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Research paper

Pregnancy swimming prevents early brain mitochondrial dysfunction and causes sex-related long-term neuroprotection following neonatal hypoxia-ischemia in rats

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ABSTRACT

Neonatal hypoxia-ischemia (HI) is a major cause of cognitive impairments in infants. Antenatal strategies improving the intrauterine environment can have high impact decreasing pregnancy-derived intercurrences. Physical exercise alters the mother-fetus unity and has been shown to prevent the energetic challenge imposed by HI. This study aimed to reveal neuroprotective mechanisms afforded by pregnancy swimming on early metabolic failure and late cognitive damage, considering animals' sex as a variable. Pregnant Wistar rats were submitted to daily swimming exercise (20′ in a tank filled with 32 °C water) during pregnancy. Neonatal HI was performed in male and female pups at postnatal day 7. Electron chain transport, mitochondrial mass and function and ROS formation were assessed in the right brain hemisphere 24 h after HI. From PND45, reference and working spatial memory were tested in the Morris water maze. MicroPET-FDG images were acquired 24 h after injury (PND8) and at PND60, following behavioral analysis. HI induced early energetic failure, decreased enzymatic activity in electron transport chain, increased production of ROS in cortex and hippocampus as well as caused brain glucose metabolism dysfunction and late cognitive impairments. Maternal swimming was able to prevent mitochondrial dysfunction and to improve spatial memory. The intergenerational effects of swimming were sex-specific, since male rats were benefited most. In conclusion, maternal swimming was able to affect the mitochondrial response to HI in the offspring's brains, preserving its function and preventing cognitive damage in a sex-dependent manner, adding relevant information on maternal exercise neuroprotection and highlighting the importance of mitochondria as a therapeutic target for HI neuropathology.

1. Introduction

Neonatal hypoxia-ischemia (HI) has an occurrence ranging from 1.5–2‰ in developed countries and can reach 26‰ in developing areas, with a lifelong impact on brain function and behavior (Douglas-Escobar and Weiss, 2015; Parikh and Juul, 2018). HI occurs due to decreased supply of oxygen and glucose to the brain and is associated with pathologies such as cerebral palsy, autism spectrum disorders, ADHD, cognitive and intellectual deficits (du Plessis and Volpe, 2002; Lapchak and Zhang, 2018). Despite advances in the understanding of HI mechanisms, until now, hypothermia is the standard treatment for its sequelae, however its benefits are limited to a small fraction of patients (Fan et al., 2010; Lee et al., 2015). The Levine-Rice method is a useful tool to evaluate HI injury mechanisms, outcomes and neuroprotective strategies (Volpe, 2008; Gunn and Thoresen, 2019). In this model, rodents are exposed to ischemia due to the permanent common carotid artery occlusion followed by a hypoxic episode. Despite its high reproducibility, injury degree is closely related to parameters such as animal's age, sex, FiO_2 and hypoxia timing exposure (for review, see Charriaut-Marlangue et al., 2017; Netto et al., 2018).

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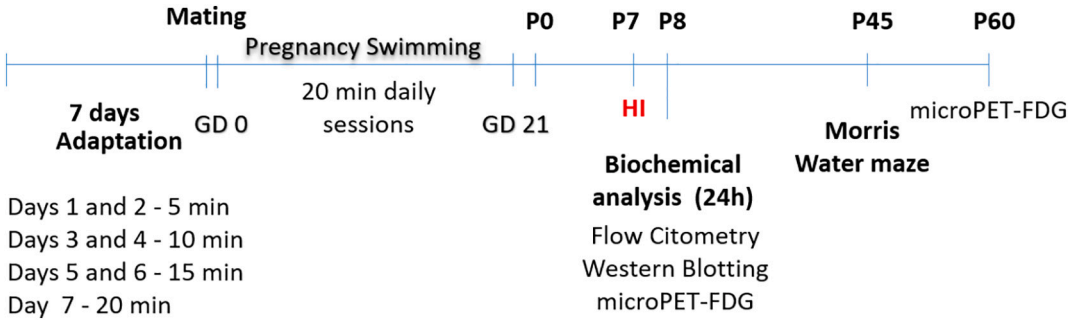

Fig. 1. - Experimental Timeline.

Public policies stimulating healthy pregnancies can have a great impact on offspring's health (Barakat, 2020). Exercise during this period prevents excessive weight gain (Ruiz et al., 2013), gestational diabetes (Barakat et al., 2015), high blood pressure (Genest et al., 2012) metabolic disorders in the offspring (AN et al., 2015), and reduces the risk of premature birth (Santana Muniz et al., 2014). Maternal exercise can elicit long-lasting and positive effects on the offspring's brain during the critical period of brain development (Robinson and Bucci, 2012). However, the link between the effects of maternal exercise on offspring's brain remains poorly understood.

Interventions in the maternal environment could be an optimal strategy for preventing neonatal damage related to HI (Durán-Carabali et al., 2018; Hassell et al., 2015; Netto et al., 2018). Physical exercise can mitigate neurological dysfunction inhibiting apoptosis, decreasing neuroinflammation and oxidative stress (primary and secondary mechanisms of HI injury) as well as stimulating neurorestoration in rodents (Archer et al., 2012; Piao et al., 2013). In this sense, mitochondria plays a major role in numerous cellular processes, particularly in aerobic energy production (Hadj-Moussa et al., 2018). Such organelles are important for brain functioning due to the high levels of energy consumption and its inability to store large amounts of energy reserves in the form of glycogen. In the brain, mitochondria are also crucial for the processes of neuroplasticity, including neural differentiation, neurite outgrowth, neurotransmitter release and dendritic remodeling (Mattson, 2007). In pathologies involving energetic failure such as HI, mitochondria can generate reactive oxygen species (ROS) that may have toxic effects in cells (Allen et al., 2018). Since they are involved in several cell processes related to HI, such as apoptosis and calcium homeostasis (Xavier et al., 2016), mitochondrial dysfunction could be a crucial factor in the development and progress of HI.

Animal studies shows that maternal exercise could improve offspring metabolic activity and have an impact on offspring mitochondrial phenotype in several tissues such as skeletal muscle (Liu et al., 2018) and liver (Cunningham et al., 2018). Indeed, there is evidence showing the beneficial effects of maternal exercise on the central nervous system mitochondrial function in offspring, particularly in the hippocampus (McGreevy et al., 2019; Park et al., 2013). Positron emission tomography (PET) has been shown to be predictive of brain metabolic dysfunction caused by HI in clinical (Shi et al., 2012; Thorp et al., 1988); and pre-clinical settings (Odorcyk et al., 2020). This technique can be a powerful tool assessing the benefits of therapeutic strategies since it allows longitudinal studies, improving the validity of the experimental models as well as the search for new therapies.

Also, the beneficial effects of maternal physical exercise could be sex-specific (Titterness et al., 2011). In experimental conditions, males and females present differentiated sensitivity of brain regions to HI injury and strong experimental evidence suggests that there are differences between the sexes in cell death pathways that may favor females to the detriment of males in several disease models (McCullough et al., 2005; Netto et al., 2017; Simpson and Kelly, 2012; Villapol et al., 2017). Regarding neonatal hypoxia-ischemia, we previouly reported that after HI at postanal day 7, sex-related differences in mitochondrial function, in which females presented an overall mitochondrial activity higher than females, were observed (Weis et al., 2012). These observations are also important when designing therapeutical strategies, in which there is growing evidence of sexually dimorphic effects of treatments following HI (Tsuji et al., 2010). Interestingly, sex differences occurring in response to maternal exercise in offspring's brain after HI episode have never been examined. Our main objectives this study are 1) to test the hypothesis that maternal exercise training induces transgenerational modifications in mitochondrial function in the offsprings' brains assessing electron chain transport, ROS production, mass and mitochondrial potential; 2) to test if the decrease in the glucose consumption induced by HI is prevented by maternal swimming using 18F-FDG microPET in the early and late phases after injury; 3) to observe if the early neuroprotection afforded by maternal swimming was responsible for the late cognitive performance improvement at the adult age and 4) to test whether sexual dimorphism has a role in maternal exercise neuroprotection in the tissue response to HI injury.

2. Experimental procedures

2.1. Animal protocol

Wistar rat dams and their pups (8–12 per litter) were used. Animals were maintained at constant temperature (22 ± 2 °C) with a 12 h light/dark cycle (lights on at 07:00 am) with free access to water and standard rat chow. All procedures were in accordance to local ethical committee guidelines that follow the guidelines from the National Institute of Health (Guide for the Care and Use of Laboratory Animals and the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals) and were approved by the Institutional Ethical Committee in Animal Use - Protocol number 35785. No efforts were spared to minimize animal suffering as well as to reduce the number of animals used in the experiments. After birth, at postnatal day 7 (PND7) rat pups (weighing 14–16 g) were used for HI induction. The cages were cleaned once a week by the Universidade Federal do Rio Grande do Sul staff or by the researchers and no additional physical enrichment was provided to the standard cages. During pregnancy, the females were kept in pairs in the cages until gestational day 19 in order to avoid social isolation stress. After weaning, pups were 3 to 5 animals per cage until the end of experiments. A schematic view of the experimental design is shown in Fig. 1.

2.2. Swimming exercise protocol

Before matings, adult female rats (around 90-old days age) were randomly distributed into 2 groups, namely swimming (SW, $n = 12$) and sedentary (SE, $n = 15$). SW animals had 7 sessions of adaptation to training with increased time exposure to the tank, as previously described (Sanches et al., 2020). Pregnancy was confirmed by the presence of spermatozoids in the vaginal smear; this was considered as

gestational day (GD0). The swimming apparatus consisted of a circular acrylic water tank (110 cm × 50 cm) filled with water at 32 ± 2 °C and the exercise protocol was conducted as previously described (Lee et al., 2006; Sanches et al., 2018). After a period of acclimation to the tank (see Fig. 1), the exercise protocol consisted in 20 min daily sessions of forced swimming from day 0 to 21 (last training session). Animals were gently placed and removed from the apparatus and dried after each exercise session. SE animals were also handled and their cages were moved to the same training room in order so as to endure the daily routine stress of the r SW animals.

2.3. Neonatal hypoxia-ischemia (HI) model

At PND7, newborn rats (males and females) were subjected to hypoxia-ischemia routinely as previously reported (Sanches et al., 2015). Briefly, under 3% anesthesia, the right common carotid artery was exposed by incising the midline of the ventral cervical skin. Subsequently, the right carotid artery was occluded with silk thread (4.0) and the incision was sutured. Afterwards, pups were returned to their mothers for a 2 h recovery phase then placed in a hypoxic chamber with Fi O2 8% balanced in nitrogen for 60 min to induce HI injury. Temperature and humidity were maintained at 37 °C and 50–80% respectively throughout the experiment. After HI injury, pups were returned to their mothers. Animals in the sham group were subjected to an identical dissection with neither carotid ligation nor ischemia. For biochemical analyses, twenty-four hours post-HI insult, rats were euthanized, and their right cortex and hippocampi were collected for assessment.

2.4. Animals and group distribution

After HI procedure, animals were randomly assigned to eight groups, as follows: sedentary sham male (SESH ♂), sedentary sham female (SESH ♀), sedentary HI male (SEHI ♂) and sedentary HI female (SEHI ♀); swimming sham male (SWSH ♂), swimming sham female (SWSH ♀), swimming HI male (SWHI ♂) and swimming HI female (SWHI ♀). The specific number of animals used for each technique is described in each figure or table legend.

2.5. Biochemical analysis

2.5.1. Electron transport chain assessment

Right cortex and hippocampus were homogenized at 1:20 (*w/v*) in ice-cold SETH buffer (pH 7.4, 250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, and 50 UI/mL heparin). Homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C. Supernatants were stored at −70 °C until respiratory chain enzyme activity assessment. *Succinate DCIP-oxidoreductase (complex II) and Succinate dehydrogenase (SDH) activities* were determined as described by Fischer et al. (1985). Mitochondrial membranes were broken by thawing and refreezing of supernatants three times previously to the test in order to obtain mitochondrial enzymes. Briefly, the supernatant was pre-incubated using 16 mM succinate, 40 mM potassium phosphate, and 8 μM DCIP, pH 7.4 solution for 20 min at 30 °C. After, 7 μM rotenone, 4 mM sodium azide, and 40 μM DCIP was added to the reactional medium and the activity of Complex II was measured up to 5 min at 600 nm, with 700 nm as reference wavelength (ε = 19.1 mM cm − 1). Quickly, one mM phenazinemethosulfate was added to the reaction medium and it was measured up to 5 min later for SDH activity assay at the same wavelength. The results were expressed as nanomol/min/mg of protein (Ferreira et al., 2018). *Cytochrome c oxidase (Complex IV) activity* was determined according to Rustin et al. (1994). Briefly, four μgs of right hippocampus protein homogenate were incubated in 10 mM potassium phosphate, pH 7.4, 0.6 mM n-dodecyl-β-d-maltoside and 7.0 μg of reduced cytochrome *c*. The activity was measured up to 5 min after reaction at 550 nm with 580 nm as reference wavelength (ε = 19.1 mM cm − 1) at 25 °C for 10 min. Results were expressed as nanomol/min/mg of protein.

2.5.2. Flow cytometry assessment of oxidative stress (DCF formation) and mitochondrial damage (potential and mass)

Right cortex and hippocampi were quickly harvested, minced with a scalpel blade and then kept in Ca^{2+}-free, Mg^{2+}-free ice-cold Hanks' balanced salt solution (HBSS). Samples went through careful mechanical dissociation with a glass Pasteur pipette (150 mm) and were filtered with a 70 μm cell strainer. After centrifugation (10 min at 4 °C at 500 xg), the supernatant was removed and the pellet was re-suspended with HBSS (Odorcyk et al., 2017). Intracellular ROS formation was detected using fluorochrome 20,70-dichlorofluorescein diacetate (DCFH-DA). This probe is cell-permeable and is hydrolyzed intracellularly to the DCFH carboxylate anion that is retained in the cell. Two-electron oxidation of DCFH results in the formation of a fluorescent product, dichlorofluorescein (DCF), which can be analyzed by DCFH-DA labeling flow cytometry. Cell suspensions were incubated with DCFH-DA fluorochrome (4 μl/100 ml) in HBSS for 30 min at 37 °C and were later washed twice in buffer before loading into the flow cytometer (Arteaga et al., 2015). The emission of fluorochromes was recorded through the band-pass fluorescence filter green (FL-1; 488 nm wavelength). Fluorescence emissions were collected using logarithmic amplification. As in section 2.5.1.2.1, data from 100.000 events (intact cells) were acquired, recording its fluorescence intensity. All acquisitions and analysis were performed using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) and Flow Jo software (Weis et al., 2012). For the assessment of mitochondrial function, cell suspension was incubated with 200 nM fluorochromes MitoTracker Red (MTR or Chloromethyl-X-rosamine) and MitoTracker Green (MTG). They are used to stain mitochondria of live cells depending, respectively, on the oxidative activity (mitochondrial membrane potential) and organelle lipid content (mitochondrial mass). Negative controls (containing unstained cells, cells stained with MTG only and cells stained with MTR only) were included to set up the equipment and determine the negative dot plot region. The emission of fluorochromes was recorded through specific band-pass fluorescence filters: red (FL-3; 670 nm wavelength) or green (FL-1; 488 nm wavelength). Fluorescence emissions were collected using logarithmic amplification. In brief, data from 100.000 events (intact cells) were acquired and the number of cells in each gate was determined, as well as its fluorescence intensity. All acquisitions and analysis were performed using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) and Flow Jo software (Weis et al., 2012).

2.6. ^{18}F-FDG microPET scan

Micro positron emission tomography (microPET) scans were performed at the Preclinical Research Center of the Brain Institute of Rio Grande do Sul (BraIns). Rats were submitted to image examination at PND8 and PND60, 53 days post-HI induction, as previously described (Nunes Azevedo et al., 2020; Zanirati et al., 2018). Animals were anesthetized individually using a mixture of isoflurane and oxygen (3–4% induction and 2–3% maintenance), 250 μCi (PNPD8) or 1 mCi (PNPD60) of $^{[18F]}$ fluorodeoxyglucose (18F-FDG) was administered intraperitoneally. Animals were returned to the home cage for a 40-min period of conscious tracer uptake and were placed on a heating plate to maintain body temperature at 36 ± 1 °C. After the uptake period, the rats were placed in a headfirst prone position on the heated bed of the equipment (Triumph microPET, LabPET-4, TriFoil Imaging, Northridge, CA, USA). The static acquisition was performed under inhalational anesthesia for 10 min with the field of view (FOV: 3.75 cm) centered on the rat's head. Image reconstruction and data analysis was performed in whole brain as well as in 24 brain regions bilaterally (auditory, cingulate, entorhinal, frontal, insular, medial prefrontal (MPF), motor, orbitofrontal, parietal, retrosplenial, somatosensory, and visual cortices, acb core shell, amygdala, striatum, hippocampus, olfactory apparatus, hypothalamus, thalamus, superior colliculus, midbrain, ventral tegmental area (VTA), inferior colliculus, and cerebellum). All images were reconstructed using a 3-dimensional maximum likelihood expectation

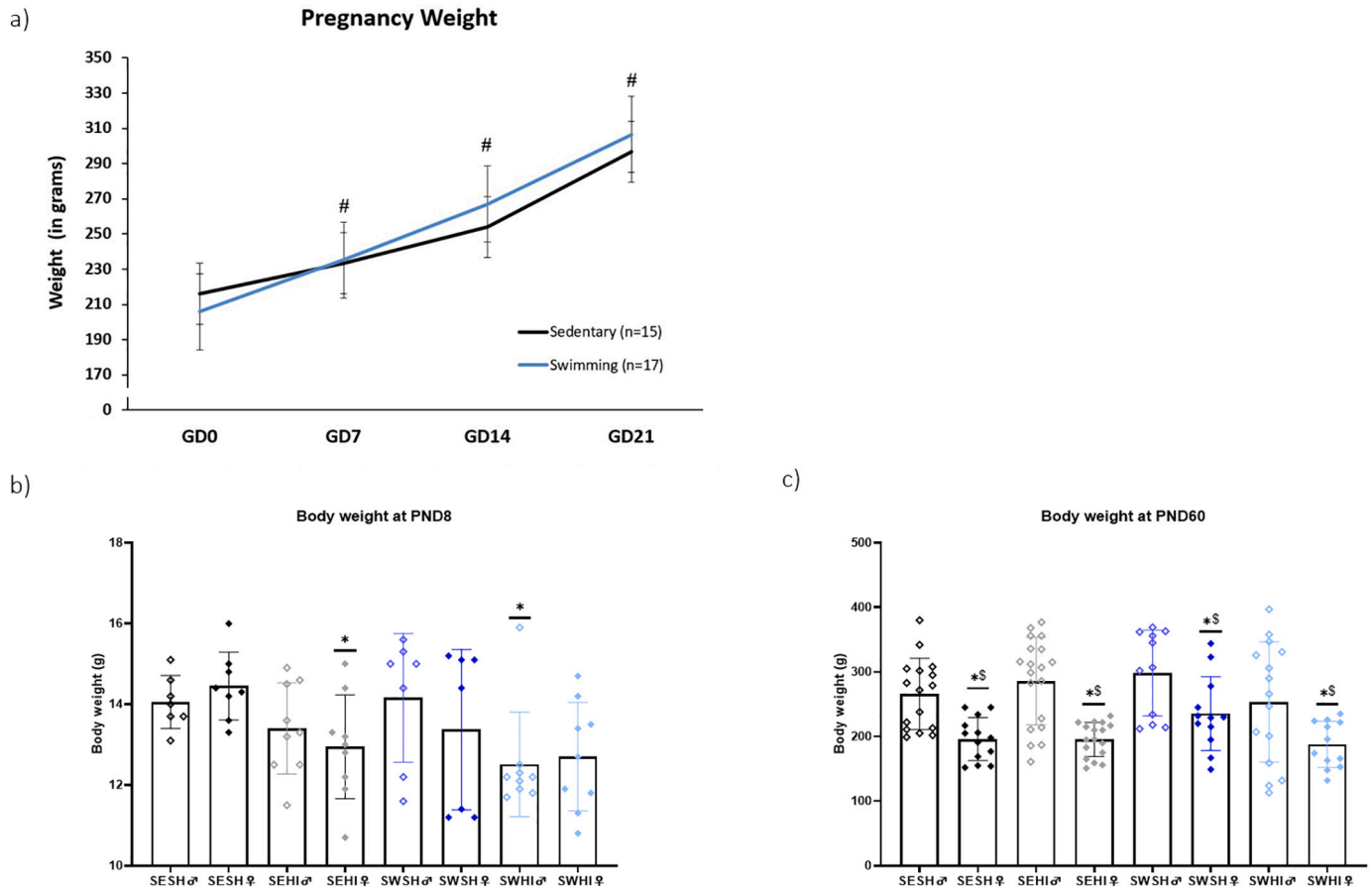

Fig. 2. - Effects of maternal swimming exercise on gestational weight during pregnancy (a) and in the offspring at PND8 (b) and PND60 (c). Results are expressed as mean ± S.E.M. Number of animals at PND8 and PND60 are respectively SESH ♂ (7 and 16), SESH ♀ (8 and 14), SEHI ♂ (9 and 19) and SEHI ♀ (9 and 17); SWSH ♂ (7 and 11), SWSH ♀ (7 and 11), SWHI ♂ (9 and 15) and SWHI ♀ (9 and 12). Repeated measures ANOVA (a). # Effect of time on animals' gain weight. Results are expressed as mean ± SD. Three-way ANOVA followed by Duncan's post hoc (b and c). Significance accepted when $p < 0.05$. * Injury effect; HI vs. respective control group; $ Effect of animal's sex.

maximization (3D-MLEM) algorithm with 20 iterations. For PND8 analysis, microPET images were manually co-registered to standard magnetic resonance imaging (MRI) histological templates corresponding to animals' age. MRI templates were obtained from the Duke Center for In Vivo Microscopy NIBIB P41 EB015897 (Calabrese et al., 2013). PND60 images were reconstructed and spatially normalized using an [18F]-FDG brain image template through PMOD 3.5 software and the Fusion toolbox (PMOD Technologies, Zurich, Switzerland). A magnetic resonance imaging (MRI) rat brain voxel of interest (VOI) template was used to overlay the normalized, previously co-rotated images to the microPET imaging database, and 24 VOIs were used. ^{18}F-FDG uptake in whole brain and expressed as standardized uptake value (SUV) (Odorcyk et al., 2020; Zanirati et al., 2018).

2.7. Behavioral testing - Morris water maze

From PND45, Morris water maze was used to assess spatial memory impairment using Reference and Working memory protocols (Sanches et al., 2015). Briefly, in the reference memory protocol, all rats underwent four 60s trials / day, during 5 consecutive days, with a 10 min intertrial interval. In the training phase, if the animals failed to find the platform, they were guided and kept on the platform for a period of 10s. Animal start positions were designated as N, S, W or E. During the 5 days, rats entered the pool facing the wall and all starting points were used in a different order each day. The platform position remained in the same location throughout the training period. The latency to find the platform during each trial was measured as a learning indicator. A probe test without the platform was performed on the 6th day and the following parameters were assessed: latency to cross the platform zone, time spent in in the platform quadrant, time spent in the opposite platform quadrant and the total distance travelled. Working memory was tested in a protocol that consisted in 4 trials/day, with a maximum 5 min intertrial interval, with a different platform position on each of the four consecutive days, with the four starting points used in a distinct order each day. The pool was set to obtain novel platform positions in a non-standard location (e.g., in the center of the pool). On the first trial of each day, rats reached the platform by chance, so those latencies would not be expected to reflect group differences. This trial constituted the 'information' stage, and the subsequent trials required matching to the novel position for that day. Working memory was assessed by the mean latencies of trial 1 (acquisition trial) and trial 4 (last test trial) in the four sessions for each animal system.

2.8. Data analysis

All statistical analyses were performed using SPSS 21.0 for Windows (SPSS Inc., Chicago, IL, USA). Data normality distribution was assessed using Kolmogorov-Smirnov test. Three-way ANOVA (injury, treatment and animal's sex as factors) was used to analyze the data. Repeated measures ANOVA was conducted for the assessment of maternal gain weight and Morris water maze reference memory training. Post hoc analyses for main effects and interactions were determined by Duncan's post hoc test. Significant interactions were *post*-tested using individual one-way ANOVAs, when appropriate. t-test was used to compare

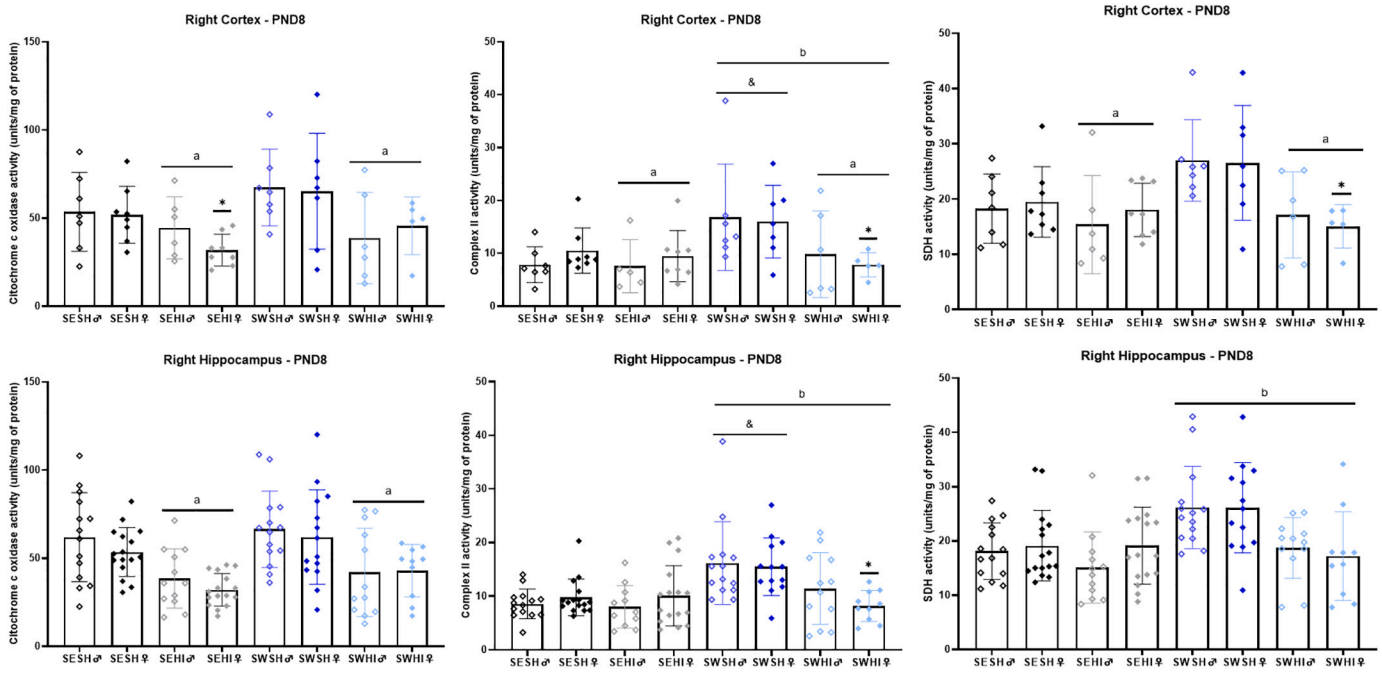

Fig. 3. - Effects of maternal swimming in the offspring's right cortex (upper panels) and hippocampus (lower panels) on the activity of complex IV (cytochrome *c* oxidase) (a and d), complex II (b and e) and SDH (c and f) at PND8. Number of animals SESH ♂ (7), SESH ♀ (8), SEHI ♂ (6) and SEHI ♀ (8); SWSH ♂ (7), SWSH ♀ (7), SWHI ♂ (6) and SWHI ♀ (5). Results are expressed as mean ± SD. Three-way ANOVA followed by Duncan's post hoc. Significance accepted when $p < 0.05$. [a] Injury effect (* HI vs. respective control); [b] Effect of gestational swimming [&] SESH vs. SWSH.

latencies in the Morris water maze, working memory protocol. Data are expressed as mean ± SD, excepting line graphs (1a and 8a) that are expressed as mean ± SEM. Significance was accepted when $p < 0.05$.

3. Results

3.1. Maternal exercise did not alter weight gain during pregnancy. Maternal swimming prevented early HI-induced weight loss mainly in female rats

Repeated measures ANOVA showed no effect of gestational swimming on weight gain in female rats during pregnancy (Fig. 2a). At PND8, three-way ANOVA evidenced an effect of injury ($F(1,64) = 11.72$, $p = 0.01$); SEHI ♀ and SWHI ♂ were smaller compared to their controls (SESH ♀ and SWSH ♂, respectively). At PND60, three-way ANOVA revealed an effect of sex ($F1,114) = 41.11$, $p < 0.001$) and an interaction between injury and treatment ($F(1,114) = 4.45$, $P = 0.03$) was observed. One-way ANOVA evidenced differences between SESH ♂ and SESH ♀, SWSH ♂ and SWSH ♀ in the controls; SEHI and SWHI animals (independent of sex) had decreased weights compared to their respective controls (Fig. 2b and c).

3.2. Neonatal HI causes electron transport chain failure in cortex and hippocampus 24 h after injury; maternal exercise prevents enzymatic activity dysfunction independently of animals' sex

Fig. 3 shows the effects of pregnancy swimming on electron transport chain enzymatic activity in cortex (Fig. 3. upper panels - **a**, **b** and **c**) and hippocampus (lower panels - **d**, **e** and **f**). At PND8, there was an effect of injury in the complex IV (also called cytochrome *c* oxidase***)*** ($F(1,53) = 1$ 0.97, $p = 0.002$), complex II ($F(1,52) = 5.73$ $p = 0.02$) and SDH ($F(1,53)$ $= 10.33$ p = 0.002) in the right cortex, in which HI animals presented activity compared to SH. HI caused a decrease in the hippocampal activity of cytochrome *c* oxidase ($F(1,53) = 21.61$ $p < 0.001$) and showed a trend to decrease the activity of Complex II ($F(1,52) = 2.95$ $p = 0.09$). There was no HI effect in hippocampal SDH activity. Regarding the effects of maternal swimming, no effects on cortical cytochrome *c* oxidase were observed. However, swimming preserved the activities of Complex II ($F(1,52) = 4.77$ $p = 0.03$) and SDH ($F(1,53) = 3.31$ $p = 0.07$) in the cortex. In the hippocampus, swimming preserved the function of Complex II ($F(1,52) = 9.54$ $p = 0.003$) and SDH ($F(1,52) = 5.83$ p = 0.02). Interactions between injury and treatment were observed in Complex II in the cortex ($F(1,52) = 4.04$ $p = 0.05$) and hippocampus ($F(1,52) = 4.16$ $p = 0.04$). Also, treatment and injury had an interaction on SDH activity in the cortex ($F(1,53) = 4.45$ p = 0.02). No effects of sex were observed in the mitochondrial electron transport chain enzymatic activity.

3.3. Neonatal HI causes mitochondrial mass and potential alterations in cortex and hippocampus 24 h after injury. Maternal exercise alters mitochondria functioning in a sex-related manner following HI

Fig. 4 shows the effects of pregnancy swimming on HI consequences in mitochondrial mass and potential at PND8 in cortex (upper panel) and hippocampus (lower panel). Three-way ANOVA showed an effect of injury in the mitochondrial mass/potential ratio in the cortex (($F(1,122)$ $= 38.14$ $p < 0.001$) and hippocampus ($F(1,122) = 48.64$ p < 0.001). Three-way ANOVA also showed an effect of pregnancy swimming on this parameter in the cortex ($F(1,122) = 9.48$ p = 0.003) and hippocampus ($F(1,122) = 32.37$) p < 0.001). Interestingly, in the cortical tissue, three-way ANOVA showed an interaction among the three variables ($F(F1,122) = 3.97$ $p = 0.009$) in which SWHI ♀ had increased ratios (FL1/FL3) compared to the other groups. No isolated effects of sex as a variable in the ratio mitochondrial mass/potential were observed.

Fig. 5 shows the effects of pregnancy swimming on DCF formation in the cortex (**a**) and hippocampus (**b**). Three-way ANOVA showed and effect of injury in the right cortex ($F(1,122) = 7.88$ $p = 0.006$) and hippocampus ($F(1,122) = 4.09$ $p = 0.04$) in which HI animals had increased production of ROS (measured by DCF intensity). In the cortex, maternal swimming (treatment effect) prevented DCF formation following HI ($F(1,122) = 6.33$ $p = 0.013$).

In order to study the effects of maternal exercise on glucose

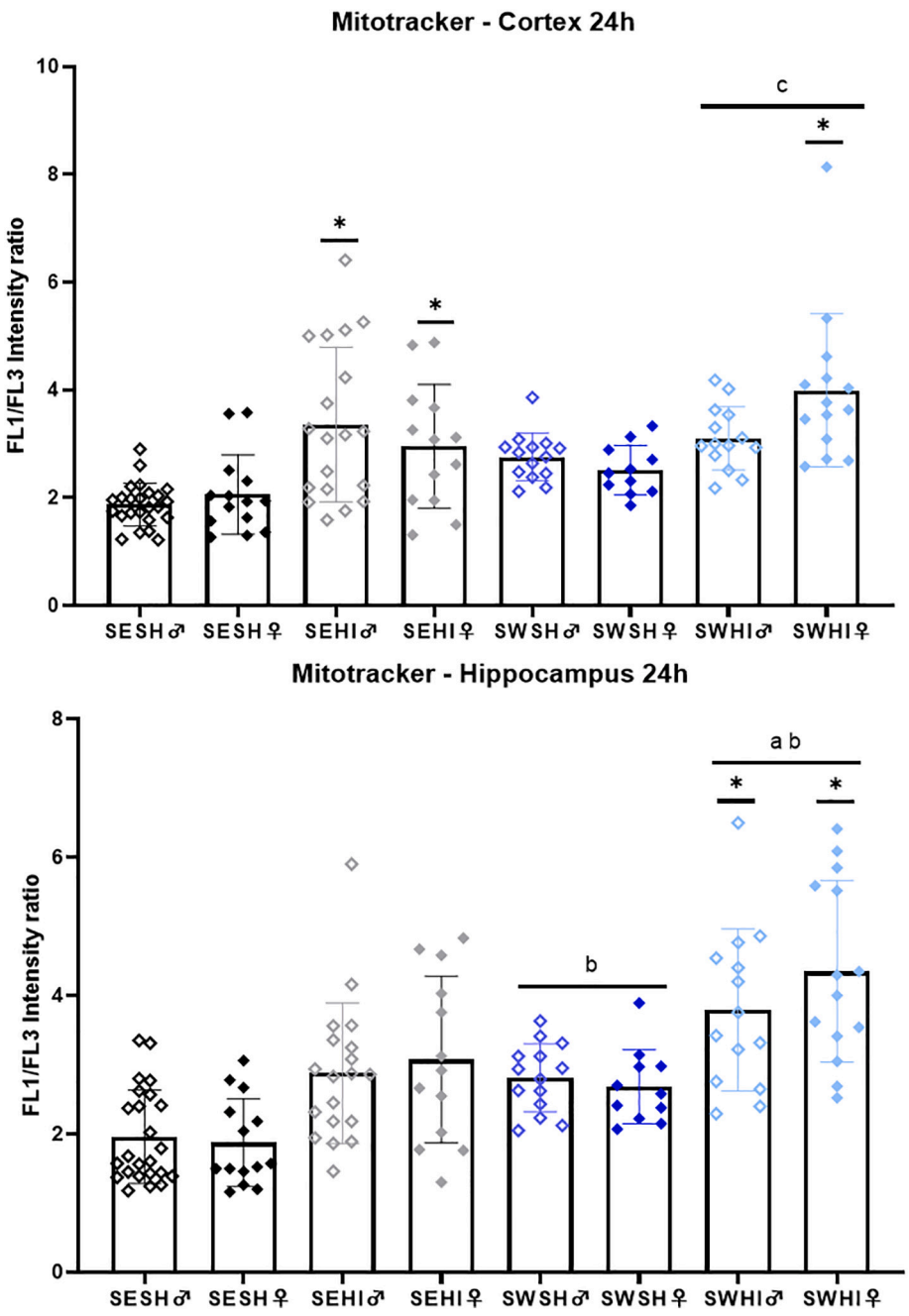

Fig. 4. - Effects of maternal swimming in the mitochondria (mass/potential ratio) in the offspring right cortex (upper panel) and hippocampus (lower panel) at PND8 (24 h after HI). Number of animals SESH ♂ (24), SESH ♀ (14), SEHI ♂ (19) and SEHI ♀ (13); SWSH ♂ (14), SWSH ♀ (11), SWHI ♂ (14) and SWHI ♀ (14). Results are expressed as mean ± SD. Three-way ANOVA followed by Duncan's post hoc. Significance accepted when $p < 0.05$. [a] Injury effect; [b] Effect of gestational swimming; [c] Effect of sex.

metabolism of the HI brain in vivo, 18F-FDG microPET images were obtained at PND8 and PND60 (Figs. 6 and 7). In the Fig. 6, total SUV in the right and left brain hemispheres at PND8 is shown. Injury had an effect in the right ($F(1,38) = 7.259$, $p = 0.01$) and left hemispheres ($F(1,38) = 8.219$ $p = 0.007$) decreasing glucose uptake in HI animals. No effects of sex or swimming were observed. At PND60, an effect of injury was observed in the right ($F(1,39) = 10.942$, $p = 0.002$) and left hemispheres ($F(1,39) = 5.304$, $p = 0.002$) in the glucose utilization measured by PETSCAN. The parameters observed were not altered by sex or treatment. When evaluating the hippocampus, injury also had an effect in the right hemisphere ($F(1,39) = 13.839$ $p = 0.001$) (Fig. 7**a** and **b**). Interestingly, a negative correlation was observed between SUV in the left hemisphere at PND8 and the performance in the Morris water maze (Area Under the Curve - AUC), where the less glucose metabolism the animal presented, the worse was its performance in the test ($y = 0.87–4.92E\text{-}4*x$; $R^2 = 0.117$, $p = 0.04$). Table 1 and Table 2 (Supplementary data) show the effects of pregnancy swimming on brain glucose Standard Uptake Value (SUV) assessed by microPET scan imaging after $^{[18F]}$ FDG injection at postnatal day 8 (PND8) and 60 (PND60).

3.4. Neonatal HI causes sex-related cognitive impairments. Maternal exercise improves offspring's performance in Morris water maze in a sex-dependent manner

Fig. 8a shows the latency to find the platform during acquisition phase in Morris water maze. Repeated measures ANOVA revealed an effect of injury ($F(1,122) = 38.89$, $p < 0.01$), treatment ($F(1,122) = 17.11$ $p < 0.01$) and sex ($F(1,122) = 4.61$ $p = 0.03$) between subjects during the five training days. Area under the curve (AUC) was assessed in order to represent a learning index of platform location over the 5 days. Three-way ANOVA showed an effect of injury ($F(1,129) = 36.49$, p

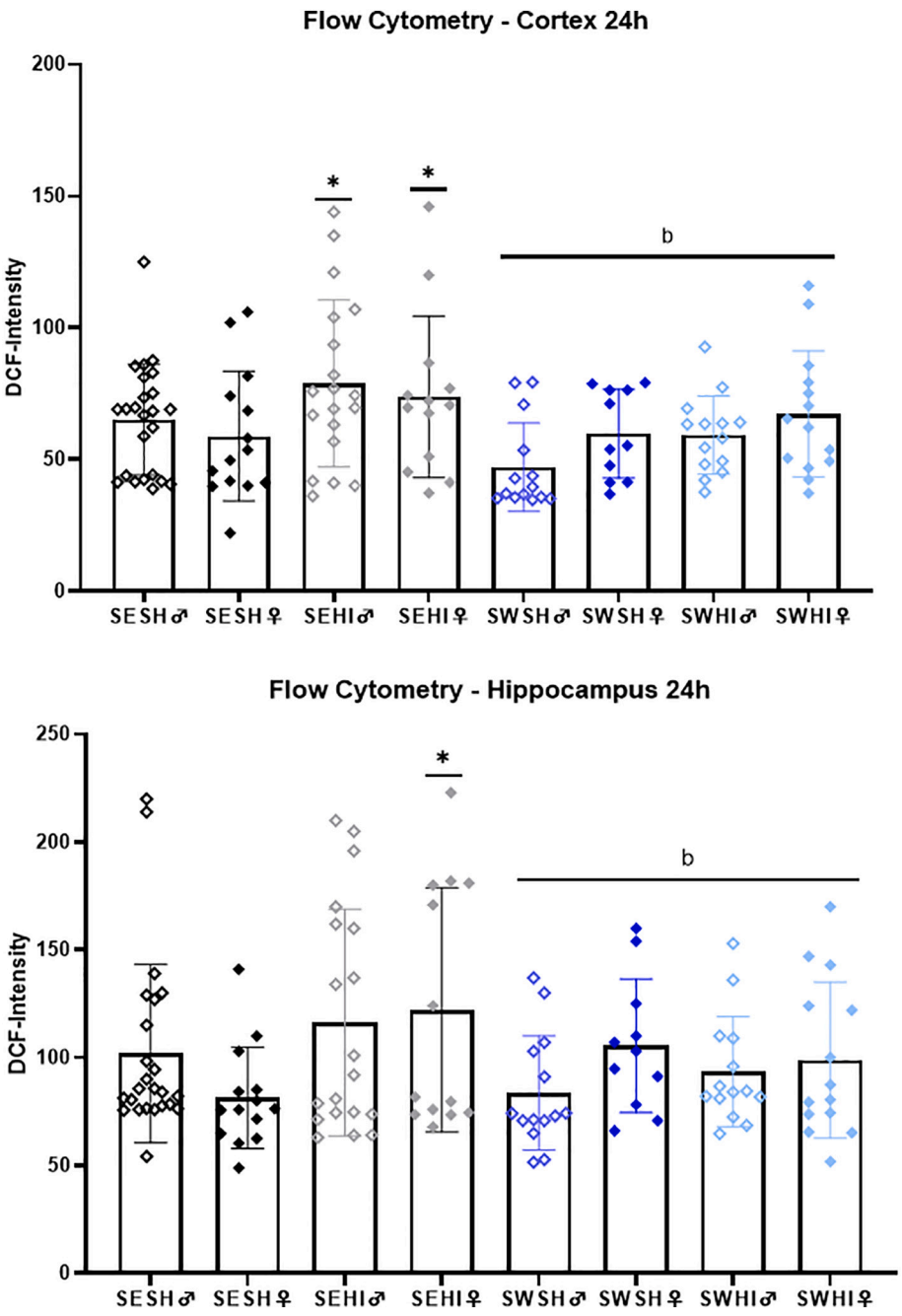

Fig. 5. - Effects of maternal swimming in the ROS production in the offspring's right cortex (upper panel) and hippocampus (lower panel) at PND8 (24 h after HI). Number of animals SESH ♂ (24), SESH ♀ (14), SEHI ♂ (19) and SEHI ♀ (13); SWSH ♂ (14), SWSH ♀ (11), SWHI ♂ (14) and SWHI ♀ (14). Results are expressed as mean ± SD. Three-way ANOVA followed by Duncan's post hoc. Significance accepted when $p < 0.05$. [a] Injury effect; [b] Effect of gestational swimming; [c] Effect of sex.

< 0.001), treatment ($F(1,129) = 16.130$ $p < 0.001$) and sex ($F(1,129) = 5.05$ $p = 0.026$) on AUC (Fig. 8b). In the probe trial, three way ANOVA revealed an effect of injury ($F(1, 129) = 36.49$, $p < 0.001$) and treatment ($F(1, 129) = 8.39$, $P = 0.004$) and an interaction among all variables ($F(1,129) = 3.773$ $p = 0.05$; in which SWHI♀ benefited most from the treatment (Fig. 8c). Fig. 9a shows the performance of animals in the working memory protocol of the Morris water maze. The protocol considered the difference between time spent to find the platform in trial 4 compared to trial 1 on the same day. Three way ANOVA showed an effect of injury ($F(1,129) = 7.186$ $p = 0.008$) in the first trial of the protocol, which could indicate trouble assuming a new strategy searching for the platform in a distinct location from that used in the reference memory protocol. In trial 4, three-way ANOVA revealed an effect of injury ($F(1, 129) = 14.508$, $p = 0.01$), treatment ($F(1, 129) = 6.781$, $P < 0.001$) and sex ($F(1,129) = 4.529$ $p = 0.03$). When analyzing the delta (T4-T1), an effect of treatment ($F(1,129) = 6.440$ $p = 0.01$) and an interaction between sex and injury was observed ($F(1,129) = 4.098) = 4.098$ $p = 0.04$) (Figs. 9b and 10). Results show that SW animals of both sexes, although worse than controls, performed better he task compared to SEHI. However, when evaluating working memory, an effect of sex was observed, in which male animals benefited more from maternal swimming compared to females, which could help explain their similar performance in the reference memory protocol.

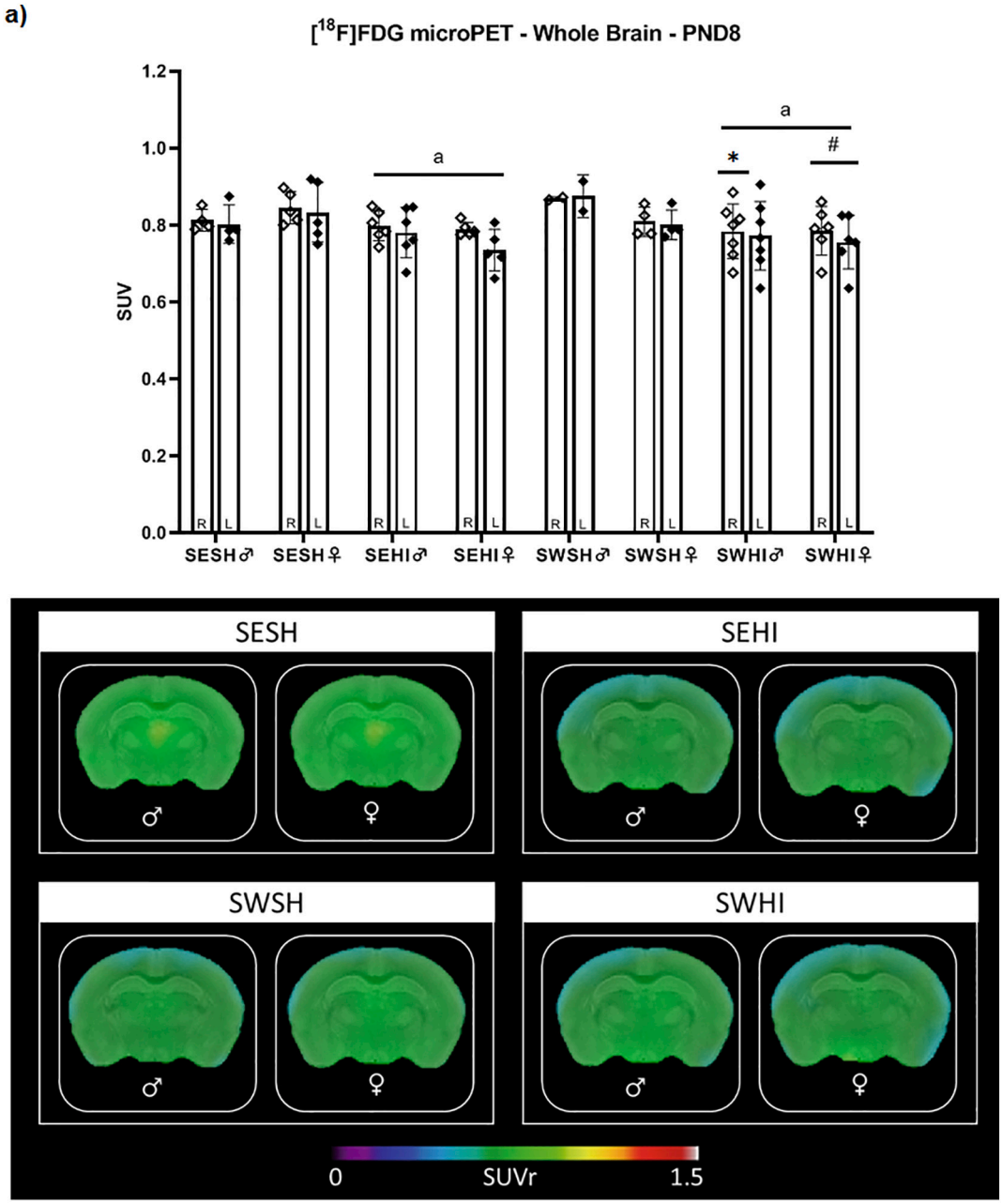

Fig. 6. - Changes in whole brain glucose metabolism in HI rats. Graph illustrates changes in ^{18}F-FDG SUV at PND8 in the Right (left bars) and Left (right bars) hemispheres. Number of animals in SESH ♂ (4), SESH ♀ (5), SEHI ♂ (6) and SEHI ♀ (5); SWSH ♂ (2), SWSH ♀ (4), SWHI ♂ (7) and SWHI ♀ (6) groups. Results are expressed as mean ± SD. Three-way ANOVA followed by Duncan's post hoc. Significance accepted when $p < 0.05$. [a] Lesion effect. * HI vs. SH. [#] Paired *t*-test (right vs. left hemisphere).

4. Discussion

In the present study, we show that pregnancy swimming induces sex-related neuroprotection following neonatal HI injury. Maternal exercise exposure decreased HI- induced damage to mitochondrial function, namely the inhibition of enzymatic activities in the mitochondrial electron transport chain and altered mitochondrial mass and membrane potential. By altering brain tissue response to HI, maternal exercise prevented cognitive deficits at adult age. Our data also suggests that the prevention of mitochondrial dysfunction in addition to early brain glucose uptake preservation could be associated with improvement in cognition in the "swimmer's moms" offspring.

Many resources are being used to treat children suffering from HI, however, up until now there are no proven effective therapies. In the experimental context used in this study, we observed a significant intergenerational effect of mother's physical activity on offspring brain function, which can stimulate public policies aiming to improve the mother-fetus unit and prevent complications during this period, thus programming offspring's brain in case of metabolic challenges such as HI as has been suggested in literature (Torabi et al., 2017). The absence of difference in weight gain between exercise and non-exercise mother are in agreement with studies in both humans and rodents, showing that physical activity during pregnancy had no detrimental effects on the offspring birth weight (Ferrari et al., 2018; Kelly et al., 2015; Barakat et al., 2009).

Mitochondrial dysfunction is believed to play a central role in the pathogenesis of several CNS pathologies, ranging from depression (Chen et al., 2017) to Alzheimer's disease (Picca et al., 2020). In the brain,

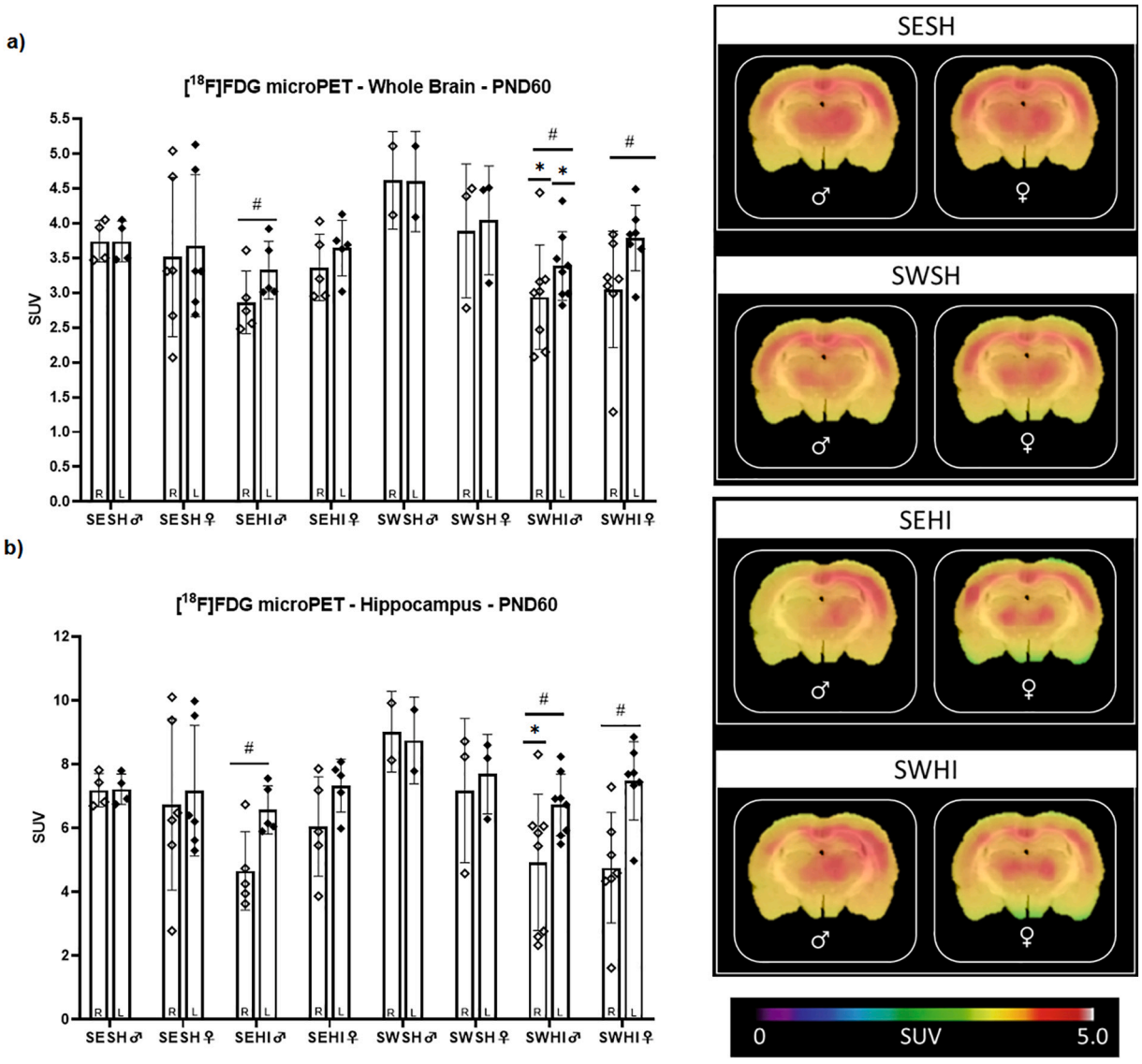

Fig. 7. – Changes in whole brain glucose metabolism (a - left upper panel) and hippocampus (b - left lower panel) in HI rats. Graphs illustrate changes in ^{18}F-FDG SUV at PND60. Number of animals SESH ♂ (4), SESH ♀ (6), SEHI ♂ (5) and SEHI ♀ (5); SWSH ♂ (2), SWSH ♀ (3), SWHI ♂ (8) and SWHI ♀ (7). Results are expressed as mean ± SD. Three-way ANOVA followed by Duncan's post hoc. Significance accepted when $p < 0.05$. * HI vs. SH. # Paired t-test (right vs. left hemisphere).

mitochondria are crucial for the processes of neuroplasticity, including neural differentiation, neurite outgrowth, neurotransmitter release and dendritic remodeling (Mattson, 2007). Given that HI is associated with abnormal neurogenesis and synaptic formation (Volpe, 2008), increasing evidence indicates that mitochondrial disturbances could be a target for neuroprotection in HI (Silachev et al., 2018). Here, we show that HI causes loss of mitochondrial membrane potential and that maternal exercise could reverse such alterations, increasing mass and mitochondrial potential at the same time. However, to the best of our knowledge, this neuroprotective effect of maternal swimming on the mitochondrial functioning in the offspring's brain has not been shown previously. Allied to this, we observed that HI causes a decrease in the enzymes activity of the electron transport chain in both cortex and hippocampus. The rate-limiting enzyme associated with oxidative phosphorylation is cytochrome *c* oxidase (Acín-Pérez et al., 2003). Rodent studies suggest that deletion of a single subunit of such enzyme results in impaired oxidative capacity in different tissues (Hüttemann et al., 2012). Here, we show that the activities of cytochrome *c* oxidase (complex IV) and Complex II are preserved in the hippocampus of SWHI ♂ rats. This, in addition to the decrease in glucose consumption observed in the microPET, decreased ROS production and mitochondrial mass/potential activity, could help to explain the better performance of SWHI ♂ in the Morris water maze. Interestingly, Du et al. (2004) demonstrated that cultured neurons derived from males are more sensitive to the toxic effects of $NO^{\cdot}$ than those derived from females. It is therfore possible to infer that part of the maternal exercise effects on pup brain neuroprotection is due to decrease in oxidative stress.

Consistent with the mitochondrial functional assessment, there was an increase in cortex and hippocampus oxidative stress, as evidenced by increase in ROS production in HI rats (irrespective of sex) and by the ratio between mitochondrial membrane potential and mass. It is well described that increased ROS content causes mitochondria damage leading to cell swelling (Weis et al., 2012). Growing evidence has shown that exercise could improve mitochondrial function and increase antioxidant enzymes in the nervous tissue (Park et al., 2013; Caldwell et al., 2020). Dos Santos et al. (2017) showed that physical exercise during development protects against brain oxidative damage caused by chronic stress exposure later in life. The mitochondrial biogenesis elevation observed in our study possibly indicates that mitochondrial autophagy/mitophagy may be elevated in offspring born from exercised

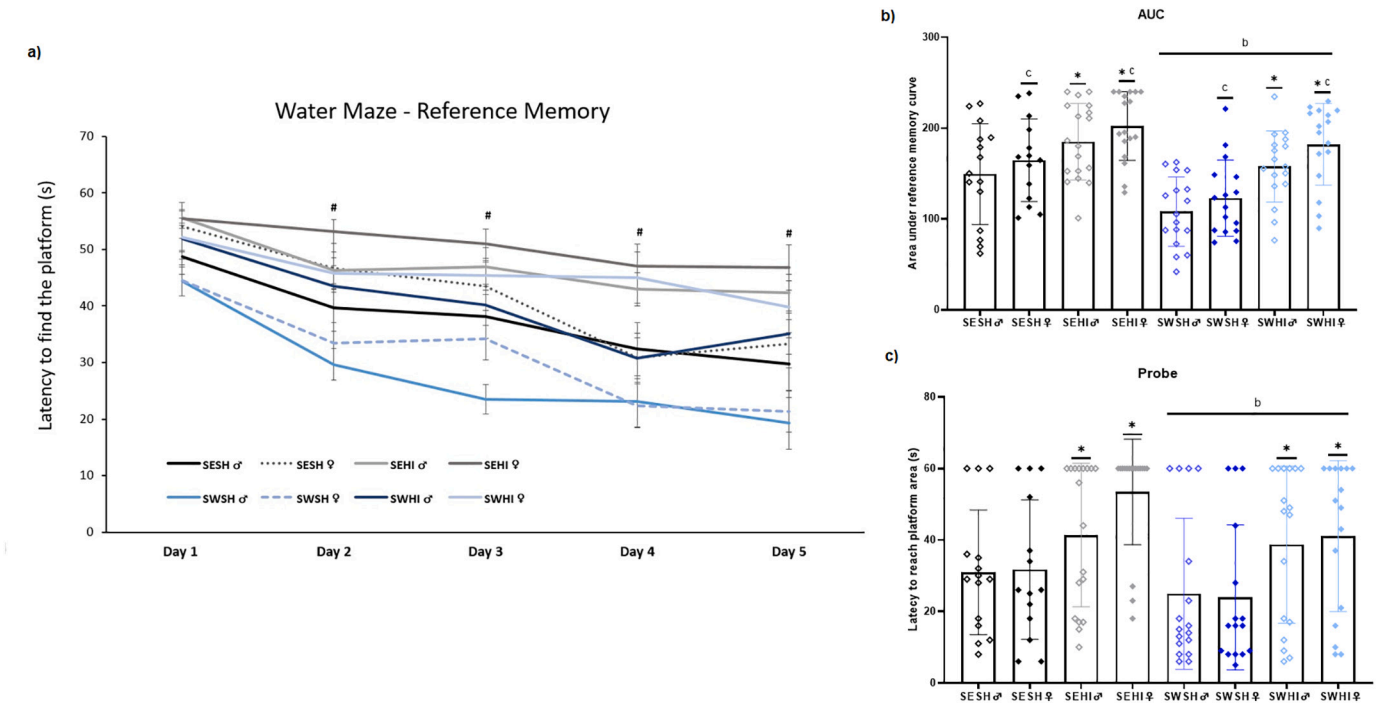

Fig. 8. - Morris water maze reference memory protocol at adult age (PND45). Average time to reach platform in the four trials on five consecutive days of training (a), (b) area under the curve (in seconds) and (c) latency to reach the platform location in the Probe trial (s). Data are expressed as mean ± SD. Values were considered significant when $p < 0.05$. Number of animals per group: SESH ♂ (15), SESH ♀ (14), SEHI ♂ (18) and SEHI ♀ (17); SWSH ♂ (17), SWSH ♀ (16), SWHI ♂ (18) and SWHI ♀ (16). Repeated measures three-way ANOVA (sex x treatment x injury). * Injury effect (HI vs. SH). # Interaction between time (days of training) and experimental groups. [b] Pregnancy swimming effect. [C] Interaction between sex and maternal swimming.

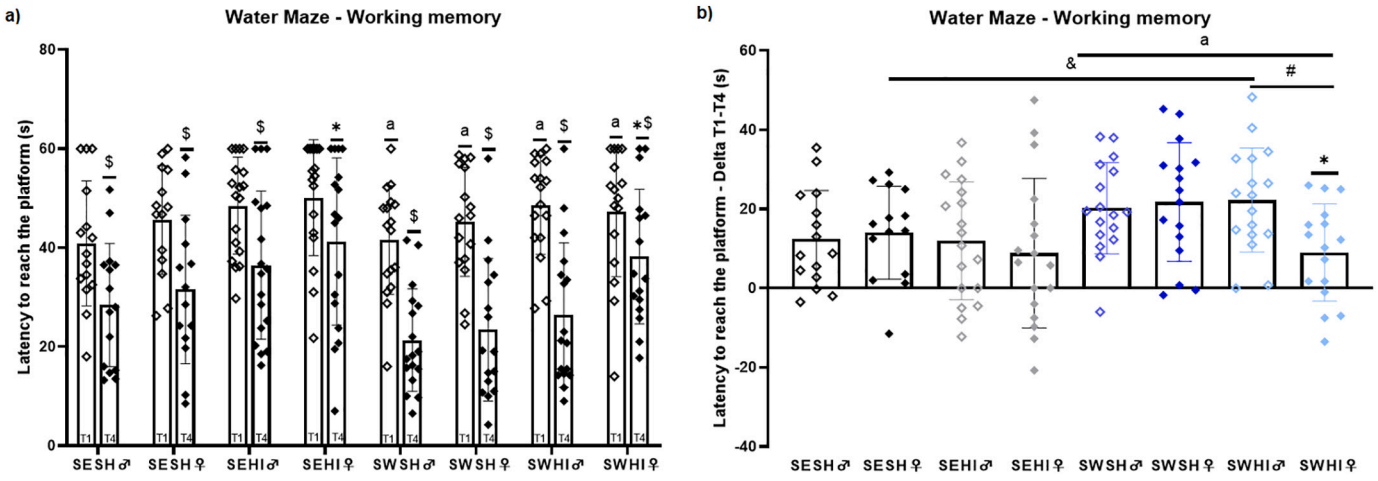

Fig. 9. - Morris water maze working memory protocol (a) and delta between T1 and T4 in the test (b). In the graph (a) left rows represent latency to find the platform in the 1st trial and right rows represent the average latency in the 4th trial during days testing. Number of animals per group: SESH ♂ (15), SESH ♀ (14), SEHI ♂ (18) and SEHI ♀ (17); SWSH ♂ (17), SWSH ♀ (16), SWHI ♂ (18) and SWHI ♀ (16). Results are expressed as mean ± SD. Three-way ANOVA followed by Duncan's post hoc. [$] Paired t-test (T1 vs. T4). [a] Pregnancy swimming effect. *Injury effect (HI vs. SH). [$] Difference between T1 and T4 (t-test). [#] SWHI ♂ vs. SWHI ♀. [&] SEHI ♂ vs. SWHI ♂.

mothers in order to improve mitochondrial turnover, in which, dysfunctional / aberrant mitochondria are removed via mitophagy and replaced in an effort to maintain mitochondrial homeostasis after injury (Simmons et al., 2020).

Physical exercise is generally related to learning and memory enhancement (Cotman and Berchtold, 2002; van Praag et al., 2005; van Praag et al., 1999). Animal studies have demonstrated that maternal physical exercise boosts cognitive performance and hippocampal neurogenesis in adult male offspring (Akhavan et al., 2012; Lee et al., 2006; Robinson and Bucci, 2012; Torabi et al., 2017). Likewise, our previous studies also showed that swimming is able to prevent early energetic failure and preserve cognitive function after HI, however such studies used animals from both sexes (Sanches et al., 2017; Sanches et al., 2018). Despite evidence showing that male rats perform cognitive tasks better than females (Charriaut-Marlangue et al., 2017), we did not observe this pattern in SH animals in this study. Regarding the effects of maternal exercise, the present study showed that apart from improving learning in HI animals, swimming had a great impact on control animals, evidencing that maternal exercise per se enchanced cognition, confirming previous data from the literature (Akhavan et al., 2012; Lee

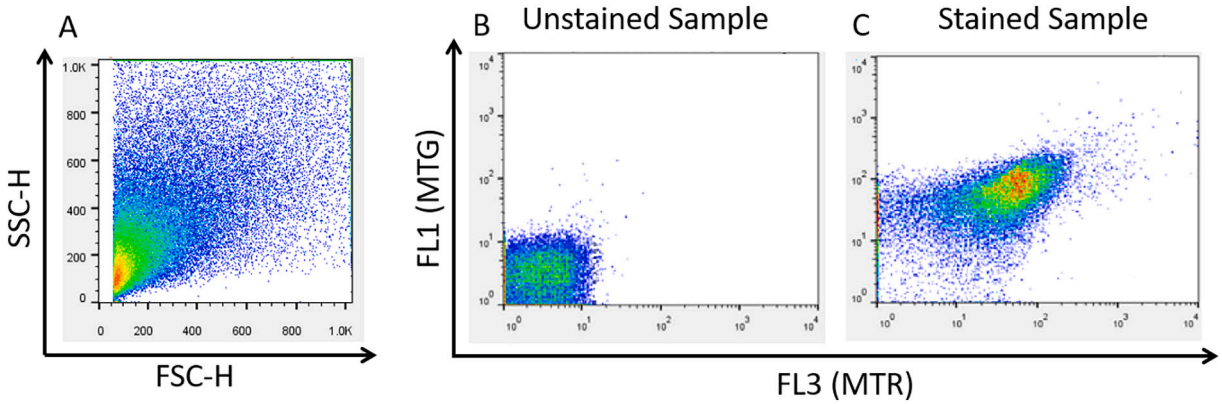

Fig. 10. - Representative images of flow cytometry analysis. Side scatter (SSC) and Forward scatter (FSC) of the assessed cell population (A). Unstained sample used as negative control for autofluorescence (B). Stained sample with both Mitotracker green (MTG) and red (MTR) used for analysis of mitochondrial mass and potential (C).

et al., 2006). However, it is interesting to note that the effects of swimming are distinct in the two protocols of the task. While in the reference protocol maternal swimming seems to improve males performance, in the working memory protocol it clearly benefits the female HI most. This, reinforces evidence from literature showing that male and female humans (Pu et al., 2020) and rodents (Qi et al., 2016) have different strategies when searching for the platform in the task and points out that the exact mechanisms through which exercise exerts its benefits need better understanding.

In the past years, efforts from our research group are being undertaken to better understand the sex-related effects caused by neonatal HI in different domains (Arteni et al., 2010; Weis et al., 2012; Sanches et al., 2013). Literature has shown that the HI outcomes are consistently altered by the sex of the animals (for review, see Netto et al., 2018, Charriaut-Marlangue et al., 2017). More recently, therapeutic strategies used for preventing and treating the consequences of neonatal HI have shown to be sexually dimorphic. It is well established that the outcomes following HI are more severe in males which points to an intrinsic disadvantage of potential therapies in this gender (Hill et al., 2011). Using classical behavioral tasks as rehabilitation following neonatal HI in rats, Tsuji et al., 2010 observed that females seem to benefit more from the treatment (Tsuji et al., 2010). Curiously, maternal exposure to environmental enrichment influences offspring in a sex-specific manner, with anxiety-like behavior increasing exclusively in males, whereas learning abilities improve in females only. These changes are likely due to modification in maternal care such as reduction of total nursing time and increase in licking/grooming behavior (Zuena et al., 2016). Durán-Carabali et al. (2019) showed that animals of both sexes reared in enriched environments recover from HI in a similar way; however, the mechanisms of recovery seem to be distinct between sexes. When using pharmacological approaches, evidence from basic and clinical settings show the same pattern (Durán-Carabali et al., 2019). Neonatal caffeine treatment between P3 and P12 improves respiratory function solely in adult male rats (Bairam et al., 2010) and in clinical trials, Ment et al. (2004) observe (in males) the efficacy of prophylactic administration of indomethacin in preventing intraventricular hemorrhage in preterm babies (Ment et al., 2004). Further studies confirmed the pattern of improvement in males after therapy (Rosenkrantz et al., 2019).

To the best of our knowledge, the sexual dimorphism observed in the neuroprotection provided by gestational swimming was never demonstrated before. In this study, we show that brain mitochondrial dysfunction caused by HI was prevented preferentially in male rats, who benefited more from the treatment when cognition was tested. Our current findings suggest that maternal physical activity improves markers of mitochondrial function in young rats early after HI, which may lay the foundation for late brain tissue preservation and behavioral improvements. These data shed light on HI lesion progression in rodents under the influence of maternal physical activity, and provide insight into sex differences on HI outcomes as well as the benefits of maternal physical activity.

In conclusion, this study expands previous information on the beneficial effects of gestational exercise on the offspring's brain function. Pregnancy swimming induces alterations in the pup brain mitochondrial functioning and in the ability of using glucose as energetic substrate maintaining the metabolic rates following HI. These early alterations are linked to long-term neuroprotective effects observed in spatial memory tasks. Here, we provide new evidence from basic science that physical exercise should be recommended in clinical settings as a promising strategy for intergenerational neuroprotection when performed during pregnancy. However, further studies are needed to establish the intensity as well as periodicity of swimming for posterior translation to clinical trials.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors contributions

EFS - Designed the study, performed animal surgeries and biochemical experiments, analyzed the data and wrote the manuscript; TMS - performed the biochemical experiments and graphical abstract illustration; FKO - performed the biochemical experiments; HU, ER and TA - performed the animal's training and behavioral experiments; APM - performed the biochemical and animals' training; EH - performed the animal's training, behavioral experiments and PETscan image reconstruction, GTV and SG - performed the PETscan, JCC - Reviewed the final version of the manuscript; CAN and ATW - Designed the study and reviewed critically the final version of the manuscript.

Declaration of Competing Interest

The authors declare that the research was carried out without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at [doi: https://doi.org/10.1016/j.expneurol.2021.113623].

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