippocampus of adult rats, by inducing the intrinsic apoptotic pathway, which is mainly regulated by mitochondria [11, 14]. Here, we show that iron induced an increase in *caspase 3* gene expression in aged rats as well. Although measuring protein levels of cleaved caspase 3 or cleaved caspase 3 substrates would have been more accurate, evidence suggest that increased *caspase 3* gene expression might be related to an activation in apoptotic pathways. Accordingly, recent studies have also shown that increased hippocampal apoptosis, confirmed by Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, was associated with increased *caspase 3* gene expression in the hippocampus [51, 52]. In the present in vivo experimental conditions, LA supplementation was unable to reverse iron-induced caspase 3 gene expression increases. Nonetheless, previous in vitro studies showed that LA presents anti-apoptotic effects through caspase 3 dependent pathway and NF-kappaB signaling in desflurane-treated hippocampal neurons [53] and that LA treatment normalized cell viability to control values, and attenuated the H_2O_2-induced apoptosis through the mitochondrial-mediated pathway in primary mouse astrocytes [54].

ROS along with transition metals can initiate and propagate oxidative reactions that are normally kept in check by a well-developed antioxidant defense system comprising antioxidant enzymes and non-enzymatic radical scavengers, including the regulation by NRF2 [46, 55]. Noteworthy, transcription factor NRF2 binds to antioxidant response elements (ARE) in the promoter region of many cell defense genes to activate their transcription. *Nrf2* (*NFE2L2)* gene contains ARE within its promoter region that renders NRF2 the ability to directly activate its own transcription, providing a positive feedback mechanism to amplify NRF2 effects [56, 57]. Here, iron overload also leads to an increased *Nrf2* gene expression in the hippocampus of aged rats, which might be attributed to over generation of oxidative stress [46]. LA supplementation, given in the old age only or in adulthood and later in life reversed iron effects in inducing *Nrf2* expression, as significant interactions were found, suggesting a protective effect against iron-induced neurotoxicity in the hippocampus.

Since iron treatment increased *Nrf2* expression, we sought to investigate its effects on expression of other ARE-containing genes, *Gpx1* and *Nqo1*. Results indicated that *Gpx1* and *Nqo1* expression accompanied increased *Nfr2* expression, thus suggesting that iron exposure in the neonatal period resulted in increased oxidant state in aged rats, which was normalized by LA supplementation. We propose that excess brain iron may induce a pro-oxidant condition that results in *Nfr2*-dependent increased gene expression of antioxidant enzymes. It is noteworthy, however, that this mechanism, as an attempt to compensate an exacerbated pro-oxidant state was not efficient, once memory deficits, increased mtDNA deletions and caspase 3 expression were observed. In pathological states, the antioxidant defense system may become overwhelmed, leading to oxidative stress. LA treatment, possibly by restoring mitochondrial functioning and scavenging free iron and toxic hydroxyl radicals [23], may have normalized antioxidant gene expression. However, to further confirm our findings, a deeper investigation involving

quantification of antioxidant enzymatic activity, or oxidative stress markers would be required. In fact, previous studies reported that LA restored lipid peroxidation and total antioxidant capacity, while upregulating *Nrf2* and *Gpx1* gene expression in the brains of rabbits exposed to cadmium [58] and also restored lipid peroxidation and protein carbonylation, which was accompanied by an increase in catalase activity in an animal model of phenylketonuria [59].

In the present study, we show that LA supplementation, even when administered at old age only was able to recover age-induced functional impairments in iron-treated rats, observed in memory testing, which was associated to beneficial effects on mtDNA deletions and antioxidant gene induction. These findings support the view that LA possesses neuroprotective effects that may be related to mitochondrial functioning, likely due to LA-induced restoration of a healthier oxidative status during aging.

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Data availability The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics approval This study was approved by the Institutional Ethics Committee for the Use of Animals of the Pontifical Catholic University (CEUA SIPESQ #7510) and all experimental procedures were performed in accordance with the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI, Brazil).

Consent for publication All listed authors have approved the manuscript before submission, including the names and order of authors.

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