Protective effect of maternal exercise against amyloid-β neurotoxicity in the male rat offspring's cerebellum

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Abstract

The Developmental Origins of Health and Disease (DOHaD) states that intrauterine maternal environment influences postnatal life by programming offspring's metabolism. Intrauterine milieu induced by exercise during pregnancy promotes long-lasting benefits to the offspring's health and seems to offer some resistance against chronic diseases in adult life. Alzheimer's disease is a public health concern with limited treatment options. In the present study, we assessed the potential of maternal exercise during pregnancy in long-term programming of young adult male rat offspring's cerebellar metabolism in conferring neuroprotection against amyloid-β (Aβ) neurotoxicity. Female Wistar rats were submitted to a swimming protocol 1 week prior mating and throughout pregnancy (five sessions/a week lasting 30 min). Aβ oligomers were infused bilaterally in the brain ventricles of 60-day-old male offspring. Fourteen days after surgery, we measured parameters related to redox state, mitochondrial function, and the immunocontent of proteins related to synaptic function. We found that maternal exercise during pregnancy attenuated several parameters in the offspring's male rat cerebellum, such as the reactive species rise, the increase of inducible nitric oxide synthase immunocontent and tau phosphorylation induced by Aβ oligomers, increased mitochondrial fission indicated by dynamin-related protein 1 (DRP1), and protein oxidation identified by carbonylation. Strikingly, we find that maternal exercise promotes changes in the rat offspring's cerebellum that are still evident in young adult life. These favorable neurochemical changes in offspring's cerebellum induced by maternal exercise may contribute to a protective phenotype against Aβ-induced neurotoxicity in young adult male rat offspring.

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Introduction

Intrauterine and early postnatal development provide a window for metabolic programming that produces long-term effects on offspring's health. In recent years, it has been demonstrated that maternal environment affects offspring metabolism, modulating the susceptibility of disease development[1,2] as is encompassed by Developmental Origins of Health and Disease (DOHaD).[3] The phenotype shaping of the developing metabolism is established through plastic processes that enable an adaptation to environment[4] by metabolic programming,[5] which rely on epigenetic alterations that modulate gene expression and oxidative environment.[6]

We and others have demonstrated that maternal exercise during pregnancy improves brain antioxidant defense system[7] and induces mitochondrial biogenesis[7,8] in 7-day-old rats, improves glucose homeostasis and insulin sensitivity in adult mice (>6 weeks old),[9] and neurogenesis in newborn rats[10] and young mice,[11] as well as increases brain-derived neurotrophic factor (BDNF) levels in the brain of young[10] and adult[12] rats. These findings highlight maternal exercise as a modulator of intrauterine environment that favors enhanced brain function. We recently showed that maternal exercise is associated with a high-quality pattern of maternal care during the dark cycle and with increased exploratory behavior of male and female offspring.[13] On basis of these wide range of metabolic and behavioral
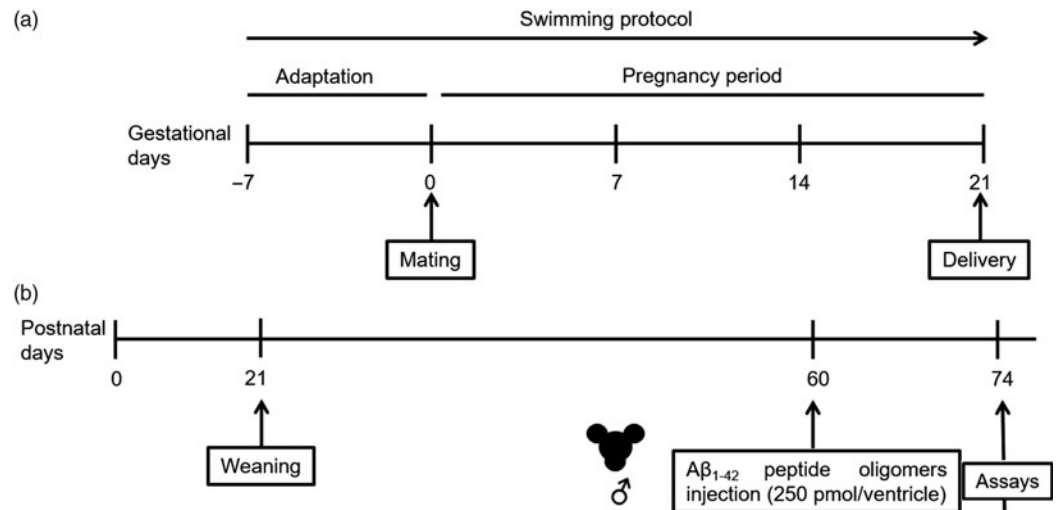
Fig. 1. Experimental design. (*a*) Maternal swimming protocol and (*b*) offspring's timeline.

programming effects, different groups have showed that maternal exercise during pregnancy is able to protect adult offspring against chronic metabolic dysfunction.[14–19]

It has been proposed that the physical exercise improves brain function, including in those with neurodegenerative disorders, such as Alzheimer's disease (AD).[20–23] Although human and animal studies have shown that physical exercise during pregnancy improves several brain functions and cognitive performance of the offspring,[10,24–28] few studies have addressed the beneficial effects of maternal exercise during pregnancy regarding neuroprotective role against neurodegeneration in the offspring at adult age.[8,14] The hallmarks of AD are the amyloid-β (Aβ) peptide accumulation and intracellular neurofibrillary tangles that lead to neuronal damage.[29] In addition, events including oxidative stress, mitochondrial dysfunction, energetic metabolism failure, and neuroinflammation are suggested to be part of the underlying molecular mechanisms of the disease.[30,31] Increased reactive species (RS) levels and downregulation of the antioxidant system contribute to Alzheimer disease (AD) pathology.[32,33] Moreover, oxidative damage occurs in the brain in the presence of amyloid β oligomers (AβO), targeting proteins in a special way, resulting in loss of function.[34] To date, there is no curative treatment to counteract the establishment of the disease since its pathophysiological mechanisms are not fully understood. In this context, the role of the cerebellum in Alzheimer's disease presents a new frontier of study. Recently, non-motor functions have been recognized in the cerebellum, such as emotional and cognitive associative learning control.[35] Several changes related to exercise adaptive responses in central nervous system such as enhance synaptic plasticity, neurogenesis, and cognition have also been observed in the cerebellum.[36]

In this study, we hypothesized that metabolic modulation in the cerebellum of 7-day-old rats by maternal swimming exercise persists up to young adulthood and that in the face of a challenge might be able to protect the rat offspring's cerebellum from metabolic alterations. Therefore, we set out to investigate whether maternal swimming before and during pregnancy promotes long-lasting metabolic effects on rat offspring's cerebellum to examine the cellular and molecular underlying mechanisms. Moreover, we investigated the potential of maternal swimming before and during pregnancy in conferring neuroprotection to young adult male rat offspring against Aβ-induced neurotoxicity.

Material and methods

Reagents

Reagents for biochemical assays were obtained from Sigma-Aldrich. Probes for flow cytometric analyses were obtained from Molecular Probes. Antibodies for Western blot were obtained from Millipore, Cell Signaling, Abcam, Santa Cruz Technologies, and GE Healthcare. All the reagents used are listed below in the techniques section.

Animals and ethical approval

Seventy-day-old female (220–260 g) and male (300–350 g) Wistar rats were obtained from in-house breeding colonies at the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil. Rats facility was under controlled light (12:12 hour light/dark cycle), temperature (22 ± 1°C), and humidity conditions (50–60%). Adult animals were housed in four per cage (approximately 41 × 34 × 16 cm) according to experimental group, while the litter was maintained under maternal care until weaning. All animals had free access to a 20% (w/w) protein commercial chow (CR1 lab chow, Nuvilab Ltda., Curitiba, Brazil) and water.

All experimental procedures were approved by the local Ethics Commission on the Use of Animals (Comissão de Ética no Uso de Animais – CEUA/UFRGS) and were performed in accordance with the National Animal Rights Regulations (Law 11.794/2008), the American National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1996), and the Directive 2010/63/EU. ARRIVE guidelines were followed in the preparation of the manuscript. All efforts were made to minimize the number of animals used and their suffering. The number of animals used in each experiment is indicated in the figures captions.

Experimental design

The study design is depicted in Fig. 1. The animals were allocated into different experimental groups by simple randomization. Adult female rats were initially divided into two groups: (1) sedentary control dams, group in which the rats were exposed to aquatic environment stress without exercising and (2) maternal exercise

dams, group in which the rats were subjected to a swimming protocol in a pool (30 width × 30 length × 90 depth cm) filled with water at $32 \pm 1°C$.[7] The animals underwent involuntary swimming, 30 min daily (performed between 9 am and 12 pm) for 4 weeks, 5 days/week, 1 week for adaptation to aquatic environment prior to mating, and during entire pregnancy period.[7] At the end of first week, two females were mated with one male, and the pregnancy was confirmed by the presence of a vaginal plug or sperm in the vaginal fluid. The pregnant rats underwent the exercise protocol during the entire pregnancy. The animals were left free to swim and were gently stimulated to swim when necessary. After all rats swam, control rats were immersed in water lasting 10 s, carefully dried, and returned to the housing boxes. Beginning on the 20th gestational day, the dams were housed individually and observed twice a day (8 am and 6 pm) to verify litter's birth. The offspring's birth was considered postnatal day (PD) 0. Within 24 h after delivery, the litter was adjusted to a number of eight pups per dam. From delivery (PD 0) until weaning (PD 21), each dam was housed together with its litter. On PD 21, female littermates were euthanized, and male littermates were housed in four per cage until the PD 60. On 60 days of age, male rat offspring were subjected to a surgical procedure for bilateral microinfusion of AβO or vehicle into the brain ventricles. Thus, the offspring was subdivided into the following groups: (1) sedentary + vehicle (control group), (2) maternal exercise + vehicle, (3) sedentary + AβO, and (4) maternal exercise + AβO. On the 14th day post-surgery, the rats were euthanized by decapitation for evaluation. The experimenters were blinded at the time of sample preparation, data analysis, and calculation. No animal died or were excluded.

Aβ1-42 peptide oligomers preparation

Soluble AβO were prepared according to Klein.[37] The Aβ (sequence 1-42) peptide (American Peptide Co., Sunnyvale, CA, USA) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma-Aldrich, St. Louis, MO, USA) in order to form monomers of Aβ peptide. After incubation lasting 1 h at room temperature and 10 min on ice, the solution was aliquoted, and the tubes containing the Aβ peptide were maintained overnight in the hood to remove HFIP followed by centrifugation in a SpeedVac system to obtain a clear film of monomeric Aβ peptide at the bottom of the tubes, which were stored at $-80°C$. Twenty-four hours before in vivo infusion, aliquots were solubilized in dimethyl sulfoxide (DMSO), diluted in phosphate buffer saline (PBS) pH 7.4, and incubated at 5°C for 24 h. After incubation, the tubes were centrifuged at $14,000g$ for 10 min at 4°C, and the AβO-containing supernatants were transferred to a new tube. The vehicle contained DMSO + PBS without any protein. Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fischer), and structural characterization was performed using the specific Aβ 1-16 antibody 6E10 (Covance) through western blotting.

Surgical procedure for AβO infusion

On the PD 60, young adult male rat offspring were anesthetized (100 mg/kg ketamine and 15 mg/kg xylazine, i.p.) and then placed in a stereotaxic frame to the following surgical procedure previously described by Hoppe et al.[38] Two trained experimenters performed the surgical manipulation. Using sterile surgical instruments, a mid-sagittal incision was made in the scalp, and bilateral holes were drilled in the skull using a dental drill over the lateral ventricles, according to Paxinos and Watson atlas coordinates: 0.8 mm posterior to bregma and 1.5 mm lateral to the sagittal suture,

and the depth of the microinfusion consisted in 3.5 mm beneath the surface of brain.[39] Rats received a single intraventricular infusion of 5 μL bilaterally of Aβ peptide oligomers (500 pmol/rat), and control rats received bilateral infusions into each lateral ventricle of equal volume of PBS and 2% DMSO. The microinfusions were performed using a 10 μL Hamilton syringe fitted with a 26-gauge needle in an infusion rate of 1 μL/min over a period of 5 min. At the end of infusion, the needle was left in place for an additional 3 to 5 min before being slowly withdrawn to allow diffusion from the tip and prevent reflux of the solution. After the infusion, the scalp was sutured, and the animals were allowed to recover from the anesthesia on a heating pad to maintain body temperature at $37.5 \pm 0.5°C$.

Sample preparation for biochemical assays

Rats were euthanized 14 days after the AβO infusion by decapitation, without anesthesia in order to avoid any interference of anesthetic on biochemical assays. For redox state parameters assessment, the cerebellum was rapidly dissected, weighed, and immediately frozen ($-80°C$) until homogenization (1:10 w/v in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl). After being homogenized, the samples were centrifuged at $750g$ for 10 min at 4°C to obtain the supernatant containing cytosol, mitochondria, and other organelles. The assays were performed in duplicate using SpectraMax microplate reader (Molecular Devices, Multi-Mode Analysis Software).

Antioxidant enzymes activities

To examine the endogenous enzymatic antioxidant system, we measured the activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). The specific activities of antioxidant enzymes were expressed as U/mg protein.

SOD (EC 1.15.1.1) activity was assayed according to Misra and Fridovich,[40] with the assay adapted for 96 wells microplates. We measured the total SOD activity by quantifying the inhibition of superoxide-dependent epinephrine autoxidation at 480 nm at 32°C for 10 min. SOD activity is expressed as the amount (equal to 1 unit) of enzyme that inhibits the oxidation of epinephrine by 50%.

CAT (EC 1.11.1.6) activity was assayed according to Aebi,[41] with the assay adapted for 96 wells microplate. We measured the CAT activity in the sample by the consumption of hydrogen peroxide added to the reaction medium (containing 20 mM H_2O_2, 0.1% Triton X-100, and 10 mM potassium phosphate buffer, pH 7.0). The decrease in the absorbance at 240 nm was measured using a UV bottom microplate. The CAT unit is defined as 1 μmol of H_2O_2 consumed per minute at 37°C for 3 min.

GPx (EC 1.11.1.9) activity was assayed according to Wendel,[42] with the assay adapted for 96 wells microplate. We measured the GPx activity in the sample in a reaction medium containing 100 mM potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, 2 mM reduced glutathione (GSH), 0.15 U/mL glutathione reductase (EC 1.8.1.7), 0.4 mM azide, 0.1 mM NADPH, and 0.5 mM tert-butyl hydroperoxide as enzyme substrate. NADPH disappearance was monitored at 340 nm at 25°C for 3 min. The GPx unit is defined as 1 μmol of NADPH consumed per minute.

Reduced GSH content

To examine the most important endogenous nonenzymatic antioxidant in the brain, we measured the content of GSH, whose

concentration was measured according to Browne and Armstrong,[43] with the assay adapted for 96 wells microplate. Initially, the proteins in supernatant were precipitated with meta-phosphoric acid (1:1, v/v), and after centrifugation, the GSH present in the supernatant was allowed to react with 7.5 mM fluorophore o-phtaldialdehyde present in the reaction medium in addition to 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA. The fluorescence was measured at excitation and emission wavelengths of 350 and 420 nm, respectively. Standard GSH curve ranging from 0.001 to 1 mM was prepared and a blank sample was performed in parallel. Data are expressed as nmol of GSH/mg protein.

Thiol content

Thiol (SH) content was measured according to Aksenov and Markesbery,[44] with the assay adapted for 96 wells microplate. The thiol content present in the sample reduces the 50-dithiobis-(2-nitrobenzoic acid) (DTNB), which become oxidized (disulfide), generating a yellow derivative (TNB). The absorbance was measured at 412 nm in a reaction medium containing 20 mM sodium phosphate buffer, pH 7.4, and 10 mM DTNB prepared in a 0.2 M potassium phosphate solution, pH 8.0. Data are expressed as nmol TNB/mg protein.

Carbonyl content

Protein carbonyl content was measured according to Reznick and Packer,[45] with the assay adapted for 96 wells microplate. Protein carbonyls react with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound that was measured at 370 nm. Data are expressed as nmol of carbonyls/mg protein.

Respiratory system complexes activities

Rats were euthanized 14 days after the AβO injection by decapitation, without anesthesia in order to avoid tissue chemical contamination. The cerebellum was rapidly dissected, weighed, and immediately frozen (-80°C) until homogenization. The cerebellum was homogenized (1:20 w/v) in SETH buffer, pH 7.4, that contains 250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base, and 50 IU/mL heparin and centrifuged at 800*g* for 10 min at 4°C. The pellet was discarded, and the supernatant was collected and submitted to three subsequent freeze–thawing.[46] Mitochondrial respiratory chain enzyme activities were measured in homogenates with a protein concentration varying from 1.5 to 5.0 mg protein/mL. The assays were performed in duplicate using SpectraMax microplate reader (Molecular Devices, Multi-Mode Analysis Software).

Succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) (EC 1.3.99.1) activity was determined according to the method of Fischer et al.[47] adapted for 96 wells microplate. Complex II enzyme activity in the sample was measured in a reaction medium containing 62.5 mM potassium phosphate buffer, pH 7.4, 250 mM succinate, and 0.5 mM DCIP, at 30°C for 10 min. Complex II activity was determined following the decrease in absorbance due to reduction of DCIP at 600 nm. The activity of complex II is expressed as nmol/min/mg protein.

Cytochrome *c* oxidase (complex IV) activity was measured according to Rustin et al.[48] adapted for 96 wells microplate. Complex IV enzyme activity in the sample was measured in a reaction medium containing 10 mM potassium phosphate buffer, pH 7.0, 125 mM n-dodecil-β-D-maltoside, and 1% cytochrome *c*.

Complex IV activity was determined following the decrease in absorbance due to reduced cytochrome *c* oxidation at 550 nm at 30°C for 5 min. The activity of complex IV is expressed as nmol/min/mg protein.

Protein determination

Protein concentration (mg of protein/mL) was measured according to the method described by Lowry et al.,[49] which was adapted for 96 wells microplate, using bovine serum albumin as standard. The absorbance was measured at 750 nm.

Flow cytometry

Flow cytometric analysis was conducted according to Marcelino et al.[7] Briefly, the tissue samples (approximately 100 mg) were dissociated in PBS containing 1 mg% of colagenase IV (Sigma-Aldrich, St. Louis, MO, USA, catalog number C5138) and 0.5 mg% of DNase (Sigma-Aldrich, St. Louis, MO, USA, catalog number D5025), filtered using the 40 μm pore size cell strainer (SPL Lifesciences Co., Naechon-Myeon Pocheon, South Korea), and then incubated at 37°C with the molecular probes. Reactive oxygen and nitrogen species levels were measured using 10 μM 2′,7′-dichlorofluorescin diacetate (H$_2$DCF-DA; Sigma-Aldrich, St. Louis, MO, USA, catalog number D6883), nitric oxide (NO•) levels were measured using 10 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM; Invitrogen, Molecular Probes, Eugene, OR, USA, catalog number D23841), mitochondrial superoxide were measured using 1 μM MitoSOX® Red (Invitrogen, Molecular Probes, Eugene, OR, USA, catalog number M36008), mitochondrial mass and membrane potential were measured using 100 nM MitoTracker® Green and 100 nM MitoTracker® Red (Invitrogen, Molecular Probes, Eugene, OR, USA, catalog numbers M7514 and M7513), respectively. Cells were gated based on the FSC and SSC pattern of the sample cells, and 30,000 events were acquired per sample in a BD FACSCalibur Flow Cytometry System; a non-labeled sample was used as negative fluorescent control. DCFH, DAF-FM, and MitoSOX data are expressed as mean fluorescence intensity (MFI), and MitoTracker® Green and MitoTracker® Red data are expressed as percentage of double-positive cells. Data were analyzed using the software FlowJo (Tree Star, Ashland, OR, USA).

Western blot assay

Cerebellum is homogenized in ice-cold lysis buffer containing 4% sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mM Tris, and 1% protease inhibitor cocktail. The homogenates were denatured 100°C for 5 min and then centrifuged at 10,000*g* for 30 min. After this, the supernatant containing the cytosolic fraction was collected, β-mercaptoethanol was added to a final concentration of 5%, and then the samples were stored at −80°C until use. Equal concentration of protein (50 μg) was loaded and immunodetected as previously described.[38] Membranes were incubated for 60 min at 4°C in blocking solution (Tris-buffered saline containing 5% non-fat milk and 0.1% Tween-20, pH 7.4) prior the incubation with the primary antibody. Membranes were incubated overnight at 4°C in blocking solution containing one of the following primary antibodies: rabbit monoclonal anti-synaptophysin (1:2000 dilution, Millipore, catalog number #AB9272), anti-PSD-95 (1:1000 dilution, Cell Signaling, catalog number #2507), anti-sirtuin 3 (SIRT3) (1:1000 dilution, Abcam, catalog number #ab189860), anti-mitofusin 1 (1:1000 dilution, Abcam, catalog number # ab104274),
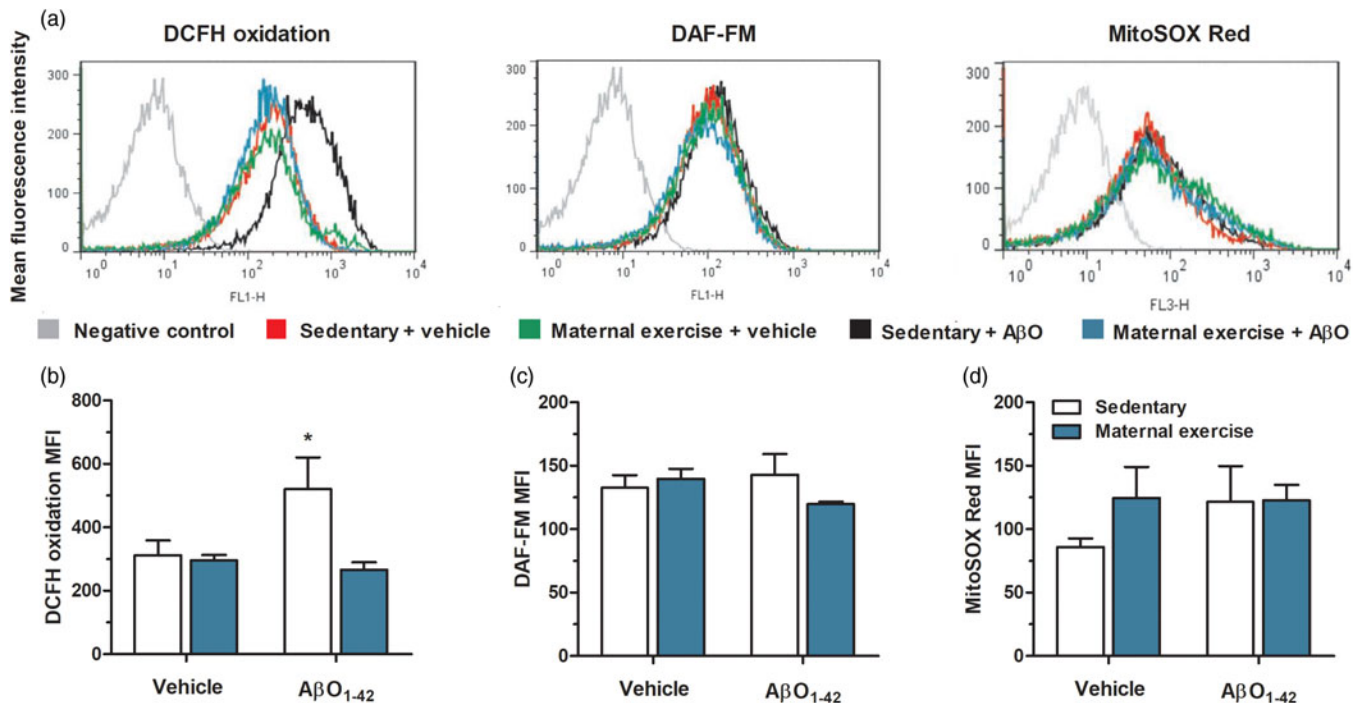
Fig. 2. Effects of maternal exercise and AβO infusion on offspring's cerebellar reactive species levels. (*a*) Representative histograms of DCFH, DAF-FM, and MitoSox mean fluorescence intensity (MFI). Bars show mean + standard error (n = 6 rats in each group) of (*b*) DCFH oxidation MFI, (*c*) DAF-FM MFI, and (*d*) MitoSOX Red MFI. Two-way ANOVA showed a main effect of maternal exercise and an interaction between maternal exercise and AβO on DCFH oxidation. * P<0.05 sedentary + AβO compared to other groups (indicated by Tukey post hoc test).

anti-dinamin-related protein 1 (1:1000 dilution, Abcam, catalog number #ab154879), anti-phospho(S9)-glycogen synthase kinase 3β (GSK-3β) (1:1000 dilution, Cell Signaling, catalog number #9336), anti-GSK-3β (1:1000 dilution, Cell Signaling, catalog number #9315), anti-NOS2 (1:250 dilution, Santa Cruz Technologies, catalog number #sc-7271), anti-phospho(S396)-Tau (1:1000 dilution, Cell Signaling, catalog number #9632), anti-Tau (1:1000 dilution, Cell Signaling, catalog number #4019), and rabbit monoclonal anti-β-actin (1:2000 dilution, Cell Signaling, catalog number #4967). After washing, the membranes were incubated with secondary antibody containing peroxidase-conjugated anti-rabbit IgG (1:1000 dilution, GE Healthcare Life Sciences, catalog number #NA934V) and anti-mouse IgG (1:1000 dilution, GE Healthcare Life Sciences, catalog number #NA931V) for 1 h. The chemiluminescence was detected using the digital imaging system Image Quant LAS 4000 (GE Healthcare Life Sciences) and analyzed using the Image J Software. The average optical density for the control group was designated as 100%.

Statistical analyses

The sample size of the study was calculated according to previous works performed in the lab (with $\alpha = 0.05$, $\beta = 0.20$) using the Minitab 16 software (Minitab Inc., State College, PA, USA). Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using the GraphPad Prism 6.0 software. All data were tested for normality using Shapiro–Wilk test. Data points outside the 95% confidence interval were treated as outliers and excluded from the data analysis. Two-way analysis of variance (ANOVA) was used to analyze the effect of the two independent variables, maternal exercise, and AβO infusion. Post hoc analysis was carried out using Tukey's test. Main effects were considered significant if $p \leq 0.05$.

### Results

Maternal exercise prevents the increase of AβO-induced RS levels

Concerning overall RS measured by DCFH oxidation, two-way ANOVA indicated an effect of maternal exercise ($p = 0.013$), and Tukey post hoc test indicated that sedentary + AβO group differs from the other groups. Two-way ANOVA also indicated an interaction between maternal exercise and AβO infusion ($p = 0.027$), indicating that AβO infusion induced an increase of RS levels and that maternal exercise was able to prevent completely such rise (Fig. 2a). Nitric oxide levels were not altered by either maternal exercise ($p = 0.473$) or AβO infusion ($p = 0.653$) in the cerebellum of young adult male rat offspring (Fig. 2b). Mitochondrial superoxide levels were not altered by any treatment in the cerebellum of young adult offspring (Fig. 2c). Neither maternal exercise ($p = 0.304$) nor AβO ($p = 0.377$) altered cerebellar mitochondrial superoxide levels. Taking together, these results indicated maternal exercise during pregnancy is able to prevent overall RS levels increase induced by AβO infusion, which is mediated mainly via elevation in peroxides, hydroxyl radicals, or peroxynitrite, and not by nitric oxides or mitochondrial superoxide.

Maternal exercise increases GSH levels in young adult male offspring's cerebellum

Two-way ANOVA indicated a significant effect of maternal exercise on GSH levels ($p = 0.0005$), which was found increased in the young adult male offspring's cerebellum (Fig. 3a). No effect of AβO infusion was found on GSH concentration ($p = 0.727$). Two-way ANOVA indicated that both factors, maternal exercise and AβO infusion, exerted no effect on activities of the enzymes SOD (Fig. 3b; $p = 0.470$ and $p = 0.459$, respectively), GPx (Fig. 3c;
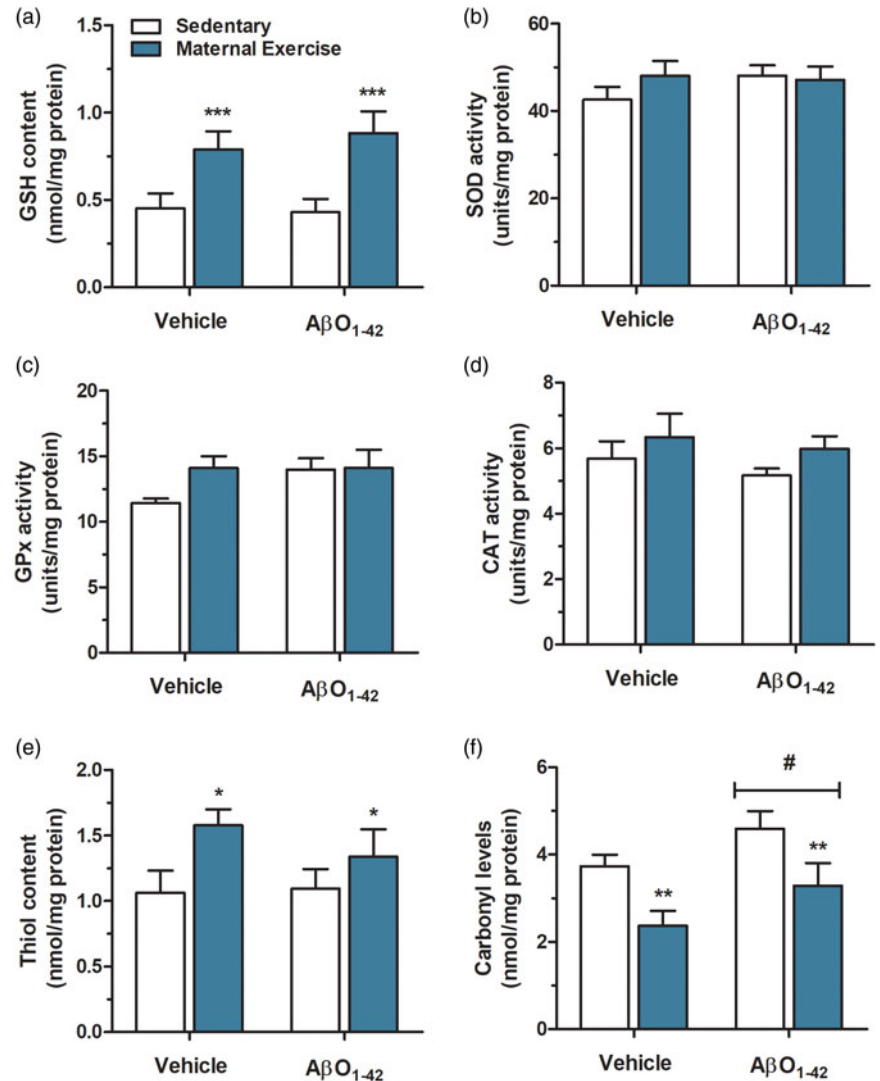
Fig. 3. Effects of maternal exercise and AβO infusion on offspring's cerebellar antioxidant system and oxidative damage. (*a*) Reduced glutathione (GSH) content; (*b*) activity of superoxide dismutase (SOD); (*c*) glutathione peroxidase (GPx); (*d*) catalase (CAT) enzymes; (*e*) thiol; and (*f*) carbonyl contents. Values are expressed as mean + standard error (n = 8 to 11 rats per group for each parameter). Two-way ANOVA showed an effect of maternal exercise on GSH, thiol, and carbonyl contents and a main effect of AβO infusion on carbonyl content. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ effects of maternal exercise (two-way ANOVA), # $P<0.05$ effect of AβO infusion (two-way ANOVA).

$p = 0.303$ and $p = 0.107$, respectively), and CAT (Fig. 3d; $p = 0.170$ and $p = 0.412$, respectively).

Increased protein carbonylation elicited by AβO is prevented by maternal exercise

Two-way ANOVA indicated an effect of maternal exercise on thiol content ($p = 0.029$), which was found increased in the offspring's cerebellum (Fig. 3e); AβO infusion exerted no effect on thiol content ($p = 0.532$). Concerning carbonyl levels, two-way ANOVA indicated an effect of both maternal exercise ($p = 0.002$) and AβO infusion ($p = 0.028$). AβO infusion increased carbonyl levels, while maternal exercise reduced carbonyl levels (Fig. 3f).

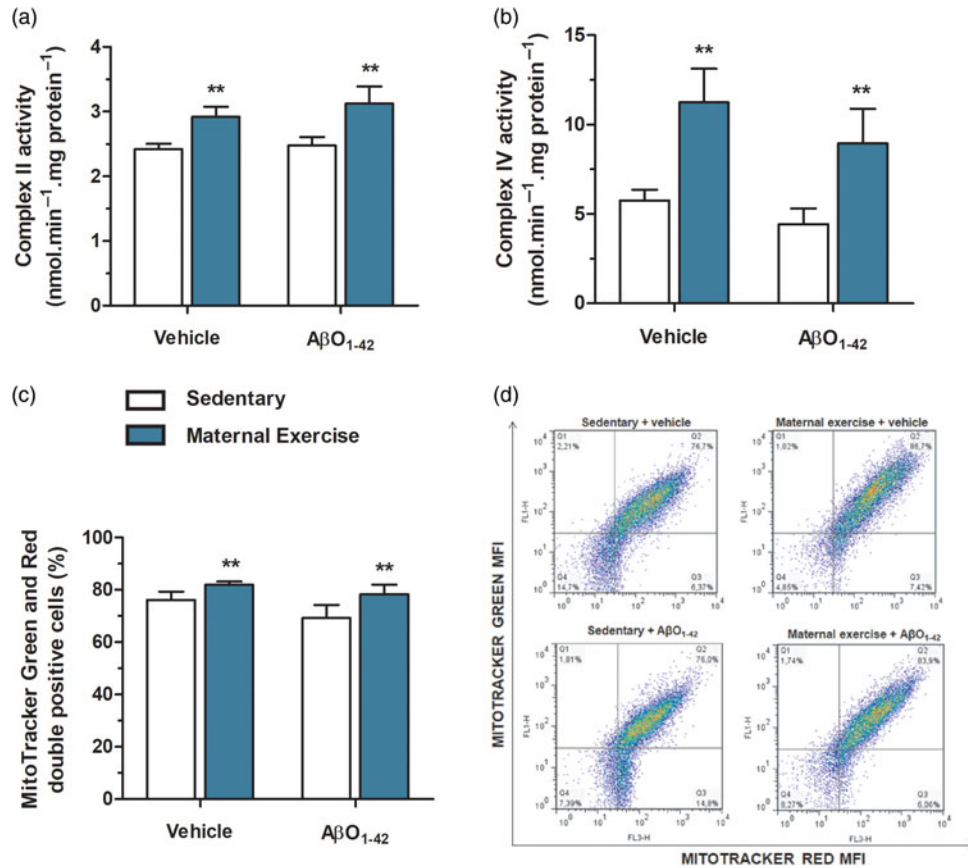### Effects of maternal exercise and AβO infusion on mitochondrial parameters

Two-way ANOVA indicated a main effect of maternal exercise on CII (Fig. 4a; $p = 0.001$) and compled IV (CIV) (Fig. 4b; $p = 0.001$), evidenced by increased activities and no effect of AβO infusion on both enzymes activities, CII ($p = 0.197$) and CIV ($p = 0.419$).

In agreement with the effect of maternal swimming, which activated the mitochondrial complexes, two-way ANOVA indicated a main effect of maternal exercise on MitoTracker Green and Red

double-positive cells ($p = 0.041$), indicating increased mitochondrial mass and membrane potential. In addition, mitochondrial mass and membrane potential were found unchanged by AβO infusion ($p = 0.141$). It is important to note that AβO infusion did not abolish the effect promoted by maternal exercise on mitochondrial function (Fig. 4c and 4d).

Two-way ANOVA indicated an interaction between maternal exercise and AβO infusion on mitofusin 1 (MFN1) immunocontent in the young adult male offspring's cerebellum (Fig. 5a) ($p = 0.037$). Maternal exercise increased offspring's cerebellar MFN1 levels; however, AβO infusion abolished the effect promoted by maternal exercise. Concerning cerebellar dynamin-related protein 1 (DRP1) immunocontent (Fig. 5b), two-way ANOVA indicated a main effect of AβO infusion ($p = 0.042$), and a borderline significant effect of maternal exercise on DRP1 levels ($p = 0.055$). These data indicate that AβO infusion increases DRP1 levels in the offspring's cerebellum, and rise of DRP1 levels in maternal exercised + AβO group reached values similar to control group.

Two-way ANOVA indicated a main effect of maternal exercise on SIRT3 levels (Fig. 5c; $p = 0.039$), increasing SIRT3 immunocontent in the cerebellum of young adult male offspring. No effect of AβO infusion was found ($p = 0.739$). Interestingly, it was observed

Fig. 4. Effects of maternal exercise and AβO infusion on mitochondrial function in the cerebellum of adult offspring. (a) Complex II, (b) Complex IV enzymes activities, (c) percentage of double-positive Mitotracker Green and Mitotracker Red labeled cells, and (d) representative dot plots of Mitotracker Green and Red-immunolabeled cells. Values are expressed as mean + standard error (n = 6 rats in each group for each parameter). A two-way ANOVA showed an effect of maternal exercise on these parameters. **$P<0.01$ effect of maternal exercise (two-way ANOVA).
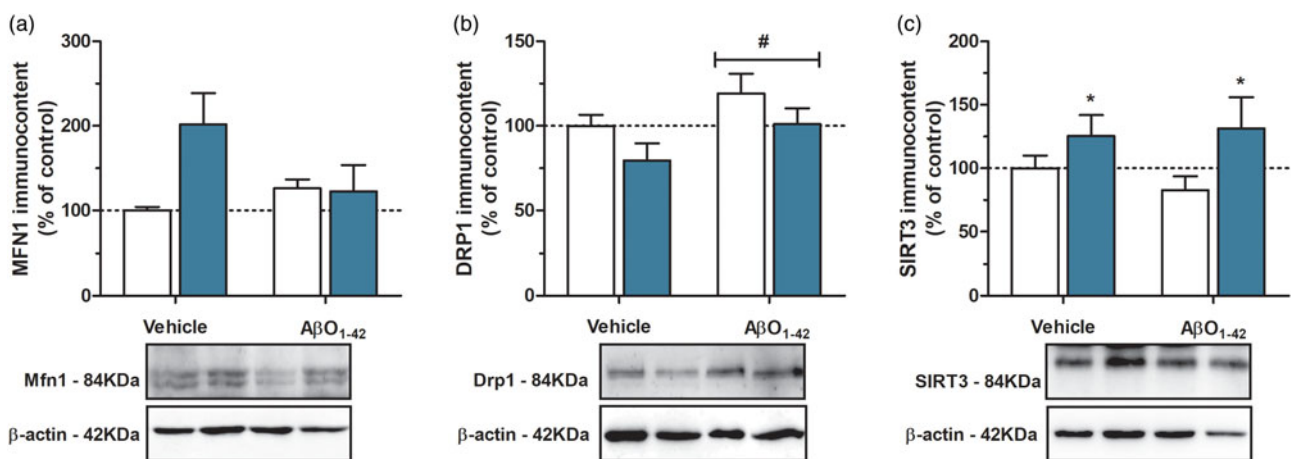
Fig. 5. Effects of maternal exercise and AβO infusion on mitochondria-related proteins levels in the cerebellum of adult offspring. Immunocontent of (a) mitofusin 1 (MFN1; n = 8 to 9 rats per group), (b) dynamin-related protein 1 (DRP1; n = 8 to 9 rats per group), and (c) SIRT3 (n = 6 rats in each group) expressed as average percentage of control. Representative quantification of proteins immunocontent normalized to b-actin protein (loading control) is shown below the graphs. Values are expressed as mean + standard error. A two-way ANOVA showed interaction between maternal exercise and AβO infusion for mitofusin (p<0.05). *$P<0.05$ effect of maternal exercise (two-way ANOVA), #$P<0.05$ effect of AβO infusion (two-way ANOVA).
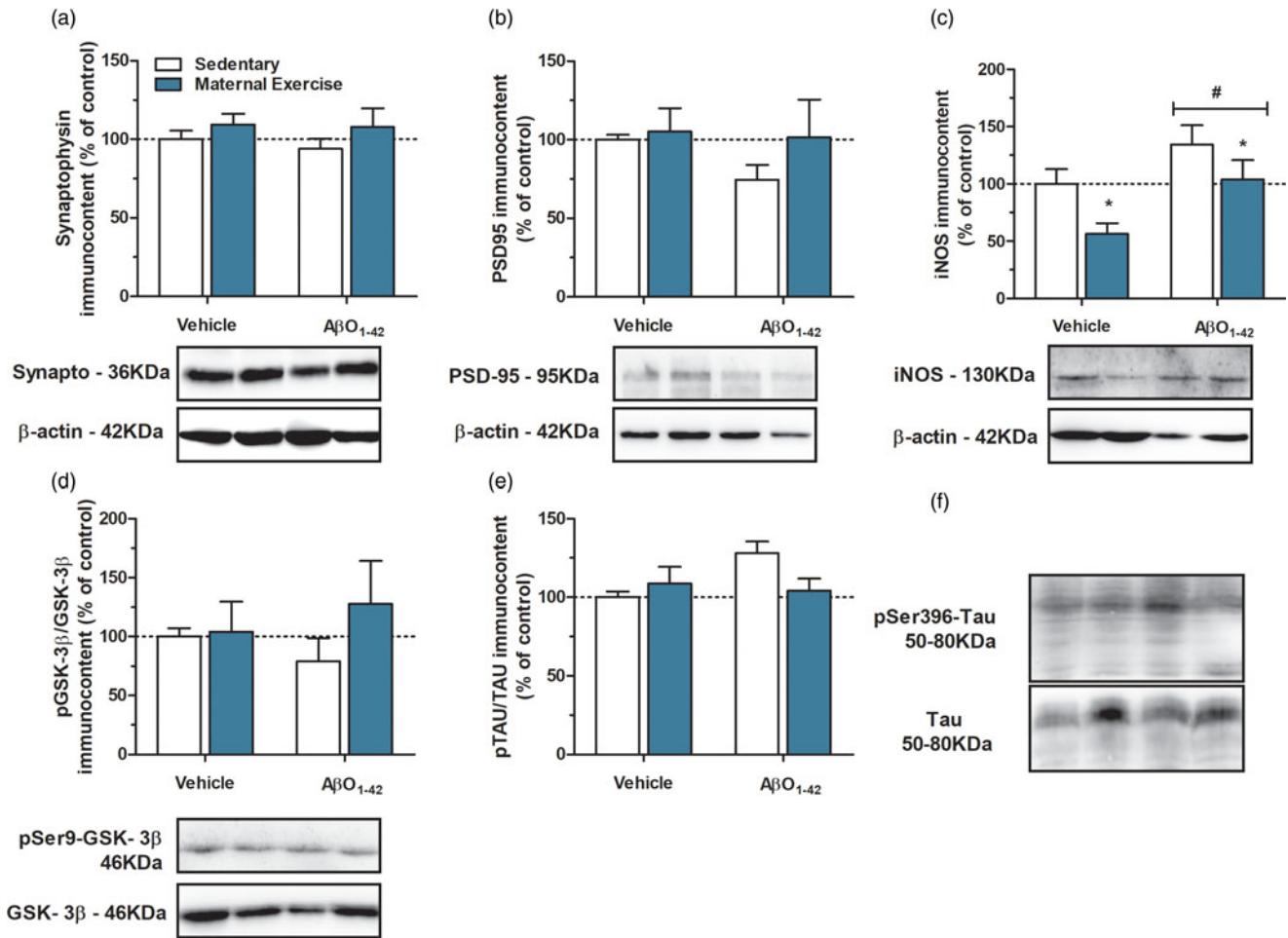
Fig. 6. Effects of maternal exercise and AβO infusion on synaptic proteins and inducible nitric oxide sintase (iNOS) in the cerebellum of adult offspring. Immunocontent of synaptic proteins (*a*) synaptophysin (n = 7 to 9 rats per group) and (*b*) post-synaptic density-95 protein (PSD95) (n = 7 to 8 rats per group). Immunocontent of (*c*) iNOS (n = 7 to 9 rats per group), (*d*) phospho-GSK-3β/GSK-3β ratio (n = 6 rats in each group), and (*e*) phospho-Tau/Tau ratio (n = 8 to 9 rats per group). Data are expressed as the average percentage of control. Representative quantification of proteins immunocontent normalized to b-actin protein (loading control) is shown below the graphs. A two-way ANOVA showed interaction between maternal exercise and AβO infusion for phospho-Tau/Tau ratio (p<0.05). *P<0.05 effect of maternal exercise (two-way ANOVA), ##P<0.01 effect of AβO infusion (two-way ANOVA).

that AβO infusion did not abolish the SIRT3-mediated adaptive ability of mitochondria to maternal exercise.

Maternal exercise prevented the increment of iNOS immunocontent elicited by AβO infusion in the cerebellum of young adult male offspring

The results concerning the immunocontent of inducible nitric oxide synthase (iNOS) in the offspring's cerebellum, two-way ANOVA indicated an effect of both maternal exercise and AβO infusion (Fig. 6c). While maternal exercise reduced iNOS levels ($p = 0.015$), AβO infusion increased iNOS levels ($p = 0.008$). Consequently, the levels of iNOS in maternal exercised + AβO group reached values similar to control group, suggesting a possible neuroprotective effect.

Synaptophysin and PSD-95 immunocontent are not altered in the cerebellum of young adult male offspring in response to AβO infusion

Two-way ANOVA indicated that neither maternal exercise nor AβO infusion exerted any effect on synaptophysin (Fig. 6a; $p = 0.146$ and $p = 0.634$, respectively) and PSD-95 levels (Fig. 6b;

$p = 0.281$ and $p = 0.325$, respectively) in the cerebellum of young adult male offspring.

Maternal swimming during pregnancy prevents the phosphorylation of tau protein in the cerebellum of young adult male offspring AβO-infused

Lastly, we assessed the phosphorylated status of two proteins that are altered in AD.[50] The ratio between phosphorylated and total content of GSK-3β was found unaltered in the cerebellum of all groups (Fig. 6d), in which neither maternal exercise nor AβO infusion exert effect on p-GSK-3β/GSK-3β ratio ($p = 0.295$ and $p = 0.958$). Concerning tau phosphorylation status, two-way ANOVA indicated an interaction between both maternal exercise and AβO infusion on p(Ser-396)-tau/tau ratio ($p = 0.041$). These data indicated that AβO infusion increases tau phosphorylation, in a GSK-3β-independent way, while maternal exercise prevented such increase (Fig. 6e).

Discussion

In a previous work, we demonstrated that maternal exercise prevented learning and memory deficits elicited by AβOs infusion, as

well as metabolic alterations in the prefrontal cortex and hippocampus of young adult male rat offspring.[8] In the present study, we extended the investigation to cerebellum and observed that maternal exercise during pregnancy promotes adaptive response in developing fetuses through programming cerebellar neurochemistry that remains evident into young adulthood. Importantly, maternal exercise-induced programming was able to prevent some pathologic consequences of AβO peptide infusion, including rise of RS, protein carbonylation, and phosphorylation of tau protein.

Brain development, including growth and maturation, extends from gestational period beyond early PDs.[51] The majority of literature data focus on cortical and hippocampal modulatory effects promoted by maternal exercise in the offspring's brain,[10–12,52] and little is known about prenatal exercise effects on cerebellum. Data of adaptive changes in the cerebellum induced by maternal exercise were recently published by our group.[7,26] We have shown that swimming during pregnancy enhances mitochondrial function and antioxidant defenses in the cerebellum, parietal cortex, hippocampus, and striatum of 7-day-old pups.[7] Increased GSH and SH content and reduced protein carbonylation in young adult male offspring's cerebellum presented reinforce our previous findings, evidencing long-term positive influence of maternal exercise on brain development. In addition, similar levels of oxidants in the cerebellum of young adult male offspring, when compared to control, contrasted to increased oxidants levels in the cerebellum of 7-day-old pups.[7] Taken together, these findings suggest that increased oxidant levels on PD7 precede the metabolic adaptation improving antioxidant system efficiency that persists in young adulthood. In accordance, Akhavan et al.[10] demonstrated that involuntary swimming exercise represents a stressful experience by mother at the end of pregnancy resulting in increased corticosterone levels in blood, which contribute to the adaptive effects of improved cell function observed in the offspring. The adaptive changes in metabolism triggered by mild levels of stress, such as forced exercise, fit the hormesis concept,[53] in which is defined as an adaptive response to low/mild levels of stress resulting in improved aptitude for physiological systems.[54]

Exercise-induced metabolic adaptation in individuals arises to regulate bioenergetics demand.[55] Likewise, fetal adaptive responses to maternal exercise are engaged to deal with energetic challenge by modulating mitochondrial function.[7,52] Mitochondria are crucial to maintain the constant supply of energy required by brain and other organs.[56] The cerebellum suffers marked metabolic adaptation in response to exercise,[57,58] even during pregnancy.[7] We found that maternal exercise increased Complex II and cytochrome c oxidase activities, which are in agreement with increased mitochondrial mass and membrane potential as well as MFN1 and SIRT3 levels, and unchanged DRP1 found in young adult male offspring's cerebellum. Currently, there are two reports in the literature that investigated the effect of maternal exercise on mitochondrial enzymes activities and biogenesis in offspring's brain.[8,52] Park et al.[52] reported that pregnant mice undergoing treadmill exercise affect positively hippocampal mitochondrial function and biogenesis of 3-day-old pups. In addition, our group recently demonstrated that maternal swimming enhances mitochondrial function in the 60-day-old offspring's prefrontal cortex and hippocampus by increasing mitochondrial mass and membrane potential and the activity of cytochrome c oxidase.[8] It has been shown that mitochondrial SIRT3 increases in response to different exercise modalities.[58] SIRT3 expression modulates adaptive responses of hippocampal neurons to exercise and strengths resistance to oxidative stress and apoptosis.[59] To the best of our knowledge, this is the first study to demonstrate that mitochondrial SIRT3 and dynamic proteins in young adult male offspring's cerebellum are modulated by maternal exercise.

NO[•] in the brain acts as a signaling molecule that regulates cerebral blood flow,[60] and as a neuromodulator that regulates synaptic transmission through NO[•]/cyclic guanosine monophosphate (NO[•]/cGMP) pathway.[57,61,62] Brain adaptive response to exercise seems to be mediated by NO[•] through upregulation of endothelial nitric oxide synthase (eNOS)[57,63] and downregulation of the calcium-independent inducible NOS (iNOS),[64] whose effects can be neurotoxic.[60] In the present study, we reported unaltered NO[•] levels and reduced iNOS immunocontent in the cerebellum of young adult male offspring delivered to exercised dams. On basis of these results, it seems that increased NO[•] levels are only necessary to induce metabolic adaptation in early postnatal brain of pups born to exercised dams, as previously demonstrated by our group.[7]

Different exercise regimens have shown to increase proteins related with neuroplasticity. Treadmill exercise in a schedule of 40 min/day, 3 days/week for 4 weeks, or 30 consecutive days increased synaptophysin immunocontent in the cerebellum of rats.[65] Similarly, Liu et al.[66] demonstrated that swimming exercise (60 min/day, 5 days/week for 4 weeks) is able to reverse the reduction of synaptophysin levels in the hippocampus of mice previously submitted to a chronic unpredictable mild stress. Here, we observed that maternal exercise does not modify pre-and post-synaptic protein levels in the cerebellum of 74-day-old young adult male rat offspring, at least in our experimental conditions.

Few studies have examined the effect of AD-associated Aβ neurotoxicity in the cerebellum.[67–69] We found an increase in overall cerebellar RS levels and tau phosphorylation following AβOs infusion, which were prevented by maternal exercise. Whereas H$_2$DCF is oxidized mainly by hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (•OH), and peroxynitrite (ONOO$^-$),[70,71] we sought to investigate the contribution of others RS, such as nitric oxide (•NO) and mitochondrial superoxide (mO$_2$•$^-$). We observed no change in mitochondrial superoxide and NO[•], as well as antioxidant enzymes after AβOs infusion, despite increased RS levels. It is possible that the overall RS measured are hydrogen peroxide (H$_2$O$_2$) species, which might be interacting with NO[•] yielding peroxynitrite explaining the increased iNOS immunocontent and absence of alteration in NO[•] levels. The positive augmentation of some metabolic marks induced by maternal exercise in the male offspring's cerebellum, such as GSH and SH content, was not abolished by AβOs infusion. AβOs infusion increased protein carbonylation, DRP1, and iNOS levels. While these parameters were significantly higher in the male rat offspring's cerebellum born to sedentary dams than male control offspring, protein carbonylation, DRP1, and iNOS levels reached control values in the offspring born to exercised dams. These data indicated that AβO infusion promoted protein oxidation in cerebellum, and maternal exercise prevented the oxidative effect of AβO, suggesting a neuroprotective potential of maternal exercise against AD-changes induced by neurotoxic AβOs in the cerebellum of young adult male offspring. Similarly, Herring et al.[14] reported that running during pregnancy alleviated cerebral oxidative stress and inflammation associated with decreased Aβ plaque in 5-month-old offspring carrying APP transgene.

Mitochondrial dysfunction is an early event that accompanies AD pathologic features underlying the major metabolic changes verified in this disease.[32] It has been shown that AβO accumulates in mitochondria,[72] thus AβO can target directly essential metabolic enzymes impairing its function.[73] Immunoprecipitation analyses
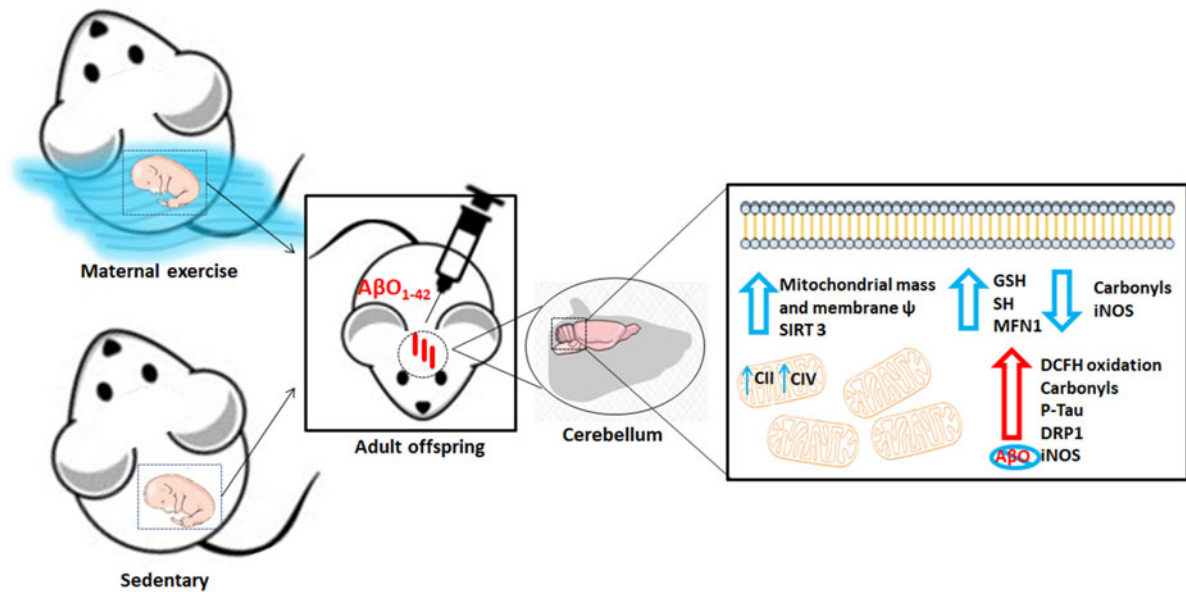
Fig. 7. Summary effects of maternal exercise and AβO infusion found on the cerebellum of adult offspring.

of mitochondrial dynamics showed that AβOs interact with DRP1 leading to abnormal mitochondrial dynamics and increased mitochondrial fragmentation, thus culminating in neuronal damage.[74] We observed that MFN1 and DRP1 levels in maternal exercised offspring infused with AβOs remained at control values, indicating a balance between fusion and fission processes despite the extinction of maternal exercise-induced MFN1 increase. In our previous work, we demonstrated that maternal exercise increased MFN1 levels in the 74-day-old male rat offspring's prefrontal cortex and prevented the increase of DRP1 levels induced by AβOs in the hippocampus.[8] Moreover, the main regulator of mitochondrial energy homeostasis, SIRT3,[75] was found increased together with enhanced mitochondrial enzymes activities, mass, and membrane potential. The increased SIRT3 might be leading to an elevation of mitochondrial activity.[76] On the other hand, an unbalance of mitochondrial dynamic seems to occur in the maternal sedentary male rat offspring once DRP1 levels were increased by AβOs, while no effect was identified on MFN1 levels. The increased mitochondrial fission protein might be responsible by the removal of damaged mitochondria[76] and, as postulated by Demetrius et al.,[77] it is possible that a compensatory mechanism, in which occurs an upregulation of oxidative phosphorylation in some mitochondria to compensate energy production and to maintain impaired cells viable, is being operated.

As increased phosphorylated tau is also involved in AD pathology,[29] the rise in pTAU/TAU ratio in the cerebellum of maternal sedentary male offspring, but not in the maternal exercised male offspring, is another indicative of neuronal damage following AβOs infusion. Despite GSK is the main enzyme phosphorylating TAU protein, herein we observe no alteration in the more active status of GSK, its phosphorylated form. In AD, beyond altered status of kinases enzymes, it is common the altered activity of phosphatases acting over TAU protein, thus contributing to the hyperphosphorylation of TAU in the pathology.[31]

The work conducted by Thal et al.[78] demonstrates that cerebellar pathological features of Aβ deposition in the cerebellum

and related clinical manifestation are evident in later stages of AD. In accordance, Kuwabara et al.[67] demonstrated that cerebellar neuropathology and accumulation of Aβ appear with disease progression in APPswe/PS1dE9 double transgenic mice. The authors also revealed that Aβ accumulation in the cerebellum was associated with impaired long-term depression but unaffected synaptic transmission,[67] which might help us to explain why AβOs did not induce reduction of synaptic proteins in the offspring's cerebellum in the present work. We previously demonstrated that AβOs reduced significantly synaptophysin levels in the hippocampus of 74-day-old male rats and that maternal exercise was able to mitigate this Aβ-induced synaptophysin reduction.[8] In this way, it is possible that we could not observe other alterations following AβO infusion as function of the time we assessed the parameters, but we cannot exclude the possibility of later appearance of AβO-associated pathological features in the cerebellum of 74-day-old male rat offspring. So far, one suggested molecular mechanism responsible by maternal exercise-promoted protective effects observed against biochemical changes caused by AβOs could be the improved mitochondrial bioenergetics.

Conclusion

In summary, we report for the first time that exercise during pregnancy exerts long-lasting effects on the cerebellar metabolism of male rat offspring by inducing adaptive changes in utero. Remarkably, these metabolic programming effects are still evident during young adulthood, on PD 74. Our findings reveal that the long-term influence of maternal exercise on offspring metabolism may offer some protection against AD changes induced by neurotoxic AβO (Fig. 7). Our study provides another example of the benefits of exercising during pregnancy to the offspring and a promising neuroprotective approach to modify the course of disease later in life.

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