● RESEARCH ARTICLE

Paternal physical exercise modulates global DNA methylation status in the hippocampus of male rat offspring

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Abstract

It is widely known that maternal physical exercise is able to induce beneficial improvements in offspring cognition; however, the effects of paternal exercise have not been explored in detail. The present study was designed to evaluate the impact of paternal physical exercise on memory and learning, neuroplasticity and DNA methylation levels in the hippocampus of male offspring. Adult male Wistar rats were divided into two groups: sedentary or exercised fathers. The paternal preconception exercise protocol consisted of treadmill running, 20 minutes daily, 5 consecutive days per week for 22 days, while the mothers were not trained. After mating, paternal sperm was collected for global DNA methylation analysis. At postnatal day 53, the offspring were euthanized, and the hippocampus was dissected to measure cell survival by 5-bromo-2′-deoxiuridine and to determine the expression of synaptophysin, reelin, brain-derived neurotrophic factor and global DNA methylation levels. To measure spatial memory and learning changes in offspring, the Morris water maze paradigm was used. There was an improvement in spatial learning, as well as a significant decrease in hippocampal global DNA methylation levels in the offspring from exercised fathers compared with those from sedentary ones; however, no changes were observed in neuroplasticity biomarkers brain-derived neurotrophic factor, reelin and 5-bromo-2′-deoxiuridine. Finally, the global DNA methylation of paternal sperm was not significantly changed by physical exercise. These results suggest a link between paternal preconception physical activity and cognitive benefit, which may be associated with hippocampal epigenetic programming in male offspring. However, the biological mechanisms of this modulation remain unclear.

Key Words: *epigenetics; preconception; methylation; learning; treadmill running; fetal programming; inheritance; sperm; nerve regeneration*

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Introduction

Epigenetic mechanisms have been defined as the dynamic regulation of gene expression in response to external stimuli without changes to the primary DNA sequence (Bale, 2015). Histone acetylation status, an important epigenetic mark, is controlled by histone acetyltransferase which add acetyl groups to lysine residues from amino- terminal tails of histones, altering chromatin conformation and enhancing transcriptional activity. Histone deacetylase enzymes, remove acetyl groups from lysine, which results in chromatin condensation and transcriptional repression (Kouzarides, 2007). On the other hand, DNA methylation is dynamically modulated by activity-dependent events, and it consists of the covalent addition of a methyl group at the 5′ position of the cytosine residue. This process is catalyzed by DNA methyltransferase enzymes and often occurs at CpG islands (Lister et al., 2009). DNA demethylation induces gene transcription and activation, while DNA hypermethylation is usually associated with silencing of genes that are of dramatic importance for cell function under homeostatic and disease conditions (Curley et al., 2011; Deichmann, 2016; Fernandes et al., 2017).

Interestingly, emerging clinical studies have been reported physical exercise as an epigenetic modulator for the maintenance of overall body and brain health (Denham et al., 2015; da Silva et al., 2017; Dorneles et al., 2017; Lavratti et al., 2017). In addition, compelling evidence has shown that both single sessions and chronic exercise protocols on a treadmill can alter DNA methylation status in the rat brain at different stages of development. This effect can modulate and influence the expression of several genes, including those related to brain plasticity, cognition and disease (Gomez-Pinilla et al., 2011; Elsner et al., 2013; Denham et al., 2015; Fernandes et al., 2017).

Furthermore, it is known that life experiences that change paternal sperm DNA methylation patterns before fertilization have the potential to alter epigenetic programming in future offspring (Mychasiuk et al., 2012, 2013; Denham et al., 2015). Denham et al. (2015) have shown that an aerobic exercise protocol performed two times per week for 3 months (covering the human spermatogenesis cycle) is able to reduce global DNA methylation levels in the sperm of healthy young adult men. In addition, it has been demonstrated that exercise causes significant DNA hypermethylation, altering the expression of specific genes related to neurological disorders such as autism, schizophrenia, Parkinson's disease, and Alzheimer's disease, as well as cardiometabolic diseases including obesity, type-2 diabetes mellitus, high blood pressure and atherosclerosis. A pater-

nal physical exercise contribution to offspring development has also been observed in brain tissue. In this sense, Yin et al. (2013) reported increased levels of brain-derived neurotrophic factor (BDNF) in association with higher cognitive performance in the offspring from exercised mouse fathers compared to sedentary ones. BDNF is involved in neuroplasticity, neurogenesis, neuronal maintenance and survival and, consequently, plays a pivotal role in learning and memory processes (Poo, 2001; Binder and Scharfman, 2004). In fact, we have recently shown that a paternal physical exercise protocol of treadmill running for eight weeks was able to promote the demethylation of hippocampal DNA in male offspring, although it did not modify cognitive and physical development (Mega et al., 2018). Altogether, these findings reinforce the idea that healthy paternal habits prior to conception influence the phenotype of the offspring.

Some research groups have demonstrated that the exercise-induced upregulation of plasticity-promoting genes, such as BDNF, occurs as the result of engaging hippocampal DNA demethylation and histone hyperacetylation in rodents (Gomez-Pinilla et al., 2011; Maejima et al., 2018). Reelin is an extracellular glycoprotein that plays an important role in hippocampal neuroplasticity and cell migration during neural development and maintenance (Lu and Figurov, 1997; Herz and Chen, 2006), and can be modulated by epigenetic mechanisms (Sui and Li, 2010; Sui et al., 2012). However, little is known about the exercise effects on reelin expression associated with other neuroplasticity markers. In view of these considerations, the present study was designed to broaden our knowledge about the effects of paternal physical exercise on offspring and the associated environmental inheritance mechanisms. We aimed to analyze the impact of a 22-day paternal exercise protocol on 1) offspring cognition, 2) global DNA methylation, BDNF levels, cellular survival, reelin and synaptophysin expression in the offspring hippocampus, and 3) global DNA methylation in paternal sperm.

Materials and Methods

Ethical considerations
All procedures in the present study were previously approved by the Animal Ethics Committee at the Universidade Federal do Rio Grande do Sul (27587) on December 8, 2014. Animal care was performed in accordance with the recommendations of the Brazilian Society for Neuroscience, Committee of the School of Veterinary Surgery, University of Buenos Aires, International Brain Research Organization (IBRO) and the National Institute of Health Guidelines for the Care and Use of Laboratory Rats (NIH Publication No. 85-23, revised 1985). All efforts were made to minimize any animal discomfort and to reduce the number of animals needed for the experiment.

Animals
Initially, 10 adult male and 10 adult female Wistar rats (60 days old) from our local breeding colony (CREAL/ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil) were used to obtain male offspring. Animals were maintained in standard Plexiglas housing boxes ($410 \times 340 \times 160$ mm^3),

with 3 to 4 rats per cage, under controlled environmental conditions (20 ± 1°C, 12-hour light/dark cycle with lights on at 7:00 a.m. and food and water available ad libitum). All procedures were performed from 2:00 p.m. to 5:00 p.m.

Experimental design and paternal preconception exercise protocol
Ten adult male rats were randomly divided into the sedentary ($n = 5$) and exercised by treadmill running group ($n = 5$). Prior to training, indirect measurements of maximum oxygen consumption (VO$_2$max) were used to determine the running speed. In this evaluation, animals were placed individually on the treadmill (INBRAMED TK 01, Porto Alegre, Brazil) and the running speed was increased progressively (3 m/min) every 3 minutes until exhaustion (the animal refused to run on the treadmill) (adapted from Spindler et al. 2014). The latency to fatigue (s) and the maximum speed (m/min) achieved by each animal were considered as performance parameters, and the mean speed of all animals in the experimental group was used as the training speed.

In the paternal exercise protocol, the males were submitted to running sessions at 60% of VO$_2$max on a horizontal motorized treadmill adapted to rodents with individual Plexiglas lanes (INBRAMED TK 01, Porto Alegre, Brazil). The duration and periodicity of the training protocol was 20 min/d, 5 consecutive days per week for a total period of 22 training days (modified from Parnpiansil et al., 2003).

On the first two training days, the rats ran at 3.09 m/min for the first 2 minutes, 4.4 m/min for the next 4 minutes, 9.39 m/min for 8 minutes, 4.4 m/min for 4 minutes, and 3.09 m/min for the last 2 minutes. On the other training days, the daily sessions were done as follows: during the first 4 minutes, the rats ran at a speed of 3.09 m/min, then from 4–16 minutes at 9.39 m/min (60% VO$_2$max) and at 3.09 m/min for the remaining 4 minutes. The sedentary rats were handled exactly the same as the exercised animals, but they were kept on the treadmill while it was turned off (without any stimulus to run) for 5 minutes (Elsner et al., 2011; Lovatel et al., 2013; Spindler et al., 2014). The treadmill running modality was chosen due to the ease of controlling the parameters, such as intensity and velocity, unlike voluntary wheel running (Arida et al., 2011). In this study, neither electric shocks nor physical stimulation were used, and the animals that refused to run received gentle manual stimuli. If the animals continued to refuse to run, they were withdrawn from the study.

Mating and standardization of litters
After 22 days of paternal exposure to physical exercise, the estrous cycle of the females was checked daily; in the proestrus phase, they were housed overnight with a male to mate. All females used in the current study were sedentary. Vaginal smears were examined the next morning after mating and the presence of spermatozoa was considered as day 0 of gestation (G0). If the onset of gestation was not confirmed within 10 days after the end of the paternal preconception exercise period, the females were excluded from the study. After the mating period, male progenitors were removed from the present study.

The day the offspring were born was denominated as postnatal day 0 (P0). Each experimental group was composed by 32–34 male offspring from 10 different litters and determined by paternal exposure to physical exercise or not during the preconception period:

(1) Male offspring of sedentary fathers and mothers (SED);

(2) Male offspring of exercised fathers and sedentary mothers (EXE).

The size of the litter was standardized between six and eight animals each (composed of males and females) to avoid litters of disparate sizes. The offspring remained with their mothers until P21, when they were weaned, and the females were removed from the study. A set of male offspring was allocated for neonatal development assessments and histological analyses, a second set for BDNF and epigenetic analyses, and a third set of animals was used in behavioral tests, with the objective of avoiding interference among the evaluations. A timeline (**Figure 1**) is provided to illustrate the experimental procedures.

Neonatal developmental evaluations

The body weight of the offspring was analyzed at P1, P7, P14 and P53 (before euthanasia), and the main developmental milestones were evaluated daily from P1 to P21, according to the methods of Marcuzzo et al. (2010). Briefly, the milestones assessed were: surface righting, cliff aversion, forelimb grasp, stability on an inclined surface (negative geotaxis), hind limb proprioceptive placing, open-field activity assessment, audio startle and eye opening, and were indicated by the average postnatal day that the group performed each task for the first time within a maximum time of 30 seconds (n = 13/group).

BrdU administration

From P20 to P23, rats destined for the developmental evaluation were treated with four intraperitoneal injections of 5-bromo-2′-deoxiuridine (BrdU; Sigma, 100 mg/kg, dissolved in 0.1 M NH_4OH, 20 mg/mL, 24 hours apart). BrdU is a compound analogous to thymidine and is incorporated into DNA during the S phase of mitosis; it can be detected by immunohistochemistry (Nowakowski et al., 1989; Taupin, 2007; Veena et al., 2009). To assess the cellular sur-

vival phase of the hippocampal neurogenesis process in the dentate gyrus (DG), animals were euthanized 30 days after the last BrdU injection (P53), as described by Piazza and collaborators (2014).

Physical performance assessment

The physical performance of offspring was evaluated at P46 by the indirect measurement of VO_2max exactly as described to determine the paternal training speed. The same animals were also submitted to the Morris water maze task (n = 11/ group).

Spatial learning and memory analysis

Male offspring were submitted to Morris water maze task with modifications (Pereira et al., 2007), at P47 to P51. The maze consisted of a circular pool (120 cm in diameter and 40 cm deep) with blurred water at 23°C divided into four equal imaginary quadrants. Four visual cues were fixed on the walls of the experimental room to be used as reference points. In the target quadrant, a platform (10 cm in diameter) was placed 2 cm under the surface of the water. The task was performed for five days; the first four days were dedicated to training and the last to the probe trial. On each training day, the animals underwent four trials, with 15-minute intervals between them.

Each trial consisted of placing the rat at a starting point with its head facing the pool wall, and the latency to find the platform was measured. If the rat did not find the platform within 1 minute, the animal was guided gently to it, and held there for 10 seconds. At the end of each trial, the rats were dried and returned to their housing boxes. The order of the starting position varied for every trial. The latency to find the platform was measured on each trial and the average latency for each training day was calculated. On the last day, the probe trial (test day) was performed. The animal was placed in the opposite quadrant to the platform location, which was removed. Thus, the time the animal spent in the opposite and target quadrants were recorded to assess the retention of information.

All the trials were recorded by a video acquisition system (Sony Action Cam, model HDR-AZ1; Sony, Tokyo, Japan) for posterior analysis. This task was conducted in a silent
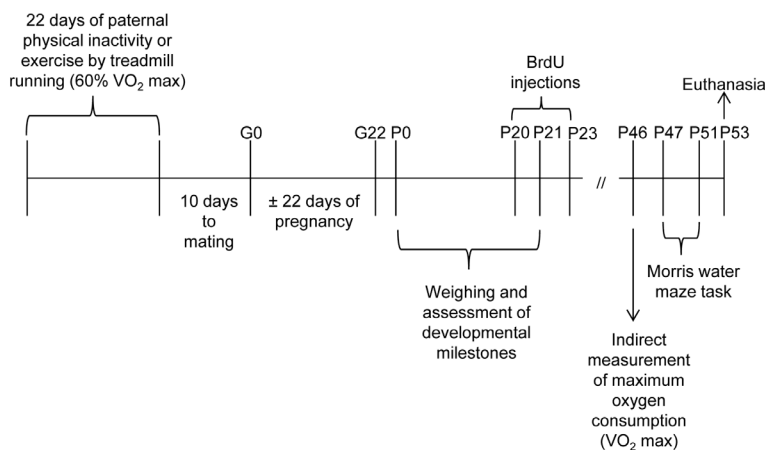

Figure 1 Schematic representation of the timeline of the experimental procedures.
G: Gestational day; P: postnatal day; BrdU: 5-bromo-2′-deoxiuridine; VO_2max: maximum oxygen consumption.

and temperature-controlled room, and the animals were handled by the same researcher on the training days and during the probe trial.

Sample preparation

The offspring were euthanized by decapitation at P53, and hippocampi were quickly dissected. The sperm samples from paternal testes were collected as described by Bielawski et al. (2002). Briefly, the epididymis and vas deferens were placed in a sterile culture dish at room temperature containing 1 mL of sterile phosphate-buffered saline. The samples were cut into small pieces with a razor and the tissues were teased apart to collect mature spermatozoa. The sperm suspension was then transferred to a microcentrifuge tube. Sperm were separated from cellular debris by centrifugation at $2000 \times g$ for 5 minutes, and the supernatant was removed. Immediately, samples were snap-frozen in liquid nitrogen and stored at –80°C until biochemical analyses.

DNA isolation

Offspring hippocampal DNA and paternal sperm DNA were isolated using a commercial tissue section DNA isolation kit (FitAmp General Tissue Section DNA Isolation Kit catalog #P-1003, Epigentek Group Inc., Farmingdale, NY, USA) according to the manufacturer's instructions. The samples were lysed in a water bath at 37°C, the DNA was purified using the enclosed columns and washed out with elution buffer.

Global DNA methylation measurement

The global DNA methylation of the offspring hippocampus and of the paternal sperm was assessed using a commercial MethylFlash™ Methylated DNA Quantification Kit (Base catalog #P-1034, Epigentek Group Inc., Farmingdale, NY, USA). Briefly, 100 ng/well of DNA from each sample was added to the plates and incubated with 28 µL of DNA ligation buffer for 40 minutes at 37°C, and then 40 minutes at 60°C. Thereafter, 150 µL of blocking buffer were added and incubated for 30 minutes at 37°C followed by washed twice with washing buffer. The diluted capture antibody (1:5000) was added (50 µL/well) and incubated for 30 minutes at room temperature, and after washed five times with washing buffer. The enhancement solution (50 µL/well) was then added and incubated for 30 minutes at room temperature followed by washed five times with washing buffer. Color developing solution (100 µL/well) was added and incubated for 1–5 minutes in the dark. The absorbance at 450 nm was measured using an automatic microplate reader (Mega et al., 2018).

BDNF analysis

BDNF analysis was performed by ChemiKine Sandwich ELISA kit (Cat. No. CYT306, Millipore, Darmstadt, Germany). Briefly, offspring hippocampi were homogenized according to the manufacturer's instructions using a specifics lysis buffer and centrifugation, and the supernatant was collected for BDNF quantification. Assays were performed in 96-well microplates that were pre-coated with mouse anti-Human BDNF Monoclonal Antibody. BDNF standard (7.8–500 pg/mL) or samples and incubated at 4°C overnight. The plates were washed four times, and a Biotinylated Mouse anti-Human BDNF Monoclonal Antibody (1:1000) was added to each well and incubated for 3 hours at room temperature. The plates were washed again, and a diluted streptavidin-horseradish peroxidase conjugate solution (1:1000) was added to each well and incubated for 1 hour at room temperature. After washing, a tetramethylbenzidine substrate solution and a stop solution were added and incubated for 15 minutes at room temperature. Each plate was immediately read at 450 nm. A standard curve was plotted for each plate. BDNF concentrations were assayed from the regression line of the BDNF standard (Mega et al., 2018). Protein concentration of each sample was measured by Bradford method using bovine serum albumin as standard (Bradford, 1976).

Histological procedures

The offspring used for the neonatal development analyses was transcardially perfused at P53 to perform immunohistochemistry for BrdU, reelin and synaptophysin. The animals were deeply anesthetized with sodium thiopental (50 mg/kg, i.p.; Cristália, São Paulo, Brazil) associated with lidocaine (10 mg/mL), injected with 1000 IU of heparin (Cristália, São Paulo Brazil) in the left ventricle and euthanized by transcardiac perfusion with 200 mL of saline solution, followed by 200 mL of a solution containing 4% paraformaldehyde diluted in 0.1 M phosphate buffer (PB; pH 7.4, Synth, São Paulo, Brazil) at room temperature, using a peristaltic pump (Milan, Brazil, 30 mL/min). The brains were removed from the skull, post-fixed in the same fixative solution for 4 hours at room temperature and cryoprotected by immersion in 15% and 30% sucrose solution (Synth, Brazil) in PB at 4°C. The brains were then quickly frozen in isopentane, cooled in liquid nitrogen and stored at –80°C until use. Coronal sections (40 µm thick) of the dorsal hippocampus were obtained using a cryostat (CM1850, Leica, Nussloch, Germany) at –20°C. Slices were serially collected (200 µm apart) on gelatin coated slides (Piazza et al., 2014). The point of origin was located approximately 2.30 to 4.52 mm posterior to bregma (Paxinos and Watson, 1982).

Immunohistochemistry for BrdU, reelin and synaptophysin

Immunostaining for BrdU, reelin and synaptophysin was performed separately at P53. The sections were washed in phosphate buffered saline (PBS), pH 7.4, and antigen retrieval was performed by heating sections in 0.01 M sodium citrate buffer (pH 6.0) in a thermostatic bath for 20 minutes at 92°C. The tissues were washed in PBS and endogenous peroxidase was inactivated with 3% hydrogen peroxide (Synth, São Paulo, Brazil) dissolved in PBS for 30 minutes. Sections were washed again in PBS, and then in PBS containing 0.4% Triton X-100 (PBS-Tx) for 15 minutes and pre-incubated with 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in PBS-Tx for 30 minutes. Then, samples were incubated with monoclonal mouse anti-BrdU antibody (1:100 diluted in nuclease, GE Healthcare, Buckinghamshire, England) for 2 hours at room temperature or at 4°C overnight, with mouse anti-reelin antibody (1:400 diluted

in 3% BSA; Millipore, Darmstadt, Germany) or with mouse anti-synaptophysin (SYP) antibody (1:200 diluted in 3% BSA; Sigma Aldrich) for 48 hours at 4°C. Sections were washed in PBS-Tx and incubated with the specific secondary antibody anti-mouse IgG conjugated with peroxidase (1:500, Sigma-Aldrich) for 2 hours at room temperature. The immunohistochemical reactions were revealed using a solution of 0.06% 3,3-diaminobenzidine (Sigma-Aldrich) and 10% hydrogen peroxide for 5 minutes. Finally, the sections were rinsed in PBS, dehydrated in ethanol, cleared with xylene and covered with synthetic Canada balsam (Synth, São Paulo, Brazil) and coverslips. The BrdU-labeled slices were counterstained with hematoxylin. Negative controls were prepared by omitting the primary antibody and replacing it with PBS. For all samples, immunohistochemistry was performed at the same time, with the same solutions and under the same conditions, with the aim of minimizing the differences in staining and background (Piazza et al., 2014).

BrdU and reelin-labeled cell quantification

Two blinded observers quantified BrdU$^+$ cells in the granule cell layer (GCL) and in the subgranular zone (SGZ) of the DG in both hemispheres of each section of offspring at P53 at 400× magnification using an BX40 microscope (Olympus, Tokyo, Japan) (Piazza et al., 2014). The SGZ was considered as a two-nucleus-wide band between the apparent border of the GCL and the hilus. The cells in the outermost focal plane were omitted (Malberg et al., 2000). In order to facilitate reelin$^+$ cell counting, digitalized images from the DG of the dorsal hippocampus of both hemispheres of each slice were obtained with an Optiphot-2 microscope (100×, Nikon, Tokyo, Japan) coupled to a Micrometric camera (Accu Scope, Commack, NY, USA). With the software Image Pro Plus 6.0 (Media Cybernetics Rockville, MD, USA) and zoom of 100%, reelin$^+$ cells were counted in the SGZ and hilus of DG of the hippocampus. The number of BrdU$^+$ or reelin$^+$ cells per section was determined and multiplied by the section periodicity to obtain the total number of cells per DG (Malberg et al., 2000; Piazza et al., 2014). For both analyses, six slices/animal were used from four animals/group.

Optical densitometry of SYP

SYP immunostaining was measured by means of regional semi-quantitative optical densitometry (OD) (Piazza et al., 2014). Digitalized images of the DG region of the dorsal hippocampus were obtained using an Optiphot-2 microscope (200×; Nikon, Tokyo, Japan) coupled to a CMOS camera (518CU; Micrometrics, Commack, NY, USA). All lighting and magnification conditions were held constant during the analysis. Using the Image Pro Plus 6.0 software (Media Cybernetics Rockville, MD, USA), the digital images were converted to an 8-bit gray scale (0–255 gray levels). In each image, the expression of SYP was measured by the mean optical densitometry of three squares inserted in the hilus region of the DG of the hippocampus, measuring 5396.118 μm^2 each (area of interest, AOI). The background was corrected, and the optical densitometry was calculated according to the

method of Xavier et al. (2005): OD (x,y) = −log[(INT(x,y)− BL)]/(INC−BL); where OD is the optical density, INT (x,y) or intensity is the intensity at the pixel (x,y), BL or black is the intensity generated when no light passes through the material, and INC is the intensity of the incidental light (Piazza et al., 2014). For this analysis, 4–9 images (both left and right sides)/animal were used from five animals per group.

Statistical analysis

Initially, the data were submitted to the Shapiro-Wilk normality test to verify a normal distribution, and Levine's test to observe the homogeneity of variance. The body weight in the neonatal period and the latency to find the platform on the four training days of Morris water maze task were analyzed by repeated measures analysis of variance. When paternal exercise or time factors presented significant F values ($P < 0.05$), the Tukey's *post hoc* test was used. The pairwise comparison of the learning curve evolution intra-groups in the Morris water maze task were submitted to the Tukey's *post hoc* test. This analysis enables an evaluation of the speed of learning in each group (Gomes Da Silva et al., 2016). All the other data analyses were submitted to unpaired Student's *t*-tests. SPSS software (version 24.0; IBM, New York, NY, USA) was used for the statistical analysis. Differences between groups were considered significant when $P < 0.05$. Data are expressed as mean ± standard error of the mean (SEM).

Results

Paternal physical exercise does not affect development of male offspring

There was no difference between the offspring from EXE and SED fathers regarding body weight at any time point evaluated (P1, P7, P14 and P53). Similarly, no differences were observed regarding the age of acquisition of any of the development milestones ($P > 0.05$; data not shown).

Paternal physical exercise does not affect physical performance of male offspring

The EXE group demonstrated equivalent performance on the VO$_2$max indirect consumption test (904.45 ± 45.01 seconds) compared with the SED group (979.18 ± 39.39 seconds) ($P > 0.05$).

Paternal physical exercise improves spatial learning of male offspring

No significant differences were detected between groups in the latency to find the platform on any of the four training days ($P > 0.05$). Interestingly, when analyzing the learning curve from each group separately, a significant statistical difference in comparison with the first training day emerged on the second training day for the EXE ($P < 0.01$) group and on the third training day for the SED group ($P < 0.01$). These findings might indicate that paternal exercise induced an improvement in spatial learning in the offspring, since the EXE group learned the task (platform location) faster than the SED group. Moreover, the latency to find the platform on the third and fourth training days continued to be lower than

that of the first day in both groups ($P < 0.001$) (**Figure 2A**).

On the fifth day of the water maze task, the platform was removed for the probe trial. Unpaired Student's t-tests showed no differences between the EXE and SED groups regarding the time spent in the opposite or target quadrants ($P > 0.05$; **Figure 2B** and **C**). Therefore, no differences in retention of information on the platform location were detected between groups.

Paternal physical exercise decreases global DNA methylation in the hippocampus of male offspring

The EXE group presented lower global DNA methylation levels in the hippocampus compared to the SED group ($P < 0.05$; **Figure 3A**). Moreover, the global DNA methylation levels in paternal sperm did not differ between groups (**Figure 3B**).

Paternal physical exercise does not affect BDNF levels in the hippocampus of male offspring

No significant changes in hippocampal BDNF levels were found between the EXE and SED groups ($P > 0.05$; **Figure 4**).

Paternal physical exercise does not affect cell survival and neuroplsticity in the DG of male offspring

No differences were observed in the amount of BrdU-labeled cell survival, in the number of reelin$^+$ cells or in the level of

synaptophysin expression in the DG between the EXE and SED groups ($P > 0.05$; **Figure 5D–F**).

Discussion

It has been well-established through experimental and clinical evidence that exercise acts as a powerful epigenetic modulator in many tissues, such as the brain, sperm, muscle and leukocytes; this response is observed in healthy and diseased individuals (McGee et al., 2009; Denham et al., 2015; da Silva et al., 2017; Dorneles et al., 2017; Elsner et al., 2017; Figueiredo et al., 2017; Lavratti et al., 2017; Korb et al., 2018). However, father-son phenotype transmission in response to exercise exposure has recently been brought to light.

In this context, the present study shows that paternal physical exercise significantly decreased the global DNA methylation levels in the hippocampus of male offspring, indicative of increased transcriptional activity and gene expression. In agreement with this, we recently demonstrated that paternal physical exercise performed for 8 weeks also demethylates the hippocampal DNA of male offspring (Mega et al., 2018). Altogether, these findings reinforce the idea that paternal exercise might modulate DNA methylation status in future generations (Denham et al., 2015) and highlight the importance of paternal lifestyle choices and their impact on offspring development.
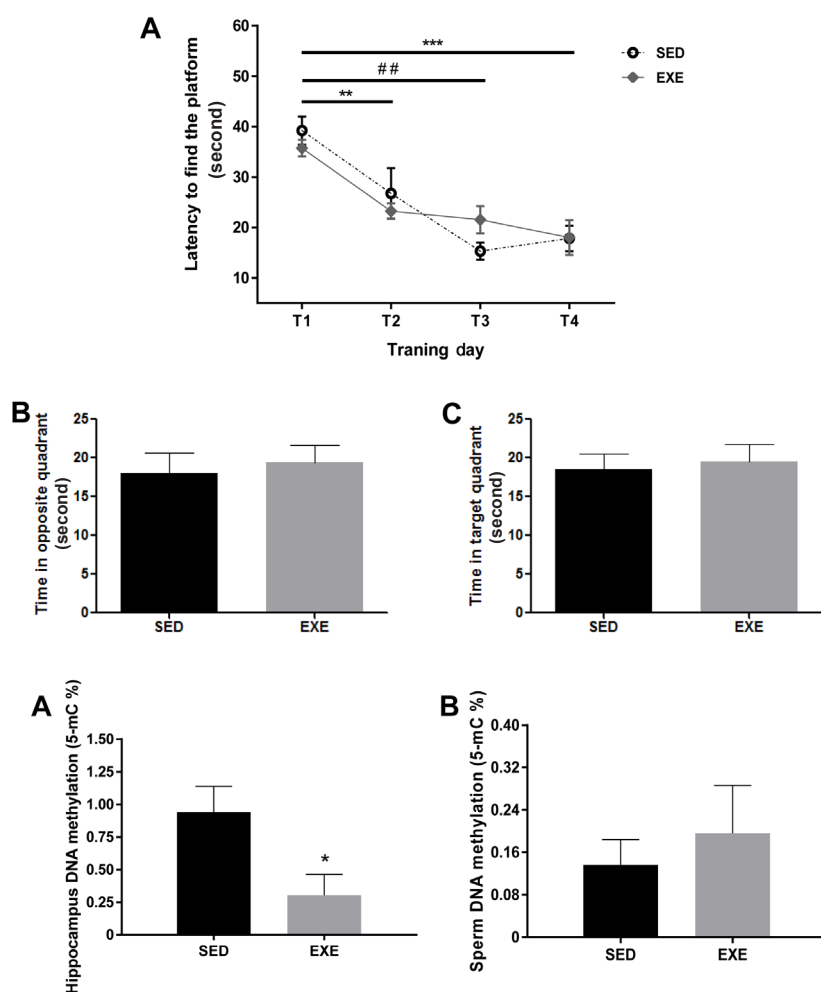

Figure 2 Spatial learning and memory evaluation in the Morris water maze task.
(A) Latency to find the platform on training days by the offspring. Repeated measures analysis of variance (paternal exercise × time): paternal exercise effect ($F_{(1,60)} = 0.004$, $P > 0.05$) and time effect ($F_{(1,60)} = 23.961$, $P < 0.001$). ***$P < 0.001$ means decrease in latency to find the platform between training days 1 *vs.* 4 in both groups. **$P < 0.01$ means decrease in latency to find the platform between training days 1 *vs.* 2 in the EXE group. The EXE group learned the task faster. ##$P < 0.01$ means decrease in latency to find the platform between training days 1 *vs.* 3 in both groups. (B) Time spent in the opposite quadrant in the probe trial (unpaired Student's t-test, $t_{(20)} = -0.395$, $P > 0.05$). (C) Time spent in the target quadrant in the probe trial ($t_{(19,7)} = -0.344$, $P > 0.05$). There were no significant differences between groups in B and C. $n = 9$–13 per group. Data are expressed as the mean ± SEM. SED: Offspring from sedentary fathers; EXE: offspring from exercised fathers; T: training day.

Figure 3 Global DNA methylation in offspring hippocampus and paternal sperm.
(A) Globaloffspring hippocampal DNA methylation. Decrease of hippocampal DNA methylationlevels in offspring from exercised fathers (EXE) *vs.* offspring from sedentary fathers (SED) group (*$P < 0.05$, unpaired Student's t-test, $t_{(8)} = 2.51$). (B) Global sperm DNA methylation levels in EXE and SED fathers ($t_{(8,97)} = -0.580$, $P > 0.05$). $n = 5$–7 per group. Data are expressed as the mean ± SEM.

Importantly, there is strong evidence demonstrating that the modulation of DNA methylation status plays a crucial role in neuronal plasticity, promoting learning and memory improvement (Feng et al., 2007; Miller et al., 2008). Although we found alterations in global DNA methylations in both studies, it is interesting to point out that this epigenetic modification was not accompanied by changes in cognitive tasks in response to the long paternal exercise protocol (8 weeks), while in the current study, we observed that a paternal exercise protocol with a duration of 22 days was able to improve the spatial learning ability of offspring. We suggest that shorter paternal exercise protocols on a treadmill are more effective to induce cognitive benefits by affecting the DNA methylation status of the offspring. Further research should be done in order to clarify this matter.

Our data did not show statistical differences between the experimental groups in the memory evaluation of the Morris water maze probe trial. However, when we analyzed the learning curves during the acquisition phase, we observed that male offspring from exercised fathers were faster learners compared to offspring from sedentary ones. Our data are in accordance with other studies (Akhavan et al., 2013; Gomes Da Silva et al., 2016) demonstrating that offspring from female rats submitted to an exercise protocol showed faster learning during the acquisition phase of the water maze but not at the memory evaluation. However, our results on learning curve are similar to a previous study, which showed that paternal treadmill exercise enhanced spatial cognition in rodents (Yin et al., 2013).

Compelling evidence has shown that there is an important association between cognitive amelioration in response to exercise and BDNF upregulation (Vaynman et al., 2003; Gomez-Pinilla et al., 2011; Karpova, 2014). Specifically, regarding the effect of maternal exercise during pregnancy on the offspring, cognitive improvement was related to higher BDNF mRNA levels in the hippocampus (Lee et al., 2006; Kim et al., 2007; Gomes Da Silva et al., 2016), although this effect seems to be time-specific. For example, Parnpiansil et al. (2003) showed that treadmill exercise in pregnant rats resulted in greater offspring hippocampal BDNF mRNA expression at P0, no difference at P14 and significantly decreased BDNF expression at P28. Interestingly, continuous physical training of the parents did not alter hippocampal BDNF mRNA expression in male offspring at 8 or 28 weeks of age (Venezia et al., 2015). This finding is similar to ours, since the first assessment point (8 weeks old) is close to P53, the age at which we evaluated the offspring in the present study.

With regard to paternal exercise only, Yin et al. (2013) reported significantly higher hippocampal BDNF expression in 25-day-old offspring from exercised fathers compared to those from sedentary fathers. In contrast, in the current study,
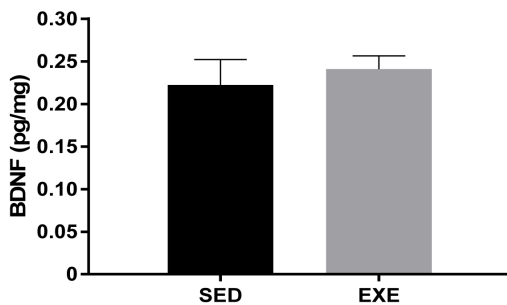

Figure 4 Offspring hippocampal BDNF levels (ELISA assay).
No differences were observed in hippocampal BDNF levels between groups (unpaired Student's *t*-test, $t_{(14)} = -0.343$, $P > 0.05$). $n = 8$ per group. Data are expressed as the mean ± SEM. ELISA: enzyme-linked immuno sorbent assay; SED: offspring from sedentary fathers; EXE: offspring from exercised fathers
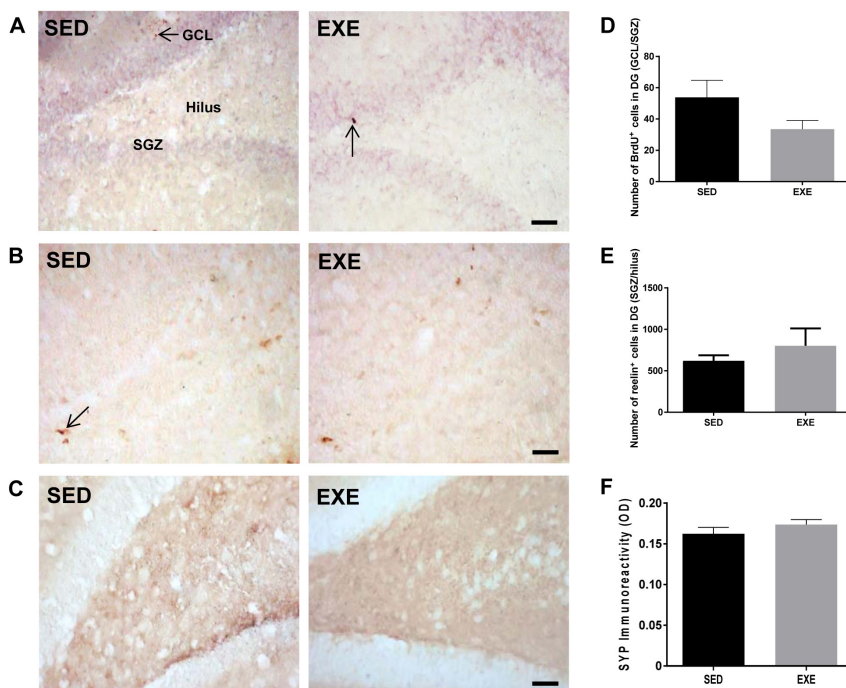

Figure 5 Survival of BrdU⁺ cells, reelin⁺ cells and SYP immunoreactivity in the DG of the hippocampus.
Digitalized images of the DG stained for BrdU showing cell survival in the SGZ and GCL (arrows; 100× magnification) (A), reelin in the hilus and SGZ (arrows; 100× magnification) (B), and SYP in the hilus (C) (200× magnification) in all groups. Scale bar = 50 μm. Quantitative analysis of the immunohistochemistry for BrdU (D) ($t_{(7)} = 1.76$, $P > 0.05$), reelin (E) ($t_{(3.33)} = -1.29$, $P > 0.05$) and SYP (F) ($t_{(8)} = -1.15$, $P > 0.05$) in different groups. There were no differences between groups. Data are expressed as the mean ± SEM ($n = 4$–5 per group). SED: Offspring from sedentary fathers; EXE: offspring from exercised fathers; DG: dentate gyrus; SGZ: subgranular zone; GCL: granular cell layer; SYP: synaptophysin; OD: optical density.

the analysis time point was almost a month later, which may explain why no differences were observed in the expression of BDNF in the offspring hippocampus. Potentially, we lost the window of observation for the regulation of BDNF expression, since the data suggest that the influence of parental exercise on the BDNF expression in the hippocampus of progeny is relatively short. It seems that the BDNF modifications are more associated to fine, transitory plastic changes.

Physical exercise in autistic-like rats has been shown to induce neurogenesis and increase reelin expression associated with ameliorated aggressive behavior and improved spatial learning memory (Seo et al., 2013, 2014). To the best of our knowledge, the relationship between paternal exercise, reelin expression and hippocampal neurogenesis in offspring has not been studied yet. In the present study, paternal exercise did not alter these biomarkers in the offspring hippocampus. However, Yin et al. (2013) found enhanced reelin expression accompanied by increased BDNF and spatial learning performance improvements in male C57BL/6J mice offspring from treadmill exercised fathers. It is important to note that the exercise regime adopted by Yin et al. (2013) consisted of 6 weeks, 60 min/d, 5 days per week at 75% VO₂max, quite different from that used in the current study (22 days, 20 min/d, 5 days per week at 60% VO₂max). Therefore, we might infer that this divergence could be related, at least in part, to previous findings suggesting that the effects of training may vary with exercise type, intensity and duration (Risedal et al., 1999; Ramsden et al., 2003; Elsner et al., 2011).

There is a growing body of evidence suggesting that maternal exercise during pregnancy can promote increased hippocampal neurogenesis associated with higher scores on memory tasks (Bick-Sander et al., 2006; Akhavan et al., 2013; Gomes Da Silva et al., 2016). In contrast, the current study, along with the findings reported by Mega and colleagues (2018), demonstrates that paternal exercise does not modulate hippocampal neurogenesis and BDNF levels in the brains of offspring. Taken together, these data led us to hypothesize that these neural benefits might be influenced more by maternal habits during pregnancy compared to paternal experiences before conception.

Another remarkable point to discuss is that our experimental aim was to observe the effects of paternal physical exercise *per se*; in this way, neither the father nor the offspring were subjected to any other unfavorable experimental conditions. It could be possible that the effects of the paternal exercise paradigm would have been more apparent if the exercise had been used to inhibit the intergenerational transmission of deleterious stimuli (Mega et al., 2018). Interestingly, paternal habits have been shown to be capable of reverting or ending the epigenetic inheritance of undesirable maternal behaviors. In a study conducted by Gapp et al. (2016), an enriched paternal environment prevented behavioral changes in the offspring from mothers who were exposed to unpredictable maternal separation and maternal stress. These behavioral changes were accompanied by increased glucocorticoid receptor expression and decreased DNA methylation of the glucocorticoid receptor promoter in the offspring hippocam-

pus. In the same way, paternal exercise also improved learning ability in male pup rats born from obese mothers, leading to an increase in cell differentiation and proliferation in the hippocampus, and the hippocampal expression of molecules involved in neuroplasticity, such as BDNF and the tyrosine kinase B receptor (Park and Kim, 2017).

Considering that germ cells are the likely vectors that transfer environmentally affected DNA methylation profiles to future generations, we also evaluated the effect of exercise on global DNA methylation in paternal sperm. The proposed paternal exercise protocol was not capable of inducing significant alterations in sperm global methylation or the epigenetic machinery. Denham et al. (2015) demonstrated that three months of running exercise in humans led to global sperm demethylation. Since sperm was the only possible source of paternal influence in our experimental design, we speculated on whether other epigenetic modifications, such as non-coding RNA or histone acetylation status could be involved in these offspring outcomes (Kim et al., 2015; Murashov et al., 2016; Short et al., 2017). Even though the present study demonstrated that paternal exercise was able to induce changes in the offspring, suggesting that paternal life experiences were somehow transmitted to the offspring, this was probably mediated by mechanisms other than global sperm DNA methylation.

Assuming that fetal programming during development depends on an intricate balance of maternal and paternal environmental influences (Curley et al., 2011; Rosa et al., 2013; Day et al., 2016), it is important to isolate the role of fathers interference on offspring development. Filling this knowledge gap may contribute to a better understanding of how individual life experiences before conception can modulate and program offspring development, resulting in phenotypes and behavioral traits that determine health and disease patterns throughout the life of the progeny. We chose to study the isolated role of paternal exercise on the offspring phenotype to highlight possible reprogramming *via* germ cells. In most mammalian species, the male parent does not care for the offspring; this behavior is practically exclusive to female progenitors. Thus, as neonatal care is also an important modulator of offspring behavioral traits, studies with only paternal interventions reveal the biological influence transmitted *via* gametes. Additionally, in the vast majority of studies on the effect of maternal exercise on offspring, physical exercise is performed during the gestational period. Exercise during the gestational period affects the uterine environment in which the fetus develops, in contrast to exercise during the pre-gestational period, which may influence parameters in the gametes. The influence of exercise on fetal development seems to have an effect on the offspring phenotype, but this does not exclude a role for pre-gestational interventions in parents in modulating offspring traits. It is important to emphasize that interventions in the mother can generate maternal behavioral changes that may influence the later care of the offspring. Thus, the offspring phenotype is a combination of exercise effects associated with behavioral changes in the mother. A study regarding the effects of interventions performed on mothers before preg-

nancy, in addition to the gestational period, on the programming of descendant phenotypes is also imperative. Taking this into consideration, further studies need to be conducted to show the effects of exposing both parents to physical activities on epigenetic modulation in the offspring brain.

In summary, the present study demonstrates that paternal treadmill exercise is capable of programming male offspring phenotype, specifically decreasing global DNA methylation levels in the hippocampus of male offspring. These data indicate that paternal physical exercise prior to conception modulates the epigenetic machinery in the brains of offspring, possibly positively interfering with offspring cognitive capacity.

One limitation of our study was that it only measured one epigenetic mark, *i.e.*, global DNA methylation. Thus, we recommend that future studies should consider the modulation of other parameters that could epigenetically respond to the effects of paternal exercise in the offspring hippocampus and paternal sperm; examples of other such parameters include histone H3 and H4 acetylation levels, modifications in histone methylation status and miRNA regulation, and the expression of specific genes. Furthermore, the utilization of inhibitors of DNA methylation (such as 5-Aza-2´-deoxycytidine) could also be considered by future studies aiming confirm the hypothesis of the role of DNA methylation in this issue. In addition, in this study we performed this analyses in whole hippocampus, however it is important to note that specific epigenetic evaluations of hippocampal subregions (*i.e.*, CA1, CA2 and CA3), and cell type specific (i.e. glial or neuronal) may be addressed by future studies. These findings might contribute to the elucidation of the exact epigenetic pathways that explain the relationship between paternal lifestyle factors, specifically the practice of exercise, and offspring neuroplasticity and behavior. Furthermore, the expression of other genes in the hippocampus not studied here may be potentially involved in the molecular mechanisms that facilitate learning; Arc (activity-regulated cytoskeletal gene) and zif268 (nerve growth factor inducible-A) are examples. Both genes play a central role in memory and synaptic plasticity (Gupta et al., 2010) and are thought to be epigenetically modulated (Cheng et al., 2015). Future studies into the intergenerational effects of parental exercise should consider this issue and should evaluate the impact of parental exercise on Arc and zif268 expression in the offspring hippocampus to elucidate the exact mechanisms mediating the observed cognitive improvements.

References

Akhavan MM, Miladi-Gorji H, Emami-Abarghoie M, Safari M, Sadighi-Moghaddam B, Vafaei AA, Rashidy-Pour A (2013) Maternal voluntary exercise during pregnancy enhances the spatial learning acquisition but not the retention of memory in rat pups via a TrkB-mediated mechanism: The role of hippocampal BDNF expression. Iran J Basic Med Sci 16:955-961.

Arida RM, Scorza FA, Da Silva SG, Cysneiros RM, Cavalheiro EA (2011) Exercise paradigms to study brain injury recovery in rodents. Am J Phys Med Rehabil 90:452-465.

Bale TL (2015) Epigenetic and transgenerational reprogramming of brain development. Nat Rev Neurosci 16:332-344.

Bick-Sander A, Steiner B, Wolf SA, Babu H, Kempermann G (2006) Running in pregnancy transiently increases postnatal hippocampal neurogenesis in the offspring. Proc Natl Acad Sci U S A 103:3852-3857.

Bielawski DM, Zaher FM, Svinarich DM, Abel EL (2002) Paternal alcohol exposure affects sperm cytosine methyltransferase messenger RNA levels. Alcohol Clin Exp Res 26:347-351.

Binder DK, Scharfman HE (2004) Brain-derived neurotrophic factor. Growth Factors 22:123-131.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. Anal Biochem 72:248-254.

Brooks GA, White TP (1978) Determination of metabolic and heart rate responses of rats to treadmill exercise. J Appl Physiol Respir Environ Exerc Physiol 45:1009-1015.

Cheng MC, Hsu SH, Chen CH (2015) Chronic methamphetamine treatment reduces synaptic plasticity genes expression and changes in their DNA methylation status in the mice brain. Brain Res 1629:126-134.

Curley JP, Mashoodh R, Champagne FA (2011) Epigenetics and the origins of paternal effects. Horm Behav 59:306-314.

da Silva IRV, de Araujo CLP, Dorneles GP, Peres A, Bard AL, Reinaldo G, Teixeira PJZ, Lago PD, Elsner VR (2017) Exercise-modulated epigenetic markers and inflammatory response in COPD individuals: A pilot study. Respir Physiol Neurobiol 242:89-95.

Day J, Savani S, Krempley BD, Nguyen M, Kitlinska JB (2016) Influence of paternal preconception exposures on their offspring: through epigenetics to phenotype. Am J Stem Cells 5:11-18.

Deichmann U (2016) Epigenetics: The origins and evolution of a fashionable topic. Dev Biol 416:249-254.

Denham J, Brien JO, Harvey JT, Charchar FJ (2015) Genome-wide sperm DNA methylation changes after 3 months of exercise training in humans. Epigenomics 8:717-731.

Dorneles GP, Boeira MCR, Schipper LL, Silva IRV, Elsner VR, Dal Lago P, Peres A, Romão PRT (2017) Acute strenuous exercise induces an imbalance on histone H4 acetylation/histone deacetylase 2 and increases the proinflammatory profile of PBMC of obese individuals. Oxid Med Cell Longev 2017:1530230.

Elsner VR, Basso C, Bertoldi K, de Meireles LCF, Cechinel LR, Siqueira IR (2017) Differential effect of treadmill exercise on histone deacetylase activity in rat striatum at different stages of development. J Physiol Sci 67:387-394.

Elsner VR, Lovatel GA, Bertoldi K, Vanzella C, Santos FM, Spindler C, de Almeida EF, Nardin P, Siqueira IR (2011) Effect of different exercise protocols on histone acetyltransferases and histone deacetylases activities in rat hippocampus. Neuroscience 192:580-587.

Elsner VR, Lovatel GA, Moysés F, Bertoldi K, Spindler C, Cechinel LR, Muotri AR, Siqueira IR (2013) Exercise induces age-dependent changes on epigenetic parameters in rat hippocampus: A preliminary study. Exp Gerontol 48:136-139.

Feng J, Fouse S, Fan G (2007) Epigenetic regulation of neural gene expression and neuronal function. Pediatr Res 61(5 Pt 2):58R-63R.

Fernandes J, Arida RM, Gomez-Pinilla F (2017) Physical exercise as an epigenetic modulator of brain plasticity and cognition. Neurosci Biobehav Rev 80:443-456.

Gapp K, Bohacek J, Grossmann J, Brunner AM, Manuella F, Nanni P, Mansuy IM (2016) Potential of environmental enrichment to prevent transgenerational effects of paternal trauma. Neuropsychopharmacology 41:2749-2758.

Gomes Da Silva S, De Almeida AA, Fernandes J, Lopim GM, Cabral FR, Scerni DA, De Oliveira-Pinto AV, Lent R, Arida RM (2016) Maternal exercise during pregnancy increases BDNF levels and cell numbers in the hippocampal formation but not in the cerebral cortex of adult rat offspring. PLoS One 11:1-15.

Gomez-Pinilla F, Zhuang Y, Feng J, Ying Z, Fan G (2011) Exercise impacts brain-derived neurotrophic factor plasticity by engaging mechanisms of epigenetic regulation. Eur J Neurosci 33:383-390.

Gupta S, Kim SY, Artis S, Molfese DL, Schumacher A, Sweatt JD, Paylor RE, Lubin FD (2010) Histone methylation regulates memory formation. J Neurosci 30:3589-3599.

Herz J, Chen Y (2006) Reelin, lipoprotein receptors and synaptic plasticity. Nat Rev Neurosci 7:850-859.

Karpova NN (2014) Role of BDNF epigenetics in activity-dependent neuronal plasticity. Neuropharmacology 76:709-718.

Kim SK, Jee BC, Kim SH (2015) Histone methylation and acetylation in ejaculated human sperm: Effects of swim-up and smoking. Fertil Steril 103:1425-1431.

Kouzarides T (2007) Chromatin modifications and their function. Cell 128:693-705.

Lavratti C, Dorneles G, Pochmann D, Peres A, Bard A, de Lima Schipper L, Dal Lago P, Wagner LC, Elsner VR (2017) Exercise-induced modulation of histone H4 acetylation status and cytokines levels in patients with schizophrenia. Physiol Behav 168:84-90.

Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462:315-322.

Lovatel GA, Elsner VR, Bertoldi K, Vanzella C, Moysés F dos S, Vizuete A, Spindler C, Cechinel LR, Netto CA, Muotri AR, Siqueira IR (2013) Treadmill exercise induces age-related changes in aversive memory, neuroinflammatory and epigenetic processes in the rat hippocampus. Neurobiol Learn Mem 101:94-102.

Lu B, Figurov A (1997) Role of neurotrophins in synapse development and plasticity. Rev Neurosci 8:1-12.

Maejima H, Kanemura N, Kokubun T, Murata K, Takayanagi K (2018) Exercise enhances cognitive function and neurotrophin expression in the hippocampus accompanied by changes in epigenetic programming in senescence-accelerated mice. Neurosci Lett 665:67-73.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 20:9104-9110.

Marcuzzo S, Dutra MF, Stigger F, do Nascimento PS, Ilha J, Kalil-Gaspar PI, Achaval M (2010) Different effects of anoxia and hind-limb immobilization on sensorimotor development and cell numbers in the somatosensory cortex in rats. Brain Dev 32:323-331.

McGee SL, Fairlie E, Garnham AP, Hargreaves M (2009) Exercise-induced histone modifications in human skeletal muscle. J Physiol 587:5951-5958.

Mega F, de Meireles ALF, Piazza FV, Spindler C, Segabinazi E, dos Santos Salvalaggio G, Achaval M, Marcuzzo S (2018) Paternal physical exercise demethylates the hippocampal DNA of male pups without modifying the cognitive and physical development. Behav Brain Res 348:1-8.

Miller CA, Campbell SL, Sweatt JD (2008) DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. Neurobiol Learn Mem 89:599-603.

Murashov AK, Pak ES, Koury M, Ajmera A, Jeyakumar M, Parker M, Williams O, Ding J, Walters D, Neufer PD (2016) Paternal long-term exercise programs offspring for low energy expenditure and increased risk for obesity in mice. FASEB J 30:775-784.

Mychasiuk R, Harker A, Ilnytskyy S, Gibb R (2013) Paternal stress prior to conception alters DNA methylation and behaviour of developing rat offspring. Neuroscience 241:100-105

Mychasiuk R, Zahir S, Schmold N, Ilnytskyy S, Kovalchuk O, Gibb R (2012) Parental enrichment and offspring development: Modifications to brain, behavior and the epigenome. Behav Brain Res 228:294-298.

Nowakowski RS, Lewin SB, Miller MW (1989) Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA- synthetic phase for an anatomically defined population. J Neurocytol 18:311-318.

Park H, Kim T (2017) Paternal physical exercise improves spatial learning ability by enhancing hippocampal neuroplasticity in male pups born from obese maternal rats. J Exerc Rehabil 13:266-272.

Parnpiansil P, Jutapakdeegul N, Chentanez T, Kotchabhakdi N (2003) Exercise during pregnancy increases hippocampal brain-derived neurotrophic factor mRNA expression and spatial learning in neonatal rat pup. Neurosci Lett 352:45-48.

Paxinos G, Watson C (1982) The rat brain in stereotaxic coordinates, 2nd ed. Sydney: Academic Press.

Pereira LO, Arteni NS, Petersen RC, da Rocha AP, Achaval M, Netto CA (2007) Effects of daily environmental enrichment on memory deficits and brain injury following neonatal hypoxia-ischemia in the rat. Neurobiol Learn Mem 87:101-108.

Piazza FV, Segabinazi E, Centenaro LA, do Nascimento PS, Achaval M, Marcuzzo S (2014) Enriched environment induces beneficial effects on memory deficits and microglial activation in the hippocampus of type 1 diabetic rats. Metab Brain Dis 29:93-104.

Poo MM (2001) Neurotrophins as synaptic modulators. Nat Rev Neurosci 2:24-32.

Ramsden M, Berchtold NC, Patrick Kesslak J, Cotman CW, Pike CJ (2003) Exercise increases the vulnerability of rat hippocampal neurons to kainate lesion. Brain Res 971:239-244.

Risedal A, Zeng J, Johansson BB (1999) Early training may exacerbate brain damage after focal brain ischemia in the rat. J Cereb Blood Flow Metab 19:997-1003.

Rosa BV, Blair HT, Vickers MH, Dittmer KE, Morel PC, Knight CG, Firth EC (2013) Moderate exercise during pregnancy in Wistar rats alters bone and body composition of the adult offspring in a sex-dependent manner. PLoS One 8:e82378.

Seo DY, Lee SR, Kim N, Ko KS, Rhee BD, Han J (2014) Humanized animal exercise model for clinical implication. Pflugers Arch 466:1673-1687.

Short AK, Yeshurun S, Powell R, Perreau VM, Fox A, Kim JH, Pang TY, Hannan AJ (2017) Exercise alters mouse sperm small noncoding RNAs and induces a transgenerational modification of male offspring conditioned fear and anxiety. Transl Psychiatry 7:e1114.

Spindler C, Cechinel LR, Basso C, Moysés F, Bertoldi K, Roesler R, Lovatel GA, Rostirola Elsner V, Siqueira IR (2014) Treadmill exercise alters histone acetyltransferases and histone deacetylases activities in frontal cortices from Wistar rats. Cell Mol Neurobiol 34:1097-1101.

Sui L, Li BM (2010) Effects of perinatal hypothyroidism on regulation of reelin and brain-derived neurotrophic factor gene expression in rat hippocampus: Role of DNA methylation and histone acetylation. Steroids 75:988-997.

Sui L, Wang Y, Ju LH, Chen M (2012) Epigenetic regulation of reelin and brain-derived neurotrophic factor genes in long-term potentiation in rat medial prefrontal cortex. Neurobiol Learn Mem 97:425-440.

Taupin P (2007) Brdu immunohistochemistry for studying adult neurogenesis: Paradigms, pitfalls, limitations, and validation. Brain Res Rev 53:198-214.

Vaynman S, Ying Z, Gomez-Pinilla F (2003) Interplay between brain-derived neurotrophic factor and signal transduction modulators in the regulation of the effects of exercise on synaptic-plasticity. Neuroscience 122:647-657.

Veena J, Srikumar BN, Mahati K, Bhagya V, Raju TR, Rao BS (2009) Enriched environment restores hippocampal cell proliferation and ameliorates cognitive deficits in chronically stressed rats. J Neurosci Res 87:831-843.

Venezia AC, Guth LM, Spangenburg EE, Roth SM (2015) Lifelong parental voluntary wheel running increases offspring hippocampal Pgc-1α mRNA expression but not mitochondrial content or Bdnf expression. Neuroreport 26:467-472.

Xavier LL, Viola GG, Ferraz AC, Da Cunha C, Deonizio JMD, Netto CA, Achaval M (2005) A simple and fast densitometric method for the analysis of tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta and in the ventral tegmental area. Brain Res Protoc 16:58-64.

Yin MM, Wang W, Sun J, Liu S, Liu XL, Niu YM, Yuan HR, Yang FY, Fu L (2013) Paternal treadmill exercise enhances spatial learning and memory related to hippocampus among male offspring. Behav Brain Res 253:297-304.

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