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EFEITOS DO EXERCÍCIO FÍSICO PARENTAL SOBRE PARÂMETROS COMPORTAMENTAIS E DE NEUROPLASTICIDADE EM FILHOTES DE RATOS WISTAR

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Tese apresentada ao Programa de Pós-Graduação em Neurociências do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutor em Neurociências.

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SUMÁRIO

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RESUMO

O encéfalo pode sofrer alterações funcionais e morfológicas em resposta às mudanças do ambiente. Esta capacidade, denominada neuroplasticidade, pode exercer um importante papel nos processos de adaptação e reorganização do microambiente encefálico, resultando em padrões de comportamento distintos. Evidências clínicas e experimentais demonstraram que condições tanto maternas quanto paternas podem interferir no processo de neuroplasticidade nos descendentes, predispondo o desenvolvimento de doenças ou promovendo efeitos benéficos na prole após o nascimento e na vida adulta. Nesta tese investigamos os efeitos de protocolos de exercício físico em esteira ergométrica adaptada para ratos sobre marcadores de neuroplasticidade e comportamentais na prole. Ratos Wistar adultos machos e fêmeas foram utilizados e divididos em dois estudos: (1) paterno e (2) materno. Os ratos machos foram divididos em dois grupos experimentais, sendo um grupo exercitado (22 dias, 5 vezes por semana, intensidade 60%VO2, durante 20 minutos) e outro sedentário (animais expostos à esteira ergométrica desligada pelo mesmo tempo que o grupo exercitado), neste estudo as fêmeas foram mantidas totalmente sedentárias. A prole destes animais foi dividida em dois grupos experimentais, de acordo com o protocolo paterno (EXE) para os descendentes de pais exercitados e (SED) para os descendentes de pais sedentários. Os níveis de metilação global de DNA no esperma dos pais foram analisados. Parâmetros de neuroplasticidade (BDNF, Relina e sobrevivência celular) e epigenética (metilação global de DNA) foram medidos nos hipocampos dos filhotes. Os marcos do desenvolvimento dos filhotes foram observados. Testes comportamentais de desempenho físico e memória espacial foram realizados nos filhotes adultos. Houve uma melhora do aprendizado espacial nos filhotes do grupo EXE, acompanhada de uma diminuição dos níveis de metilação global do DNA no hipocampo. Os animais não apresentaram alteração nos marcos do desenvolvimento. Nenhum dos marcadores neuroplásticos apresentou diferença estatística entre os grupos estudados. Não houve diferença nos níveis de metilação no esperma dos pais. Na segunda parte deste trabalho, a prole das ratas fêmeas exercitadas e dos pais sedentários foi dividida em 4 grupos, conforme o período de treino materno: sedentárias antes e durante a gestação (SS); sedentárias antes e exercitadas durante a gestação (SE), exercitadas antes e sedentárias na gestação (ES) e por fim, exercitadas nos dois períodos. Os mesmos parâmetros avaliados no estudo paterno foram realizados nos filhotes. Nehuma diferença nos marcos do desenvolvimento foi observada. Todos os grupos descendentes de mães exercitadas, SE, ES e EE apresentaram melhores escores de aprendizado em relação ao grupo SS. Os grupos SE e ES apresentaram maior proliferação celular e conteúdo de relina hipocampal. Somente o grupo ES apresentou diminuição significativa nos níveis de metilação de DNA hipocampal em relação ao grupo SS. Conclui-se que o exercício físico, tanto materno quanto paterno, foi capaz de gerar uma programação no encéfalo da prole, aumentando a capacidade de aprendizado. Ainda os protocolos maternos pré- gestacional e gestacional se mostraram seguros para o desenvolvimento da prole e devem ser estimulados. Além disso o exercício paterno se mostrou uma experiência positiva uma vez que gerou uma melhora cognitiva na prole.

ABSTRACT

The brain is vulnerable to functional and morphological alterations in response do environmental changes. This capacity, know as neuroplasticity, can play an important role in adaptions and reorganization encephalic microenvironment. Clinical and preclinical evidences shown that both paternal and maternal experiences can modulate the neuroplasticity process on the offspring, increasing the risk of diseases or generating positive effects on descendants. Here, we investigate the effects of treadmill running protocols in neuroplasticity and behavioral markers on the progenies. Adult male and female Wistar rats were divided into two studies: (1) Paternal and (2) Maternal. In the first study of this thesis we divided the male rats into two groups: Exercised (EXE) (20 minutes daily, 5 consecutive days per week for 22 days) and sedentary (SED), while the mothers were not trained. After mating, paternal sperm was collected for global DNA methylation analysis. The development milestones were observed. At postnatal day 53, the offspring were euthanized, and the hippocampus was dissected to measure cell survival by 5-bromo-2′-deoxiuridine (BrdU) 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. No differences in developmental milestones were observed. 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 cell survival. Finally, the global DNA methylation of paternal sperm was not significantly changed by physical exercise. In the second part of this thesis, we evaluated the effects of maternal aerobic exercise initiated before and maintained during gestation, or performed in these isolated periods, on cognition and plasticity in the hippocampus of offspring. Groups of male pups were categorized by the exposure of their mothers to: treadmill off (sedentary, SS), pregestational exercise (ES), gestational exercise (SE) or combined protocols (EE). The analysis of offspring cognition and plasticity in the hippocampus followed the same experimental design described at paternal work. The offspring from ES, SE and EE mothers demonstrated improved spatial learning compared to SS, but hippocampal DNA methylation was significantly modified only in the offspring from ES mothers. The offspring from ES and SE mothers presented higher number of BrdU+ and reelin+ hippocampal cells than EE and SS. No differences were observed in the BDNF levels among the groups. The maternal pregestational and gestational isolated exercise protocols showed similar effects for offspring plasticity and spatial cognitive ability, while the combined protocol simply improved their spatial learning. Interestingly, only pregestational exercise was able to induce plasticity in the offspring hippocampus associated with modulation of global DNA methylation. In conclusion, we demonstrated that both maternal and paternal treadmill running were capable of program the offspring brain, increasing scores of spatial learning and neuroplastic parameters. Furthermore, these positive outputs are linked to a modulation of global DNA methylation in hippocampus of offspring.

APRESENTAÇÃO

A presente tese teve como objetivo avaliar os efeitos do exercício físico parental sobre parâmetros comportamentais e de neuroplasticidade em filhotes de ratos Wistar. Os resultados deste estudo estão apresentados sob a forma de artigos científicos e foram divididos em dois capítulos.

No capítulo I encontra-se o primeiro artigo intitulado: *Paternal physical exercise modulates global DNA methylation status inhippocampus of male pups.*

No capítulo II encontra-se segundo artigo intitulado: *Effects of maternal physical exercise on global DNA methylation and hippocampal plasticity of rat male offspring.*

LISTA DE ABREVIATURAS

5-mc: 5- metilcitosina

ACOG: Colégio Americano de Geriatria e Obstetrícia

ANOVA: Análise de variância

ApoER2 :receptor de Apolipoproteína E – 2

BDNF: Fator Neurotrófico Derivado do Encéfalo

BrdU: Bromodesoxiuridina

BSA: Albumina sérica bovina

CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico

CREAL: Centro de Reprodução de Animais de Laboratório

DAB: Diaminobenzidina

DNA: Ácido Desoxiribonucleico

DNMT: DNA metil transferase

EE: Grupo experimental descendentes de fêmeas exercitadas em ambos os períodos, pré gestacional e gestacional

ES: Grupo experimental descendentes de fêmeas exercitadas antes da gestação e mantidas sedentárias no período gestacional

FAPERGS: Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul

GCL: Camada celular granular

H3: Histona H3

H4: Histona H4

HAT: Histona acetiltransferase

HDAC: Histona desacetilase

I.P.: Intraperitoneal

IGF-1: Fator de crescimento semelhante à Insulina tipo 1

IgG: Imunoglobulina G

LTP: Potenciação de longa duração

M: Molar

Min: Minutos

ml: Mililitro

mm: Milímetros

ng: Nanograma

ºC: Celsius

PBS: Tampão Fosfato-Salino

pg: Picograma

pH: Potencial De Hidrogênio

RNA: Ácido Ribonucleico

S: Segundos

SE: Grupo experimental descendentes de fêmeas que foram mantidas sedentárias antes da gestação e exercitadas durante o período gestacional

SGZ: Zona subgranular

SS: Grupo experimental descendentes de fêmeas que foram mantidas sedentárias antes e durante a gestação

TRKB: Receptor Tirosina Quinase B

UFRGS: Universidade Federal do Rio Grande do Sul

VLDLR: Receptor de lipoproteína de muito baixa densidade

VO_{2max}: Volume máximo de oxigênio

µm: Micrômetro

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INTRODUÇÃO

O encéfalo pode sofrer alterações funcionais e morfológicas em resposta às mudanças do ambiente. Esta capacidade, denominada neuroplasticidade, pode exercer um importante papel nos processos de adaptação e reorganização do microambiente encefálico, resultando em padrões de comportamento distintos (Keenan et al., 2018; Lindsay et al., 2018). Embora o encéfalo apresente neuroplasticidade durante toda a vida, alguns períodos do seu desenvolvimento são considerados de maior vulnerabilidade, desencadeando importantes mudanças morfológicas, bioquímicas e comportamentais. Os períodos intrauterino, perinatal, neonatal e a puberdade são exemplos destas janelas de vulnerabilidade. Experiências negativas podem favorecer o desenvolvimento de doenças. Por outro lado, um ambiente saudável pode promover neuroproteção e proporcionar melhores respostas contextuais a eventos estressores ou adversidades (Raineki et al., 2016). Evidências clínicas e experimentais demonstraram que condições tanto maternas quanto paternas podem interferir no processo de neuroplasticidade nos descendentes, predispondo o desenvolvimento de doenças ou promovendo efeitos benéficos na prole após o nascimento e na vida adulta (Bale et al., 2010; Lindsay et al., 2018).

Fatores nutricionais maternos estão associados ao desenvolvimento de doenças cardiovasculares e metabólicas nos filhos (Painter et al., 2008). Estudos experimentais mostraram que a restrição calórica durante a gestação pode alterar o eixo hipotálamo-hipófise-adrenal dos filhotes. Essas alterações modificam a expressão e a função de receptores de glicocorticóides favorecendo o desenvolvimento da obesidade e da síndrome metabólica nos filhotes (Correia-branco et al., 2014). A obesidade materna e a dieta rica em gorduras representam importantes fatores de risco para o desenvolvimento de doenças como o transtorno do déficit de atenção e hiperatividade, esquizofrenia, transtornos depressivos, de ansiedade e reduzem a capacidade de aprendizado dos filhotes (Bilbo and Tsang, n.d.; Ramírez et al., 2012; Sullivan et al., 2016). Outro fator ambiental estudado durante a gestação é o tabagismo. A exposição à fumaça de cigarro em ratas prenhas resultou em atraso no desenvolvimento motor e cognitivo na vida pós-natal dos filhotes(Abbott et al., 2012). Esta condição também foi associada com redução da fertilidade, alterações respiratórias, baixo peso ao nascer e síndrome da morte súbita (Tehranifar et al., 2017). Da mesma maneira que o tabagismo, o consumo de álcool durante a gestação afeta negativamente a saúde da prole, podendo causar baixo peso ao nascer, síndrome alcoólica fetal e aumento do risco de prematuridade (Lundsberg et al., 2016; Martínez-galiano et al., 2018). Embora as condições de saúde materna sejam fundamentais nos desfechos fisiopatológicos e comportamentais da prole, um destaque também tem sido dado às condições paternas (Curley et al., 2011).

Estudos avaliaram a relação do estilo de vida paterno sobre o desenvolvimento da prole, indicando que as experiências dos machos, como a idade, a dieta, a exposição a drogas de abuso e toxinas, podem induzir alterações transgeracionais, celulares e fenotípicas, nos filhos e também nos netos (Curley et al., 2011). Em combinação com esses efeitos paternos diretos, a experiência do macho antes do acasalamento pode influenciar na preferência da fêmea no momento do acasalamento e no cuidado materno (Figura 1). Estes fatores ambientais podem desempenhar um papel significativo na fisiopatologia das doenças crônicas da prole na vida adulta, como resistência à insulina e diabetes, doenças cardiovasculares e redução da sobrevida. Por exemplo, a privação alimentar em ratos machos, antes do acasalamento, induziu alterações em níveis séricos de glicose, corticosterona e hormônio do crescimento semelhante a Insulina tipo 1 (IGF-1) nos filhotes (Anderson et al., 2006). De forma semelhante, a exposição de ratos machos à uma dieta pobre em proteínas aumentou o risco de doenças metabólicas e cardiovasculares na prole (Watkins and Sinclair, 2018). Além disso, filhotes de ratos machos obesos, quando avaliados na vida adulta, apresentam maior probabilidade de desenvolver síndrome metabólica (Ng et al., 2010). Os efeitos neurotóxicos do álcool e tabaco também foram estudados e demonstraram capacidade de induzir alterações transgeracionais, a utilização de álcool ou tabaco pelos pais geraram descendentes com prejuízos no desempenho em testes de memória, aprendizado, função motora, redução do volume cortical e maior taxa de mortalidade (Abel, 2004; Mcpherson and Aitken, 2014).

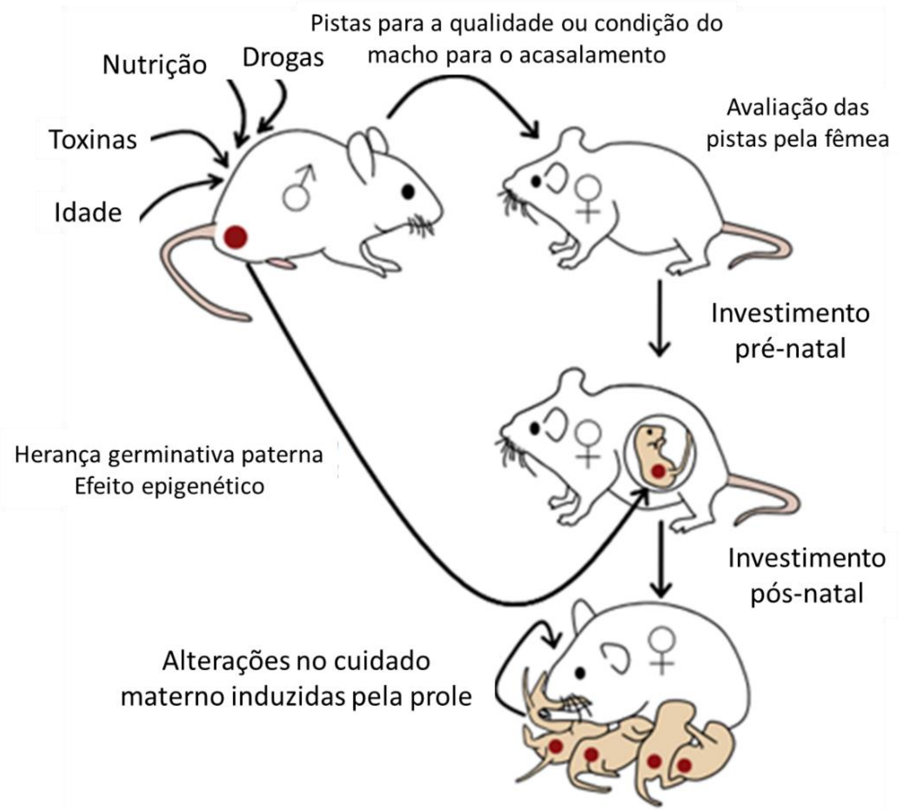

Figura 1. Influência dos fatores de exposição do macho sobre a prole após o nascimento. Adaptado de Curley et al., 2011.

Além dos estudos evidenciando a influência de experiências negativas, tanto maternas quanto paternas, no desenvolvimento de doenças na prole, experiências positivas capazes de promover neuroproteção e minimizar efeitos deletérios gerados por insultos durante este período também têm sido realizadas. Ênfase tem sido dada sobre os efeitos benéficos do exercício físico sobre parâmentros moleculares e comportamentais na prole.

Exercício físico

Os efeitos benéficos do exercício físico foram demonstrados em inúmeros estudos nas últimas décadas e ainda são alvos de pesquisas recentes devido ao seu potencial na prevenção e tratamento de doenças. Além disso, o baixo custo e o fácil acesso são fatores importantes na promoção da saúde da população. Estudos prospectivos fornecem evidências diretas de que um estilo de vida fisicamente ativo reduz a mortalidade por todas as causas e diminui 40% da mortalidade por doenças cardiovasculares (Green and Loprinzi, 2018). Além disso, a prática de exercício físico está associada a redução do risco do desenvolvimento de doenças neurodegenerativas, endócrinas, depressão e obesidade (Intlekofer and Cotman, 2013; Ren et al., 2018) Indivíduos exercitados apresentam melhor função cognitiva e motora comparado a sedentários (Duzel et al., 2016; Gomez-pinilla and Hillman, 2013). Estudos experimentais mostraram que o exercício é capaz de melhorar o desempenho de animais em diferentes paradigmas comportamentais. Protocolos de exercício forçado como natação e corrida em esteira ergométrica foram capazes de induzir melhoras na memória espacial e aversiva em roedores(Alomari et al., 2013; Lovatel et al., 2013). A capacidade de aprendizado também foi aprimorada, uma vez que ratos exercitados apresentaram uma curva de aprendizado maior comparado aos sedentários no teste do labirinto aquático (Sampedro- Taati et al., 2014).

A prática de exercício físico durante a gestação foi negligenciada por muitos anos. Acreditava-se que o exercício poderia aumentar o risco de prematuridade devido ao aumento da atividade uterina. Recentemente, destaca-se a importância da atividade física regular em gestantes, tanto para a saúde da mulher como para a do bebê (American College of Obstetricians and Gynecologists, 2015). Um número cada vez maior de evidências demonstra que, na ausência de contraindicações médicas, o exercício aeróbico regular de intensidade moderada (como a caminhada diária por mais de 30 minutos) é benéfico para a saúde materna (Seneviratne et al., 2014).Exercícios regulares têm sido associados à redução do risco de ganho excessivo de peso durante a gestação, o desenvolvimento de diabetes gestacional e hipertensão (Barakat et al., 2016; Muktabhant et al., 2015). Além disso, filhos de mulheres que se exercitaram durante a gravidez tiveram melhor desempenho cognitivo geral e nas habilidades de linguagem comparado a crianças de mães sedentárias (Fernandes et al., 2017). Estudos experimentais mostraram que diferentes protocolos de exercícios realizados durante a gestação afetam as funções encefálicas nos filhotes. Ratas submetidas a exercício tanto forçado quanto voluntário durante a gestação tiveram filhotes com melhores escores em testes de aprendizado, memória espacial e reconhecimento de objetos, comparados a filhotes das ratas sedentárias (Dayi et al., 2012a; Robinson and Bucci, 2014).

Além dos efeitos do exercício durante o período gestacional, as experiências pré-gestacionais dos progenitores parecem ter um papel importante no desenvolvimento da prole. Embora existam poucos estudos, algumas evidências sugerem que a prática de exercício prévio à gestação, tanto materna quanto paterna, está associada a efeitos benéficos sobre a prole.

Foi demonstrado que filhos de mulheres que se exercitaram no período pré-gestacional apresentam escores acadêmicos superiores aos filhos de mães sedentárias (Esteban-Cornejo et al., 2016). O exercício pré-gestacional também foi associado ao aumento no metabolismo mitocondrial e reprogramação epigenética de embriões de roedores. Melhora da função cognitiva associada a neurogênese hipocampal e aumento dos níveis de fatores neurotróficos e de plasticidade foram observadas em filhotes de ratas exercitadas durante a gestação (Dayi et al., 2012b; Gomes Da Silva et al., 2016; Herring et al., 2012).

A influência transgeracional das experiências paternas sobre o fenótipo da prole, também têm sido estudada (Short et al., 2017). Dados recentes do nosso grupo de pesquisa demonstraram que exercício de corrida em esteira por 8 semanas, realizado por ratos machos antes do acasalamento, foi capaz de promover alterações moleculares evidenciadas pela diminuição da metilação global no hipocampo dos filhotes. No entanto, essas alterações bioquímicas não foram acompanhadas por mudanças comportamentais nos testes de memória e de desenvolvimento físico (Mega et al., 2018). Corroborando com estes achados, Yin e colaboradores (2013) mostraram que exercício físico de corrida em esteira ergométrica, realizado somente nos camundongos machos, foi capaz de gerar alterações no encéfalo dos filhotes. O exercício foi capaz de melhorar o desempenho motor, o aprendizado e a memória espacial associado ao aumento dos níveis de fatores neurotróficos e de plasticidade no hipocampo dos filhotes. Além disso, filhotes de camundongos machos submetidos a exercício voluntário por 4 semanas antes do acasalamento apresentaram menos reação de medo condicionado e de ansiedade do que os filhotes de ratos sedentários (Short et al., 2017). Ainda, um estudo com modelo de obesidade paterna em camundongo mostrou que os filhotes desenvolveram hiperlipidemia, intolerância à glicose, insensibilidade à insulina e alteração da expressão de micro RNA´s pancreáticos. Quando os camundongos obesos foram submetidos a exercício de natação e exercicio voluntário, antes do acasalamento, houve melhora em todos esses parâmetros (McPherson et al., 2017; Krout et al., 2018).

Os mecanismos de ação pelos quais o exercicio fisico exerce seus efeitos benéficos não estão totalmente esclarecidos. Sabe-se que o exercício aumenta os níveis de fator neurotrófico derivado do encéfalo (BDNF, do inglês *Brain-derived Neurotrophic Factor*), promove neuroplasticidade e neurogênese (Jeon and Ha, 2017). Também foi observado que exercício físico pode modular mecanismos epigenéticos, promovendo mudanças na metilação do DNA e na acetilação de histonas no encéfalo (Fernandes et al., 2017).

O BDNF é uma neurotrofina e sua expressão é regulada por diversos estímulos como o exercício físico, a dieta e o estresse (Giacobbo et al., 2018). A ação do BDNF se dá pela sua ligação com o receptor de tirosina quinase B (*TrkB*, do inglês *receptor tyrosine kinase B*) desencadeando ativação de cascatas de sinalização intracelular. Este fator neurotrófico é abundante no encéfalo e está relacionado com a sobrevivência e a proliferação neuronal. Além disso, mecanismos de neuroplasticidade têm sido associados ao aumento do BDNF assim como modulação de neurotransmissores e melhora da memória e do aprendizado (Giacobbo et al., 2018; Hesari and Sales, 2013).

Diferentes protocolos de exercício físico, como a natação, a corrida na roda de livre acesso e a corrida em esteira ergométrica, podem alterar os níveis de BDNF, induzindo aumento da sua expressão no hipocampo de animais (Hopkins and Bucci, 2010; Jeon and Ha, 2017). Entretanto, as respostas induzidas pelo exercício físico podem variar de acordo com as especificidades de cada protocolo, uma vez que alguns protocolos de exercício físico não apresentaram efeito sobre a expressão desta neurotrofina (Ferreira et al., 2011). Assim, embora o BDNF esteja bem estabelecido na promoção da neuroplasticidade, o seu papel como mecanismo de ação dos efeitos benéficos do exercício precisa ser melhor explorado.

Outra molécula importante implicada nos efeitos positivos sobre memória e aprendizado e que pode ser modulada pelo exercício físico é a relina. Trata-se de uma glicoproteína da matriz extracelular, secretada principalmente por neurônios denominados Cajal-Retzius na zona marginal do córtex e do hipocampo. A sua ação é mediada pelos receptores de lipoproteína de baixa densidade (VLDLR, do inglês *very low–density lipoprotein receptor*) e de apolipoproteína E2 (ApoER2). A ativação dos receptores ativa uma cascata intracelular que promove a sinalização dos neurônios para locais em desenvolvimento (Hirota and Nakajima, 2017). A relina apresenta um papel crucial na plasticidade sináptica, no controle da migração neuronal e na formação das camadas corticais durante o desenvolvimento do encéfalo (Pujadas et al., 2010). No sistema nervoso adulto, a relina é expressa e liberada especialmente por neurônios gabaérgicos e sua ação está associada a densidade pós-sináptica, formação de espinhos dendríticos e plasticidade hipocampal (Rogers et al., 2011). A deficiência desta glicoproteína e de seus receptores pode induzir prejuízos na memória espacial e associativa, deterioração da potenciação de longa duração e alterações morfológicas nos espinhos dendríticos (Rogers et al., 2011). Estudos sugerem que o exercício físico pode influenciar a síntese e a liberação desta glicoproteína no hipocampo, no entanto, sua modulação é dependente do tipo de protocolo. Um estudo com roedores mostrou que corrida em esteira, por 28 dias, aumentou os níveis de relina associado à melhora no desempenho em teste de memória espacial (Seo et al., 2013). Enquanto outro estudo, com protocolo de corrida em esteira por 14 dias, não apresentou aumentos significativos na expressão desta glicoproteína.

Mecanismos epigenéticos

A atuação dos mecanismos epigenéticos na regulação dos eventos celulares e moleculares vem sendo cada vez mais estudada e compreendida. Hipóteses sobre o funcionamento do DNA e a regulação da expressão gênica têm sido exploradas, e a epigenética passou a ser considerada um ponto chave nesse processo. As interações epigenéticas que controlam a expressão gênica podem ocorrer no DNA e na cromatina e são responsáveis pela ativação e repressão dos genes. A cromatina é um complexo formado por DNA e proteínas, denominadas histonas, mudanças na sua estrutura podem influenciar na expressão gênica. (Bird, 2007). Quando a estrutura da cromatina está menos condensada a transcrição de genes do DNA é facilitada e quando a estrutura se encontra mais condensada, a transcrição é reprimida. Essas alterações podem ser induzidas por processos de acetilação, metilação, ubiquinação e fosforilação.

A acetilação das histonas é controlada pelas enzimas denominadas histona acetil transferase (HAT) e a histona desacetilase (HDAC). As HATs catalisam a adição de um grupo acetil às histonas, promovendo a diminuição da força de ligação com o DNA. Esta reação torna a estrutura da cromatina menos condensada e consequentemente facilita o processo de transcrição gênica. Por outro lado, as HDAC´s retiram o grupamento acetil, promovendo o processo inverso e tornando o DNA mais condensado, tornando mais difíceis os processos de transcrição gênica (Puckett and Lubin, 2011; Figura 2).

A metilação do DNA é caracterizada pela adição de um grupo metil ao nucleotídeo da citosina, formando a 5-metilcitosina (Horsburgh et al., 2015). Esse processo ocorre em locais específicos, chamados ilhas CpGs, que são pequenas sequências de citosina e guanina dispostas ao longo da sequência de DNA. A metilação das citosinas do DNA normalmente reprime a transcrição gênica e indiretamente leva a condensação da cromatina, o que facilita essa repressão. Esse processo é controlado por uma família de enzimas denominadas DNA-metil-transferases (Denham et al., 2013; Puckett et al., 2011; Figura 2).

Os mecanismos epigenéticos participam das funções encefálicas, estando implicadas em processos de formação de sinapses, LTP e memória (Puckett et al., 2011; Figura 2). As alterações epigenéticas podem ser induzidas por vários fatores ambientais, entre eles destaca-se o exercício físico. Estudos sugerem que as alterações epigenéticas, incluindo a acetilação de histonas e a metilação do DNA, induzidas pelo exercício apresentam um papel importante na regulação da plasticidade sináptica, aprendizagem e memória (Abel and Rissman, 2013; Intlekofer and Cotman, 2013). O aumento da acetilação de histonas foi observado em animais submetidos a protocolos de exercício de corrida em esteira por uma sessão e por 2 semanas. Este resultado foi acompanhado por melhora na memória dos animais (Elsner et al., 2011; Lovatel et al., 2013). Exercício de corrida em esteria também foi capaz de regular a metilação do DNA através da modulação das enzimas metiltransferases (Elsner et al., 2013)

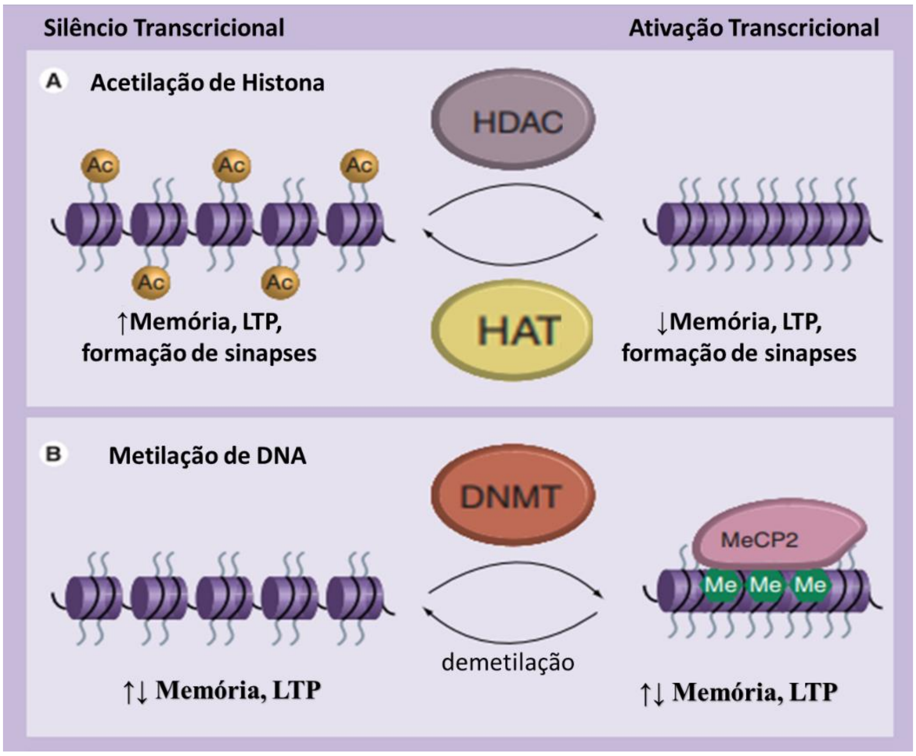

Figura 2. Mecanismos epigenéticos de modulação da transcrição gênica (Adaptado de Puckett et al., 2011). DNMT: DNA metiltransferase; HAT: Histona acetiltransferase; HDAC: Histona deacetslase.

Sabe-se que os fatores ambientais, tanto maternos quanto paternos, são determinantes para o desenvolvimento encefálico saudável da prole. No entanto, poucos estudos têm sido conduzidos afim de explorar os efeitos do exercício físico e seus mecanismos de ação em filhotes de pais exercitados antes e durante a gestação. Considerando a importância da prática do exercício maternos e paternos e as lacunas na literatura sobre seus efeitos comportamentais e bioquímicos sobre o encéfalo em desenvolvimento, o presente estudo buscou investigar se o exercício paterno antes do acasalamento bem como materno antes e durante a gestação pode afetar o comportamento e ou promover alterações bioquímicas no encéfalo dos filhotes.

OBJETIVOS

Objetivo geral

Investigar os efeitos do exercício físico parental sobre parâmetros comportamentais e de neuroplasticidade na prole de ratos Wistar.

Objetivos específicos

- Avaliar o efeito do exercício físico paterno antes do acasalamento e materno nos períodos pré-gestacional e gestacional sobre o peso corporal e o desenvolvimento sensório-motor dos filhotes;

- Avaliar o efeito do exercício físico paterno antes do acasalamento e materno nos períodos pré-gestacional e gestacional sobre a memória espacial nos filhotes;

- Avaliar o efeito do exercício físico paterno antes do acasalamento e materno nos períodos pré-gestacional e gestacional sobre a o desempenho físico (VO_{2max}) dos filhotes;

- Avaliar o efeito do exercício físico paterno antes do acasalamento e materno nos períodos pré-gestacional e gestacional sobre a neurogênese hipocampal nos filhotes;

- Avaliar o efeito do exercício físico paterno antes do acasalamento e materno nos períodos pré-gestacional e gestacional sobre metilação global de DNA hipocampal nos filhotes;

- Avaliar o efeito do exercício físico paterno antes do acasalamento e materno nos períodos pré-gestacional e gestacional sobre a plasticidade sináptica nos filhotes.

RESULTADOS

CAPÍTULO I

NEURAL REGENERATION RESEARCH www.nrronline.org

● 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 paternal 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 × 340 × 160 mm), 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_2max) 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_2max 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_2max) 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 survival 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

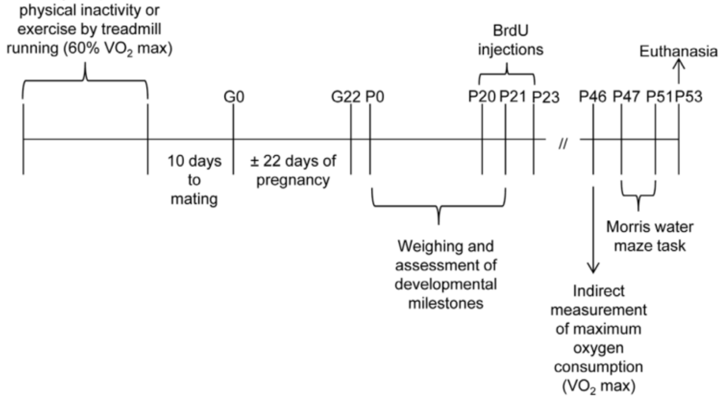

Figure 1 Schematic representation of the timeline of the experimental procedures.
G: Gestational day; P: postnatal day; BrdU: 5-bromo-2′-deoxiuridine.

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 × *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_2max 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.

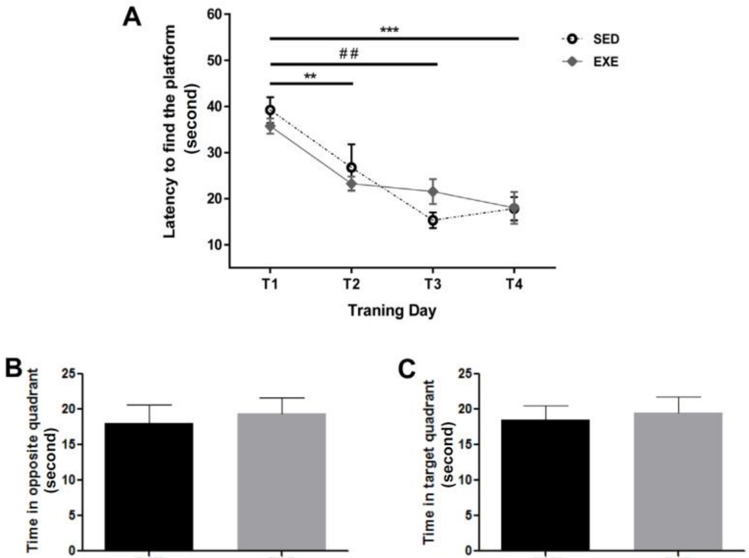

Figure 2 Spatial learning and memory evaluation in the Morris water maze task.
(A) Latency to find the platform on training days by the offspring. Repeated measures analysis of variance (paternal exercise × time): paternal exercise effect ($F_{(1,60)} = 0.004$, $P > 0.05$) and time effect ($F_{(1,60)} = 23.961$, $P < 0.001$). ***$P < 0.001$ 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.

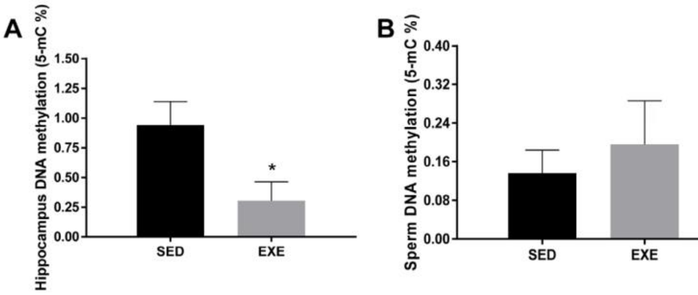

Figure 3 Global DNA methylation in offspring hippocampus and paternal sperm.
(A) Globaloffspring hippocampal DNA methylation. Decrease of hippocampal DNA methylationlevels in offspring from exercised fathers (EXE) *vs*. offspring from sedentary fathers (SED) group (*$P < 0.05$, unpaired Student's *t*-test, $t_{(8)} = 2.51$). (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).

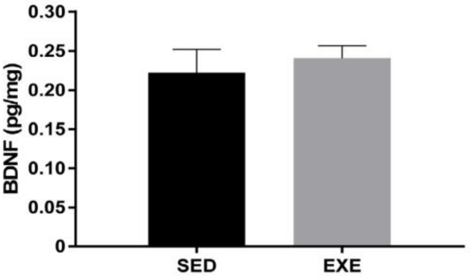

Figure 4 Offspring hippocampal BDNF levels (ELISA assay).
No differences were observed in hippocampal BDNF levels between groups (unpaired Student's *t*-test, $t_{(14)} = -0.343$, $P > 0.05$). $n = 8$ per group. Data are expressed as the mean ± SEM. ELISA: enzyme-linked immuno sorbent assay; SED: offspring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

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,

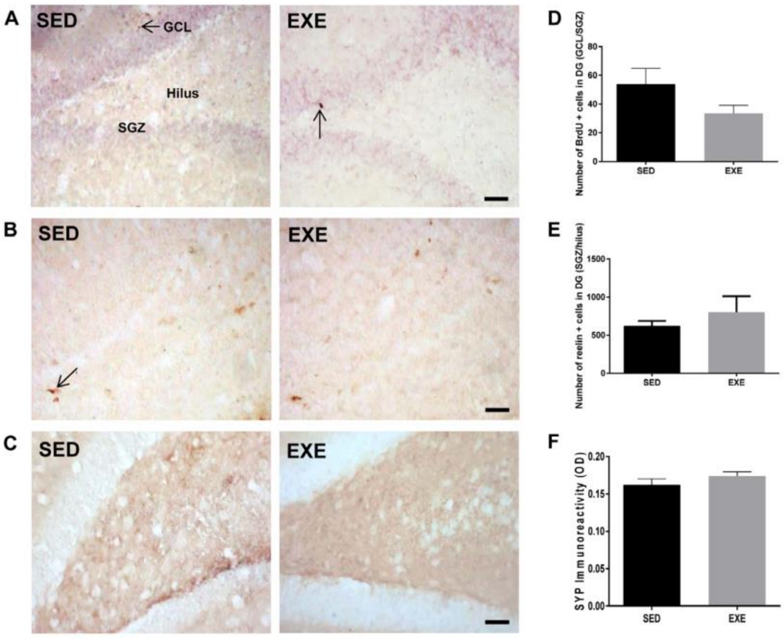

Figure 5 Survival of BrdU^{+} cells, reelin^{+} cells and SYP immunoreactivity in the DG of the hippocampus.
Digitalized images of the DG stained for BrdU showing cell survival in the SGZ and GCL (arrows; 100× magnification) (A), reelin in the hilus and SGZ (arrows; 100× magnification) (B), and SYP in 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% VO_2max, quite different from that used in the current study (22 days, 20 min/d, 5 days per week at 60% VO_2max). 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 hippocampus. 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 pregnancy, 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.

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CAPÍTULO II

Effects of maternal physical exercise on global DNA methylation and hippocampal plasticity of rat male offspring

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Highlights

Pregestational maternal exercise decreases offspring hippocampal DNA methylation.

Pregestational exercise has similar beneficial effects to gestational exercise for pups.

Pregestational exercise raises reelin levels and cell survival in pup hippocampus.

Exercise done before and during pregnancy does not have synergism for isolated periods.

Pregestational exercise induced hippocampal epigenetic reprogramming in male progeny.

Abstract

Intrauterine exposure to exercise is beneficial to cognition of the offspring. Although it is advisable to start practicing physical exercise during pregnancy, it is probable that practitioners or sedentary women keep their previous habits during gestation. This study was designed to evaluate the effects of maternal aerobic exercise initiated before and maintained during gestation, or performed in these isolated periods, on cognition and plasticity in the hippocampus of offspring. Groups of male pups were categorized by the exposure of their mothers to: treadmill off (sedentary, SS), pregestational exercise (ES), gestational exercise (SE) or combined protocols (EE). Between postnatal day 20 (P20) and P23 the offspring received one daily 5-bromo-2′-deoxiuridine (BrdU) injection and, from P47 to P51, were evaluated by the Morris water maze task. At P53, hippocampal global DNA methylation, survival of progenitor cells (BrdU), Brain-derived Neurotrophic Factor (BDNF) and reelin levels were measured. The offspring from ES, SE and EE mothers demonstrated improved spatial learning compared to SS, but hippocampal DNA methylation was significantly modified only in the offspring from ES mothers. The offspring from ES and SE mothers presented higher number of BrdU+ and reelin+ hippocampal cells than EE and SS. No differences were observed in the BDNF levels among the groups. The maternal pregestational and gestational isolated exercise protocols showed similar effects for offspring plasticity and spatial cognitive ability, while the combined protocol simply improved their spatial learning. Interestingly, only pregestational exercise was able to induce plasticity in the offspring hippocampus associated with modulation of global DNA methylation.

Keywords: epigenetic; preconception; memory; maternal treadmill running; neurogenesis; synaptic plasticity

Introduction

Brain development requires the regulation of molecular events controlled by gene expression and environmental stimuli (Stiles and Jernigan, 2010). An environmental stimulus, such as maternal physical exercise, has the potential to influence the trajectory of brain development and the offspring phenotype (da Silva and Arida, 2015). Evidence has shown that children of mothers who practiced physical exercise during pregnancy had better scores in intelligence and language scales (Clapp et al., 1998; Domingues et al., 2015; Polańska et al., 2015). In the same way, experimental studies observed that exercise in pregnant rodents increased brain-derived neurotrophic factor (BDNF) and reelin levels and neurogenesis in the hippocampus of pups, providing evidence of some of the biological mechanisms involved in these phenotypical modifications (Da Silva et al., 2016; Herring et al., 2012).

It is easy to perceive how mothers can transmit biomolecules (e.g. nutrients or hormones), environmental influences (e.g. temperature or intrauterine environment) or behavior (e.g. maternal care or anxiety) to the offspring during pregnancy and postnatal life (Crean and Bonduriansky, 2014). Nonetheless, in the pregestational period, physical exercise practiced by mothers has also been linked to benefits to offspring such as higher scores in academic performance indicators (Esteban-Cornejo et al., 2016). Likewise, pregestational exercise is associated with better rates of embryonic development, increased mitochondrial metabolism and amplified epigenetic reprogramming of early embryos as observed *in vitro* (Xu et al., 2017). Reinforcing the idea that life experiences prior to conception can provide benefits to progeny, one study with paternal physical exercise showed positive influences on offspring, such as the improvement of their spatial memory and hippocampal plasticity (Yin et al., 2013). In fathers, the mechanisms of transmission of exercise’s effects to offspring, at least in part, could be explained by alteration in methylation patterns in sperm (Denham et al., 2015; Denham, 2017).

Altogether, these outcomes indicate that physical exercise prior to conception and during pregnancy has the potential to transfer maternal traits to offspring through reprogramming of germ or somatic cells, causing positive influences on their phenotype. However, as observed by Evenson and Wen (2010), only a low percentage of women report being more active during gestation than before, even though exercise in this period is recommended. Hence, it is more common for active women to remain active during gestation than sedentary women to become active while pregnant (Evenson and Wen, 2010; Venezia et al., 2015). Nevertheless, the experimental studies focused on the effects of exercise in the gestational period. Thus, the aim of this study was to elucidate the effects of the maternal exercise initiated before and maintained during the pregnancy or performed in these isolated periods on learning and memory, neuroplasticity (BDNF, progenitor cells survival and reelin expression) and global DNA methylation in the hippocampus of offspring.

Experimental procedures

Ethical considerations

All procedures for this study were approved by the Ethical Committee at the Universidade Federal do Rio Grande do Sul (27587). Animal care was performed in accordance with the guidelines 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's Guidelines for Care and Use of Laboratory Rats (publication nº 85–23, revised 1985). All efforts were made to diminish the number of animals used and their discomfort as much as possible.

Animals

In this study, 60 days old male and female Wistar rats (*n* = 15 and 29, respectively) were used from the local breeding colony (CREAL/ICBS, Universidade Federal do Rio Grande do Sul, Brazil) with the aim of obtaining male progeny. Animals were kept in standard Plexiglas home cages (410 x 340 x 160 mm), 3–4 rats per box, under controlled environmental conditions (20 ± 2ºC, 12 h light/dark cycle with lights on at 7:00 a.m., food and water offered *ad libitum*). All experiments were performed from 2:00 p.m. to 5:00 p.m.

Experimental procedure and maternal exercise protocols

Initially, female rats were randomly divided into four groups: 1) sedentary mothers during the pregestational and gestational periods (SS), 2) mothers exercised only during the pregestational period (ES), 3) mothers exercised only during the gestational period (SE) and 4) mothers exercised during the pregestational and gestational periods (EE). The sedentary females were manipulated with the same frequency as the exercised ones, but were exposed to the treadmill with it off for 5 min/day, without any stimulus to run, for the total duration of the exercise protocols (Lovatel et al., 2013; Spindler et al., 2014). Treadmill running was the chosen modality since it allows standardization of parameters such as intensity and frequency of exercise. No electric shock or aversive physical stimulation was used, and the rats that refused to run were stimulated with gentle touches. If they persisted in refusing to run, they were discarded from the study.

Before the training by treadmill running, the indirect measurement of maximum oxygen consumption (VO_2 max) test was used to define the females' training speed. In this test, rats were positioned separately on the treadmill and the training speed was augmented gradually (3 m/min) every 3 min until fatigue (the females were no longer able to continue running on the treadmill). The latency to fatigue (s) and the maximum speed (m/min) reached by every single female were considered as performance parameters, and their average was used to determine the running speed (Mega et al., 2018).

In the pregestational exercise protocol, the females were subjected to training sessions at 60% of VO_2 max on a horizontal motorized treadmill adapted to rodents, in separate Plexiglas lanes (INBRAMED TK 01, Porto Alegre, Brazil). The exercise protocol consisted of running for 20 min/day, 5 consecutive days/week for a total period of 22 training days (adapted from Parnpiansil et al., 2003); Fig. 1). After the pregestational exercise period, the estrous cycle of the females was verified daily and in proestrous phase they were left overnight with a sedentary male for breeding. If spermatozoa were detected in the vaginal smear the morning following copulation, that day was considered as the fertilization day or day 0 of gestation (G0). And, if the animals did not become pregnant up to 10 days after the end of the pregestational exercise period, the females were withdrawn from the study (Fig. 1). After the breeding period, male progenitors were also discarded from the present study. The gestational exercise protocol was performed with the same frequency and duration (from G1 to G22) as the pregestational exercise, but at 30% of VO_2 max (adapted from Parnpiansil et al. 2003; Fig. 1).

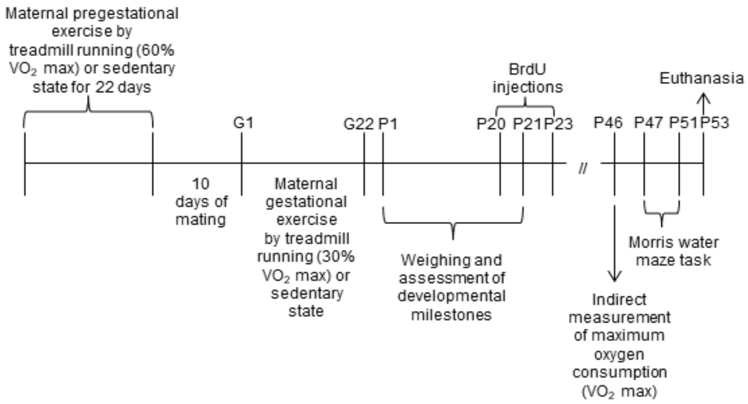

Fig.1. Timeline of experimental procedures. G: gestational day. P: postnatal day.

The day of the pups' birth was considered as postnatal day 0 (P0). Every experimental group was formed by 31–36 male offspring from 29 litters that were destined for distinct analyses. Thus, the offspring were divided into four groups, according to the period in which their mothers were exercised:

1) Offspring from mothers sedentary during the pregestational and gestational periods (SS).
2) Offspring from mothers exercised only during the pregestational period (ES).
3) Offspring from mothers exercised only during the gestational period (SE).
4) Offspring from mothers exercised during the pregestational and gestational periods (EE).

The number of pups per litter was standardized between 6 and 8 offspring including males and females, to prevent litters of discrepant sizes. At P21, the male offspring were weaned and their female siblings were discarded from the study. Only male offspring were used in this study. A cohort of rats was designated for neonatal development evaluations (*n* = 9–13 rats per group) and for histological analyses (*n* = 4 rats per group), while the remaining offspring were divided in two other cohorts destined for behavioral tests (VO_2 max and Morris water maze task; *n* = 9–15 rats per group) or for biochemical analyses (*n* = 9–10 rats per group) to prevent bias among the assessments. Hence, the molecular evaluations were not performed on the same animals that went through Morris water maze testing.

Moreover, one week after weaning, 6–7 mothers per group were euthanized by decapitation. Their right and left adrenal glands were removed and the relative weight was calculated (adrenal glands weight/body weight ratio) and considered as an indirect measure of maternal stress status possibly induced by physical exercise (Marcuzzo et al. 2010; Piazza et al., 2011).

Neonatal development

At P1, P7, P14 and P53 (before euthanasia), offspring body weight gain was ascertained. Furthermore, between P1 and P21 the following developmental milestones were examined daily, according to Marcuzzo et al. (2010): 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 (Fig. 1). The postnatal day that every pup realized each task within 30 s for the first time was recorded (n = 9–13 animals per group).

BrdU administration

From P20 to P23, some rats of the cohort designed to the neonatal evaluations received one BrdU injection daily (5-bromo-2′-deoxiuridine; Sigma, 100 mg/kg, intraperitoneally, dissolved in 0.1 M NH_4OH, 20 mg/mL; Fig. 1). BrdU is a compound analogous to thymidine and is inserted into DNA during cell division and can be identified by immunohistochemistry (Nowakowski et al., 1989; Taupin, 2007; Veena et al., 2009). The offspring were euthanized 30 days after the last BrdU administration (P53) to analyze the cellular survival phase of hippocampal neurogenesis (Piazza et al., 2014).

Physical performance assessment

At P46, the physical performance of progeny was measured by the indirect measurement of the VO_2 max test as previously described by Mega et al. (2018; Fig. 1). This analysis was performed to investigate if maternal exercise could influence the offspring’s capacity to exercise. Each group was composed of 10–15 rats that were also subjected to the Morris water maze task.

Spatial learning and memory analysis

The spatial learning and memory of pups were analyzed from P47 to P51 in the Morris water maze task (adapted from Pereira et al., 2007; Fig. 1). The apparatus consisted of a circular pool (120 cm in diameter and 40 cm deep) with blurred water at 23°C divided into four equal fictional quadrants. On the walls of the experimental room, four visual cues were fixed to be used as reference points. In the target quadrant, a platform 10 cm in diameter was 2 cm underwater. For 4 days, the animals were trained to find the platform and on the fifth were subjected to the probe trial. On every training day, the rats executed four trials, with 15 min intervals between them.

In each trial, the rat was put at a starting point with its head directed to the pool wall, and the latency to find the platform was recorded. The animals that did not find it within 60 s were gently conducted to it, where stayed for 10 s. After each trial, the animals were parched and taken back to their home cages. Every day, the order of starting position changed in each trial. The latency to find the platform was registered in each trial and the mean latency for every training day was determinate.

On the fifth day of the task, the platform was removed for the probe trial, for which the rats were put on the opposite quadrant from the platform location. This trial lasted 1 min, where the time in which the rat remains in the target and opposite quadrants were computed as a measure of retention of information. All the trials were recorded by a video acquisition system for posterior analysis. This evaluation was performed in a soundless and temperature controlled environment, and the rats were handled by the same researcher during the 5 days of the task. Each group was composed of 9–13 rats.

Preparation of samples

At P53, the pups were euthanized by decapitation without anesthesia, between 10 a.m. and 3 p.m. Their entire hippocampi were rapidly collected (Fig. 1). Instantaneously, the samples were frozen in liquid nitrogen and then kept at -80 °C until processing for global DNA methylation and BDNF assays.

DNA purification

The pups' hippocampal DNA was purified with a commercial tissue section DNA isolation kit (FitAmp General Tissue Section DNA Isolation Kit, catalog #P-1003, Epigentek Group Inc., Farmingdale, NY, USA), in accordance with the producer's guidelines. Concisely, the samples were lysed in a water bath at 37°C, then the DNA was isolated with the enclosed columns and washed out with elution buffer.

Global hippocampal DNA methylation

The pups' global hippocampal DNA methylation was analyzed using a commercial kit MethylFlash™ methylated DNA quantification kit (Base catalog #P-1034) as described by Mega et al. (2018). It was measured, specifically, the 5-methylcytosine (5-mC) levels, resulting of a covalent addition of a methyl group at the 5-carbon of the cytosine ring by DNA methyltransferases. The offspring global hippocampal DNA methylation was expressed as percentage of 5-mC in total DNA. Each group was composed of 4–5 rats.

BDNF analysis

The levels of offspring hippocampal BDNF were analyzed by ChemiKine Sandwich ELISA kit (Cat. No. CYT306, Millipore, USA) according to the protocol described by Mega et al. (2018) and Spindler et al. (2019). The samples were collected between 10 a.m. and 3 p.m., homogenized according to the producer's guidelines using a specific lysis buffer, centrifuged, and the supernatant was separated for BDNF measurement. The analysis was executed in 96-well microplates that were pre-coated with mouse anti-Human BDNF Monoclonal Antibody. BDNF standard (7.8–500 pg/mL) or samples were incubated at 4 °C overnight. The plates were washed away four times, and a Biotinylated Mouse anti-Human BDNF Monoclonal Antibody (1:1000) was added to each well and incubated for three hours at room temperature. The plates were washed one more time, and a diluted streptavidin-horseradish peroxidase conjugate solution (1:1000) was added to each well and incubated for one hour at room temperature. In the sequence, a tetramethylbenzidine substrate solution and a stop solution were added and incubated for fifteen minutes at room temperature too. Every plate was read instantaneously at 450 nm. A standard curve was plotted for each plate. The BDNF concentrations were evaluated from the regression line of the BDNF standard. The protein concentration of samples was quantified by Bradford method using bovine serum albumin as standard (Bradford, 1976). Each group was composed of 9–10 rats.

Histological procedures

At P53, some rats of the cohort designated for neonatal development evaluations (4 rats from each group) were euthanized for immunohistochemistry of BrdU and reelin (Fig. 1). They received sodium thiopental (50 mg/kg, i.p.; Cristália, Brazil) combined with lidocaine (10 mg/mL) and, posteriorly, 1000 IU heparin (Cristália, Brazil) via the left ventricle. Soon after, rats underwent 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, Brazil) at room temperature, using a peristaltic pump (Milan, Brazil, 30 mL/min). The brains were collected, post-fixed in the same fixative solution at room temperature for 4 h and cryoprotected by immersion in 15% and 30% sucrose solution (Synth, Brazil) in PB at 4°C until they sank. Then, they were fast congealed in isopentane, cooled in liquid nitrogen and kept at -80°C until processing. Coronal sections from the dorsal hippocampus of 40 µm thickness were acquired using a cryostat (CM1850, Leica, Germany) at -20°C. The sections were successively collected on gelatin treated slides with an interval of 200 µm between them (Piazza et al. 2014). The point of origin was around 2.30 to 4.52 mm posterior to the bregma (Paxinos and Watson, 1982).

Immunohistochemistry for BrdU and reelin

Each immunohistochemical procedure was executed independently. The slices were washed in phosphate buffer saline (PBS, pH 7.4) and then heated for 20 min in 0.01 M sodium citrate buffer (pH 6.0) in a thermostatic bath at 92°C. The sections were washed in PBS and the endogenous peroxidase was inactivated with 3% hydrogen peroxide (Synth, Brazil) diluted in PBS for 30 min and washed in PBS, afterward they were washed in PBS with 0.4% Triton X-100 (PBS-Tx) for 15 min and preincubated with 3% bovine serum albumin (BSA; Sigma Aldrich, USA) in PBS-Tx for 30 min. After this sequence, the tissues were incubated with a monoclonal mouse anti-BrdU antibody (diluted 1:100 in nuclease, GE Healthcare, Amersham Biosciences, USA) at room temperature for 2 h and at 4°C overnight or with mouse anti-reelin antibody (diluted 1:400 in 3% BSA; Millipore, USA) for 48 h at 4ºC. Then, the slices were washed in PBS-Tx and incubated with the secondary antibody anti-mouse IgG conjugated with peroxidase (1:500, Sigma Aldrich, USA) at room temperature for 2 h. Both immunohistochemical reactions were visualized with a solution of 0.06% 3.3-diaminobenzidine (DAB; Sigma Aldrich, USA) dissolved in 10% hydrogen peroxide for 5 min. Lastly, the slices were washed in PBS, dehydrated in ethanol, cleared with xylene and coated with synthetic Canada balsam (Chemical Reaction, Brazil) and coverslips. The BrdU-stained sections were hematoxylin-counterstained. Negative control was performed by switching the primary antibody with PBS (Piazza et al., 2014).

BrdU and reelin-labeled cell quantification

Two blinded observers counted the BrdU-labeled cells in the granule cell layer (GCL) and in the subgranular zone (SGZ) of the dentate gyrus (DG) in both hemispheres of every slice at a 400x magnification on an Olympus BX40 microscope (Piazza et al., 2014). For both immunohistochemistry analyses, the SGZ was defined as a two-nucleus-wide band between the apparent border of the GCL and the hilus. With the purpose of simplifying the quantification of reelin-labeled cells, digitalized images from the DG of the dorsal hippocampus of both hemispheres of every section were acquired with a Nikon Optiphot-2 microscope (100×, Tokyo, Japan) coupled to a Micrometric camera (Accu Scope, Commack, NY, USA). The reelin-labeled cells were quantified in the SGZ and hilus of DG of the hippocampus using the software Image Pro Plus 6.0 and zoom of 100%. To determine the total number of cells per DG, the number of BrdU+ or reelin+ cells per slice were counted and multiplied by the interval between them (5 slices) (Malberg et al., 2000; Piazza et al., 2014). In each evaluation five sections/rat and four offspring/group were used.

Statistical analysis

The normality of data was analyzed by Kolmogorov-Smirnov test. The body weight gain and latency to find the platform of Morris water maze task were evaluated by two-way repeated-measures analysis of variance (ANOVA) with pregestational and gestational exercise as the independent variables, and postnatal or training day as repeated measures. The other data were analyzed by two-way ANOVA and Tukey's *post hoc* test. Statistical analysis was performed with the Statistica 12.5 software package (StatSoft, Inc., USA). Graphs were plotted using Graph Pad Prism 5. Differences between groups were considered significant when $p < 0.05$. Data is expressed as mean ± standard error of mean (SEM).

Results

Maternal adrenal gland weights and neonatal development

There was no significant difference in the relative adrenal weights among the maternal groups, expressed in milligrams (SS: 0.316 ± 0.025, ES: 0.336 ± 0.042, SE: 0.360 ± 0.041, EE: 0.357 ± 0.033). In addition, there were also no differences regarding offspring body weight at any time point evaluated (P1, P7, P14 and P53). Two-way ANOVA revealed that the offspring from mothers exercised only during the gestational period presented the forelimb grasp earlier than the other offspring (Table 1). However, there were no differences among the groups in the day of acquisition of the other development milestones (Table 1).

Table 1. Mean ± SEM of postnatal day that each developmental milestone was observed for the first time in the different groups.

Evaluation	SS	ES	SE	EE
Surface Righting	1.08 ± 0.08	1.44 ± 0.18	1 ± 0	1.77 ± 0.39
Cliff Aversion	3.46 ± 0.42	4.44 ± 0.44	2.73 ± 0.19	3.31 ± 0.43
Forelimb Grasp	7.85 ± 0.37	8.78 ± 0.81	5.64 ± 0.59 *	7.69 ± 0,50
Negative Geotáxis	7.62 ± 0.51	6.56 ± 0.53	7.45 ± 0.91	8.15 ± 0.52
Hind Limb Proprioceptive Placing	5.85 ± 0.68	5.44 ± 0.67	5.91 ± 0.21	4.77 ± 0.38
Open-Field Activity Assessment	11.92 ± 0.45	13.78 ± 0.36	12.55 ± 0.56	12.08 ± 0.46
Audio Startle	12.38 ± 0.18	12.56 ± 0.29	12.27 ± 0.36	12.23 ± 0,32
Eye-Opening	14.46 ± 0.18	14.11 ± 0.11	14.27 ± 0.36	14.77 ± 0.17

Two-way ANOVA revealed significant effects only for the factors pregestational exercise (F (1, 42) = 7.187; $p < 0.01$) and gestational exercise (F (1, 42) = 8.744; $p < 0.01$) for the forelimb grasp milestone. * $p<0.05$ indicates that the offspring from SE group achieved the forelimb grasp earlier than other groups. Two-way ANOVA followed by Tukey's *post hoc* test. n = 9–13 per group. SS: offspring from sedentary mothers during the pregestational and gestational periods; ES: offspring from mothers exercised only during the pregestational period; SE: offspring from mothers exercised only during the gestational period; EE: offspring from mothers exercised during the pregestational and gestational periods;

Physical performance

The four groups of male pups showed similar latency to fatigue in the indirect measurement of VO_2 max test, expressed in seconds (SS: 979.181 ± 39.397, ES: 956. 923 ± 34.860, SE: 844.2 ± 63.417, EE: 866.4 ± 41.950; $p > 0.05$).

Spatial learning and memory

The Morris water maze task was performed on 5 days. No significant differences were detected among the groups in the latency to find the platform on any of the 4 training days. Interestingly, when evaluating the learning curve from every group individually, a statistical difference in relation to the latency on the first training day arose on the second training day for the offspring from exercised mothers regardless of the exercise period (ES, SE and EE) ($p < 0.01$). For the offspring from sedentary mothers (SS), this difference appeared on the third training day ($p < 0.001$) (Fig. 2A). Moreover, the latency to find the platform on the third and fourth training days continued to be lower than on the first day in all groups ($p < 0.001$) (Fig. 2A).

On the fifth day of the Morris water maze task, a probe trial was performed in the absence of the platform. There were no differences in the time spent in the target and opposite quadrants (retention of information) between offspring from exercised (ES, SE and EE) and sedentary mothers (SS) (Fig. 2B and C, respectively). Nevertheless, ES offspring spent more time in the target quadrant and, consequently, less time in the opposite quadrant than the EE group ($p < 0.001$; $p < 0.05$, respectively).

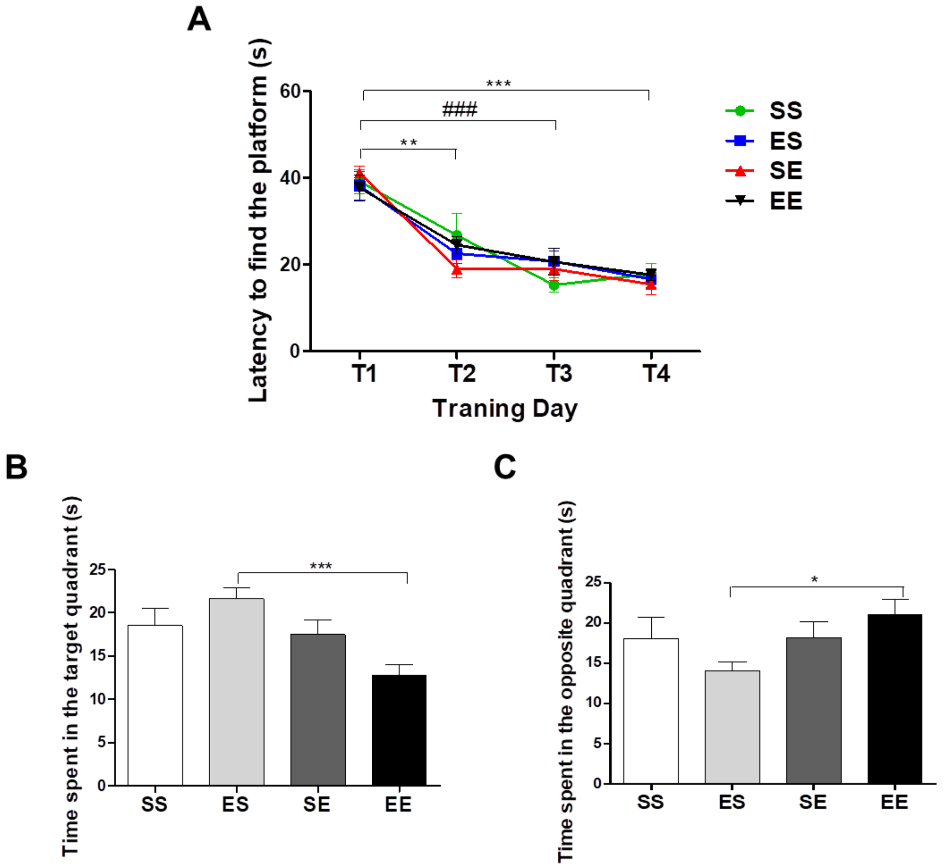

Fig.2. Spatial learning and memory assessment in the Morris water maze task. (A) Latency to find the platform on training days by the male offspring (learning curve). Two-way repeated-measures ANOVA (pregestational x gestational exercise x time): time effect (F (3,132) = 68.309; $p < 0.05$). **$p < 0.01$ indicates decreased latency to find the platform between training day 1 *vs* 2 only in the groups of offspring from exercised mothers (ES, SE and EE). $^{\#\#\#}p < 0.001$ indicates decreased latency to find the platform between training day 1 *vs* 3 in all groups. ***$p < 0.001$ indicates decreased latency to find the platform between training day 1 *vs* 4 in all groups. (B) Time spent in the target quadrant in the probe trial. Two-way ANOVA (pregestational x gestational exercise): gestational exercise effect (F (1, 44) = 10.528; $p < 0.01$) and pregestational x gestational exercise interaction effect (F (1, 44) = 6.653; $p < 0.05$). ***$p < 0.001$ indicates increased time spent by ES group in the target quadrant *vs*. EE group. (C) Time spent in the opposite quadrant in the probe trial. *$p < 0.05$ indicates decreased time spent by ES group in the opposite quadrant *vs*. EE group. Two-way ANOVA followed by Tukey's *post hoc* test. n = 9–13 per group. Data are expressed as mean ± standard error of mean. SS: offspring from sedentary mothers during the pregestational and gestational periods; ES: offspring from mothers exercised only during the pregestational period; SE: offspring from mothers exercised only during the gestational period; EE: offspring from mothers exercised during the pregestational and gestational periods; T: training day.

Global hippocampal DNA methylation and BDNF levels

The offspring from mothers exercised only in the pregestational period (ES) presented lower global DNA methylation levels when compared to the offspring from sedentary mothers (SS) ($p < 0.05$; Fig. 3A). No significant difference in hippocampal BDNF levels was observed among the groups (Fig. 3B).

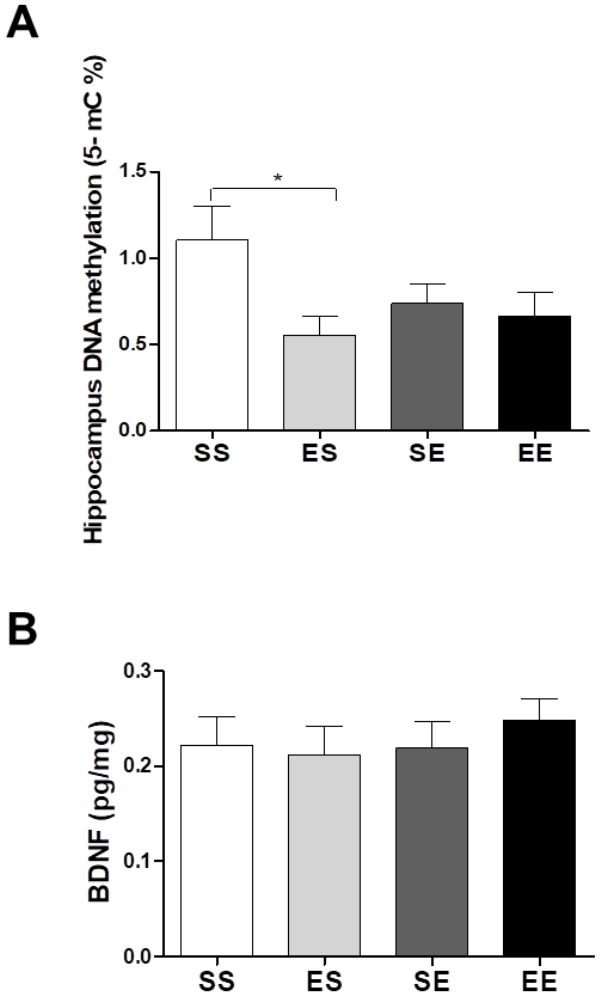

Fig.3. Global DNA methylation and BDNF levels in offspring hippocampus. (A) Global hippocampal DNA methylation of male progeny. Two-way ANOVA (pregestational x gestational exercise): pregestational effect (F (1, 14) = 4.919; $p < 0.05$). *$p < 0.05$ indicates decreased hippocampal DNA methylation levels in the ES group *vs.* SS. n = 4–5 per group. (B) Hippocampal BDNF levels of male progeny. No differences in hippocampal BDNF levels were observed among the groups. Two-way ANOVA. n = 9–10 per group. Data are expressed as mean ± standard error of mean. SS: offspring from sedentary mothers during the pregestational and gestational periods; ES: offspring from mothers exercised only during the pregestational period; SE: offspring from mothers exercised

only during the gestational period; EE: offspring from mothers exercised during the pregestational and gestational periods; 5-mC: 5-methylcytosine.

Quantification of BrdU and reelin-labeled cells

The offspring from mothers exercised only in pregestation (ES) and only in the gestational period (SE) presented higher numbers of BrdU+ cells in the DG of the hippocampus (Fig. 4A) compared to the control (SS) and to the offspring of mothers exercised in both periods (EE) ($p < 0.05$; Fig. 4B). Moreover, the ES and SE groups also showed an enhancement on the number of reelin+ cells (Fig. 5A) compared to SS and EE ($p < 0.05$; Fig. 5B).

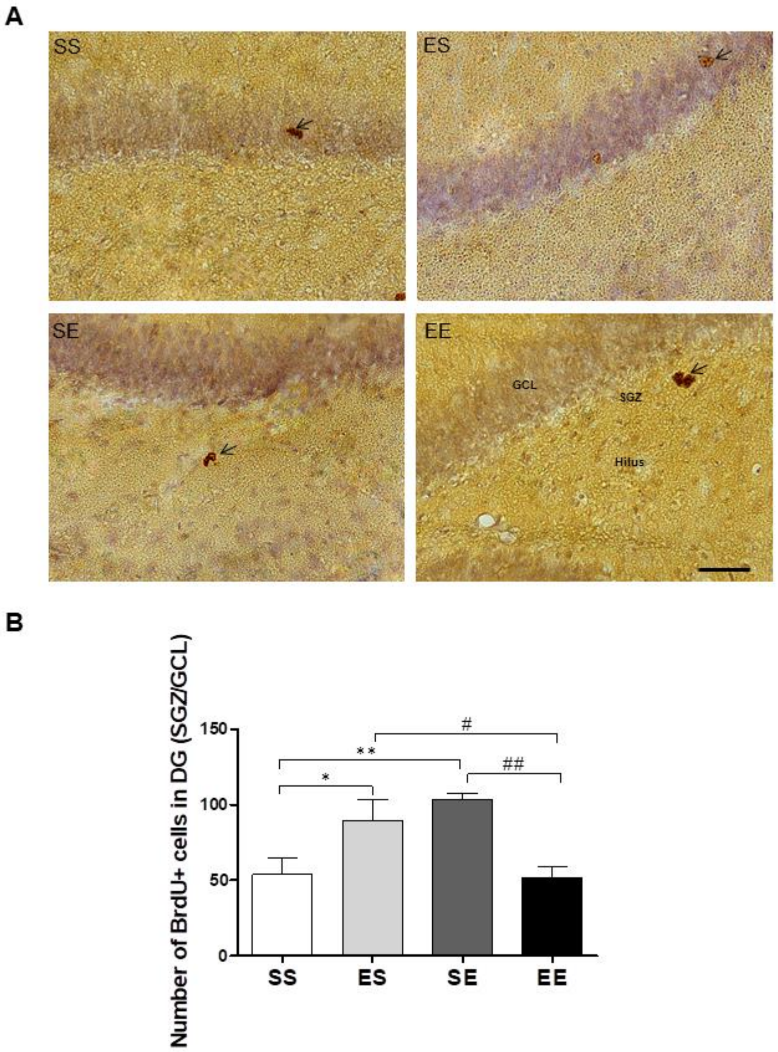

Fig. 4. Survival of BrdU+ cells in the DG of the hippocampus. A) Digitalized images of the DG stained for BrdU showing cell survival in the SGZ and GCL (arrows; 400× magnification). B) Quantitative analysis of the immunohistochemistry for BrdU. Two-way ANOVA (pregestational x gestational exercise): pregestational x

gestational exercise interaction (F (1, 12) = 19.405; $p < 0.001$).*$p < 0.05$ indicates increase in the number in BrdU-labeled cells in DG of hippocampus of ES vs. SS group. **$p < 0.01$ indicates increase in the number of BrdU-labeled cells in DG of hippocampus of SE *vs.* SS group. $^{\#}p < 0.05$ indicates increase in the number of BrdU-labeled cells in DG of hippocampus of ES vs. EE group. $^{\#\#}p < 0.01$ indicates increase in the number of BrdU-labeled cells in DG of hippocampus of SE *vs.* EE group. Data are expressed as mean ± standard error of mean. $n = 4$ per group. Scale bar = 50 μm. SS: offspring from sedentary mothers during the pregestational and gestational periods; ES: offspring from mothers exercised only during the pregestational period; SE: offspring from mothers exercised only during the gestational period; EE: offspring from mothers exercised during the pregestational and gestational periods; DG: dentate gyrus; SGZ: subgranular zone; GCL: granule cell layer.

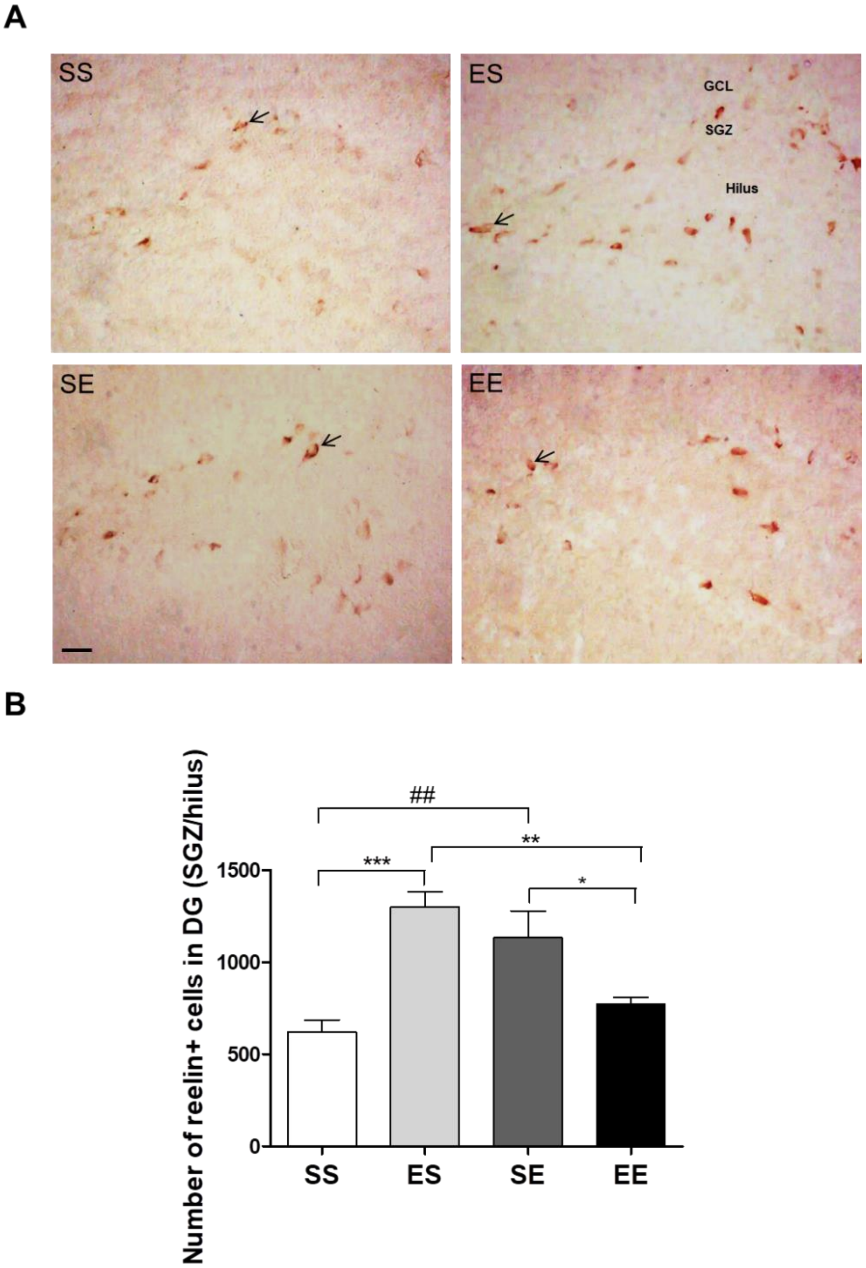

Fig. 5. Counting of reelin+ cells in the DG of the hippocampus. A) Digitalized images of the DG stained for reelin in the hilus and SGZ (arrows; 100× magnification). B) Quantitative analysis of the immunohistochemistry for reelin. Two-way ANOVA (pregestational x gestational exercise): pregestational x gestational exercise interaction (F (1, 12) = 31.479; $p < 0.001$).*$p < 0.05$ indicates increase in the number of reelin-labeled cells in DG of hippocampus of SE *vs.* EE group. **$p < 0.01$ indicates increase in the number of reelin-labeled cells in DG of hippocampus of ES *vs.* EE group. ***$p < 0.001$ indicates increase in the number of reelin-labeled cells in DG of hippocampus of ES *vs.* SS group. $^{\#\#}p < 0.01$ indicates increase in the number of reelin-labeled cells in DG of hippocampus of SE *vs.* SS group. Two-way ANOVA followed by Tukey's *post hoc* test. Data are expressed as

mean ± standard error of mean. *n* = 4 per group. Scale bar = 50 μm. SS: offspring from sedentary mothers during the pregestational and gestational periods; ES: offspring from mothers exercised only during the pregestational period; SE: offspring from mothers exercised only during the gestational period; EE: offspring from mothers exercised during the pregestational and gestational periods; DG: dentate gyrus; SGZ: subgranular zone; GCL: granule cell layer.

Discussion

The purpose of this study was to highlight the effects of pregestational maternal exercise on the hippocampus of offspring and to compare them with exercise practice started at gestation. In addition, the possibility of synergism between these effects was analyzed. The data showed that pregestational exercise induced similar positive effects on the offspring to those caused by gestational exercise. The hypothesis of synergism was not supported. While pregestational and gestational exercise, in isolation, led to a superior performance in the learning task accompanied by increased hippocampal progenitor cell survival and expression of reelin, continuous maternal exercise did not cause these effects on neuroplasticity parameters, resembling the control offspring.

Maternal exercise, independent of the period, induced a better learning curve (faster learning). Nevertheless, there was no difference observed among the groups in spatial memory (evaluated by the time spent in the target or opposite quadrant in the Morris water maze task). Interestingly, there was a subtle beneficial effect of exercise performed before gestation more so than continuous maternal exercise on the spatial memory of offspring (see Fig. 2B and C). There are no preclinical studies about the effects of pregestational exercise, specifically on pup’s cognition, to discuss with regards to the current outcome. Nevertheless, in humans, sons of mothers who exercised before or/and during pregnancy had significantly higher scores in academic performance indicators in relation to the sedentary ones (Esteban-Cornejo et al., 2016). This corroborates with the present findings and strengthens their translational value. Additionally, the data for gestational exercise are consistent with other studies that observed better cognitive performance (comparing the learning curve) and no differences in retention of spatial information in the Morris water maze task (Akhavan et al., 2013; Da Silva et al., 2016).The learning benefits in the progeny from mothers exercised at pregestation or in the gestational period were associated with an increase in the hippocampal reelin and progenitor cell survival levels. However, the relationship between maternal exercise, neurogenesis and reelin expression in offspring hippocampus has not been investigated. One study that performed paternal treadmill exercise found enhanced reelin expression accompanied by increased BDNF and improvement of spatial memory in male offspring of C57BL/6J mice (Yin et al., 2013). Treadmill exercise was also able to increase reelin expression and neurogenesis in the hippocampus of rats subjected to an autistic-like pathology, leading to an improvement in learning and spatial memory (Seo et al., 2013). These data point to a relationship between higher reelin expression, the increment of hippocampal progenitor cell survival and the learning of the descendants of mothers exercised before or during pregnancy, although this must be studied in greater depth.

However, the neuroplastic and behavioral alterations induced by maternal exercise were not accompanied by BDNF up-regulation. Some studies suggest that parental exercise has an early and ephemeral effect on the offspring's hippocampal BDNF levels. In this way, Parnpiansil et al. (2003) demonstrated that rats that underwent treadmill exercise during gestation have offspring with an elevated hippocampal BDNF mRNA expression at P0, no difference at P14 and significantly lower BDNF expression at P28. In contrast, Kim et al. (2007) and Lee et al. (2006) showed that hippocampal BDNF mRNA expression was elevated in offspring of treadmill and swim trained mothers, respectively, at 29 days after birth. Furthermore, an elegant study that exposed both progenitor mice to a voluntary running wheel prior to conception until lactation found no effect on hippocampal BDNF mRNA expression in male offspring at 8 or 28 weeks old (Venezia et al., 2015). It is interesting to note that the first evaluation period in this study, 8 weeks, was similar to that of this study, P53, as was the result. In another study, an enriched prereproductive maternal environment (from P21 to P72, before sexual maturation) had no effect on either neurogenesis or reelin levels in the hippocampus of offspring, but did increase BDNF expression (at P21 and P55) (Cutuli et al., 2015). It seems like the BDNF modifications are more associated with transitory fine plastic changes necessary for neuronal structure (such as branching and dendritic spine modifications) and changes in neurotransmission, long-term potentiation and memory consolidation prior to neurogenesis increment.

The pregestational exercise in isolation was able to significantly reduce the global DNA methylation levels in the hippocampus of offspring (the pups of mothers exercised during pregnancy or in both periods also showed a reduction in these levels, but it was not statistically significant). As the modulation of DNA demethylation has a crucial role in neuronal plasticity, promoting learning and memory improvement, the behavioral and molecular effects can, at least in part, be related to programming of the F1 generation via epigenetic alterations of maternal germ cells before conception (Feng et al., 2007; Miller et al., 2008). Therefore, this study provides the first evidence that maternal pregestational exercise influences the epigenome of the progeny's somatic cells, as a mechanism of environmental inheritance. More studies exploring oocyte epigenetic and the expression of other specific genes involved in synaptic plasticity and memory would increase the understanding of these results. Furthermore, one limitation of this study was that it has not been tested whether a methyltransferase DNA inhibitor, as RG108 or 5-Aza-2´-deoxycytidine, would be able to mimic the effects of pre-gestational maternal exercise on hippocampal neuroplasticity and cognition of the progeny and possibly confirm the hypothesis of the role of DNA methylation in this issue. It is strongly recommended that the next studies in this field take into account this type of approach.

Another limitation of this study was that the *BDNF* mRNA levels in the offspring hippocampus have not been analyzed, which would confirm stable expression of *BDNF* despite global DNA hypomethylation. Since BDNF levels may oscillate during the circadian cycle, the BDNF dosage in the peripheral blood of the animals could also help to validate the data observed in the hippocampus. In addition, the tyrosine kinase B gene (*TrKB*) may be a very interesting target because it is also subject to modulation in its methylation state and consequently in its expression, which could lead to an increase in BDNF/TrKB signaling although the local production of BDNF did not change. Methylation in the promoter sequences of the *BDNF* and *TrKB* genes could also be analyzed by the Methylated DNA immunoprecipitation, which would give strength to the data. It is imperative that future studies in this area adopt this type of approach to better understand the relation among maternal exercise, neuroplasticity and offspring methylome.

On the other hand, the gestational exercise was able to increase the hippocampal neuroplastic parameters without significantly modifying the global DNA methylation in this structure. Similarly, Herring et al. (2012) observed that female pups of mice that practiced wheel running during pregnancy showed up-regulation of reelin in their hippocampus compared to the descendants of sedentary mothers. However, the authors did not observe differences in methylation of the reelin gene promoter on the hippocampus of pups, which corroborates the current finding. Therefore, future studies should analyze other epigenetic parameters in the offspring hippocampus that may respond to the effects of physical exercise in pregnancy; such as histone H3 and H4 acetylation levels, modifications in histone methylation status and microRNA regulation. Furthermore, it is also essential to study the modulation of methylation status of other specific genes that have a vital function in the synaptic plasticity and memory, such as *activity-regulated cytoskeleton-associated protein, synaptophysin, synapsin-1* and *postsynaptic density protein 95,* as a consequence of maternal exercise performed at any period.

Moreover, initially it would be obvious to assume that maternal physical exercise prior to and during gestation would result in a synergism of positive effects, but this was not supported. The results of this experimental group did not differ from the control group. A possible explanation for this can be related to the greater manipulation of mothers than those exercised in isolated periods. The longer period of daily manipulation to carry out the exercise training could have influenced maternal behavior; however, there were no obvious signs of stress, such as alterations in the maternal relative adrenal weight. It is known that even moderate variations in the early life environment have a major impact on adult hippocampal function. The involuntary exercise (swimming or treadmill running) before and during pregnancy can be stressful and damage the pup's hippocampal neurogenesis and oxidative balance, possibly due to increased maternal levels of glucocorticoids (Jang et al., 2018; Park et al., 2012; Wasinski et al., 2016). However, the pups from mothers exercised in both periods showed similar results to the control and typical neurodevelopment, discarding a possible deficit. In addition, others strategies, like prereproductive maternal environment enrichment, positively influenced maternal care and the cognitive development of their offspring, linked to enhanced BDNF (Cutuli et al., 2015).

To isolate the effects of maternal exercise itself, an experimental strategy would be to perform the biochemical analysis at birth, so that effects would be specific to the germline and the intrauterine environment. Another option would be for future studies including cross-fostering of pups, for maternal care to be performed by mothers not exposed to physical exercise and, thus, maternal care changes possibly induced by exercise would not interfere with the results. At this moment, we cannot confirm that the positive behavior presented by offspring from exercised mothers required the subsequent interaction with the mother to become manifested. Because of that, it is imperative that future studies of maternal exercise effects on the offspring included the analysis of the maternal care.

According to our knowledge, this was the first study to observe environmental inheritance of maternal exercise in the offspring nervous tissue (hippocampus) in a window of development neglected up to now (pregestational period). In light of previous reports, we suggest an important new finding; pregestational and gestational practice of maternal exercise has similar beneficial effects on the offspring hippocampus. However, some analyses not performed in this study should be emphasized in the future. Long-term follow-up may reveal other effects of maternal physical exercise. Other training protocols (modality, intensity or duration) could alter the results found in the offspring and probably in other generations too. The analysis of female pups is also necessary to explore whether behavioral and molecular changes induced by maternal exercise are sex-specific.

In summary, this study demonstrated that maternal pregestational treadmill exercise can program the hippocampus of male offspring by modulation of their epigenetic machinery resulting in positive cognitive and neuroplastic outputs similar to those obtained extensively with gestational exercise. In addition, continuous maternal exercise was safe for the offspring. With the due reservations that the translation of basic research imposes for clinical practice, these findings might contribute to elucidate epigenetic pathways that could explain the relationship between maternal lifestyle factors, such as physical exercise, and the outcomes in the offspring's neuroplasticity and behavior.

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Author contributions

Performed the exercise protocols, neonatal analyses and Morris water maze task: ES, CS, ALFM, FM and GSS. Executed BDNF and global DNA methylation assays: CS and ALFM. Did the histological procedures and analyses: ES and FVP. Statistical data analysis: ES. Designed the experiments and wrote the paper: ES, CS, ALFM, FVP, SM and MA.

Declarations of interest

None.

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DISCUSSÃO

O propósito desta tese foi avaliar os efeitos do exercício físico parental forçado em esteira ergométrica sobre parâmetros cognitivos e de neuroplasticidade da prole. O estudo foi dividido em dois artigos, um focado no exercício físico paterno e outro no exercício físico materno, este em duas janelas de tempos diferentes, antes da gestação e durante o período gestacional, bem como um possível sinergismo durante os dois períodos.

De maneira geral, nossos resultados mostram que os protocolos de exercício físico não alteraram o desenvolvimento da prole, uma vez que os filhotes de pais exercitados, e de mães exercitadas em todos os tempos não apresentaram alterações de neurodesenvolvimento motor quando avaliados os marcos do desenvolvimento. Estes achados corroboram com diversos autores que afirmam que a prática de exercício físico durante o período gestacional sendo ele agudo ou crônico não gera nenhum tipo de malefício para o feto (Dipietro and Bornstein, 2008; Moyer et al., 2016). Ainda, é interessante ressaltar que ao analisarmos o peso das glândulas adrenais maternas, percebemos que não houve diferença entre as mães exercitadas em quaisquer grupos em relação às ratas mantidas sedentárias. Estes achados demonstram que os protocolos de exercício físico não geraram estresse nas progenitoras, uma vez que o peso destas glândulas é considerado uma medida indireta do estresse no roedor.

Neste estudo analisamos os efeitos dos protocolos de exercício físico parental sobre parâmetros de memória e aprendizado nos filhotes utilizando a análise das respostas ou teste do Labirinto Aquático de Morris. Os dados não mostraram diferenças entre os grupos exercitados e sedentários em relação à memória espacial, uma vez que não houve diferença estatística entre os grupos no teste da plataforma. Entretanto, os animais filhos de pais ou mãe exercitadas em um dos períodos (pré-gestacional ou gestacional) apresentaram uma curva de aprendizado melhor durante os treinos do paradigma. Ou seja, observamos que o exercício físico foi capaz de induzir uma melhora cognitiva na prole, melhorando a curva de aprendizado dos filhotes. Estes resultados corroboram com um estudo clínico que demonstra que filhos de mães exercitadas antes ou durante a gestação apresentaram escores mais altos em testes de desempenho acadêmico quando comparados às crianças filhas de mães que se mantiveram sedentárias. Nossos achados são semelhantes a estudos pré-clínicos que observaram o efeito do exercício materno sobre parâmetros de memória. No estudo conduzido por Gomes Da Silva e colaboradores (2016), os autores avaliaram o efeito do exercício físico em esteira ergométrica sobre a memória e aprendizado dos filhotes utilizando o Labirinto Aquático de Morris. Os dados mostraram que não houve diferença na retenção da memória espacial, entretanto observaram que os filhos de mães exercitadas apresentavam um aprendizado mais rápido da tarefa proposta. De maneira semelhante, um estudo recente também avaliou efeito do exercício físico voluntário, durante a gestação, em rodas de corrida de livre acesso sobre o mesmo paradigma de memória e aprendizado utilizado em nosso estudo. De maneira semelhante ao nosso estudo, os autores demonstraram que o protocolo de exercício voluntário não gerou diferenças entre os grupos nos escores de memória espacial, entretanto, corroborando nossos resultados, os animais descendentes de mães exercitadas também apresentaram uma melhor curva de aprendizado (Akhavan et al., 2013). Ainda, recentemente nosso grupo demonstrou que um protocolo mais extenso de exercício paternal, durante 8 semanas, também não foi capaz de alterar os escores de memória nos filhotes (Mega et al., 2018). Entretanto, é importante frisar que apenas um estudo observou o efeito do exercício físico paterno sobre escores de memória e aprendizado nos filhotes. Neste estudo camundongos foram submetidos a um protocolo de exercício físico forçado em esteira ergométrica , e os autores observaram melhores escores de aprendizado e memória nos filhotes, utilizando o mesmo paradigma do nosso estudo (Yin et al., 2013).

Os efeitos benéficos do exercício parental vêm sendo relacionados por diversos autores com o aumento do BDNF. Entretanto, nossos resultados de aprendizado nos filhotes não foram acompanhados por aumento desta neurotrofina. No estudo citado acima, conduzido por Yin e colaboradores (2013), contrapondo nossos resultados, os achados comportamentais foram acompanhados de aumentos na expressão do BDNF no hipocampo destes filhotes. Porém a janela de tempo em que o BDNF foi analisado em nosso trabalho e no estudo de Yin, apresentam quase um mês de diferença, bem como a diferença na espécie utilizada nos dois estudos podem explicar a diferença nos achados.

Por outro lado, outros estudos também observaram alterações na expressão desta neurotrofina em filhotes de mães exercitadas. Parnpiansil e colaboradores (2003) submeteram ratas à um protocolo de exercício em esteira ergométrica e observaram uma alteração flutuante das concentrações de RNAm do BDNF hipocampal nos filhotes de mães exercitadas. Os autores revelam que que a concentração hipocampal de RNAm do BDNF estava elevada em P0, sem diferença em P14 e significativamente baixa em P28. Outro estudo que submeteu os dois progenitores, machos e fêmeas, a uma roda de corrida de livre acesso antes da concepção até o período de lactação, é semelhante ao nosso estudo pois não encontrou diferenças no RNAm BDNF nos hipocampos da prole com 8 ou 28 semanas de idade (Venezia et al., 2016). Nesta tese avaliamos o conteúdo de BDNF hipocampal da prole no P53, ou seja, em um período próximo ao de 8 semanas utilizado no trabalho de Venezia (2016), e da mesma maneira, não encontramos diferenças estatísticas no conteúdo desta neurotrofina nos animais dos grupos exercitados em relação ao grupo sedentário. Uma das hipóteses para explicar o resultado da expressão do BDNF é a janela de tempo da análise, realizada com os animais já em fase adulta (P53), o que poderia sugerir que nosso grupo perdeu a janela de análise onde seria possível observar a regulação da expressão do BDNF, um vez que os dados disponíveis sugerem que os níveis de BDNF estão mais altos em períodos mais precoces, como observado nos estudos Kim et al. (2012) e Lee et al. (2016), no quais os animais descendentes de mães exercitadas apresentaram níveis maiores de RNAm de BDNF no hipocampo em P29.

Ainda, ao analisarmos a proliferação celular e expressão da relina observamos que o exercício paterno não foi capaz de induzir alterações na proliferação nem na expressão dessa proteína. Se contrapondo ao nosso estudo, o único trabalho que observou os efeitos do exercício paterno sobre estes parâmetros foi o estudo de Yin e colaboradores (2013). O autor demonstra que o melhor desempenho nos testes de memória nos filhotes foi acompanhado por aumento na expressão de relina e BDNF hipocampal. Entretanto é preciso observar as grandes diferenças entre os estudos. Os protocolos de exercício utilizado por estes autores era maior (6 semanas contra 22 dias do nosso estudo), apresentava uma sessão maior (60 minutos/ dia durante 5 dias por semana versus 20 minutos por dia durante 5 dias por semanas) e com uma carga maior, uma vez que os autores utilizaram 75%VO_{2max} enquanto que em nosso trabalho utilizamos apenas 60%VO_{2max}, além disso a espécie utilizada também diferiu nos estudos, enquanto Yin e colaboradores utilizaram camundongos C57BL/6J, o presente estudo avaliou ratos Wistar. Estas diferenças entre ambos os estudos podem explicar, ao menos em parte, as diferenças encontradas nos resultados entre os trabalhos, uma vez que os efeitos induzidos pelo exercício físico variam conforme o tipo, intensidade e duração (Elsner et al., 2011; Ramsden et al., 2003; Risedal and Johansson, 1999).

Por outro lado, os dados de proliferação celular e conteúdo de relina no hipocampo de animais filhos de mães submetidas ao protocolo de exercício físico apresentou um resultado interessante. Observamos um aumento nos grupos exercitados antes (ES) e durante (SE) a gestação, mas não no grupo que se exercitou pelos dois períodos (EE). Possivelmente os dados encontrados na análise de sobrevivência celular e de relina estão relacionados aos achados comportamentais induzidos pelo exercício materno, entretanto essa relação ainda não está totalmente clara na literatura e necessita ser melhor estudada. A relina é descrita como uma glicoproteína que no SNC adulto está envolvida com processos de plasticidade e funções sinápticas. Ainda, estudos demonstraram que a deficiência desta proteína e/ou de um de seus receptores específicos (VLDLR e ApoER2) geram diminuição da memória associativa e espacial, déficit na função das LTP no hipocampo e até mesmo alterações morfológicas nos dendritos neuronais (Pesold et al., 1998; Pujadas et al., 2010; Rogers et al., 2011). Estes dados sugerem que a relina pode estar envolvida, ao menos em parte, com os resultados de aprendizado obtidos pelo nosso grupo, entretanto, a relação entre relina e aumento da sobrevivência celular ainda deve ser melhor estudada.

Por fim, com o objetivo de observar uma possível via para a programação fetal induzida pelo exercício físico parental, avaliamos a metilação global de DNA no hipocampo dos filhotes. A contribuição parental para o desenvolvimento dos descendentes acontece de diversas maneiras. Há muito tempo é estabelecida a relação causal entre os progenitores e a prole, o que de certa forma nos dá a base da hereditariedade, ou seja, a transmissão de algum traço através das gerações. Entretanto alguns autores diferenciam os efeitos hereditários, que são mediados pela transmissão genética dos alelos daqueles efeitos mediados pela transmissão de fatores não genéticos, caracterizando uma herança não gênica (Bonduriansky and Day, n.d.). Segundo Crean and Bonduriansky (2014), os efeitos parentais não gênicos podem ser mediados pela transmissão de variações ambientais, comportamentais, morfológicas, somáticas e ou epigenéticas da vida do progenitor antes da concepção (Bonduriansky and Day.; Crean and Bonduriansky, 2014).

Nesta tese nós observamos que o exercício foi capaz de alterar o status de metilação nos grupos descendentes de pais exercitados e de mães exercitadas somente antes da gestação (ES), embora exista uma diminuição da metilação nos grupos maternos EE e SE, essa diminuição não é estatisticamente diferente do grupo controle (SS). Considerando que diversos autores já relacionaram a metilação de DNA com plasticidade neural e melhoras em tarefas de aprendizado e memória, é possível supor que nossos achados comportamentais e de neuroplasticidade possam ter sido programados no feto devido à alteração no status de metilação de DNA hipocampal (Feng et al., 2007; Heyward and Sweatt, 2016; Morris and Monteggia, 2014).

Ainda, a diminuição da metilação global de DNA hipocampal observado no grupo ES revela que o estímulo causado pelo exercício em esteira pode ter alterado as células germinativas maternas antes da concepção, transferindo então essa programação para a prole. Entretanto, para confirmar tal hipótese, estudos mais específicos do epigenoma das células germinativas de ratas submetidas ao exercício em esteira devem ser realizados. Contudo, o grupo descendente das ratas submetidas ao exercício durante a gestação apresentou alterações de neuroplasticidade sem alterações da metilação global de DNA no hipocampo. Apresentando dados semelhantes, Herring e colaboradores (2012) demonstraram que um protocolo de exercício em roda de livre acesso foi capaz de induzir um aumento na expressão da relina hipocampal na prole, porém, da mesma forma que nosso estudo, não apresentou alterações na metilação do gene promotor da Relina. Apesar de termos hipotetizado uma sinergia entre os períodos de exercício físico materno, essa hipótese não se confirmou e o grupo que foi submetido ao exercício durante os dois períodos estudados não gerou uma prole com maiores efeitos comportamentais e de neuroplasticidade quando comparada aos outros grupos. Embora o exercício forçado seja considerado por alguns autores como uma forma de estresse prenatal, e seja relacionado com alterações prejudiciais ao desenvolvimento das funções hipocampais, em nosso estudo não foi percebido nenhum sinal de estresse nos animais durante ou depois da execução do protocolo, bem como a análise do peso das glândulas adrenais, uma medida indireta de estresse animal, não demonstrou diferenças entre os grupos. (Park et al. 2013; Wasinski et al. 2016; Jang et al. 2018).

Por outro lado, o exercício paterno foi capaz de induzir um padrão de desmetilação no hipocampo dos filhotes. Esse achado corrobora com um resultado recente de nosso grupo que demonstrou que um protocolo de exercício físico prolongado (8 semanas) também gerou uma diminuição da metilação global de DNA em filhotes. O que sugere que o exercício paterno é capaz de alterar o epigenoma do hipocampo dos descendentes. Contudo diferentemente do estudo aqui apresentado, este protocolo de 8 semanas não foi capaz de gerar alterações cognitivas na prole, o que sugere que um protocolo mais curto (22) dias pode ser mais eficaz para induzir benefícios cognitivos na prole.

Entretanto em nosso estudo essa alteração não foi precedida de uma alteração na metilação do esperma dos pais, já que não foi observado diferença entre os níveis de metilação dos grupos sedentários e exercitados. Em contraponto, em um estudo clínico conduzido por Denham (2017), foi observado que após 3 meses de treinamento físico, os homens apresentaram alteração do status de metilação de DNA no esperma. O que em conjunto nos leva a crer que a programação induzida pelo exercício paterno apresenta outra via de transmissão. Uma possibilidade são outras alterações epigenéticas no esperma que, durante a transmissão podem resultar em uma alteração no epigenoma do feto. Um estudo interessante, avaliou o efeito de um protocolo de exercício voluntário sobre parâmetros comportamentais, e observou que filhos de pais corredores apresentaram efeitos ansiolíticos, e ainda, os autores revelaram que o exercício voluntário induziu alterações em RNA não codificantes no esperma dos pais. (Short et al., 2017). É possível que outros mecanismos como os RNAs não codificantes ou outras alterações epigenéticas estejam envolvidas na alteração observada em nosso estudo, no entanto mais estudos são necessários para esclarecer esse ponto.

CONCLUSÕES

Esta tese foi dividida em dois artigos, um focado no efeito do exercício físico materno antes e durante a gestação e o segundo com enfoque no efeito do exercício paterno sobre o desenvolvimento da prole.

O estudo de um protocolo de exercício físico forçado em esteira ergométrica, antes da concepção e durante o período gestacional, se mostrou seguro para o desenvolvimento dos filhotes, uma vez que nossos dados não demonstraram que os protocolos utilizados geraram estresse nas mães ou atraso no desenvolvimento neuropsicomotor na prole. Os animais que foram submetidos à um programa de corrida em esteira antes da gestação geraram filhotes com alterações neuroplásticas positivas no hipocampo, (diminuição da metilação global do DNA, aumento do conteúdo de relina e aumento da sobrevivência neuronal) que parecem estar relacionados com a melhora do aprendizado observada no teste comportamental do labirinto aquático de Morris. Estes dados inéditos demonstrados no segundo artigo desta tese demonstraram que as experiências maternas antes da concepção são capazes de programar o encéfalo dos seus descendentes e promover alterações bioquímicas e comportamentais na vida adulta da prole. Entretanto, não observamos alterações significativas na prole descendente de mães que fizeram exercício continuamente nos dois períodos estudados (pré gestacional e gestacional). Estes achados em conjunto nos levam a concluir que a corrida em esteira ergométrica antes da gestação é capaz de gerar uma programação fetal positiva. A continuidade da atividade física durante os dois períodos se mostrou segura para o desenvolvimento dos filhotes, mesmo que não tenha gerado efeitos neuroplásticos ou comportamentais na prole. Estes achados sugerem maior proteção na prescrição do exercício físico durante o período gestacional, e abrem um novo campo de estudos envolvendo exercício físico e os períodos pré gestacional e gestacional.

Ao analisar o exercício paterno, observamos nos filhotes um padrão semelhante de melhora de aprendizado no paradigma do labirinto aquático de Morris, somados a uma diminuição da metilação global de DNA no hipocampo. Porém, os níveis de metilação global de DNA observados nos descendentes de pais exercitados não foi transmitido para a prole via metilação de DNA no esperma. Sugere-se que o exercício pode modular o epigenoma dos descendentes, entretanto essa transmissão deve acontecer por outro fenômeno que não a metilação, e mais estudos podem esclarecer esta via. De nosso conhecimento, esta é a primeira evidência que demonstra a capacidade do exercício físico paterno de programar o epigenoma no hipocampo de filhotes.

Desta forma, os resultados em conjunto do presente estudo sugerem a ação benéfica de exercício físico tanto materna quanto paterna sobre a saúde da prole. É importante ressaltar que os hábitos saudáveis dos pais, e não somente das mães, devem ser estimulados, uma vez que com base nos dados descritos por esta tese, os fatores ambientais paternos também geram uma programação favorável dos descendentes.

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