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Research report

Novel insights into mitochondrial molecular targets of iron-induced neurodegeneration: Reversal by cannabidiol

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ABSTRACT

Evidence has demonstrated iron accumulation in specific brain regions of patients suffering from neurodegenerative disorders, and this metal has been recognized as a contributing factor for neurodegeneration. Using an experimental model of brain iron accumulation, we have shown that iron induces severe memory deficits that are accompanied by oxidative stress, increased apoptotic markers, and decreased synaptophysin in the hippocampus of rats. The present study aims to characterize iron loading effects as well as to determine the molecular targets of cannabidiol (CBD), the main non-psychomimetic compound of *Cannabis sativa*, on mitochondria. Rats received iron in the neonatal period and CBD for 14 days in adulthood. Iron induced mitochondrial DNA (mtDNA) deletions, decreased epigenetic modulation of mtDNA, mitochondrial ferritin levels, and succinate dehydrogenase activity. CBD rescued mitochondrial ferritin and epigenetic modulation of mtDNA, and restored succinate dehydrogenase activity in iron-treated rats. These findings provide new insights into molecular targets of iron neurotoxicity and give support for the use of CBD as a disease modifying agent in the treatment of neurodegenerative diseases.

1. Introduction

The etiology of neurodegenerative diseases has not been completely elucidated, but it is widely accepted that oxidative damage, linked to accumulation of transition metals, can contribute to neurodegeneration (Salvador et al., 2010; Kim et al., 2015). Progressive iron accumulation in the brain has been described during the normal aging process. Remarkably, in neurological diseases such as Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD) diseases, iron accumulates in brain areas relevant to disease-associated neurodegenerative processes (Stankiewicz and Brass, 2009; Mills et al., 2010). For instance, it has been demonstrated that iron selectively accumulates in the *substantia nigra pars compacta* in PD patients (Dexter et al., 1991; Sofic et al., 1991), while it builds up around and within amyloid plaques and neurofibrillary tangles in brains from AD patients (Connor et al., 1992; Lovell et al., 1998).

In previous studies, aiming to examine the mechanisms of iron neurotoxicity in neurodegenerative diseases, we have established an animal model of brain iron loading, with oral administration of iron during the neonatal period, which is the period of maximal iron uptake by the brain (Taylor and Morgan, 1990). Iron neonatal treatment induces emotional memory deficits, tested in the inhibitory avoidance task (Schröder et al., 2001; Fagherazzi et al., 2012; Figueiredo et al., 2016) as well as recognition memory impairments (de Lima et al., 2005; Fagherazzi et al., 2012; Figueiredo et al., 2016). These memory deficits are accompanied by increased thiobarbituric acid reactive species (TBARS), protein carbonylation and superoxide production (Dal-Pizzol et al., 2001; de Lima et al., 2005), increased levels of apoptotic markers,

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Par4 and caspase 3 (Miwa et al., 2011, da Silva et al., 2014), and reactive astrogliosis (Fernandez et al., 2011). In addition, recent studies have shown that iron overload leads to accumulation of ubiquitinated proteins (Figueiredo et al., 2016), decreased levels of synaptophysin, as well as alterations in the levels of DNM1L, a protein critically involved in mitochondrial fission (da Silva et al., 2014), resembling common alterations observed in neurodegenerative disorders. Kaur et al. (2007) reported that mice, treated with iron for eight days during the neonatal period with a dose four times higher than the one used in our model, developed histological and neurochemical alterations relevant to PD pathology, i.e., increased oxidative stress, decreased striatal dopamine content and nigral tyrosine hydroxylase (TH) positive cells.

Neurons are highly differentiated cells with high energy requirements, which mainly come from mitochondria, warranting neuronal survival and many essential functions, including axonal growth and branching, generation of action potentials, and synaptic transmission and plasticity (Lin and Sheng, 2015). Therefore, it has been suggested that dysfunctions in these organelles play a role in the pathogenesis of neurological disorders (Mattson et al., 2008). Mitochondrial DNA damage has already been related to aging and neurodegenerative diseases and contribute to mitochondrial disruption, which in turn may lead to cell injury, particularly in the central nervous system (CNS) (Siddiqui et al., 2012; Mao et al., 2012; Grünewald et al., 2016). Recently, studies have shown that mtDNA is also subject of epigenetic regulation, including methylation (5mC) and hydroxymethylation (5hmC) (Manev and Dzitoyeva, 2013). Increases and decreases of DNA methylation have been observed during aging, but most of the studies are limited to nuclear DNA (Richardson, 2003). Mitochondrial epigenetics are in nascent form and should be better and extensively studied (Manev et al., 2012).

Mitochondria maintain cellular energy reserves, which are extremely important to the CNS, by keepping respiratory chain and Krebs cycle under strict control (Basha and Poojary, 2014). Creatine kinases catalyze reversible transfer of phosphoryl groups between ATP and creatine, mainly in high energetic consumption tissues, being essential to energy homeostasis (Pilla et al., 2003). Mitochondrial dysfunction compromises energetic metabolism, resulting in overproduction of reactive oxygen species (ROS) and bioenergetic failure to the cells, contributing to many neurodegenerative diseases (Arun et al., 2016).

Since mitochondria are the major sources of cellular iron utilization, these organelles play a key role in maintaining iron homeostasis (Napier et al., 2005). Iron entry across the mitochondrial inner membrane requires one of two homologous proteins of the mitochondrial solute carrier family, called mitoferrin 1 and mitoferrin 2 (Paradkar et al., 2009). Mitoferrin 1 is the main mitochondrial iron importer in haematopoietic tissues, while mitoferrin 2 contributes to iron acquisition in non-erythroid tissues (Shaw et al., 2006; Paradkar et al., 2009). In 2001, Levi and coworkers (Levi et al., 2001) identified an iron storage protein inside mitochondria, mitochondrial ferritin, with a similar structure to the cytosolic ferritin. It has been described that mitochondrial ferritin is expressed preferentially in tissues with high oxygen consumption and has a role in protecting mitochondria from oxidative damage induced by free iron rather than storing iron (Levi and Arosio, 2004; Santambrogio et al., 2007). Although it has been proposed that both mitochondrial disruption and iron accumulation are involved in the pathophysiology of neurodegenerative disorders, there is restricted information about the regulation of mitochondrial mechanisms of iron transport and storage in these diseases.

Cannabidiol (CBD) is the main non-psychotropic constituent of *Cannabis sativa*, corresponding to approximately 40% of plant extract (Campos et al., 2012; Zuardi, 2008). Evidence indicates that CBD possesses antioxidant, antiapoptotic, and neuroprotective properties (Hampson et al., 1998; Iuvone et al., 2004; García-Arencibia et al., 2007; Castillo et al., 2010, Pazos et al., 2012). We have previously shown that CBD completely reverses iron-induced memory deficits (Fagherazzi et al., 2012) and normalizes hippocampal levels of caspase 3, synaptophysin, and mitochondrial fission protein DNM1L in rats with brain iron overload (da Silva et al., 2014).

The aim of the present study was to characterize the effects of iron loading on mitochondrial physiology by measuring mtDNA deletions and mtDNA epigenetic modifications in the hippocampal formation, a brain region critically involved in learning and memory, known to be primarily affected in AD. We also wanted to determine if neonatal iron loading would hinder mitochondrial iron handling, by altering the expression of mitoferrin 2 and mitochondrial ferritin later in life. Relevant functional parameters of energy metabolism, succinate dehydrogenase and creatine kinase activities, were also analyzed in the hippocampus of iron-loaded rats. Considering that CBD proved to ameliorate iron-induced memory deficits, which are relevant in the context of aging and neurodegenerative disorders, and the demand for neuroprotective treatments, we further investigated possible targets of CBD action on iron-induced mitochondrial alterations.

2. Material and methods

2.1. Animals

Pregnant Wistar rats (CrlCembe:WI) were obtained from the Centro de Modelos Biológicos Experimentais (CeMBE), Pontifical Catholic University, Porto Alegre, RS, Brazil. After birth each litter was adjusted within 48 h to eight rat pups, and to contain offspring of both genders in about equal proportions, 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 postnatal treatments, animals were given standardized pellet food and tap water *ad libitum*.

All experimental procedures were performed in accordance to the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI, Brazil) and approved by the Institutional Ethics Committee for the Use of Animals of the Pontifical Catholic University (CEUA 14/00409). All efforts were made to minimize the number of animals.

2.2. Treatments

2.2.1. Neonatal iron treatment

The neonatal iron treatment has been described in detail elsewhere (da Silva et al., 2014; Fagherazzi et al., 2012). Briefly, 12-day-old rat pups received orally a single daily dose 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).

2.2.2. Cannabidiol

Adult (3 month-old) rats, treated neonatally with vehicle or iron, as described above, received a daily intraperitoneal injection of vehicle (Tween 80–saline solution 1:16 v/v) or CBD (10 mg/kg, approximately 99.9% pure; kindly supplied by BSPG-Pharm, Sandwich, UK) for 14 consecutive days. Drug solutions were freshly prepared immediately prior to administration (Fagherazzi et al., 2012; da Silva et al., 2014).

Rats were euthanized by decapitation at 24 h after the last injection of CBD treatment. Brains were quickly dissected and hippocampi were isolated and stored at −80 °C for subsequent RT-qPCR, Western Blotting, and enzymatic activity assays. For analyses of mtDNA methylation and hydroxymethylation, hippocampal mitochondria were freshly extracted according to a commercial protocol (XIT™ Mitochondrial DNA, G-Biosciences, St. Louis, USA) and mtDNA was isolated.

2.3. Molecular analyses

2.3.1. Real-time PCR analysis of mitochondrial Complex I deletion

The assays were performed as previously described by Ochoa et al. (2011) with adaptations. Two different regions of the mitochondrial genome were studied, one that is rarely affected by deletions (*nd1*) and another that is frequently deleted (*nd4*) both in humans and in rats (Van Tuyle et al., 1996; He et al., 2002). Total DNA was isolated from hippocampus using the Qiagen® DNeasy tissue kit (Hilden, GER) in accordance with the manufacturer's instructions. DNA purity (Abs 260/280 nm ~1.8) and concentration were determined by Nanodrop® (Thermo Fisher Scientific Inc, Waltham, USA). Quantitative PCR was performed using SYBR® Green I (Invitrogen, Carlsbad, USA), 0.25 ng/μL DNA, and mitochondrial DNA primers for *nd1* (forward primer CGCC CCAACCCTCTCC, reverse primer GTATGCCTAGGTTGAGGTTGATA AGG) and *nd4* (forward primer CATTTTCCTGATCGAACCCCTCTAT, reverse primer AGTTTTCCTCGTTGGGTTGTGATAA) on the 7500 Real-time PCR System (Applied Biosystems, Foster City, USA). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C to confirm the specificity of primers and absence of primer-dimers and showed in all cases one single peak. All real time assays were carried out in sextuplicate and, in all cases, a reverse transcriptase negative control was included. Both reactions (that of *nd1* and that of *nd4*) propagated with high efficiency (close to 1). The relative deletion was calculated using XCq *nd4*/XCq *nd1*. Similar efficiency values between the two reactions mean that Ct values can be used as a measure of input DNA and to quantify the relative amount of *nd1* to *nd4* because Ct does not depend on the dilution series.

2.3.2. mtDNA 5hmC and 5mC content

The 5mC content of mitochondrial DNA was measured using a methylated DNA quantification kit (MethylFlash™ Methylated DNA Quantification Kit, Epigentek, Farmingdale, USA). 100 ng of DNA from each sample were used in the 96-well plate and quantified colorimetrically by reading the absorbance at 450 nm in a microplate spectrophotometer. The 5hmC content was measured using a hydroxymethylated DNA quantification kit (MethylFlash™ Hydroxymethylated DNA Quantification Kit, Epigentek, Farmingdale, USA). 200 ng of DNA from each sample were used in the 96-well plate and quantified colorimetrically by reading the absorbance at 450 nm in a microplate spectrophotometer (adapted from Dzitoyeva et al., 2012).

2.3.3. Western Blot analyses

Proteins were extracted as previously described by da Silva et al. (2014). The supernatant was collected and the protein content was determined using Bradford assay (Bradford, 1976). Aliquots were stored at −20 °C.

Fifty μg of protein was separated on a 10% SDS polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. Membranes were blocked with 5% albumin in TBS containing 0.05% Tween 20 and were incubated overnight with one of the following antibodies: anti-Mitocondrial ferritin (Abcam, Cambridge, UK) at 1:700; anti-Mitoferrin 2 (Abcam, Cambridge, UK) at 1:500, and anti-Tubulin (Abcam, Cambridge, UK) at 1:20000. Goat polyclonal anti-rabbit IgG H&L (HPR) (Abcam, Cambridge, UK) secondary antibody was used and detected using ECL Western Blotting Substrate Kit (Abcam, Cambridge, UK). Pre-stained molecular weight protein markers (SuperSignal Molecular Weight Protein Ladder, Thermo Scientific, Rockford, USA) were used to determine the detected bands molecular weight and confirm antibodies target specificity. The densitometric quantification was performed using Chemiluminescent photo finder (Kodak/Carestream, model GL2200). Total blotting protein levels of samples were normalized according to each sample's Tubulin levels (adapted from da Silva et al., 2014).

2.3.4. RT-qPCR analysis

The gene expression of *Ftmt* (mitochondrial ferritin) and *Mitoferrin*-2 were determined by RT-qPCR. The total RNA was isolated from hippocampus with TRIzol® Reagent (Life Technologies, Carlsbad, USA) in accordance with the manufacturer's instructions. RNA purity (Abs 260/280 nm ~2.0) and concentration were determined by Nanodrop® and after treated with Deoxyribonuclease I (Sigma-Aldrich, São Paulo, Brazil) to eliminate genomic DNA contamination in accordance with the manufacturer's instructions. The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega, Madison, USA) from 1 μg of the total RNA, following the manufacturer's instruction. Quantitative PCR was performed using SYBR® Green I (Invitrogen, Carlsbad, USA) to detect double-strand cDNA synthesis on the 7500 Real-time PCR System (Applied Biosystems, Foster City, USA). Primers were designed by authors (*Ftmt:* forward primer TCCTGGACTTGCATA CTCTGGCCTCAG, reverse primer GCTTGTCGAAAAGATACTCCGCTA GG; *Mitoferrin 2*: forward primer AACACCCAGGAGTCCCTGGCCTTG, reverse primer CATGCGATGGCTGTGGAGGGGATC). The PCR cycling conditions were the same as described above in *Real-time PCR analysis of mitochondrial Complex I deletion* section. All real time assays were carried out in quadruplicate and, in all cases, a reverse transcriptase negative control was included. *Hprt1* was used as reference gene for normalization. The efficiency per sample was calculated using LinRegPCR 2016.1 Software (http://LinRegPCR.nl) and the stability of the references genes, and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Cq}$ method (adapted from da Silva et al., 2014).

2.3.5. Sample preparation for enzymatic activity determination

For the determination of succinate dehydrogenase and creatine kinase enzyme activities, hippocampi were homogenized (1:20, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 UI mL^{-1} heparin). The homogenates were centrifuged at 800 × *g* for 10 min at 4 °C and the supernatants were used for enzyme activity determination. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard (adapted from Schuck et al., 2013).

2.3.6. Succinate dehydrogenase activity

Succinate dehydrogenase activity was measured according to the method of Fischer et al. (1985) by following the decrease in absorbance due to the reduction of 2,6-di-chloroindophenol (2,6-DCIP) at 600 nm with 700 nm as reference wavelength (ε = 19.1 mM − 1 cm − 1) in the presence of phenazine methasulphate (PMS). The reaction mixture, consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 μM 2,6-DCIP, was preincubated with 40–80 μg homogenate protein at 30 °C for 20 min. Subsequently, 4 mM sodium azide, 7 μM rotenone and 40 μM 2,6-DCIP were added and the reaction was initiated by adding 1 mM PMS. The reaction was monitored for 5 min. The activity of succinate dehydrogenase is expressed as nmol.Min − 1. mg protein − 1 (adapted from Teodorak et al., 2017).

2.3.7. Activity of creatine kinase

Total creatine kinase activity in brain homogenates, pretreated with 0.625 mM lauryl maltoside, was measured. The reaction mixture consisted of 60 mM Tris–HCl, 7 mM phosphocreatine, 9 mM MgSO4, pH 7.5, and approximately 0.4–1.2 μg protein in a final volume of 100 μL. After 15 min of pre-incubation at 37 °C, the reaction was started by adding ADP 3.2 mmol. The reaction was stopped after 10 min by adding 1 μmol of p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by adding 100 μL 2% α-naphtol and 100 μL 0.05% diacetyl in a final volume of 1 mL and finally read spectrophotometrically after 20 min at 540 nm (adapted from Teodorak et al., 2017).

2.4. Statistical analysis

The results were expressed as mean ± S.E.M and were analyzed using SPSS 20.0 software. Levene's Test of Equality of Variances was used in order to test the assumption of homogeneity of variance. Statistical comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, when necessary. In all comparisons, *p* values less than 0.05 were considered to indicate statistical significance.

3. Results

We first decided to investigate the impact of excessive iron on the integrity of mitochondrial DNA, by quantifying the frequency of *nd1* and *nd4* mitochondrial genes, which code Complex I proteins. Statistical comparisons of relative mtDNA deletion levels demonstrated a statistically significant difference among the groups ($F_{(3,20)} = 165.59$, $p < 0.0001$, Fig. 1). When groups were compared using Tukey's *post hoc* test, results revealed that neonatal iron treatment induced a significant increase in mtDNA deletion levels, when compared to the control group (sorb-veh, $p < 0.0001$). The iron-treated group that received CBD in the adulthood had also significantly higher deletion levels when compared to the control group ($p < 0.0001$).

We next decided to further investigate the effects of iron loading and CBD treatment on epigenetic modifications of mtDNA. Interestingly, statistical comparisons have indicated that both methylation and hydroxymethylation are diminished by iron treatment. Accordingly, ANOVA revealed a significant difference among the groups regarding mtDNA methylation ($F_{(3,12)} = 39.72$, $p < 0.0001$, Fig. 2A) and hydroxymethylation ($F_{(3,16)} = 11.31$, $p < 0.0001$, Fig. 2B). Tukey's *post hoc* comparison indicated that the group that received iron in the neonatal period and vehicle in the adulthood presented a significantly reduced mtDNA methylation in comparison to the control group ($p < 0.0001$). The group that received iron in the neonatal period and CBD (iron-CBD) in the adulthood has also presented significantly reduced methylation in relation to the control group ($p < 0.0001$). Additionally, the iron-CBD group was not statistically different from the iron-veh group ($p = 0.142$). Statistical analyses of mtDNA hydroxymethylation also revealed that rats treated with iron in the neonatal period presented significantly reduced hydroxymethylation in comparison to the control group (Sorb-veh; $p = 0.001$). Interestingly, CBD in adulthood reversed this effect, since iron-treated rats that received CBD presented higher hydroxymethylation levels than the iron-veh group ($p = 0.001$) and similar levels in comparison to the control group ($p = 0.98$).

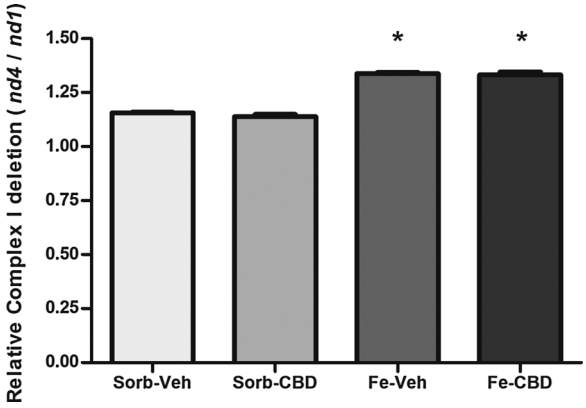

Fig. 1. Iron significantly increased relative mtDNA deletions in the hippocampus. Relative deletion in complex I deleted mtDNA in the hippocampus of rats treated with sorbitol or iron neonatally and treated with vehicle or CBD chronically in adulthood (at 3 months of age). Samples were obtained from 6 animals in each group and analyses were carried out in sextuplicate. Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc. Data expressed as mean ± S.E.M. * $p < 0.0001$ indicates significantly higher mtDNA deletion levels in iron-vehicle (Fe-Veh) and iron-CBD (Fe-CBD) groups in comparison to the sorbitol-vehicle (Sorb-Veh) group.

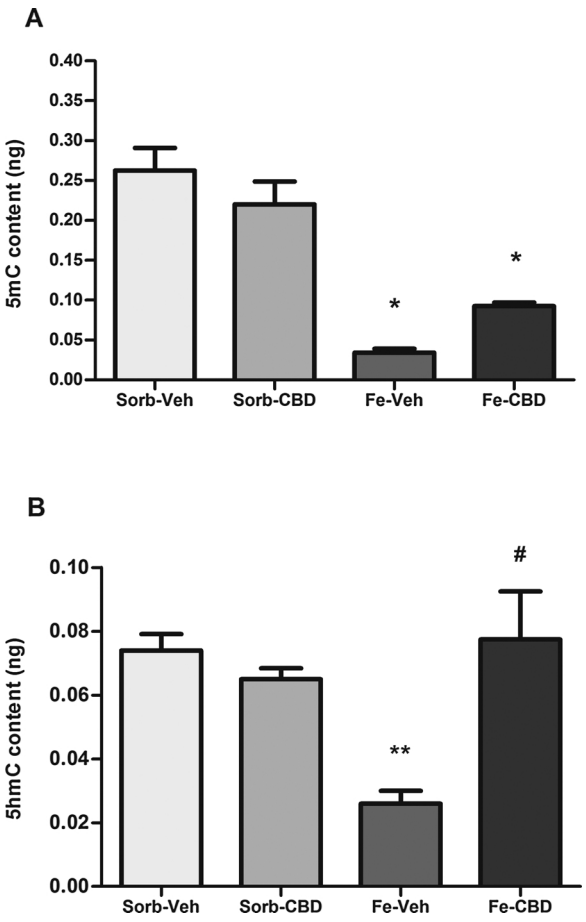

Fig. 2. Iron significantly decreased mtDNA methylation and hydroxymethylation. 5-methylcytosine (5mC) (A) 5-hydroxymethylcytosine (5hmC) (B) content in mtDNA from hippocampus of rats treated with sorbitol or iron neonatally and treated with vehicle or CBD chronically in adulthood (at 3 months of age). DNA was extracted and equal amounts of mtDNA were analyzed with 5mC and 5hmC enzyme-linked immunosorbent assay (ELISA). Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc. Data expressed as mean ± S.E.M. $N = 6$ per group. * $p < 0.0001$ indicates significantly lower levels of mtDNA methylation in iron-vehicle (Fe-Veh) and iron-CBD (Fe-CBD) groups in comparison to controls; ** $p < 0.001$ indicates significantly lower levels of mtDNA hydroxymethylation in iron-vehicle (Fe-Veh) group in comparison to controls; # $p < 0.001$ indicates significantly higher levels of mtDNA hydroxymethylation in the iron-CBD (Fe-CBD) in comparison to the iron-vehicle (Fe-Veh) group.

We also aimed to investigate the long-term consequences of neonatal iron loading and adult treatment with CBD on mitochondrial iron handling, by measuring protein levels and gene expression of mitochondrial proteins involved in iron metabolism, i.e. the mitochondrial iron transporter, mitoferrin 2, and mitochondrial ferritin. Statistical comparison using one-way ANOVA showed no significant differences among the groups when protein levels of mitoferrin 2, measured by western blot ($F_{(3,19)} = 1.55$, $p = 0.233$; Fig. 3A), or its gene expression ($F_{(3,16)} = 2.70$, $p = 0.080$; Fig. 3B) were compared. In contrast, when mitochondrial ferritin levels where compared, statistically significant differences among the groups were revealed, both in protein levels ($F_{(3,15)} = 20.49$, $p < 0.0001$) and gene expression ($F_{(3,13)} = 9.32$, $p = 0.001$). *Post hoc* comparisons of protein levels between groups indicated that neonatal iron treatment induced a significant reduction in ferritin levels in comparison to the control group (sorb–veh) ($p < 0.0001$; Fig. 4A), and in comparison to the group that received sorbitol in the neonatal period and CBD when adult ($p = 0.001$; Fig. 4A). Moreover, the iron-treated group that received vehicle in the

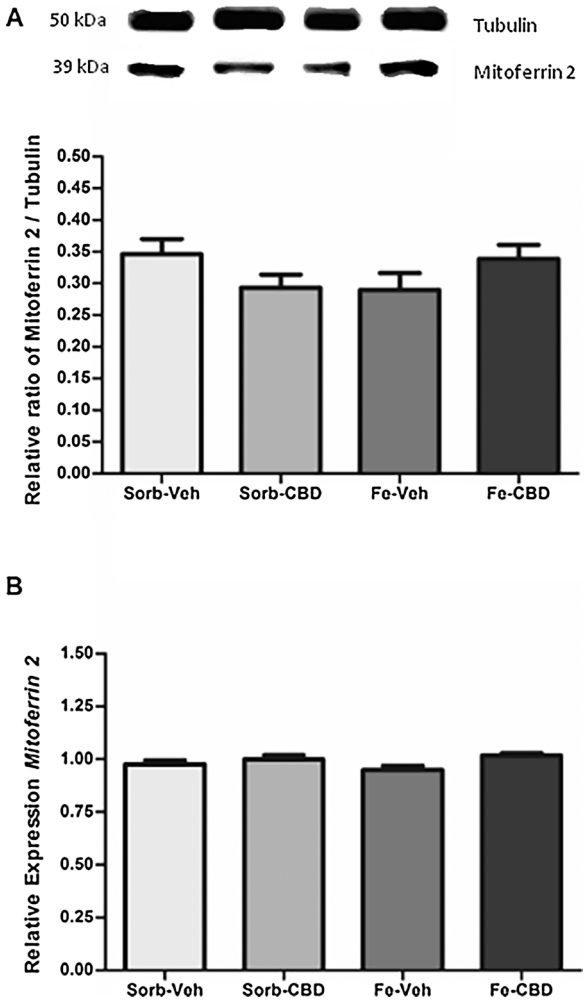

Fig. 3. Iron or CBD treatments did not alter Mitoferrin 2 expression.
(A) Western Blotting of Mitoferrin 2 and (B) relative *Mitoferrin 2* gene expression in the hippocampus of rats treated with sorbitol or iron neonatally and treated with vehicle or CBD chronically in the adulthood (3 months of age). Western blotting samples were performed with 50 µg of protein levels, normalized to Tubulin, separated on SDS-PAGE and probed with specific antibodies. Representative Western Blots for Mitoferrin 2 and Tubulin are shown. RT-qPCR samples were carried out in quadruplicate and *hprt1* was used as reference gene for normalization. Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc. Data expressed as mean ± S.E.M. $N = 5–6$ per group. No significant differences were found among groups.

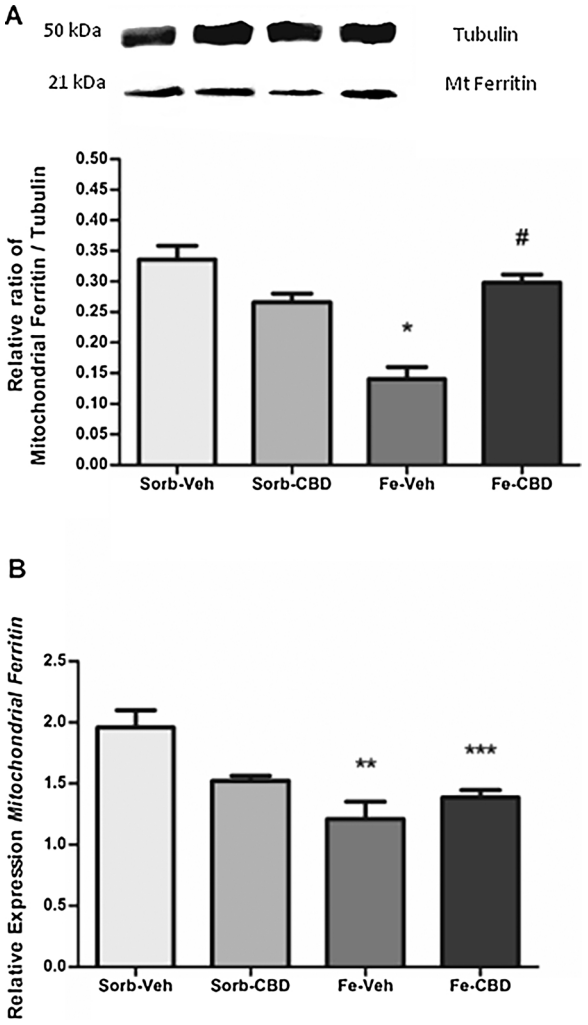

Fig. 4. Iron significantly decreased Mitochondrial ferritin protein levels and mRNA expression.
(A) Western Blotting of Mitochondrial ferritin and (B) relative *Mitochondrial ferritin* gene expression in the hippocampus of rats treated with sorbitol or iron neonatally and treated with vehicle or CBD chronically in the adulthood (3 months of age). Western blotting samples were performed with 50 µg of protein levels, normalized to Tubulin, separated on SDS-PAGE and probed with specific antibodies. Representative Western Blots for Mitochondrial ferritin and Tubulin are shown. RT-qPCR samples were carried out in quadruplicate and *hprt1* was used as reference gene for normalization. Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc test. Data expressed as mean ± S.E.M. $N = 4–5$ per group. * $p < 0.0001$ indicates a significant reduction in Mitochondrial ferritin protein levels in the iron-vehicle (Fe-Veh) group in comparison to controls; # $p < 0.0001$ indicates that the iron-CBD (Fe-CBD) group shows significantly higher levels of Mitochondrial ferritin in comparison to the iron-vehicle group (Fe-Veh); ** $p < 0.001$ indicates a significant reduction in *Mitochondrial ferritin* mRNA expression in the iron-vehicle (Fe-Veh) group compared to controls; *** $p < 0.01$ indicates a significant reduction in *Mitochondrial ferritin* mRNA expression in the iron-CBD (Fe-CBD) group compared to controls.

adult age also presented significantly lower ferritin levels when compared to the iron-treated group that received CBD in the adulthood ($p < 0.0001$; Fig. 4A). In addition, the iron-CBD group presented no significant differences when compared to the control group. At the gene expression level, results were comparable. *Post hoc* analyses revealed that ferritin gene expression was decreased in iron-treated rats that received vehicle in the adulthood in comparison to the control group ($p = 0.001$; Fig. 4B). However, the group that received iron in the neonatal period and CBD in the adulthood (iron-CBD) also presented significantly reduced ferritin gene expression in comparison to the control group ($p = 0.007$) and was not statistically different from the group that received iron in the neonatal period and vehicle in the adult age ($p = 0.609$).

We also analyzed the effects of neonatal iron loading and adult treatment with CBD on important parameters of energy metabolism in hippocampus of rats, namely succinate dehydrogenase and creatine kinase activities. Statistical comparison using one-way ANOVA has revealed significant differences in succinate dehydrogenase activity among the groups ($F_{(3,17)} = 4.518$, $p < 0.017$). *Post hoc* comparisons of enzymatic activities between groups indicated that neonatal iron treatment induced a significant decrease in succinate dehydrogenase activity in comparison to the control group, that received sorbitol in the neonatal period and vehicle in adulthood ($p = 0.022$; Fig. 5A), and in comparison to the group that received sorbitol in the neonatal period and CBD when adult ($p = 0.046$; Fig. 5A). There were no statistically significant differences in succinate dehydrogenase activity between the group that received iron in the neonatal period and CBD in the adulthood (iron-CBD) and the control group ($p = 0.742$). Although creatine kinase activity was also reduced in the iron-vehicle group, this effect did not reach statistical significance ($F_{(3,14)} = 2.815$, $p = 0.078$; Fig. 5B).

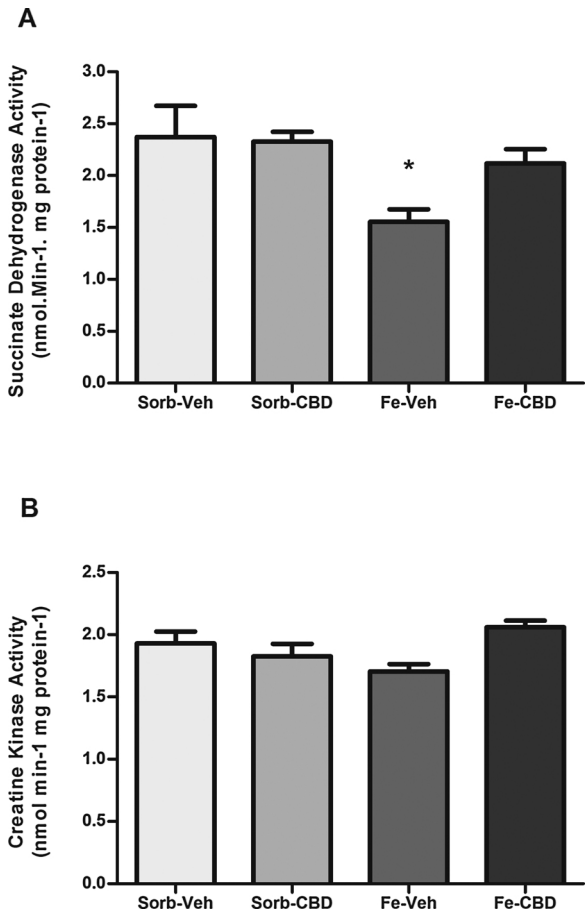

Fig. 5. Iron significantly decreased Succinate dehydrogenase activity without affecting Creatine kinase activity.
(A) Succinate dehydrogenase and (B) Creatine kinase activities in the hippocampus of rats treated with sorbitol or iron neonatally and treated with vehicle or CBD chronically in the adulthood (3 months of age). Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc. Data expressed as mean ± S.E.M, $N = 4–6$ per group, for independent experiments performed in duplicate. * $p < 0.05$ indicates a significantly reduced succinate dehydrogenase activity in the iron-vehicle (Fe-Veh) in comparison to sorbitol-vehicle (Sorb-Veh) and sorbitol-CBD (Sorb-CBD) groups.

4. Discussion

Compelling evidence suggest that iron accumulation is involved in the pathogenesis of neurodegenerative diseases, mainly due to its ability to generate highly toxic free radicals, inducing oxidative damage (Li and Reichmann, 2016). Mitochondrial metabolism represents a main source of free radical production and this organelle has been recently linked to cellular iron homeostasis. Here we showed for the first time that iron overload in the neonatal period induced higher relative mtDNA deletions in the hippocampus of adult rats in comparison to controls, implicating iron excess in mitochondrial dysfunctions.

Previous studies have shown that mtDNA deletions accumulate with aging in rats (Parkinson et al., 2014; Cahif et al., 2013; Loshchenova et al., 2015) and humans (Kazachkova et al., 2013). In addition, increased mtDNA deletions in *substantia nigra* have been found in PD patients (Bender et al., 2006) and in hippocampal neurons from AD patients (Müller et al., 2013). MtDNA damage, including deletions most likely occur during repair of oxidatively damaged molecules (Krishnan et al., 2008). We have previously characterized that iron overload induces oxidative stress (de Lima et al., 2005, Dal-Pizzol et al., 2001), including increased superoxide production in mitochondrial fraction (de Lima et al., 2005) in cortex, *substantia nigra*, and hippocampus. High levels of ROS may lead to double strand breaks which, if repaired incorrectly, result in mtDNA deletions (Greaves et al., 2012). Although several evidences suggest that CBD may possess antioxidant properties, in the present conditions, CBD treatment later in life was not able to reverse iron-induced mtDNA deletions.

Interestingly, iron administered in the neonatal period produced dramatic decreases in mtDNA methylation and hydroxymethylation in the hippocampus of adult rats. Despite the increasing concentration of studies on epigenetic mechanisms in CNS physiology and pathology, the great majority has focused on nuclear DNA epigenetics. Dzitoyeva et al. (2012) investigated mitochondrial epigenetics in the mammalian CNS using a mouse model of aging and mouse primary neurons in culture, and demonstrated that aging decreases mtDNA 5hmC in the frontal cortex. Although mitochondrial epigenetic modulation is obscure and more studies are required to understand mechanisms of regulation and functioning, we can speculate on a role played by iron excess, since iron accumulates in the CNS with aging and in neurodegenerative diseases. Devall et al. (2014) suggested that mitochondrial dysfunctions associated to AD might be related to an altered pattern of epigenetic modulations. CBD was able to modify mitochondrial hydroxymethylation profile of rats treated neonatally with iron, bringing 5hmC content to levels comparable to controls. One study (Pucci et al., 2013) investigated the possible epigenetic regulation of skin differentiation genes by phytocannabinoids, including CBD. They showed that CBD reduced the expression of all the genes tested by increasing DNA methylation, supporting the view that CBD's mechanism of action may include epigenetic modulation.

Taking into account the present findings showing that iron overload leads to mtDNA injury, we sought to further investigate on how this organelle handled excessive iron. We found that mitochondria failed to increase ferritin expression in response to iron overload, while no alterations were observed in the iron transporter protein, mitoferrin 2. Mitochondrial ferritin has been recently described as a specific mitochondrial iron storage protein that contributes to cellular iron homeostasis. Despite its large similarity to cytosolic ferritin, little is known about physiological functions and regulation of this protein (Arosio and Levi, 2010; Wang et al., 2011). It has been suggested that mitochondrial ferritin expression is not primarily related to iron storage, but to protection of mitochondria against oxidative damage (Levi and Arosio, 2004; Santambrogio et al., 2007; Arosio and Levi, 2010). Due to the fact that we have not used a mitochondrial protein to normalize protein content, we cannot completely rule out the possibility that reductions in mitochondrial ferritin levels observed might be related to iron-induced changes in cellular mitochondrial content rather than specific changes in this protein. Although abnormalities of mitochondrial function and iron metabolism have been reported in neurodegenerative diseases, only recently, investigations relating mitochondrial ferritin and neurodegenerative disorders started to appear. Wang et al. (2011) reported that mitochondrial ferritin expression was increased in the cerebral cortex of DA patients, and that H_2O_2 alone or in combination with β-amyloid increased mitochondrial ferritin in human neuroblastoma cell line. Wu et al. (2013) showed that knocking mitochondrial ferritin expression down enhanced Aβ-induced neurotoxicity, oxidative stress, and cell apoptosis and opposite results were obtained with overexpression of this protein. Recently, it was demonstrated that mitochondrial ferritin deletion in mice exacerbated the effects of Aβ infusion on learning and memory and increased apoptosis (Wang et al., 2017). Interestingly, in the present study, we found reduced expression of mitochondrial ferritin in rats that received iron in the neonatal period. The precise mechanisms that regulate mitochondrial ferritin expression are poorly understood. We, hypothesize that the insult of iron overload in the neonatal period may have induced early alterations, possibly increasing mitochondrial ferritin expression early in life, leading to a decreased expression when iron intake was normalized throughout life. It is possible that the inability to keep increased mitochondrial ferritin levels may contribute to iron-induced mitochondrial damages, which subsidize mtDNA deletions observed in the present study, as well as increased levels of apoptotic markers

induced by iron previously described (da Silva et al., 2014). Studies investigating the ontogenetic pattern of mitochondrial ferritin expression under iron loading conditions would contribute to better characterize the role of this protein in iron homeostasis.

Our results showed that chronic CBD in adulthood was able to rescue mitochondrial ferritin protein levels in iron-treated rats. The mechanisms by which CBD was able to modulate mitochondrial ferritin levels remain unknown. We propose that the ability of CBD in recovering ferritin levels in iron-treated rats may account, at least in part, to its anti-oxidant and neuroprotective effects.

As expected, iron-induced mitochondrial alterations resulted in functional bioenergetic failures as well. Succinate dehydrogenase, an enzyme that links the tricarboxylic cycle with the electron transport chain, showed reduced activity in hippocampus of iron-treated rats, whereas, a tendency of reduction in creatine kinase, an enzyme that plays a key role in energy transfer in cells with high energy flux requirements, was also observed. Studies have shown that oxidative damage changes bioenergetic metabolism, decreasing Krebs cycle enzymes and electron transport chain complexes activities in some regions of CNS in rats (Basha and Poojary, 2014; Mehan et al., 2017; Teodorak et al., 2017). CBD was able to partially reverse iron-induced succinate dehydrogenase's activity reduction. Although this is the first evidence that CBD improves the activity of succinate dehydrogenase, a recent study has shown that CBD enhances mitochondrial bioenergetics, and modulates glucose metabolism via the pentose-phosphate pathway, preserving both energy and the redox balance in an *in vitro* model of oxygen-glucose-deprivation/reperfusion (OGD/R) in mouse hippocampal neuronal cell line (Sun et al., 2017). Furthermore, *in vivo* acute and chronic CBD increased the activity of the mitochondrial complexes (I, II, II–III, and IV) and creatine kinase in the rat brain (Valvassori et al., 2013). The dose of CBD used in the present study was lower than those utilized by Valvassori and coworkers, possibly explaining why in the present study CBD did not alter creatine kinase activity.

In conclusion, here we observed that iron overload in the neonatal period changes mitochondrial function, increasing mtDNA deletions, reducing epigenetic modulation of mtDNA, decreasing mitochondrial ferritin levels, and decreasing succinate dehydrogenase activity, which may altogether compromise proper cellular functioning, and contribute to neurodegeneration. Considering that the neonatal period is critical for iron uptake by the CNS and iron-fortified infant formulas contain much higher iron content than the breast milk, the long-term effects of iron supplementation still need to be determined. It has been proposed that elevated iron intake during the neonatal period in humans may represent a risk factor to the development of neurodegenerative diseases later in life (Hare et al., 2015).

We showed for the first time that CBD was able to restore hippocampal epigenetic modulation of mtDNA and to increase mitochondrial ferritin levels, which may be directly related to its neuroprotective properties. Also, CBD rescued succinate dehydrogenase activity in iron-treated rats, contributing for bioenergetic recovery, thereby promoting neural cell survival. These findings provide new insights in the molecular targets of CBD, and give support for its use as disease modifying agent in the treatment of neurodegenerative diseases.

Disclosures

AWZ, JECH and JAC are co-inventors (Mechoulam R, JC, Guimarães FS, AZ, JH, Breuer A) of the patent "Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023" Def. US no. Reg. 62193296; 29/07/2015; INPI on 19/08/2015 (BR1120150164927). The University of São Paulo has licensed the patent to Phytecs Pharm (USP Resolution No. 15.1.130002.1.1). The University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil) to "develop a pharmaceutical product containing synthetic cannabidiol and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson's disease, and anxiety disorders". JAC and JECH received travel support from and are medical advisors of BSPG-Pharm.

All the other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The funding sources had no role in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

Conflict of interest

The authors declare that there are no conflict of interest.

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