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ORIGINAL ARTICLE

Maternal Swimming Exercise Induces Sex-Dependent Changes in Mitochondrial Metabolism and Dynamics in the Cerebellum of Wistar Rat Offspring

Manuela Menegotto,[a] Laura Ilha,[a] Simone Vargas,[a] João Victor Zanella,[b] Maria Luisa Diehl,[b] Ariadni Peres,[a] Bruna Valdameri,[a] Rachel Krolow,[a,b] Carla Dalmaz,[a,b] and Cristiane Matté[a,b]

[a] *Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil*

[b] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil*

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Background and Aims. The field of Developmental Origins of Health and Disease (DOHaD) investigates how environmental interventions during critical periods of development can permanently modify offspring metabolism. In this context, beneficial interventions such as maternal physical exercise during pregnancy may program offspring metabolism and act as a protective metabolic programming strategy linked to positive phenotypic plasticity. However, the cellular mechanisms underlying the effects of maternal exercise during pregnancy on the offspring remain to be fully elucidated. This study aimed to investigate the neuroadaptive effects of maternal physical exercise during pregnancy on the expression of genes and the immunocontent of proteins related to mitochondrial metabolism and dynamics in the offspring cerebellum.

Methods. Female Wistar rats (120 d old) were subjected to five weekly sessions of 30 min of swimming, beginning one week before mating and continuing throughout pregnancy until delivery. On postnatal day 7 (PND7), the offspring of both sexes were euthanized, and the cerebellum was collected and stored at –80°C for subsequent analyses.

Results. Maternal exercise increased the expression of genes related to mitochondrial biogenesis (*Ppargc1a, Tfam, Nrf1, Nfe2l2, Cs*), dynamics (*Mfn1, Mfn2, Opa1, Dnm1l*), and *Fndc5* exclusively in female offspring, while no significant differences in protein immunocontent were observed in either sex.

Conclusion. These findings suggest that maternal exercise during pregnancy promotes early metabolic programming in the cerebellum of female offspring by modulating mitochondrial gene expression in a sex-dependent manner. The observed effects contribute to the understanding of sex-specific neurodevelopmental programming induced by gestational exercise.

Key Words: DOHaD, Maternal exercise, Mitochondria, Cerebellum, Female offspring, Phenotypic plasticity.

Corresponding author: Cristiane Matté, Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Departamento de Bioquímica, Porto Alegre, Rio Grande do Sul, Brazil; E-mail: matte@ufrgs.br

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Introduction

The Developmental Origins of Health and Disease (DOHaD) framework links environmental exposures, especially, but not exclusively, during the first 1,000 d of life to the risk of non-communicable chronic diseases (NCDs) throughout life (1). During this critical period, the developing organism exhibits high metabolic plasticity, enabling adaptive responses to environmental conditions (2). Accordingly, pregnancy represents a critical window for metabolic programming, in which positive interventions can promote offspring health (3). In this context, maternal physical exercise is recommended during uncomplicated pregnancies, with at least 150 min of moderate-intensity activity per week starting in the first trimester, including aerobic, resistance, and aquatic modalities (4). Maternal exercise, when practiced during pregnancy, positively modulates the intrauterine environment, acting as a non-pharmacological strategy to program offspring metabolism and neurodevelopment (5). Studies conducted by our research group using a maternal swimming exercise model during pregnancy demonstrated enhanced cerebral antioxidant capacity and increased mitochondrial biogenesis. This was evidenced by greater mitochondrial mass and membrane potential, an indicator of mitochondrial functional activity, in the offspring's cerebellum and parietal cortex (6). In adulthood, offspring also exhibited improvements in learning and memory (7), as well as enhanced mitochondrial dynamics and metabolism markers, evidenced by increased levels of the mitochondrial fusion protein mitofusin 1 (MFN1) in the prefrontal cortex and increased activities of the mitochondrial electron transport chain in the cerebellum, prefrontal cortex, and hippocampus (7,8). Moreover, maternal swimming exercise during gestation prevented the redox imbalance induced by a high-fat diet in the hippocampus of adult offspring (9) and, to a certain extent, in a hypoxic-ischemic model in neonatal rats (10).

Physical exercise during pregnancy is also known to optimize mitochondrial function in offspring (11). Mitochondrial functionality depends on mitochondrial dynamics, which are key processes that ensure energy production and cellular adaptation (12). In this sense, exercise is one of the most potent stimuli for mitochondrial biogenesis, primarily through the activation of PGC-1α, the master regulator of this process. PGC-1α induces the nuclear respiratory factors NRF1 and NRF2 which stimulate the expression of mitochondrial transcription factor A (TFAM), essential for mitochondrial DNA (mtDNA) replication (13). Exercise also regulates mitochondrial fusion, which can enhance ATP-synthesizing capacity and is mediated by MFN1/2 and OPA1, as well as mitochondrial fission, which contributes to the elimination of dysfunctional mitochondrial segments and is mediated by DRP1, encoded by the Dnm1l gene, along with MFF and FIS1, eliminates mitochondrial dysfunctional segments (14,15). Thus, a coordinated balance among the biogenesis, fusion and fission processes is essential for maintaining the quality of the mitochondrial pool (16). Increased PGC-1α expression, elevated mitochondrial DNA content and altered markers of mitochondrial fusion and fission have been documented in the skeletal muscle of the offspring following maternal exercise (11). In addition, exercise promotes the secretion of myokines by skeletal muscle that can act on distal tissues, including the brain. The PGC-1α–FNDC5– BDNF axis is activated by exercise and influences the secretion of the myokine irisin. Studies suggest that irisin crosses the blood–brain barrier and induces BDNF expression in the hippocampus, leading to enhanced neuronal plasticity (17). In this study, we chose to investigate the cerebellum. This region is involved in postural control, coordination and motor learning, and is highly sensitive to exercise-induced alterations. Cerebellar neurons have high energy demands and consequently produce elevated levels of reactive oxygen species (ROS), making the cerebellum particularly susceptible to biochemical changes arising from exercise-induced increases in energy expenditure. Moderate exercise reduces oxidative damage and enhances mitochondrial function in the cerebellum (18). Previous studies have shown that gestational exercise increases insulin-like growth factor 1 (IGF-1) in the maternal cerebellum (19). Other findings have reported modulation of mitochondrial dynamic proteins, including increased MFN2 and decreased DRP1, as well as increased activity of complexes I, III, and V, in the cerebellum of rats following 12 weeks of exercise (20).

Considering that physical exercise is a well-established health-promoting intervention with recognized impacts on neurodevelopment, we aimed to investigate how maternal exercise during pregnancy influences the expression of genes that encode proteins regulating mitochondrial metabolism and dynamics in the cerebellum of Wistar rat offspring. We hypothesize that mitochondria are sensitive to prenatal interventions, and that maternal exercise induces early-life programming of gene expression related to mitochondrial activity and dynamics in the offspring."

Materials and Methods

Animals and Reagents

A total of 18 adult female (120 d old) and 9 adult male Wistar rats (120 d old) were obtained from the Animal Facility of the Department of Biochemistry of the Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained under controlled environmental conditions (12:12 h light/dark cycle; temperature: 22 ± 1°C). During the experimental period, rats were housed three per cage, and from 1 d before delivery, females were housed individually. Throughout the experiment, all animals had free ac-

cess to a commercial chow containing 20% (w/w) protein and water *ad libitum*. All experimental procedures were approved by the local Animal Ethics Committee (Comissão de Ética no Uso de Animais – CEUA/UFRGS), under protocol number 37628. The experiments were conducted and reported in accordance with the ARRIVE guidelines (ARRIVE 2.0) and in compliance with the national animal rights legislations (Law 11.794/2008, Decree 6.899/2009, and applicable CONCEA guidelines), the Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th Ed., revised 2011), and Directive 2010/63/EU. All efforts were made to minimize animal use and suffering. All reagents were purchased from the following companies: Sigma-Aldrich (St. Louis, MO, USA), *Invitrogen* (Thermo Fisher Scientific, Carlsbad, CA, USA), Promega Corporation (Madison, WI, USA), Bio-Rad Laboratories (Hercules, CA, USA), Cell Signaling Technology (Danvers, MA, USA), Abcam (Cambridge, UK) and Millipore (Burlington, MA, USA).

Experimental Design

Pregnancy. For mating, two females were housed with one male per cage. Pregnancy was confirmed by vaginal plug detection. Inclusion criteria were successful pregnancy and normal litter delivery, and animals were excluded only in cases of unsuccessful pregnancy. Nine females became pregnant (four from the control group and five from the exercised group). Pregnant rats were housed in groups of three per cage until gestational day 20, when they were individually housed for delivery and monitored twice daily (at 9:00 and 16:00) to identify the day of birth. The day of delivery was designated as postnatal day 0 (PND 0). Pups remained with their dams until postnatal day 7 (PND7). On this day, after a 12 h fasting period, body weight was recorded, and the animals were euthanized by rapid decapitation performed by experienced personnel using a sharp guillotine device. No anesthesia was used to avoid interference with molecular outcomes. To minimize stress, animals were euthanized individually in a dedicated room, with cleaning procedures conducted between animals, including equipment sanitation, glove replacement, and environmental cleaning to reduce blood odor. Pups remained with their dams in a separate room until the time of euthanasia. The cerebellum was collected and stored at –80°C for subsequent analysis. To avoid litter effects, one or two male and female offspring from each litter were used for molecular analyses, resulting in $n = 3$–6 animals per group. The offspring were divided into four experimental groups according to maternal condition and sex: sedentary male, sedentary female, exercised male, and exercised female. For the analysis of gestational parameters, all offspring derived from four sedentary litters and five exercised litters were included. The researchers performing the biochemical analyses were blinded to group allocation.

Swimming Exercise Protocol. The maternal exercise protocol had been previously developed (6), based on adaptations of models described in the literature (21,22). The swimming pool (30 cm width × 30 cm length × 90 cm depth) was filled with water at 32 ± 1°C to maintain thermal comfort during the sessions. Female rats were randomly assigned to the sedentary or swimming exercise groups using a simple randomization procedure prior to mating (sedentary, $n = 8$; swimming exercise, $n = 10$). Their body weight was recorded daily before each swimming session. Rats in the swimming group underwent free swimming sessions for 30 min per day (between 2:00 and 7:00 p.m., during the light phase), 5 d per week, for a total of four weeks. The protocol began one week before mating to allow the rats to adapt to the aquatic environment. This ensured that any potential increase in maternal corticosterone levels induced by physical exercise occurred before gestation, and it was maintained throughout the entire gestational period (Figure 1). After each swimming session, the animals were gently removed from the tank, dried with a soft towel, and returned to their home cages. Sedentary group rats were also briefly immersed in water to ensure equal exposure to the aquatic environment, carefully dried, and then returned to their cages. This swimming protocol is classified as moderate intensity.

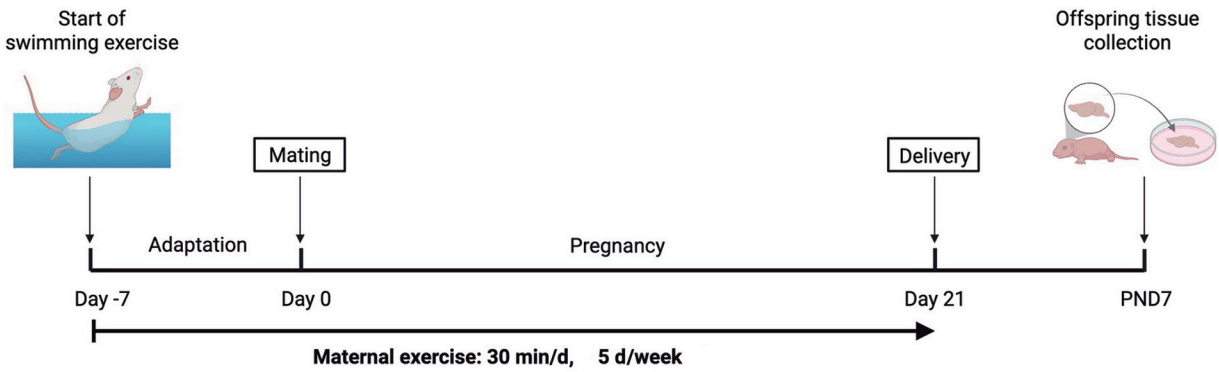

Figure 1. Maternal exercise experimental protocol and offspring's sample collection. *Created in BioRender.*

Table 1. Primer sequences for gene amplification.

Target gene	Forward primer (5‘- 3‘)	Reverse primer (5‘- 3‘)
Ppargc1a	GTGCAGCCAAGACTCTGTATGG	GTCCAGGTCATTCACATCAAGTTC
Tfam	GCCTGTCAGCCTTATCTGTATT	TGCATCTGGGTGTTTAGCTTTA
Nrf1	CATCTCGTACCATCACAGACAG	CCATCAGCCACAGCAGAATA
Nfe2l2	TGGATGAAGAGACCGGAGAA	TCAACGTGGCTGGGAATATC
Cs	CAGAAGGAAGTCGGCAAAGA	CAAACTCTCGCTGACAGGAATA
Mfn1	GGAGGGAACTGATGGAGATAAAG	TGTACCTGGGCTGTCTACTAA
Mfn2	CGTCACCGTCAAGAAGGATAAG	CTGTATTCCTGTGGGTGTCTTC
Opa1	GGAGACCCTACAAGACGAATTT	CGTCCCACTGTTGCTTATCT
Dnm1l	GCTGATCCCGGTCATCAATAA	TGGACCAGCTGCAGAATAAG
Fndc5	AGGACAACGAGCCCAATAAC	CATATCTTGCTTCGGAGGAGAC
Actb	ATTGCTGACAGGATGCAGAA	TAGAGCCACCAATCCACACAG

Ppargc1a: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *Tfam*: Mitochondrial transcription factor A; *Nrf1*: Nuclear respiratory factor 1; *Nfe2l2*: Nuclear factor erythroid 2-like 2; *Cs*: Citrate synthase; *Mfn1*: Mitofusin 1; *Mfn2*: Mitofusin 2; *Opa1*: Optic atrophy protein 1; *Dnm1l*: Dynamin-1-like protein; *Fndc5*: Fibronectin type III domain-containing protein 5; *Actb:* β-actin.

Biochemical Assays

Reverse Transcription Quantitative PCR (RT-qPCR). Total RNA was extracted from cerebellum samples using the Trizol reagent (Invitrogen). RNA purity and concentration were assessed using an i-Quant Touch spectrophotometer (Loccus). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using the GoScript™ Reverse Transcriptase Kit (Promega). The resulting cDNA was diluted 1:200, and 4 μL of the diluted cDNA was used for each quantitative PCR (qPCR) reaction. The qPCR analyses were performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems) and the GoTaq® qPCR Master Mix (Promega). Each 20 μL reaction contained 1X GoTaq® qPCR Master Mix with BRYT Green® Dye, 300 nM of CXR Reference Dye, 150 nM of each primer (forward and reverse), and 4 μL of 1:200 diluted cDNA. The primer sequences used for gene amplification are reported in Table 1. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Dissociation (melting) curves were generated at the end of the amplification cycles to confirm primer specificity. All reactions were carried out in technical duplicates. Gene expression was analyzed for *Ppargc1α, Tfam, Nrf1, Nfe2l2, Cs, Mfn1, Mfn2, Opa1, Dnm1l,* and *Fndc5,* and normalized to *Actb*, which was used as the reference gene**.** Relative gene expression levels were calculated using the $2^{\wedge-\Delta\Delta Ct}$ method (23).

Western Blotting. Cerebellum samples were homogenized in extraction buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 1 mM KCl, 1 mM sodium orthovanadate, 10 mM NaF, 0.5% IGEPAL, and 1 mM DTT). The samples were then centrifuged at 850 g for 10 min at 4°C, and the supernatant, corresponding to the cytoplasmic fraction, was collected. One volume of 2 × Laemmli sample buffer (Bio-Rad Laboratories), containing 5% β-mercaptoethanol, was added to each sample. The samples were then boiled at 100°C for 5 min and stored at –20°C until further use. Protein concentration was determined using a commercial BCA Protein Assay kit (Invitrogen) and was expressed in μg/μL. A total of 40 μg of protein were loaded onto a NuPAGE® 4–12% acrylamide gel immersed in a running buffer and subjected to electrophoresis. The proteins were then transferred to nitrocellulose membranes using the Trans-Blot® Turbo Transfer System (Bio-Rad Laboratories). The membranes were blocked for 2 h at room temperature in M-TBST (Tris-buffered saline with 0.1% Tween-20, pH 7.4, containing 5% nonfat dry milk). After blocking, the membranes were incubated overnight at 4°C with one of the following primary antibodies, diluted in TBST: anti-AMPKα (1:1000, Cell Signaling, #2532), anti-phospho-AMPKα (1:1000, Invitrogen, #PA517831), anti-PGC-1α (1:1000, Invitrogen, #PA572948), anti-TFAM (1:1000, Invitrogen, PA529571), anti-NRF2 (1:1000, Cell Signaling, #12721), anti-DNM1L (1:1000, Invitrogen, #PA116987), anti-MFN1 (1:1000, Abcam, #ab126575), anti-MFN2 (1:1000, Invitrogen, #PA572811), anti-OPA1 (1:1000, Abcam, #ab119685), anti-CS (1:1000, Sigma-Aldrich, #SAB2701077), anti-FNDC5 (1:2000, Invitrogen, PA5116799) and anti-β-actin (1:50000, Sigma-Aldrich, #A3854). The membranes were then washed with TBST and incubated for 2 h at room temperature with either peroxidase-conjugated anti-rabbit IgG (1:1000, Millipore, #AP307P) or anti-mouse IgG (1:2000, Cell Signaling, #7076). Chemiluminescence detection was performed using the ChemiDoc MP Imaging System (Bio-Rad Laboratories) and band intensities were quantified using Image Lab software (Bio-Rad Laboratories). Protein expression levels were expressed as the ratio of the intensity of the protein of interest to β-actin levels on the same membrane.

Statistical Analysis

Data normality was assessed using the Shapiro-Wilk test. Gestational parameters with normal distribution were analyzed using Student’s *t*-test, and non-normally distributed

Table 2. Effect of maternal interventions on gestational parameters.

Parameters	Sedentary	Maternal exercise	*p*
Pre-pregnancy weight (g) - mean ± SEM	282.3 ± 13.0	273.2 ± 6.3	0.5234
Weight gain during pregnancy (%) - median [IQR]	29.9 [16.3–31.9]	19.3 [18.1–28.9]	0.7302
Pregnancy rate (%)	50	50	-
Litter size - mean ± SEM	7.5 ± 1,0	7.4 ± 1.2	0.9521
Sex ratio (proportion of males) - mean ± SEM	0.29 ± 0.11	0.54 ± 0.10	0.1380
Pup weight PND7 (g) - median [IQR]			
Female	18.00 [17.00–19.00]	16.50 [16.00–19.50]*	0.0470
Male	19.00 [18.00–21.75]	16.00 [16.00–17.00]*	0.0227

Data are presented as mean ± SEM or median [IQR]. Maternal measures: $n = 4$ sedentary; $n = 5$ exercised. Offspring measures: $n = 22$ sedentary females; $n = 8$ sedentary males; $n = 16$ exercised females; $n = 21$ exercised males. Offspring were derived from 4 sedentary litters and 5 exercised litters, respectively. Parametric data were analyzed using Student's *t*-test, and non-parametric data using the Mann-Whitney test. Indicates the maternal exercise effect: $^{*}p < 0.05$.

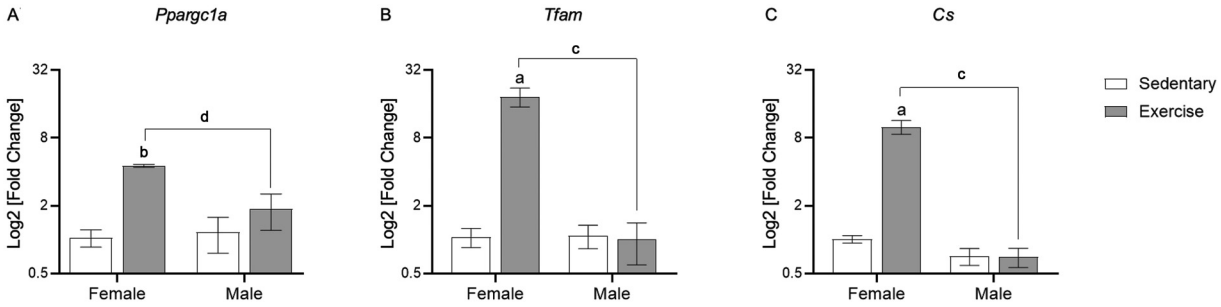

Figure 2. Effect of maternal exercise during gestation on the mRNA expression of A. *Ppargc1α*, B. *Tfam,* and C. *Cs* in the cerebellum of offspring on PND7. Data are presented as mean ± SEM with $n = 3$–4 per group. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test. Indicates the maternal exercise effect: $^{a}p < 0.001$; $^{b}p < 0.01$. Represents the sex effect: $^{c}p < 0.001$; $^{d}p < 0.01$.

parameters using the Mann-Whitney test. We analyzed qPCR and Western blotting data using two-way ANOVA followed by Tukey's post hoc test (factors: exercise and sex). Results are expressed as mean ± SEM or median [IQR], with statistical significance set at $p \leq 0.05$. All analyses were performed using GraphPad Prism 8.0 software.

Results

Maternal Exercise Had No Impact on Gestational Parameters, while a Slight Decrease in offspring Body Weight was Observed

Maternal exercise did not alter weight gain during pregnancy, pregnancy rate, litter size or sex ratio as shown in Table 2. Offspring weight at PND7 was reduced in both male (–15.6%) and female (–8.33%) pups from mothers in the exercise group (Table 2).

Maternal Swimming Exercise Enhances Mitochondrial Biogenesis Regulators in the Cerebellum of Female Offspring

To investigate the influence of maternal exercise on mitochondrial biogenesis in the offspring's cerebellum, we first analyzed the mRNA expression of key genes that regulate this pathway (Figure 2). The results showed that the expression of *Ppargc1a, Tfam*, and *Cs* was significantly influenced by maternal exercise and sex, indicating a sex-dependent effect. For *Ppargc1a*, which encodes PGC-1α, maternal exercise increased transcriptional levels specifically in the cerebellum of female offspring (exercise effect: $F[1,11] = 22.59$, $p = 0.0006$; sex effect: $F[1,11] = 8.14$, $p = 0.0157$; interaction: $F[1,11] = 9.82$, $p = 0.0095$) (Figure 2A), while no significant changes were observed in males. Similar effects were observed for *Tfam* (exercise effect: $F[1,11] = 39.16$, $p < 0.001$; sex effect: $F[1,11] = 39.61$, $p < 0.001$; interaction: $F[1,11] = 39.91$, $p < 0.001$) (Figure 2B) and *Cs* (exercise effect: $F[1,11] = 34.08$, $p < 0.001$; sex effect: $F[1,11] = 38.91$, $p < 0.001$; interaction: $F[1,11] = 34.27$, $p < 0.001$) (Figure 2C). These findings indicate a significant interaction between maternal exercise and sex.

Next, we investigated the mRNA expression of the transcription factor genes *Nrf1* and *Nfe2l2* (Figure 3), which regulate mitochondrial protein expression necessary for mtDNA replication. Maternal exercise significantly increased the expression of both *Nrf1* (exercise effect: $F[1,12] = 12.30$, $p = 0.0043$; sex effect: $F[1,12] = 35.64$, $p < 0.001$; interaction: $F[1,12] = 39.81$, $p < 0.001$) (Figure 3A), and *Nfe2l2* (exercise effect: $F[1,11] = 11.72$, $p = 0.0057$; sex effect: $F[1,11] = 9.88$, $p = 0.0094$; interaction: $F[1,11] = 17.60$, $p = 0.0015$) (Figure 3B) in the cerebellum of female offspring, whereas no significant

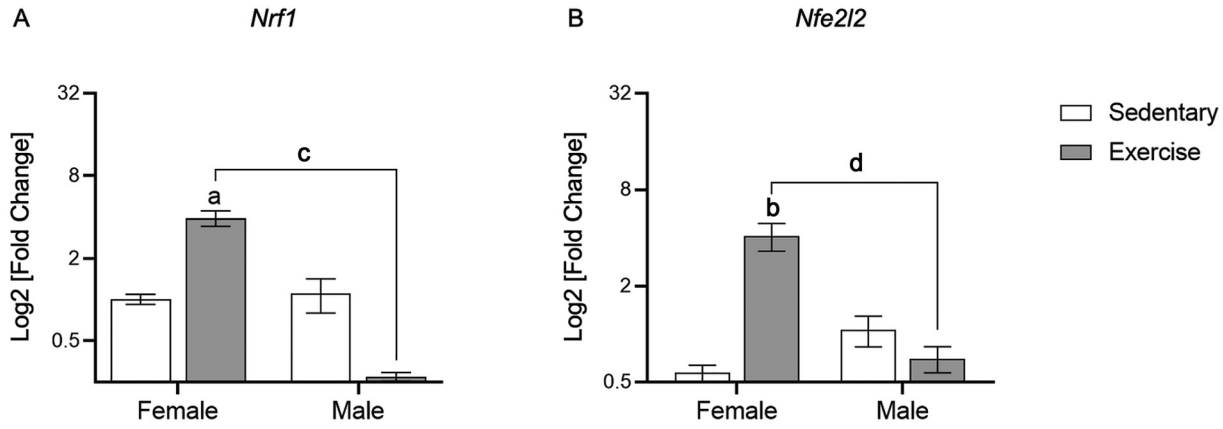

Figure 3. Effect of maternal exercise during gestation on the mRNA expression of A. *Nrf1* and B. *Nfe2l2* in the cerebellum of offspring on PND7. Data are presented as mean ± SEM with $n = 3$–4 per group. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test. Indicates the maternal exercise effect: $^{a}p < 0.001$; $^{b}p < 0.01$. Represents the sex effect: $^{c}p < 0.001$; $^{d}p < 0.01$.

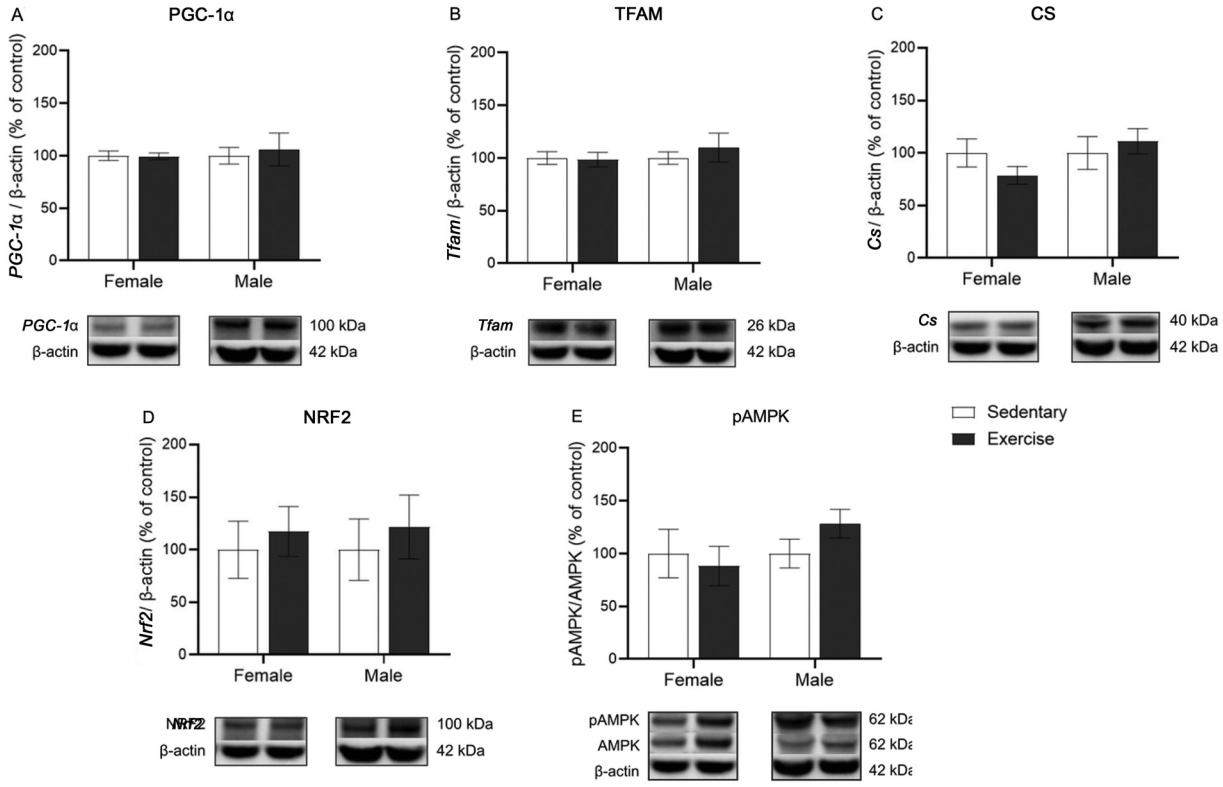

Figure 4. Effect of maternal exercise during gestation on the immunocontent of A. PGC-1α, B. TFAM, C. CS, D. NRF2, and E. pAMPK in the cerebellum of offspring on PND7. Data are presented as mean ± SEM with $n = 4$–6 per group. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test.

changes were observed in males, supporting a significant exercise × sex interaction.

To corroborate these transcriptional findings, we used Western blot to assess the protein content of PGC-1α, TFAM, CS, NRF2, and pAMPK in the offspring's cerebellum, given its key role in activating PGC-1α and stimulating mitochondrial biogenesis (Figures 4A–4E). No significant differences in protein levels were observed in response to maternal exercise. Taken together, these findings demonstrate that maternal exercise upregulates key mitochondrial biogenesis genes in a sex-dependent manner, with transcriptional increases occurring only in female offspring.

Maternal Swimming Exercise Modulates Mitochondrial Dynamics Genes in the Cerebellum of Female Offspring

To further investigate whether maternal exercise during gestation induces alterations in mitochondrial dynamics in

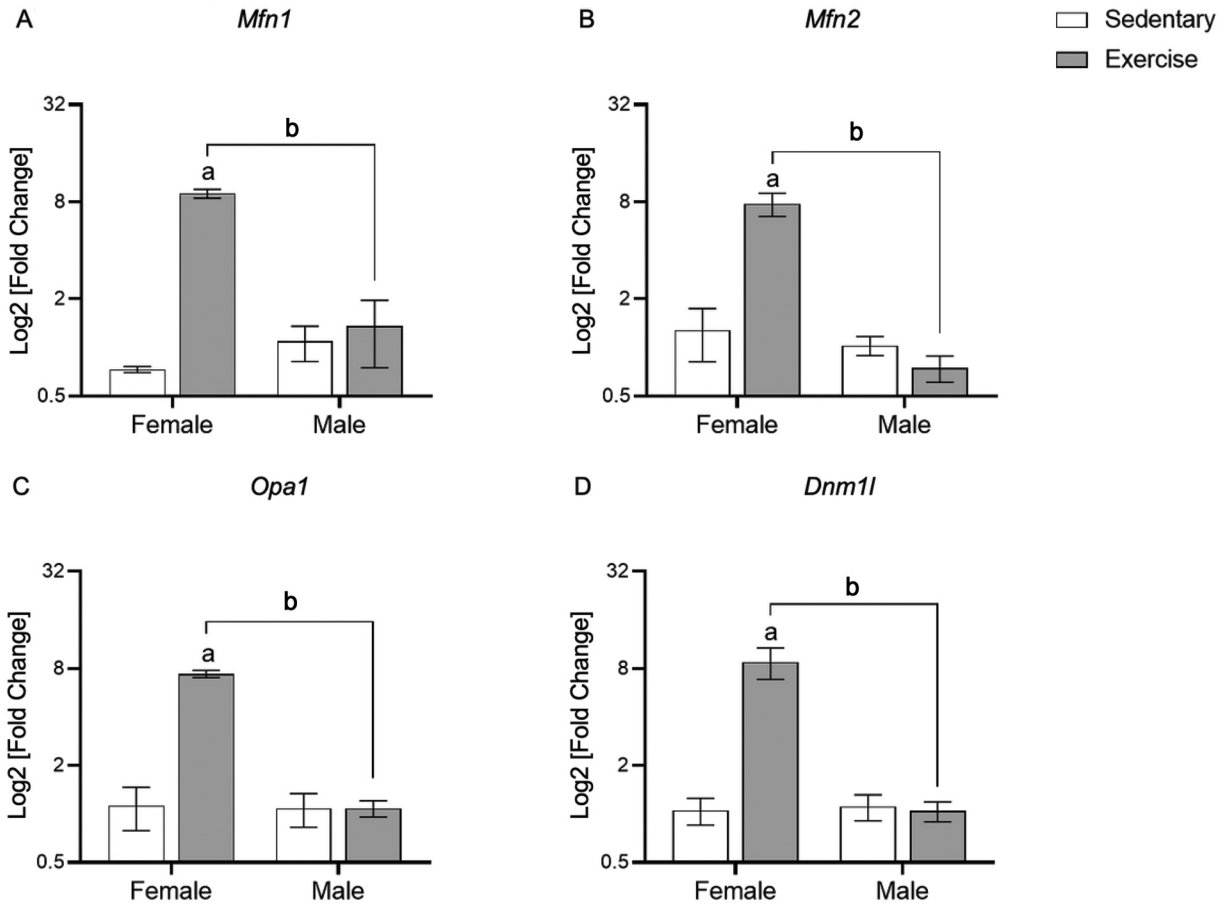

Figure 5. Effect of maternal exercise during gestation on the mRNA expression of A. *Mfn1*, B. *Mfn2*, C. *Opa1,* and D. *Dnm1l* in the cerebellum of offspring on PND7. Data are presented as mean ± SEM with $n = 3$–4 per group. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test. Indicates the maternal exercise effect: $^{a}p < 0.001$. Represents the sex effect: $^{b}p < 0.001$.

the offspring's cerebellum, we evaluated the expression of genes involved in fusion, including *Mfn1, Mfn2, Opa1* and fission, including *Dnm1l* (Figure 5). Maternal exercise significantly increased *Mfn1* expression (exercise effect: $F[1,11] = 80.57$, $p < 0.001$; sex effect: $F[1,11] = 58.73$, $p < 0.001$; interaction: $F[1,11] = 70.81$, $p < 0.001$) (Figure 5A), *Mfn2* (exercise effect: $F[1,11] = 30.47$, $p < 0.001$; sex effect: $F[1,11] = 41.89$, $p < 0.001$; interaction: $F[1,11] = 36.28$, $p < 0.001$) (Figure 5B), *Opa1* (exercise effect: $F[1,12] = 118.4$, $p < 0.001$; sex effect: $F[1,12] = 121.7$, $p < 0.001$; interaction: $F[1,12] = 118.2$, $p < 0.001$) (Figure 5C), and *Dnm1l* (exercise effect: $F[1,12] = 15.44$, $p = 0.0020$; sex effect: $F[1,12] = 15.52$, $p = 0.0020$; interaction: $F[1,12] = 16.00$, $p = 0.0018$) (Figure 5D), in the cerebellum of female offspring, with no significant changes observed in males, indicating a significant exercise × sex interaction.

In contrast to the transcriptional modulation observed, maternal exercise did not alter the protein content of MFN1, MFN2, OPA1, or DRP1 (Figures 6A–6D) in the offspring's cerebellum by Western blot. Overall, these findings reveal that maternal exercise upregulates mitochondrial dynamics regulators in the offspring's cerebellum, and female offspring show a markedly enhanced mitochondrial adaptive response at the transcriptional level.

Maternal Swimming Exercise Upregulates Fndc5/Irisin Gene Expression in the Cerebellum of Female Offspring

Based on these observations, we expanded our analysis to include genes that encode exercise-responsive proteins with established roles in brain function, such as *Fndc5* (Figure 7), the precursor of irisin. Maternal exercise significantly increased *Fndc5* mRNA levels in the cerebellum of female offspring (exercise effect: $F[1,11] = 8.705$, $p = 0.0132$; sex effect: $F[1,11] = 6.718$, $p = 0.0251$; interaction $F[1,11] = 9.219$, $p = 0.0113$), while no significant changes were observed in males. Although FNDC5 protein content by Western blot remained unchanged (Figure 8), these findings suggest that maternal exercise enhances the expression of the exercise-responsive gene *Fndc5*, potentially contributing to cerebellar neuroplasticity in a sex-dependent manner.

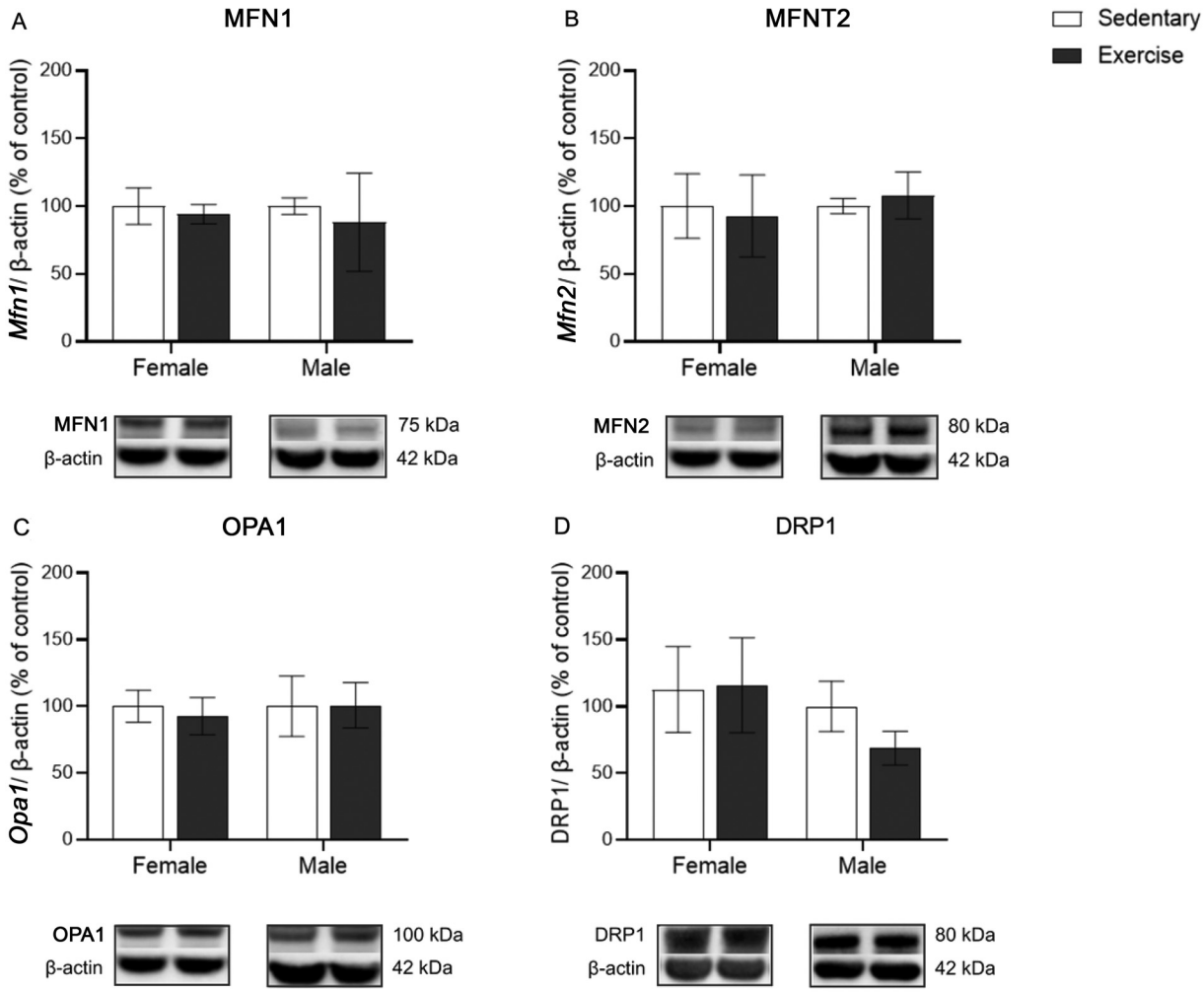

Figure 6. Effect of maternal exercise during gestation on the immunocontent of A. MFN1, B. MFN2, C. OPA1, and D. DRP1 in the cerebellum of offspring on PND7. Data are presented as mean ± SEM with $n = 4$–6 per group. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test.

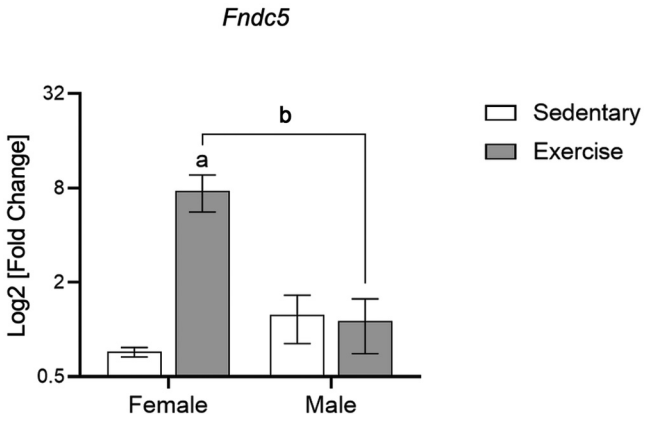

Figure 7. Effect of maternal exercise during gestation on the mRNA expression of *Fndc5* in the cerebellum of offspring on PND7. Data are presented as mean ± SEM with $n = 3$–4 per group. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test. Indicates the maternal exercise effect: $^{a}p < 0.01$. Represents the sex effect: $^{b}p < 0.01$.

Discussion

This study investigated some mitochondrial molecular mechanisms associated with the effects of maternal exercise during pregnancy on cerebellar development in the offspring. We demonstrated that maternal swimming exercise did not alter gestational parameters. Although a modest reduction in offspring body weight was observed at PND7 in the offspring of exercised dams, this finding is consistent with previous work from our research group using the same gestational exercise model (6). Importantly, this transient reduction in body weight was not accompanied by alterations in other reproductive parameters, such as the number of implantations or litter size. This suggests that maternal exercise did not adversely affect overall reproductive outcomes (6).

Given that mitochondrial function is particularly sensitive to physical exercise, we hypothesized that the maternal stimulus would modulate genes involved in mito-

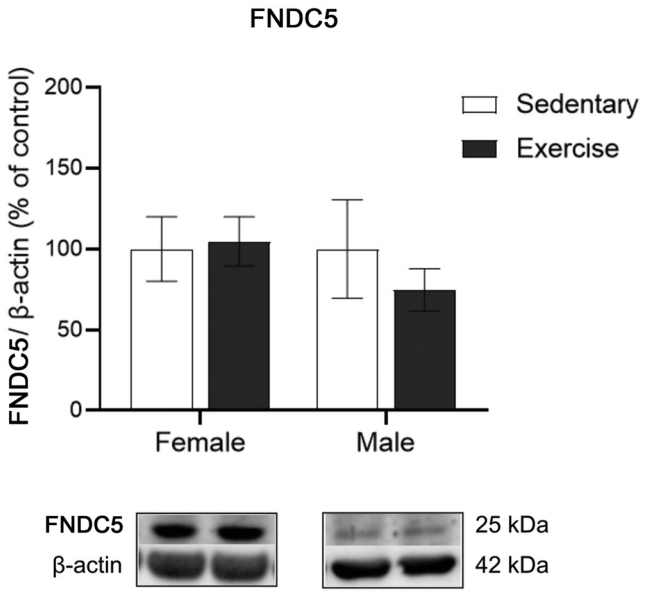

Figure 8. Effect of maternal exercise during gestation on the immunocontent of FNDC5 in the cerebellum of offspring on PND7. Data are presented as mean ± SEM with $n = 4$–6 per group. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test.

chondrial pathways during the offspring's early life. We observed that all of the genes we examined that are related to mitochondrial biogenesis (*Ppargc1a, Tfam, Cs, Nrf1,* and *Nfe2l2*) and dynamics (*Mfn1, Mfn2, Opa1,* and *Dnm1l*), as well as *Fndc5*, were upregulated in the cerebellum of female offspring, indicating early metabolic programming in a sex-dependent manner. However, these transcriptional changes did not result in alterations in protein content, indicating possible post-transcriptional regulation or a temporal mismatch between transcription and translation. Our findings corroborate previous rodent studies showing that maternal exercise modulates mitochondrial gene expression in offspring across different tissues, including increased expression of *Nrf1, Nfe2l2,* and *Mfn2* in the heart (24); *Ppargc1a, Tfam, Mfn1, Mfn2* and *Dnm1l* in the liver (25,26); and *Ppargc1a, Tfam* and *Nrf1* in skeletal muscle (27). In contrast, some of these studies also reported increased protein levels of PGC-1α and TFAM, whereas we detected the upregulation only at the mRNA level. Importantly, evidence regarding the effects of maternal exercise on mitochondrial pathways in specific brain regions remains scarce. This gap in the literature limits direct comparisons with our findings and highlights the novelty of our results. Rodents are born with a markedly immature central nervous system, and PND7 may constitute a sensitive period of neural plasticity during which key neurodevelopmental processes are established in the offspring. During the early postnatal period, brain structures such as the cerebellum undergo substantial secondary neurogenic processes that extends until PND10, reflecting ongoing structural maturation (28). Moreover, the first three postnatal weeks encompass dynamic epigenomic changes in the developing brain, including shifts in DNA methylation patterns and related regulatory mechanisms that are thought to contribute to neural circuit formation and maturation (29). We hypothesize that post-transcriptional mechanisms, such as the temporal dissociation between transcription and translation (30), may explain our findings. Moreover, increased mRNA levels without changes in protein abundance may reflect a preparatory state for future translation, a mechanism known as "on demand translation", which allows for rapid protein synthesis in response to specific signals (31). Together, these observations support the notion that PND7 may represent a preparatory developmental state. Previous studies examining the brains of neonates exposed to maternal exercise demonstrated that the effects on gene expression appear to depend on both the duration of the exercise protocol and the offspring's age. Increases in *Ppargc1a, Nrf1,* and *Tfam* were detected in the offspring of mothers who exercised for 40 min/d throughout gestation, whereas no transcriptional changes were observed with the 20 or 30 min sessions (32). Age-related differences were also described in the hippocampus of male offspring exposed to maternal exercise during gestation and lactation, with increased *Ppargc1a* expression at eight weeks but not at 28 weeks (33). Taken together, these studies demonstrate that maternal exercise affects mitochondrial gene expression in the offspring across multiple tissues. Furthermore, the type and duration of exercise, as well as offspring age, appear to influence mitochondrial regulation. Notably, most studies have reported effects in both sexes, which contrasts with our findings. Here, we demonstrate that maternal swimming exercise modulates mitochondrial gene expression in the offspring cerebellum in a sex-dependent manner, with female offspring exhibiting greater sensitivity to this regulation. However, a limitation of our study is the lack of assessments at later developmental stages; thus, we cannot exclude the possibility that male offspring may exhibit mitochondrial adaptations later in life. Another possibility is that maternal exercise-induced adaptations in males occur in alternative metabolic pathways. Studies using maternal swimming during gestation have already reported sex-dependent effects on offspring cognition, with males exhibiting better spatial memory compared to females in adulthood (34). Our group has also demonstrated that maternal swimming exercise improves spatial and working memory in male offspring in adulthood (7). These findings suggest sex-specific modulation of cognitive function induced by maternal exercise.

In the present study, we observed increased *Fndc5* expression in the offspring cerebellum following maternal exercise. This finding suggests that maternal exercise influences molecular pathways associated with neuroplasticity, given that the FNDC5 protein undergoes cleavage to produce irisin, a myokine linked to neural plasticity via the FNDC5-Irisin-BDNF axis. *Fndc5* expression is regulated

by PGC-1α, and studies have shown increased *Fndc5* in the hippocampus of mice following 30 d of voluntary exercise, as well as elevated BDNF levels after peripheral FNDC5 administration (35). These findings demonstrate that FNDC5 positively regulates BDNF and depends on PGC-1α for activation, which reinforces the role of exercise in brain plasticity. During pregnancy, circulating irisin levels increase; however, conditions such as preeclampsia and gestational diabetes reduce its concentration (36). Maternal exercise elevates circulating irisin and improves metabolic parameters (37). Despite this, little is known about the impact of maternal irisin on offspring. The literature primarily focuses on BDNF, with reports of increased hippocampal BDNF in adolescent male offspring (PND 34) (38), in adulthood (PND 60) (39) and at birth (PND 0), associated with improved spatial learning (40). In our study, we observed increased *Fndc5* expression in the offspring cerebellum. This finding is consistent with immunohistochemical evidence that indicates Purkinje cells are immunoreactive for FNDC5 and irisin (41), reinforcing the relevance of this pathway in the cerebellum. Therefore, our results align with the existing literature, indicating that maternal exercise acts as a neurometabolic programming mechanism that influences *Fndc5* expression in the cerebellum of female offspring. Modulation of the FNDC5-Irisin-BDNF axis triggered by exercise during pregnancy may support neuroplasticity processes in the offspring. Thus, the biochemical adaptations induced by maternal exercise observed in our study suggest adaptive neuroplasticity in the offspring cerebellum, thereby potentially promoting greater neuroprotection and motor function.

Conclusion

In summary, maternal swimming exercise during pregnancy modulates the expression of genes related to mitochondrial biogenesis, dynamics, and neuroplasticity in the offspring cerebellum at early life. These effects were sex-specific: increased gene expression was observed in female offspring, while no significant changes were detected in males, indicating a sex-dependent response to maternal intervention. Notably, these transcriptional alterations were not accompanied by corresponding changes in protein levels, suggesting the involvement of post-transcriptional regulatory mechanisms. Overall, our findings support maternal exercise as a potential non-pharmacological strategy to influence early-life neurodevelopment. However, further studies are needed to determine whether these effects persist into later stages of life.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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