UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Exercício Físico Materno como Estratégia Neuroprotetora na Doença de Alzheimer

CAROLINE PERES KLEIN

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de Doutor em Bioquímica.

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"...somos aquilo que recordamos, literalmente."

"As nossas memórias fazem com que cada ser humano ou animal seja um ser único, um indivíduo."

Iván Izquierdo

Dedico esta tese, com uma infinita gratidão, à minha família,

que sempre me apoiou incondicionalmente nas minhas escolhas:

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APRESENTAÇÃO

Esta tese está dividida em partes e organizada em sessões, como descrito a seguir.

Os trabalhos elaborados nesta tese foram desenvolvidos no laboratório de Programação Metabólica, no Departamento de Bioquímica da Universidade Federal do Rio Grande do Sul (UFRGS), sob a orientação da Dra. Cristiane Matté, assim como foram desenvolvidos no Laboratório de Neuroproteção e Sinalização Celular, no Departamento de Bioquímica da UFRGS, sob a co-orientação da Dra. Christianne Gazzana Salbego.

Parte I. Compreende as sessões de Lista de abreviaturas, Lista de tabelas, Lista de figuras, Resumo, Resumo em inglês (*abstract*), Introdução, Justificativa e Objetivos.

A sessão *Introdução* apresenta o embasamento teórico que levou à formulação da proposta desta tese. A sessão *Justificativa* apresenta a importância, a relevância e a viabilidade da execução da pesquisa objetivada nesta tese.

Parte II. Compreende a sessão de Capítulos (I a IV).

A sessão *Capítulos* compreende os *Capítulos I, II, III* e *IV*, os quais são referentes aos artigos científicos publicados, submetidos ou em fase de preparação para publicação. Os capítulos descritos nesta sessão apresentam objetivos específicos, materiais, métodos, resultados, discussão e referências específicas para cada Capítulo (I a IV).

Parte III. Compreende as sessões de Discussão, Conclusão, Perspectivas, Referências e Anexos.

A sessão *Discussão* engloba uma interpretação geral dos resultados obtidos na tese, os quais estão descritos na sessão *Capítulos*, enquanto que a sessão *Conclusões* descreve as conclusões gerais da tese, as quais foram obtidas a partir da análise dos resultados. A sessão *Perspectivas* aborda possibilidades para o desenvolvimento de novos trabalhos com base nos achados descritos na presente tese. A sessão *Referências* lista as referências citadas nas sessões *Introdução* e *Discussão*. A sessão *Anexos* traz a carta de aprovação da Comissão de Ética no Uso de Animais (CEUA/UFRGS), documento imprescindível para a execução desta pesquisa.

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LISTA DE ABREVIATURAS

α-KGHD, α-cetoglutarato desidrogenase

Aβ, beta-amiloide

Aβ1-42, beta-amiloide contendo 42 aminoácidos

AβO ou AβO1-42, beta-amiloide oligomérico

ACOG, Colégio Americano de Obstetrícia e Ginecologia

ACSM, Colégio Americano de Medicina Esportiva

Akt, proteína-cinase B

AMP, adenosina monofosfato

AMPK, proteína-cinase ativada por AMP

ApoE, apolipoproteína E

ARE, elemento de resposta antioxidante

BDNF, fator neurotrófico derivado do encéfalo

CAT, catalase

CSF-GM, fator estimulador de colônia de granulócitos e macrófagos

CREB, proteína de ligação ao element de resposta ao AMP cíclico

DA, doença de Alzheimer

DOHaD, origem do desenvolvimento da saúde e da doença

Drp, proteína relacionada à dinamina

ER, espécies reativas

FDG-PET, tomografia por emissão de pósitrons marcada com [18F]- fluordesoxiglicose

FGF-2, fator de crescimento de fibroblastos

FoxO, fator de transcrição forkhead box O

GPx, glutationa peroxidase;

GFAP, proteína fibrilar ácida da glia

GSH, glutationa reduzida

GSK-3β, glicogênio-sintase-cinase 3β

Icv, intracerebroventricular

IDE, enzima de degradação da insulina

IDH, isocitrato-desidrogenase

IGF-1, fator de crescimento similar a insulina

IL, interleucina

iNOS, óxido-nítrico-sintase induzível

LKB1, AMPK cinase

MAPK, proteína-cinase ativadas por mitógenos

MDH, malato-desidrogenase

Mfn, mitofusina

mO_2•-, superóxido mitocondrial

mtDNA, DNA mitocondrial

NAD+, dinucleotídeo de nicotinamida adenina

NGF, fator de crescimento neuronal

NO•, óxido nítrico

Nrf2, fator de transcrição fator 2 relacionado ao fator nuclear eritroide 2

NRF1/2, fator de respiração nuclear

NO_2, dióxido de nitrogênio

NFκB, fator nuclear Kappa B

$ONOO^-$, peroxinitrito

Opa1, proteína atrofia ótica 1

PET, tomografia por emissão de pósitrons

PGC-1α, co-ativador 1α do receptor γ ativado por proliferação de peroxissomos

PKA, proteína-cinase A

PN, dia pós-natal

RAGE, receptores de produtos de glicação avançada

SCARA, receptores scavengers A

SH, grupamentos tióis

SIRT, sirtuina

SNC, sistema nervoso central

SOD1, superóxido-dismutase

SOD2, Mn-SOD

STE, sistema de transporte de elétrons

TCA, ciclo do ácido tricarboxílico

TFAM, fator de transcrição mitocondrial A

TNF, fator de necrose tumoral

TLR, receptores toll-like

VEGF, fator de crescimento do endotélio vascular

RESUMO

O período perinatal de desenvolvimento de um organismo é conhecido como janela crítica de susceptibilidade ou oportunidade, onde o fenótipo é estabelecido sob a influência do ambiente intrauterino ou da lactação. Dessa forma, o ambiente oferecido pelo estilo de vida materno influencia o desenvolvimento ao programar o metabolismo fetal. O exercício físico promove adaptações metabólicas, mesmo durante o período de gravidez, promovendo benefícios para o feto e contribuindo para a prevenção de doenças crônicas na vida adulta. Nesse sentido, o exercício materno afeta positivamente o metabolismo cerebral da prole, conferindo resistência às condições adversas no período pós-natal. No entanto, os mecanismos adaptativos envolvidos na determinação fenotípica do metabolismo da prole em resposta ao exercício materno ainda precisam ser determinados. A doença de Alzheimer é a principal doença neurodegenerativa associada com o envelhecimento, caracterizada por demência e declínio cognitivo. Bioquimicamente, o peptídeo β-amiloide tem papel relevante na neurotoxicidade característica da doença de Alzheimer. Em adição, o estresse oxidativo e a disfunção mitocondrial são algumas das características patofisiológicas dessa doença neurodegenerativa. A investigação de mecanismos envolvidos nesse processo é essencial para elucidar a origem e a evolução da doença, considerando o desafio do diagnóstico pré-clínico e o desenvolvimento de novas estratégias de tratamento. Na presente tese, foi investigado o papel neuroprotetor do exercício materno antes e durante a gestação contra as modificações neuroquímicas e comportamentais na prole adulta submetida à injeção intracerebroventricular de oligômeros de peptídeo β-amiloide, em um modelo animal da doença de Alzheimer. Inicialmente, avaliamos e descrevemos o comportamento materno, qualitativo e quantitativo, das ratas exercitadas (30 min/dia, 5 vezes/semana), bem como parâmetros de aparecimento das características físicas, dos reflexos motores e do desenvolvimento locomotor da prole. Além disso, investigamos algumas vias de sinalização associadas aos efeitos adaptativos do exercício materno no cérebro da prole aos 7 dias de vida pós-natal. Por fim, avaliamos o potencial do exercício materno em prevenir as alterações comportamentais, relacionadas às tarefas de aprendizado e memória, e neuroquímicas, relacionadas ao estado redox, função mitocondrial e função sináptica, induzidas pela neurotoxicidade de oligômeros de peptídeos β-amiloide 1-42 no córtex pré-frontal, hipocampo e cerebelo da prole adulta. Na presente tese, foi demonstrado que o exercício físico materno antes e durante a gestação não altera o comportamento materno, porém aumenta a frequência do comportamento exploratório da prole. A via da Akt-GSK-3β e as sirtuínas 1 e 3 são moduladas positivamente pelo exercício materno no cerebelo da prole. Interessantemente, o exercício materno preveniu diversas alterações neuroquímicas provocadas pela infusão de peptídeo β-amiloide1-42 oligomérico, como a redução de sinaptofisina e o aumento de Drp1 no hipocampo, o aumento dos níveis de espécies reativas, o aumento do imunoconteúdo da enzima óxido nítrico sintase e da proteína tau fosforilada no cerebelo da prole. Por fim, nossos resultados evidenciam o potencial efeito protetor do exercício físico materno contra prejuízos na memória induzidos pela infusão de peptídeo β-amiloide1-42 oligomérico na prole adulta ao modular positivamente a função mitocondrial. Os resultados apresentados ressaltam o potencial efeito neuroprotetor do exercício materno em um modelo animal, e essa abordagem baseada na mudança do estilo de vida pode ser extrapolada para a área clínica oferecendo a vantagem de prevenir o desenvolvimento de doenças crônicas na vida adulta.

ABSTRACT

The perinatal period of development is known as a critical window of susceptibility or opportunity, in which the phenotype is stablished under the influence of intrauterine or lactational environment. Maternal lifestyle influences the development through programming the fetal metabolism. Physical exercise promotes metabolic adaptations, even during pregnancy, promoting benefits to the fetus and preventing chronic diseases in adulthood. In this way, maternal exercise positively affects offspring's cerebral metabolism, conferring resistance to adverse conditions during postnatal life. However, the adaptive mechanisms involved in the phenotypical establishment of the offspring's metabolism in response to maternal exercise remain to be elucidated. Alzheimer's disease is the main aging-associated neurodegenerative disorder, psychologically characterized by dementia and cognitive decline. In this context, the amyloid-β peptide plays a significant role in the neurotoxicity of Alzheimer's disease. In addition, the oxidative stress and mitochondrial dysfunction are also pathological features present in the disease. Investigating the underlying mechanisms is crucial to unveil the disease origin and progression, considering the challenging preclinical diagnosis and novel therapeutic strategies development. In this thesis, it was investigated the neuroprotective role of maternal exercise before and during pregnancy against neurochemical and behavioral alterations in the offspring injected with amyloid-β oligomers, in an Alzheimer's disease-like model. Initially, we assessed and described the maternal behavior of exercised dams (30 min/day, 5 days/week); as well as the appearance of physical landmarks, motoric reflexes and ontogeny of locomotor behavior of female and male offspring. Furthermore, we assessed some signaling pathways related to adaptive effects of maternal exercise in the brain of 7-days-old pups. Finally, we assessed the potential effect of maternal exercise in preventing behavioral alterations, related to learning and memory tasks; as well as neurochemical changes, related to redox state, mitochondrial function and synaptic function, induced by neurotoxic infusion of oligomeric amyloid-β peptide 1-42 in the adult offspring's prefrontal cortex, hippocampus and cerebellum. Herein, we demonstrated that maternal exercise before and during pregnancy does not alter maternal behavior but increases offspring's exploratory behavior. The Akt-GSK-3β and sirtuin 1 and 3 pathways were positively modulated by maternal exercise in the offspring's cerebellum. Strikingly, maternal exercise prevented several neurochemical alterations elicited by the infusion of oligomeric amyloid-β peptide, such as the reduction of synaptophysin immunocontent and the increase of Drp1 in the offspring's hippocampus, the rise of reactive species, the immunocontent of the inducible nitric oxide synthase enzyme and tau phosphorylated in the offspring's cerebellum. Our findings also evidence the protective effect of maternal exercise against memory impairment induced by amyloid-β peptide infusion in the adult offspring, by modulating mitochondrial function. The results presented here highlight the potential neuroprotective effect of maternal exercise in an animal model, and this approach based on the lifestyle change can be extrapolated to the clinical area by offering the advantageous to prevent the development of chronic diseases in adulthood.

INTRODUÇÃO

Na última década, o interesse pelo entendimento das bases desenvolvimentistas da saúde e da doença tem crescido entre a população cientifica. A ideia por trás do estabelecimento do conceito de *Developmental Origins of Health and Disease* (DOHaD) se baseia na influência que o ambiente exerce sobre o desenvolvimento do organismo, durante os períodos intrauterino e pós-natal, ao promover modificações permanentes no metabolismo, as quais podem alterar a susceptibilidade ao desenvolvimento de doenças ao longo da vida. Durante os períodos críticos de desenvolvimento, também conhecidos como janela de susceptibilidade, o estilo de vida da mãe e outros estímulos ambientais modulam o estabelecimento do fenótipo da estrutura e da função dos órgãos. Estudos epidemiológicos e experimentais têm demonstrado que a programação do metabolismo que ocorre durante os períodos críticos do desenvolvimento pode influenciar o desenvolvimento de diversas doenças crônicas em longo prazo.

Programação metabólica

A gestação é um período crucial para o desenvolvimento e o crescimento do feto, bem como para a programação do metabolismo e epigenoma, os quais irão ditar o estado de saúde e/ou a susceptibilidade ao desenvolvimento de doenças na vida adulta (Bale, 2015). A programação do metabolismo do feto sofre influência multifatorial; a combinação de fatores genéticos, epigenéticos e ambientais ao longo dos diferentes estágios de desenvolvimento são determinantes para a programação da resposta do organismo frente a estímulos na vida pós-natal (Bateson *et al.*, 2014; Bale, 2015). A exposição a determinados fatores ambientais durante os estágios de desenvolvimento pré-natal e na infância pode programar a resposta do metabolismo na vida pós-natal frente a adversidades (Hanson e Gluckman, 2014), conferindo resistência ou susceptibilidade à perturbação homeostática, que pode desencadear processos patológicos (Bale, 2015). Ao longo das últimas décadas, vários estudos demonstraram associações entre o estilo de vida materno e a saúde da prole (Roseboom *et al.*, 2000; Horton, 2005; Salonen *et al.*, 2011). Dessa forma, surgiu

a área da programação metabólica, a qual abrange a programação do metabolismo, durante períodos críticos do desenvolvimento, para o período pós-natal.

O reconhecimento da área veio após uma série de publicações, com base em dados epidemiológicos, por David Barker e colaboradores, entre as décadas de 80 e 90. No entanto, publicações anteriores já apontavam evidências de que o ambiente vivenciado antes do nascimento e durante a infância exercia influências sobre a saúde do indivíduo através de abordagens epidemiológica e demográfica (Barker e Record, 1967; Forsdahl, 1977; Notkola *et al.*, 1985; Wadsworth *et al.*, 1985; Kermack *et al.*, 2001). O termo "die programmierung" (programação) foi introduzido na década de 70 pelo grupo de pesquisa do alemão Günter Dörner (Dörner *et al.*, 2008). Em seus trabalhos, Dörner e colaboradores demonstraram que as condições ambientais antes e logo após o nascimento estavam relacionadas ao risco de diabetes, obesidade e doenças cardiovasculares na idade adulta em humanos (Dorner e Mohnike, 1976; Dorner *et al.*, 1988; Dorner e Plagemann, 1994) e em modelos experimentais (Gotz *et al.*, 1986; Dorner *et al.*, 1987; Plagemann *et al.*, 1992).

Após anos de extensivo trabalho para o entendimento da etiologia das doenças com crescente incidência, Barker e Osmond (1986) observaram uma associação entre a alta taxa de mortalidade infantil, devido ao baixo peso no nascimento, e a incidência de doenças cardiovasculares em adultos, décadas depois, em uma mesma localização geográfica. Essa associação levantou a hipótese de que a desnutrição materna pode modificar de forma permanente o metabolismo do feto e influenciar o desenvolvimento de doenças coronarianas na idade adulta (Barker e Osmond, 1986). Os estudos de Barker foram a base para o desenvolvimento da área de programação metabólica. Diversas terminologias foram propostas para descrever a influência que o ambiente ao longo do desenvolvimento exerce sobre a susceptibilidade a doenças ao longo da vida. A hipótese de que doenças têm sua origem durante o período de desenvolvimento intrauterino proporcionou a criação do conceito da DOHaD (Gluckman *et al.*, 2010).

O conceito atual da DOHaD foi desenvolvida por Barker e, inicialmente, foi proposta como a hipótese do fenótipo poupador (*Thrifty Phenotype hypothesis*) (Hales e Barker, 1992), também conhecida como hipótese de Barker (*Barker's hypothesis*) ou hipótese da origem fetal da doença (*Fetal Origins of Disease hypothesis*) (Hales e Barker, 2001). O termo "programação" passou a ser utilizado por Barker e outros pesquisadores da DOHaD (Gluckman e Hanson, 2004b). A programação do desenvolvimento (*Developmental Programming*) define o processo pelo qual um estímulo que ocorre durante períodos críticos do desenvolvimento influencia o metabolismo de um organismo (Lucas, 1994). No entanto, a hipótese inicial de Barker considerou a influência do ambiente sobre o desenvolvimento apenas durante o período fetal como fator para a susceptibilidade ao desenvolvimento de doenças (Barker, 1998). A percepção de que outros períodos cruciais do desenvolvimento, como os períodos preconcepção e pós-natal, sofrem influência do ambiente levou à substituição do termo Origem Fetal da Doença por Origem Desenvolvimentista da Doença (*Developmental Origins of Disease*).

A partir desse momento, estudos em modelos animais contribuíram para o entendimento da programação do organismo em desenvolvimento, onde os estudos em ratos demonstraram que a interação entre os ambientes pré- e pós-natal determina o resultado da programação e, que os desafios não devem ser necessariamente nutricionais (Gluckman, 2004). Com isso, Bateson (2001) e Gluckman e Hanson (2004b), sugeriram que o feto e a criança são capazes de extrair informações do ambiente e, de acordo com isso, ajustar a trajetória do seu desenvolvimento com consequências duradouras que irão determinar a capacidade do organismo em lidar com o ambiente pós-natal. O organismo em desenvolvimento responde ao ambiente como uma estratégia adaptativa na expectativa de um ambiente previsto para o período pós-natal, ao invés de obter vantagem imediata (Gluckman e Hanson, 2004a; Gluckman, Hanson e Pinal, 2005). Com base nisso, Gluckman (2004) propuseram o modelo da Resposta Adaptativa Preditiva (*Predictive Adaptative Responses*) para explicar a programação do organismo e o estabelecimento fenotípico (Gluckman *et al.*, 2010). Esse modelo sugere que o risco de doença é a consequência do grau de incompatibilidade entre os

ambientes que o organismo é exposto durante as fases de plasticidade e pós-plasticidade (Gluckman, Hanson e Pinal, 2005). Dessa forma, o risco de desenvolvimento de doenças aumenta ao longo da vida como resultado da diminuição da plasticidade celular e dos efeitos cumulativos decorrentes de respostas inadequadas frente a novos desafios, no entanto, é a capacidade plástica estabelecida pelo ambiente materno antes e durante a gestação o principal determinante desse risco (Godfrey *et al.*, 2016).

A plasticidade do desenvolvimento explica que o organismo em desenvolvimento modifica o seu fenótipo em resposta ao ambiente (Godfrey *et al.*, 2007), ou seja, o organismo em desenvolvimento é capaz de detectar sinais ambientais (nutrição e hormônios maternos durante a gestação e a lactação) por mecanismos moleculares, os quais são traduzidos em informações bioquímicas que programam o desenvolvimento, de forma a conferir máxima aptidão para as condições presentes e para o ambiente futuro (Gluckman, Hanson e Spencer, 2005; Gluckman *et al.*, 2009). Os processos pelos quais a plasticidade fenotípica ocorre ainda não foram completamente elucidados; no entanto, alterações epigenéticas, as quais modulam a expressão gênica, e desafios oxidativos, os quais modulam o estado redox, são os mecanismos propostos e os quais têm sido investigados na tentativa de esclarecer como a plasticidade do metabolismo se adapta em resposta às condições do ambiente (Godfrey *et al.*, 2007; Barnes e Ozanne, 2011; Gluckman *et al.*, 2011). Por definição, epigenética explica as modificações químicas que ocorrem no DNA e nas histonas, sem que ocorra alteração da sequência do genoma, resultando em modulação da expressão gênica (Mazzio e Soliman, 2012). A plasticidade fenotípica conferida pelas modificações epigenéticas permite a adaptação do metabolismo em resposta ao ambiente através da programação de genes específicos (Gluckman, 2004; Haberland *et al.*, 2009). Por fim, o reconhecimento, com base em estudos epidemiológicos, clínicos e experimentais, de que os processos plásticos são essenciais para o desenvolvimento normal do organismo levou à modificação do conceito atualmente consolidado com o nome DOHaD (Barker, 2004; Gluckman, Hanson e Pinal, 2005).

Atualmente, o conceito de DOHaD vem sendo disseminado entre as áreas médicas e de ciência básica. Sabe-se que a fisiologia, a dieta e o estilo de vida materno exercem influências duradouras na saúde e susceptibilidade a doenças da prole, em longo prazo (Fleming *et al.*, 2018). Tendo em vista que estilo de vida da mãe durante o período de gestação e lactação prepara o metabolismo do feto e do recém-nascido para o futuro, modificações no estilo de vida materno, em favor de hábitos saudáveis, parece uma estratégia promissora à programação do metabolismo em desenvolvimento de forma a conferir resistência ao desenvolvimento de doenças. Com base nisso, intervenções como o exercício físico podem causar modificações que sejam favoráveis a uma vida saudável e, ainda, podem proteger os filhos contra o desenvolvimento de doenças metabólicas crônicas não-transmissíveis na adolescência até a idade adulta (Fidalgo *et al.*, 2013; Maliszewska-Cyna *et al.*, 2017; Ribeiro *et al.*, 2017).

Exercício físico

A atividade física é definida por qualquer movimento do corpo produzido pela musculatura esquelética e que resulta em gasto energético, enquanto o exercício físico é definido pela atividade física que é planejada, estruturada e repetitiva cujo objetivo é a melhora e manutenção da aptidão física (Caspersen *et al.*, 1985).

O estilo de vida desempenha um papel importante no estado de saúde de um indivíduo. Atualmente, o estilo de vida da população global é caracterizado pelo consumo excessivo de dietas ricas em calorias e sedentarismo (Dishman *et al.*, 2006; Thornton *et al.*, 2016; Calder *et al.*, 2018). Esse estilo de vida tem sido associado a consequências negativas à saúde, como o desenvolvimento de diversas doenças crônicas (Etchegoyen *et al.*, 2018), como as doenças cardiovasculares e metabólicas, câncer, doenças neurodegenerativas, e doenças afetivas (Garber *et al.*, 2011), em decorrência de distúrbios do balanço energético (Dishman *et al.*, 2006). Mudanças no estilo de vida representam um desafio para a saúde pública e programas de incentivo à prática de exercício

físico e hábitos de dieta saudáveis têm sido encorajados (Wanigatunga *et al.*, 2017; Etchegoyen *et al.*, 2018).

Os efeitos do exercício físico à saúde têm sido amplamente demonstrados na literatura. A prática de exercício físico é associada a numerosos benefícios para a saúde física, cognitiva e mental de homens e mulheres, em diferentes idades (Ruscheweyh *et al.*, 2011), apresentando potencial preventivo e terapêutico contra diversas doenças (Dishman *et al.*, 2006; Vina e Gomez-Cabrera, 2014; Calder *et al.*, 2018). O Colégio Americano de Medicina Esportiva (*American College of Sports Medicine – ACSM*) recomenda que a maioria dos adultos pratiquem exercício de intensidade cardiorrespiratória moderada, com duração mínima de 30 minutos, 5 vezes na semana, para melhorar a aptidão física e a saúde (Garber *et al.*, 2011).

O exercício físico é considerado um estressor fisiológico que é capaz de regular o metabolismo energético do organismo (Ronn e Ling, 2013; Mooren e Kruger, 2015). O exercício moderado (estresse moderado) melhora a habilidade funcional com a qual o organismo irá lidar frente a um estresse subsequente ou mais severo (Le Bourg e Rattan, 2014). Portanto, o exercício físico tem sido considerado uma estratégia indutora do efeito de hormese (*hormesis*) (Radak *et al.*, 2005), que é o fenômeno definido pelo efeito adaptativo benéfico promovido por baixas doses de substâncias potencialmente danosas (Calabrese e Baldwin, 2001).

O exercício físico promove adaptações metabólicas em diversos tecidos do organismo, inclusive no sistema nervoso central (SNC) (Cotman *et al.*, 2007; Vina e Gomez-Cabrera, 2014; Radak *et al.*, 2016). Diversas evidências apontam para a melhora da função cognitiva e para os efeitos neuroprotetores do exercício contra doenças neurológicas e psiquiátricas (Cotman e Berchtold, 2002; Dishman, 2006). Portanto, a neurobiologia do exercício emergiu para investigar os aspectos neurais da fisiologia do exercício (Dishman, 2006), permitindo o entendimento de diversos mecanismos celulares e moleculares envolvidos nos efeitos do exercício físico sobre o metabolismo cerebral, e os processos de neurogênese, sinaptogênese e angiogênese (Dalsgaard,

2006; Dishman *et al.*, 2006). Visto que o SNC controla processos fisiológicos, comportamentais e mentais, os processos adaptativos que ocorrem no SNC em resposta ao exercício podem, além de prevenir a depressão, ansiedade e o declínio cognitivo associado ao envelhecimento, reduzir fatores de risco periféricos que podem comprometer negativamente a função cerebral, como diabetes, hipertensão, obesidade e doenças cardiovasculares (Cotman e Berchtold, 2002; Dishman, 2006). É importante ressaltar que existe uma relação dose-resposta entre a duração e a intensidade do exercício, onde os efeitos adaptativos benéficos estão associados ao exercício moderado (Cotman e Berchtold, 2002). Várias regiões do cérebro sofrem mudanças estruturais e funcionais para se adaptar ao exercício e, dessa forma, melhorar as funções cerebrais (Cotman e Berchtold, 2002).

O cérebro utiliza como principal substrato a glicose, e obtém energia a partir da sua completa oxidação, o que depende do metabolismo mitocondrial (Mergenthaler *et al.*, 2013). A obtenção de energia a partir da oxidação da glicose para formar ATP é imprescindível para sustentar a função cerebral relacionada à neurotransmissão e à manutenção do potencial de membrana (Mergenthaler *et al.*, 2013). A função e localização mitocondrial é crucial para a manutenção neuroenergética e a sobrevivência celular (Nicholls *et al.*, 2015). Nos neurônios, um grande pool de mitocôndrias está estrategicamente localizado próximo aos terminais sinápticos em função da grande demanda por moléculas de ATP para a manutenção da neurotransmissão e plasticidade sináptica (Cai e Tammineni, 2017). Dessa forma, a bioenergética mitocondrial controla diversos processos fisiológicos do SNC (Mattson, 2012). Durante os processos normais do metabolismo energético, ocorre a produção de espécies reativas (ER) em todos os compartimentos celulares; contudo, o sistema de transporte de elétrons (STE) é a principal fonte de produção de ER devido ao grande fluxo de elétrons entre os complexos enzimáticos mitocondriais (Halliwell, 2006). O exercício físico aumenta a taxa metabólica do organismo de forma sistêmica, exigindo uma maior produção de ATP, e aumentando a atividade das enzimas do STE no SNC, que resulta no aumento da produção de ER (Radak *et al.*, 2008). As ER

desempenham importantes papeis em processos fisiológicos, como sinalização celular, proteção contra patógenos, indução de apoptose, indução do sistema antioxidante endógeno e do sistema de degradação de proteínas (Banerjee, 2012). As ER, radicais livres ou não radicais, são instáveis e altamente reativas, oxidando biomoléculas a fim de alcançar sua estabilidade (Halliwell, 2006). A homeostase redox é caracterizada pelo equilíbrio entre a produção e a eliminação das ER, pois dependendo da concentração e do tempo de exposição, as ER podem ter efeitos benéficos ou deletérios nos sistemas biológicos (Sies, 2015). A produção excessiva de ER em associação a um sistema de defesa antioxidante insuficiente para a remoção das mesmas pode culminar no acúmulo de ER e, consequentemente, no dano a biomoléculas, caracterizando o estresse oxidativo/desequilíbrio do estado redox, o qual está envolvido em processos patológicos, como câncer, inflamação, doenças cardiovasculares e neurodegenerativas (Webb *et al.*, 2017).

O metabolismo energético e o estado redox são processos intrinsecamente relacionados (Griffiths *et al.*, 2017), e o exercício é capaz de modular várias moléculas que são sensíveis a mudanças da demanda energética e do estado redox (Radak *et al.*, 2013). A formação de ER decorrentes da prática de exercício físico moderado medeia os processos adaptativos ao mesmo (Radak *et al.*, 2008; Marques-Aleixo *et al.*, 2012), através da estimulação do sistema antioxidante, ativação proteassomal para o reparo de dano oxidativo às proteínas (Radak *et al.*, 2001), e indução de biogênese mitocondrial (Vina *et al.*, 2009). O fator de transcrição fator 2 relacionado ao fator nuclear eritroide 2 (Nrf2) é encontrado no citosol na sua forma inativa associado à proteína Keap 1, a qual funciona como regulador negativo de Nrf2. Durante o estado celular oxidativo, Keap1 sofre um ataque eletrofílico e se dissocia do Nrf2 (Suzuki e Yamamoto, 2015), o qual é translocado ao núcleo, onde irá estimular a transcrição de moléculas antioxidantes, dentre elas as enzimas da via de síntese da glutationa reduzida (GSH), ao se ligar ao elemento de resposta antioxidante (ARE) na região promotora de diversos genes alvos (Dinkova-Kostova e Abramov, 2015; Merry e Ristow, 2016).

O aumento da demanda energética em resposta ao exercício, indicado pelo aumento dos níveis de adenosina monofosfato (AMP) e NAD^+, promove a ativação de sensores intracelulares do metabolismo energético, como a proteína-cinase ativada por adenosina monofosfato (AMPK) e as proteínas desacetilases dependentes de NAD^+, como as sirtuínas (SIRT; *silent information regulator*) (Guerrieri *et al.*, 2017). A AMPK pode ser ativada diretamente pelas ER, através dos resíduos sensíveis à mudança do estado redox presentes na subunidade α da enzima, e pode ser ativada, indiretamente, pelo aumento da atividade da enzima AMPK cinase (LKB1) desacetilada pela SIRT1 (Griffiths *et al.*, 2017). Tanto a AMPK quanto as SIRTs regulam a atividade de fatores transcricionais relacionados ao metabolismo energético (Guerrieri *et al.*, 2017). A SIRT1, isoforma localizada no citoplasma e núcleo da célula, é o principal fator responsável por desacetilar e ativar o fator de transcrição *Forkhead box O* (FOXO) e o co-ativador 1α do receptor γ ativado por proliferação de peroxissomos (PGC-1α) (Brenmoehl e Hoeflich, 2013; Eijkelenboom e Burgering, 2013; Correia *et al.*, 2017). As proteínas FOXOs regulam a expressão de enzimas antioxidantes como a superóxido-dismutase (SOD) e a catalase (CAT) para aumentar a resistência ao estresse oxidativo (Eijkelenboom e Burgering, 2013). De forma similar, o exercício modula a ativação da SIRT3, isoforma presente na mitocôndria (Brenmoehl e Hoeflich, 2013). A SIRT3 desempenha um importante papel na homeostase redox, pois ela desacetila várias proteínas mitocondriais e regula suas funções. Por exemplo, a desacetilação pela SIRT3 da SOD2 é essencial para a sua atividade enzimática (Tao *et al.*, 2010). Processos metabólicos como a fosforilação oxidativa, o ciclo do ácido tricarboxílico (TCA) e a oxidação de ácidos-graxos são ativados diretamente pela SIRT3, enquanto o PGC-1α e a AMPK são ativados indiretamente pela SIRT3 (Brenmoehl e Hoeflich, 2013).

As mitocôndrias são organelas dinâmicas que sofrem constante remodelamento estrutural através dos processos de fusão e fissão, para manter as funções bioenergéticas adequadas às necessidades celulares (Webb *et al.*, 2017). Os processos de fusão e fissão são orquestrados, principalmente, pelas proteínas mitofusinas (Mfn) e *dinamin-related protein* (Drp),

respectivamente, que apresentam atividade GTPase e são responsáveis pela regulação do número, tamanho e movimento das mitocôndrias (Raefsky e Mattson, 2017). A dinâmica e as funções mitocondriais são amplamente moduladas pelo exercício e a biogênese mitocondrial é uma adaptação do metabolismo energético favorável ao aumento da capacidade metabólica oxidativa celular (Fealy *et al.*, 2014; Zorzano *et al.*, 2015; Gottlieb e Bernstein, 2016). A biogênese mitocondrial, definida pelo aumento do número de mitocôndrias a partir de mitocôndrias já existentes em resposta a variações energéticas (Gottlieb e Bernstein, 2016), é um processo complexo que envolve a expressão coordenada de genes codificados pelo DNA nuclear e mitocondrial (mtDNA) (Scarpulla, 2011). O aumento do número de mitocôndrias, em condições de produzir os mesmos níveis de ATP, permitirá que a atividade respiratória funcione em uma taxa mais baixa já que haverá distribuição do consumo de oxigênio entre os complexos do STE para produção dos mesmos níveis de ATP, resultando na redução da produção de ER (Radak *et al.*, 2016). A biogênese mitocondrial é regulada pelo PGC-1α, que medeia a transcrição do fator de respiração nuclear (NRF) e do fator responsável pela transcrição e duplicação do mtDNA, o fator de transcrição mitocondrial A (TFAM) (Zhu *et al.*, 2013). O TFAM é traduzido no citosol e transportado para a mitocôndria, onde induz a transcrição de enzimas mitocondriais codificadas pelo mtDNA (Ventura-Clapier *et al.*, 2008).

O exercício ativa também vias de sinalização envolvidas em processos fisiológicos, como neurogênese, plasticidade e memória (Mattson, 2012). O exercício aumenta os níveis de fatores tróficos, tais como fator neurotrófico derivado do encéfalo (BDNF), fator de crescimento neuronal (NGF), fator de crescimento de fibroblastos 2 (FGF-2), fator de crescimento similar a insulina 1 (IGF-1) e fator de crescimento do endotélio vascular (VEGF) (Intlekofer e Cotman, 2013; Radak *et al.*, 2014). O BDNF regula o desenvolvimento do cérebro, neuroplasticidade, neurogênese, plasticidade sináptica e sobrevivência celular, através das vias de sinalização da proteína cinase B (Akt)/proteína de ligação ao elemento de resposta ao AMP cíclico (CREB) e da proteína-cinase ativada por mitógenos (MAPK)/CREB, as quais estão envolvidas na formação da memória

(Mattson, 2012). A sinalização mediada pela ligação do BDNF ao receptor de tropomiosina cinase B (TrkB) leva à ativação do fator de transcrição chamado de CREB, o qual induz o aumento da expressão de BDNF, criando uma retroalimentação positiva (Radak *et al.*, 2014).

As vias de sinalização descritas acima são responsáveis pelos efeitos benéficos do exercício físico sobre os processos cognitivos (De Felice e Ferreira, 2014; Biessels e Reagan, 2015), exercendo impacto significante sobre a qualidade de vida, influenciando de forma positiva a memória e o bem-estar (Janecka, 2017). Modelos experimentais, em roedores, que utilizam diferentes modalidades de exercício físico, como exercício voluntário em rodas de correr (Ding *et al.*, 2006; Vaynman *et al.*, 2007) ou involuntário como corrida em esteira (Cassilhas *et al.*, 2012) e natação (Stone *et al.*, 2015), já estão bem estabelecidos na literatura. Os efeitos do exercício demonstrados nesses estudos promovem melhora do desempenho dos animais em diversas tarefas de aprendizado e memória, como o labirinto aquático de Morris (Cassilhas *et al.*, 2012), labirinto de braços radiais (Jin *et al.*, 2017), labirinto em T e Y, medo condicionado (Lin *et al.*, 2012), teste de reconhecimento de objetos, entre outros. Além disso, diversos estudos conduzidos com modelos animais reforçam o potencial neuroprotetor do exercício contra o desenvolvimento de doenças crônicas. Dessa forma, a prática de exercício físico tem despontado como uma estratégia não-farmacológica promissora para a prevenção de doenças neurodegenerativas como a Doença de Alzheimer (DA) (Dao *et al.*, 2013; Barnard *et al.*, 2014).

Exercício materno

A mudança de um estilo de vida sedentário para um estilo de vida ativo, caracterizado pela prática regular de atividade física, em qualquer momento da vida pode trazer benefícios à saúde. Contudo, o impacto dessa mudança à saúde é maior quando ocorre em períodos de plasticidade do desenvolvimento (Calder *et al.*, 2018). Pesquisas relacionadas à programação metabólica estão engajadas em desvendar os mecanismos pelos quais o estilo de vida materno, durante períodos

críticos do desenvolvimento, é capaz de programar o metabolismo do feto, e como isso pode estar relacionado à prevenção ou à susceptibilidade a doenças na vida adulta.

A prática regular de atividade física é indicada em todas as fases da vida, incluindo a gestação (Lotgering, 2014; Acog, 2015). O Colégio Americano de Obstetras e Ginecologistas (ACOG) recomenda que gestantes, que não apresentem contraindicações médicas, se exercitem durante 20-30 minutos por dia em quase todos os dias da semana. Gestantes são encorajadas a continuar ou iniciar a prática de atividade física como caminhada, natação, pilates, yoga ou outras atividades de baixo impacto (Acog, 2015). O exercício durante a gestação não oferece riscos ao feto, pois mecanismos de compensação metabólica ocorrem, dependendo do tipo, duração e intensidade do exercício (Artal, 2016). No entanto, atividades aquáticas, como a natação, são amplamente recomendáveis para mulheres gestantes, pois oferecem diversas vantagens em comparações a outras modalidades de exercício (Katz, 2003; Lynch *et al.*, 2007). O ambiente aquático funciona como termorregulador pois permite que o aumento da temperatura induzido pelo exercício seja dissipado durante a atividade física e, dessa forma, protege o feto do superaquecimento e os efeitos teratogênicos associados ao aumento da temperatura (Hartmann e Bung, 1999; Mottola, 2016). Além disso, a frequência cardíaca é reduzida na água, a expansão do volume sanguíneo que acompanha a imersão ajuda a manter o fluxo sanguíneo útero-placenta garantindo a disponibilidade de oxigênio e mantendo a taxa metabólica fetal (Lynch *et al.*, 2007). A propriedade flutuante da água é benéfica para as articulações da mãe e não oferece risco de queda (Hartmann e Bung, 1999). Dentre os benefícios que o exercício oferece à saúde da gestante pode-se destacar a prevenção de doenças como diabetes gestacional, hipertensão, pré-eclâmpsia, e o auxílio no controle de ganho de peso durante a gestação, visto que o aumento excessivo de peso pode provocar problemas obstétricos (Acog, 2015; Moyer *et al.*, 2016). Além de trazer benefícios à saúde da gestante, o exercício promove benefícios à saúde do feto com efeitos evidentes ao longo dos primeiros anos pós-natal (Clapp, 1996b; Clapp *et al.*, 1998; Clapp *et al.*, 1999; Clapp *et al.*, 2000; Labonte-Lemoyne *et al.*, 2017).

Estudos clínicos (Clapp, 1996b; Jukic *et al.*, 2013; Mourtakos *et al.*, 2015; Labonte-Lemoyne *et al.*, 2017) e experimentais (Akhavan *et al.*, 2008; Marcelino *et al.*, 2013; Robinson e Bucci, 2014; Marcelino *et al.*, 2016) evidenciam que os efeitos adaptativos promovidos pelo exercício também se estendem ao feto quando a prática da atividade ocorre durante o período gestacional. Em humanos, a prática de exercício físico durante todo o período de gravidez é capaz de acelerar a maturação cerebral (Labonte-Lemoyne *et al.*, 2017) e o desenvolvimento da linguagem em crianças (Clapp, 1996b; Jukic *et al.*, 2013), bem como melhora o desenvolvimento neuromotor (Clapp *et al.*, 1998) e o comportamento de orientação do neonato (Clapp *et al.*, 1999). Em roedores, foi demonstrado que o exercício materno promove alterações fenotípicas benéficas em vários tecidos e órgãos, como o tecido muscular (Liu *et al.*, 2018) e adiposo (Wasinski *et al.*, 2015), fígado (Stanford *et al.*, 2017) e cérebro (Marcelino *et al.*, 2013), que persistem até a idade adulta e, que muitas vezes, são dependentes do gênero (Marcelino *et al.*, 2016; Bale e Epperson, 2017). O exercício materno melhora as fases de aquisição e retenção da memória espacial (Akhavan *et al.*, 2008), melhora a memória de reconhecimento (Robinson e Bucci, 2014; Marcelino *et al.*, 2016), melhora a função mitocondrial (Park *et al.*, 2013), induz neurogênese hipocampal (Bick-Sander *et al.*, 2006; Akhavan *et al.*, 2008) e aumenta a expressão de BDNF no hipocampo da prole (Lee *et al.*, 2006; Gomes Da Silva *et al.*, 2016). Além disso, o exercício físico durante a gestação promove adaptações metabólicas em diversas regiões do cérebro de filhotes de ratos, demonstrado pelo aumento na capacidade antioxidante total, aumento da atividade de enzimas antioxidantes SOD, CAT e glutationa-peroxidase (GPx), e ausência de dano oxidativo a biomoléculas (Marcelino *et al.*, 2013).

Tendo em vista a habilidade de programar o metabolismo da prole, diferentes grupos demonstraram que o exercício materno é capaz de conferir proteção à prole contra diversas condições patológicas, tais como a prevenção de obesidade (Wasinski *et al.*, 2015), esteatose hepática (Sheldon *et al.*, 2016) e tumorigênese (Camarillo *et al.*, 2014), prevenção da redução do número de neurônios hipocampais causada pela hipóxia crônica neonatal (Akhavan *et al.*, 2012),

prevenção dos efeitos deletérios da dieta rica em gordura sobre o metabolismo da glicose (Stanford *et al.*, 2015) e da dieta com restrição proteica sobre o desenvolvimento motor (Falcao-Tebas *et al.*, 2012), bem como atenuação dos efeitos de restrição de crescimento fetal desencadeadas por modelo de inflamação gestacional (Kasawara *et al.*, 2016). Interessantemente, camundongos transgênicos CRND8 para a doença de Alzheimer que foram submetidos ao exercício físico *in utero* apresentaram redução das placas amiloides, de parâmetros de estresse oxidativo e pró-inflamatórios aos 5 meses de idade, sugerindo que o ambiente intrauterino promoveu alterações que foram capazes de amenizar as características da patologia (Herring *et al.*, 2012).

Doença de Alzheimer

Ao longo das últimas décadas até a atualidade, o aumento da expectativa de vida vem acompanhando avanços médicos, sanitários, tecnológicos e econômicos (Divo *et al.*, 2014). A expectativa de vida global é estimada em 75 anos para mulheres e 69 anos para homens (Dalys e Collaborators, 2017). Em adição, é observado que a taxa de crescimento populacional segue um curso inversamente proporcional ao aumento da expectativa de vida. Em resumo, a população global vivencia um significante processo de envelhecimento (Divo *et al.*, 2014), que, muitas vezes, está associado ao aumento da morbidade. A incidência de doenças crônicas e neurodegenerativas está intimamente relacionada ao envelhecimento devido ao acúmulo de modificações que ocorrem no organismo e culminam no desenvolvimento de diversas patologias.

A prevalência global de doenças neurodegenerativas que causam demência estima que aproximadamente 46,8 milhões de pessoas são afetadas. A doença neurodegenerativa mais frequente é a DA esporádica (http://www.alz.org), cuja taxa de prevalência acompanha o aumento global (Alzheimer's, 2018). A DA é a principal causa de demência (representando de 60 a 80 % dos casos) e representa um sério problema de saúde pública que requer altos custos, considerando as suas características neurológicas debilitantes, que demandam tratamentos farmacológicos e atenção de cuidadores, além de ser responsável por uma elevada mortalidade (Balin e Hudson,

2014). Clinicamente, a DA é caracterizada por perda ou prejuízo da memória, déficit cognitivo progressivo e de outras habilidades sócio-comportamentais (Parihar e Hemnani, 2004).

A etiologia da DA é multifatorial e complexa, envolvendo a interação de fatores genéticos, ambientais, metabólicos e de estilo de vida. Dessa forma, podem estar implicados no desenvolvimento da DA fatores como: disfunção mitocondrial (Lee *et al.*, 2018), inflamação (Liu e Chan, 2014), desregulação do metabolismo do cálcio (Pierrot *et al.*, 2006), alterações no metabolismo energético (Demetrius e Simon, 2013) e consequente estresse oxidativo (Xie *et al.*, 2013). Esses fatores contribuem para o desenvolvimento das características neuropatológicas da DA, que consistem na deposição de peptídeos beta-amiloide (Aβ) no meio extracelular e, em emaranhados neurofibrilares intracelulares compostos por agregados hiperfosforilados da proteína tau (Giacobini e Gold, 2013). A neurodegeneração regional aliada às alterações fisiopatológicas no cérebro de pacientes com DA são observadas, principalmente, no córtex pré-frontal e no lobo temporal, ambas as regiões que desempenham papel crucial nos processos de formação de memória (Tromp *et al.*, 2015; Guo *et al.*, 2016).

A proteína tau é uma proteína associada aos microtúbulos, com função de estabilizar o citoesqueleto e, portanto, manter a estrutura neuronal e o transporte axonal (Fig. 1). A proteína tau está em constante transição entre os estados fosforilado e desfosforilado; a tau desfosforilada interage com α- e β-tubulina e, ao ser fosforilada, perde a habilidade de interagir com essas proteínas e, dessa forma, permite a dinâmica do citoesqueleto (De-Paula *et al.*, 2012). No entanto, em condições patológicas como a DA ocorre uma hiperfosforilação da tau, especialmente pela proteína glicogênio-sintase-3β (GSK-3β). A hiperfosforilação da tau leva a uma desestabilização dos microtúbulos e, ao consequente prejuízo do transporte axonal que pode culminar na morte neuronal (Querfurth e Laferla, 2010).

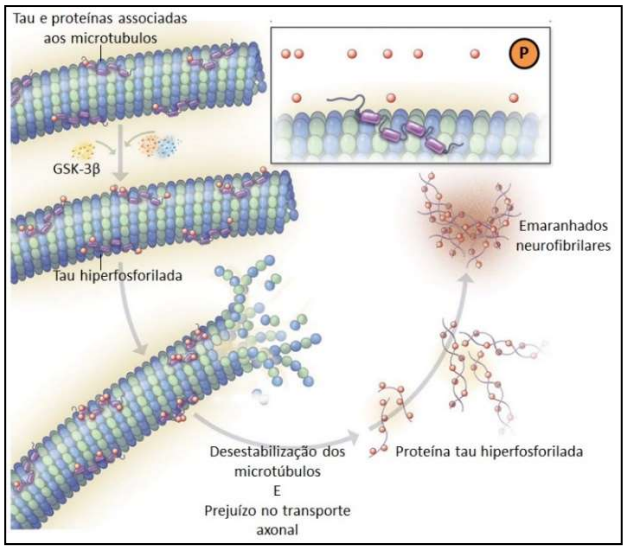

Figura 1. Função da proteína tau na (des)estabilização dos microtúbulos. Imagem adaptada de (Querfurth e Laferla, 2010). Abreviaturas: GSK-3β, glicogênio sintase cinase 3β; P, grupos fosforil.

A proteína precursora amiloide (APP), a qual origina os peptídeos Aβ, pode ser metabolizada por duas vias: a via não-amiloidogênica e a via amiloidogênica (Fig. 2). A não-amiloidogênica envolve a ação sequencial das enzimas α- e γ-secretase; a primeira cliva a APP dentro da região que contém a sequência do peptídeo Aβ e, em seguida, a APP é clivada pela enzima γ-secretase liberando o fragmento p3. A via amiloidogênica envolve o processamento da APP a partir da ação sequencial das enzimas β- e γ-secretase; a enzima β-secretase cliva a APP e promove a liberação de um pequeno fragmento N-terminal, deixando um longo fragmento C-terminal, no qual está a sequência do peptídeo Aβ; o fragmento C-terminal será clivado pela enzima γ-secretase, liberando os peptídeos Aβ (Querfurth e Laferla, 2010; De-Paula *et al.*, 2012). A toxicidade atribuída ao peptídeo Aβ inicia quando ocorre um desequilíbrio entre a produção e eliminação do mesmo (Balin e Hudson, 2014). Os peptídeos formados possuem tamanho variável, podendo conter 43 aminoácidos; dentre eles, o peptídeo Aβ composto por 42 aminoácidos, que é considerado o mais tóxico (Querfurth e Laferla, 2010). Os peptídeos Aβ se acumulam e agregam podendo formar oligômeros (AβO) solúveis ou fibrilas insolúveis. Os oligômeros formados pela agregação de peptídeos Aβ são responsáveis pela neurotoxicidade na DA (Querfurth e Laferla, 2010). As diferentes formas de peptídeos, (monômeros, oligômeros ou fibrilas) podem se ligar a

diversos tipos de receptores e, assim, interagem com vários tipos de células no SNC, como os neurônios, microglia, astrócitos e células endoteliais (Jarosz-Griffiths *et al.*, 2016). A ligação de monômeros ou fibrilas aos receptores de superfície celular, geralmente desencadeia uma resposta para eliminar os peptídeos Aβ formados; por outro lado, a ligação de AβO aos receptores de superfície geralmente desencadeiam seus efeitos neurotóxicos (Jarosz-Griffiths *et al.*, 2016).

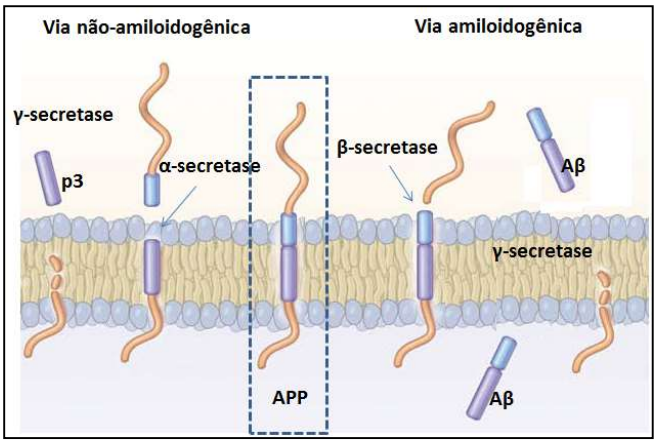

Figura 2. Processamento da proteína precursora amiloide. Imagem adaptada de (Querfurth e Laferla, 2010). Abreviaturas: APP, proteína precursora amiloide; Aβ, peptídeo β-amiloide.

Nos locais de acúmulo de peptídeos Aβ, são encontradas células microgliais e astrócitos, os quais desempenham papel imunomodulador. As células microgliais são as células de defesa do SNC, enquanto que os astrócitos desempenham uma ampla gama de funções, além da imunomodulação. Os astrócitos dão o suporte trófico para os neurônios, induzem sinaptogênese, regulam a plasticidade e a transmissão sináptica (Morales *et al.*, 2014). Os peptídeos Aβ se ligam aos receptores de superfície dessas células gliais e desencadeiam respostas que podem ser a favor da eliminação dos peptídeos Aβ, ou ainda, que promovam um processo inflamatório. A microglia desempenha esse papel dicotômico na DA dependendo do tipo de receptor ao qual o peptídeo Aβ se liga e, são encontradas na sua forma ativa em regiões com acúmulo de Aβ (Jarosz-Griffiths *et al.*, 2016). Receptores scavengers A (SCARA), receptores de macrófagos com estrutura colágena (MARCO) e receptores de lipoproteínas de baixa densidade (LDLR) são alguns dos receptores de superfície de astrócitos ou microglia que favorecem a eliminação dos peptídeos Aβ. Por outro lado,

a ligação dos Aβ aos receptores de produtos de glicação avançada (RAGEs) e receptores toll-like (TLR) na superfície dos astrócitos e microglia induz a expressão de mediadores inflamatórios, como as citocinas pró-inflamatórias interleucina (IL) 1β, IL-6 e fator de necrose tumoral α (TNF-α), e produzem ER (Jarosz-Griffiths *et al.*, 2016). Sendo assim, a presença de AβO no SNC promove neuroinflamação através da ativação das células gliais que, com o acúmulo progressivo de AβO contribuem para um processo inflamatório sustentado e de reatividade glial com expressão aumentada da proteína fibrilar ácida da glia (GFAP) pelos astrócitos; esses eventos podem levar à morte neuronal e, como consequência, podem promover déficit cognitivo e neurodegeneração (Doens e Fernandez, 2014).

A desregulação do metabolismo da glicose e da sinalização da insulina também contribui para a progressão da DA. Os AβO podem interagir com os receptores de insulina (IR), induzir a remoção dos mesmos da superfície celular e promover alterações na sinalização intracelular, prejudicando o metabolismo energético e os processos de plasticidade sináptica (De Felice *et al.*, 2009; Jarosz-Griffiths *et al.*, 2016). A sinalização da insulina está intimamente relacionada aos processos de plasticidade sináptica e aos processos de aprendizagem e formação de memória. Muitos autores têm demonstrado conexões entre a DA e o diabetes mellitus tipo 2 (DM2) uma vez que a hiperglicemia, hiperinsulinemia e inflamação são características compartilhadas entre as duas desordens, bem como déficits cognitivos e redução do volume hipocampal (De Felice e Ferreira, 2014). Além do envolvimento do metabolismo da glicose para a fisiopatologia da DA, o metabolismo do colesterol também contribui para o desenvolvimento da doença. Indivíduos que possuem o alelo da apolipoproteína E4 (ApoE4) apresentam maior susceptibilidade de desenvolver a DA (Lim, Y. Y. *et al.*, 2015). As isoformas das ApoE estão envolvidas no transporte e clearance do colesterol; no entanto, a ApoE4 é a isoforma menos efetiva nesses processos (Buttini *et al.*, 2002). A ApoE4 pode interagir com os AβO e promover o acúmulo e a agregação dos mesmos prejudicando a plasticidade sináptica e contribuindo para os déficits cognitivos (Lim *et al.*, 2016). Os efeitos da ApoE4 sobre o processo de neurodegeneração na DA também podem ocorrer devido

a redução do metabolismo do colesterol, o qual pode levar à resistência insulínica que também está presente na DA (Ong *et al.*, 2014).

As características fisiopatológicas precoces no cérebro de pacientes com DA abrangem alterações do metabolismo da glicose, disfunção da bioenergética mitocondrial e estresse oxidativo (Abolhassani et al., 2016). A alteração do metabolismo energético cerebral resulta em disfunção sináptica e está associada com a progressão da DA (Abolhassani *et al.*, 2017). Considerando que a energia fornecida pelo metabolismo oxidativo da glicose suporta os processos de aprendizado e memória (Mergenthaler *et al.*, 2013), o hipometabolismo da glicose está associado à neurodegeneração e aos déficits cognitivos (Piaceri *et al.*, 2012; Vigneron *et al.*, 2016). A determinação do metabolismo cerebral da glicose em pacientes com DA, através da técnica de tomografia por emissão de pósitrons (PET) marcada com [18F]- fluordesoxiglicose (FDG-PET), indica que a redução do metabolismo não está presente apenas nos locais de acúmulo de peptídeos Aβ, mas também em regiões que não são intensamente afetadas; essas observações foram atribuídas à diminuição da conectividade funcional entre as regiões cerebrais devido à toxicidade dos AβO (Klupp *et al.*, 2014). A desregulação do metabolismo do cálcio decorrente da interação dos AβO com os receptores NMDA exacerba o estado oxidativo na célula e afeta a função mitocondrial (Kamat *et al.*, 2016). Os AβO podem se acumular na mitocôndria (Fig. 3) e afetar sua função ao interagir com enzimas essenciais para o metabolismo energético da célula, como enzimas do TCA e do STE, afetando a sua atividade e alterando a dinâmica mitocondrial (Manczak *et al.*, 2006; Kandimalla e Reddy, 2016; Abolhassani *et al.*, 2017). Portanto, ao afetar a função mitocondrial, os AβO afetam a homeostase energética e o suprimento de ATP aos neurônios, promovendo alterações na transmissão sináptica que podem levar à morte neuronal e, consequentemente, à neurodegeneração (Hu *et al.*, 2008; Reddy *et al.*, 2010).

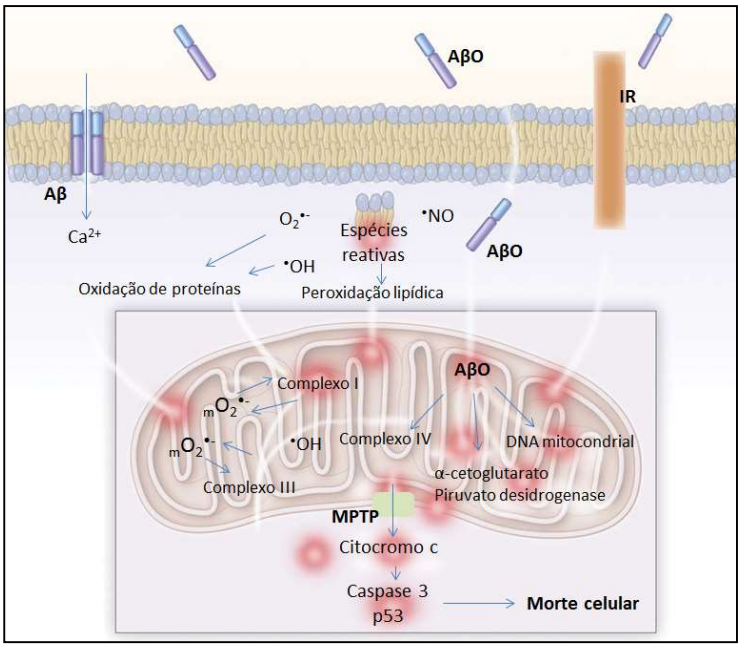

Figura 3. Ação dos peptídeos β-amiloide sobre as proteínas mitocondriais e efeitos das espécies reativas sobre as biomoléculas, culminando na morte celular. Imagem adaptada de Querfurth e Laferla (2010). Abreviaturas: Aβ, peptídeo β-amiloide; IR, receptor de insulina; MPTP, poro de transição de permeabilidade mitocondrial.

Embora já existam tratamentos disponíveis para a DA, todos apresentam efeitos limitados além de mínimos resultados clínicos (Giacobini e Gold, 2013). Sendo assim, o desenvolvimento de novas abordagens terapêuticas que impeçam a progressão da doença ou previnam o seu desenvolvimento é urgente. Estudos em modelos animais de DA têm sido desenvolvidos (Garcia-Alloza *et al.*, 2006; Bernardi *et al.*, 2012; Puzzo *et al.*, 2014) a fim de facilitar o curso das pesquisas translacionais e o desenvolvimento de novas terapias (Laferla e Green, 2012). Além disso, diversos compostos polifenólicos naturais, como resveratrol e curcumina, tem se mostrado eficazes na redução dos efeitos neurotóxicos do peptídeo Aβ em ratos (Hoppe *et al.*, 2010; Frozza *et al.*, 2013; Hoppe *et al.*, 2013). Agregado ao conhecimento obtido por meio desses modelos animais, é que hoje em dia muitos estudos clínicos têm avaliado a utilização de terapias inovadoras, tais como o uso de resveratrol (https://clinicaltrials.gov; NCT02502253; NCT01504854), imunoglobulinas contra o peptídeo Aβ (https://clinicaltrials.gov; NCT00818662; NCT01677572), fator de crescimento neuronal (NGF) (http://www.adcs.org/Studies/NGF.aspx), fator estimulador de colônia de granulócitos e macrófagos (CSF-GM) (https://clinicaltrials.gov; NCT01409915) e o

exercício físico (https://clinicaltrials.gov; NCT02000583; NCT01954550; NCT02708485; NCT01264614).

Há cerca de duas décadas que a prática de exercício físico tem sido proposta como estratégia neuroprotetora favorecendo a função cerebral em indivíduos saudáveis ou acometidos por alguma doença, incluindo a DA (Palleschi *et al.*, 1996; Vidoni *et al.*, 2015; Kim *et al.*, 2016; Maliszewska-Cyna *et al.*, 2017). Dados interessantes, em humanos (Yaffe *et al.*, 2001; Kramer *et al.*, 2005) e animais (Jin *et al.*, 2017), demonstram a habilidade do exercício físico de reduzir o prejuízo cognitivo associado ao envelhecimento. O envelhecimento é acompanhado por alterações estruturais e fisiológicas devido à perda da capacidade plástica das células, e, dessa forma, comprometendo a função cerebral (Marques-Aleixo *et al.*, 2012). Estudos clínicos, embora ainda incipientes, oferecem resultados promissores que indicam a existência de correlação inversa entre a realização de atividade física durante a vida e o desenvolvimento de demência e DA (Erickson *et al.*, 2012; Hooghiemstra *et al.*, 2012; Santos-Lozano *et al.*, 2016). A prática de atividade física é uma estratégia não-farmacológica promissora na prevenção da DA (Dao *et al.*, 2013) com a vantagem de ser uma estratégia efetiva de baixo custo (Wolff *et al.*, 2011). No entanto, apenas um estudo avaliou os efeitos neuroprotetores do exercício materno contra a neurodegeneração (Herring et al., 2012).

JUSTIFICATIVA

A transição demográfica vivenciada pela maioria dos países do mundo aponta para a mudança de um padrão de alta taxa de fertilidade/natalidade e mortalidade para um padrão de taxas mais baixas. Por outro lado, a transição epidemiológica considera a distribuição de doenças na população e aponta para mudanças no padrão de problemas de saúde e causa de morte, de doenças infecciosas para doenças crônicas (Mccracken e Phillips, 2017). As transições demográfica e epidemiológica atuais coincidem para um panorama onde a população apresenta baixa taxa de natalidade e mortalidade, maior expectativa de vida e maior prevalência de doenças crônicas associadas ao envelhecimento, de longa duração e lenta progressão. Dentre essas doenças pode-se destacar as doenças neurodegenerativas, como a DA.

Esse panorama alarma os setores de saúde pública que buscam estratégias preventivas para melhorar a qualidade de vida da população e reduzir a incidência de doenças crônicas. As prospecções para o futuro relacionadas à DA são preocupantes, e a busca por novos tratamentos que contemplem a enorme gama de alterações que ocorrem no SNC durante o processo neurodegenerativo da DA é incessante. A DA não tem cura e, até a atualidade, os tratamentos disponíveis para amenizar a sintomatologia debilitante e evitar a progressão da doença apresentam eficácia limitada. Os prejuízos socioeconômicos decorrentes da DA são enormes, pois além de representar um sério problema de saúde pública, o manejo e os cuidados diários dos pacientes requerem altos custos.

A combinação da ideia de prevenção da DA aos emergentes conhecimentos do âmbito da programação do metabolismo durante o período de desenvolvimento intrauterino surge como uma promissora abordagem. No entanto, essa área é muito recente e ainda é necessário aprofundar os conhecimentos dos mecanismos moleculares responsáveis. A atividade física materna pode determinar o fenótipo do indivíduo em favor da prevenção do desenvolvimento de doenças neurodegenerativas, tais como a DA, na vida adulta? Diante do exposto, torna-se interessante

aprofundar os conhecimentos dos mecanismos moleculares que promovem a prevenção nesta doença, ressaltando o exercício materno como uma importante abordagem neuroprotetora e preventiva para a DA.

OBJETIVOS

Objetivo geral

Avaliar o potencial neuroprotetor do exercício físico materno sobre alterações comportamentais e neuroquímicas encontradas em um modelo *in vivo* da doença de Alzheimer na prole de ratas Wistar.

Objetivos específicos

- Avaliar parâmetros qualitativos e quantitativos de cuidado materno apresentados por ratas exercitadas antes e durante a gestação;

- Avaliar parâmetros de desenvolvimento das características físicas na prole de ratas submetidas ao exercício materno antes e durante a gestação: peso corporal, abertura dos olhos, abertura do ouvido externo, dia do aparecimento dos dentes incisivos, cobertura total do corpo por pelos, deiscência dos testículos e abertura vaginal;

- Avaliar parâmetros de desenvolvimento dos reflexos motores da prole de ratas submetidas ao exercício materno antes e durante a gestação através dos testes de endireitamento de superfície, geotaxia negativa, aversão à queda e barra suspensa;

- Avaliar parâmetros de desenvolvimento locomotor da prole de ratas submetidas ao exercício materno antes e durante a gestação: tempo de imobilidade, movimento de rastejar, girar em torno do próprio eixo, caminhar, *grooming* e *rearing*;

- Avaliar o envolvimento da via da Akt/GSK-3β no cerebelo da prole de ratas submetidas ao exercício materno antes e durante a gestação, no dia embrionário 20 e no dia pós-natal 7;

- Avaliar o imunoconteúdo das proteínas SIRT1, SIRT3, Mfn1, Drp1 e Tfam no cerebelo da prole de ratas submetidas ao exercício materno antes e durante a gestação, no dia pós-natal 7;

- Determinar os níveis de BDNF maduro no cerebelo da prole de ratas submetidas ao exercício materno antes e durante a gestação, no dia pós-natal 7;

- Avaliar parâmetros comportamentais da prole de ratas submetidas ao exercício materno antes e durante a gestação, na idade adulta após administração intracerebroventricular (icv) do peptídeo $A\beta O_{1-42}$, por meios dos testes de campo aberto, reconhecimento de objetos e labirinto aquático de Morris;

- Determinar os níveis de BDNF maduro no hipocampo e no córtex pré-frontal da prole de ratas submetidas ao exercício materno antes e durante a gestação, na idade adulta após administração icv do peptídeo $A\beta O_{1-42}$;

- Determinar os níveis de espécies reativas no hipocampo, córtex pré-frontal e cerebelo da prole de ratas submetidas ao exercício materno antes e durante a gestação, na idade adulta após administração icv do peptídeo $A\beta O_{1-42}$;

- Avaliar parâmetros de função mitocondrial em hipocampo, córtex pré-frontal e cerebelo da prole de ratas submetidas ao exercício materno antes e durante a gestação, na idade adulta após administração icv do peptídeo $A\beta O_{1-42}$: massa e potencial de membrana mitocondrial;

- Avaliar a atividade das enzimas do TCA e do STE em hipocampo e córtex pré-frontal da prole de ratas submetidas ao exercício materno antes e durante a gestação, na idade adulta após administração icv do peptídeo $A\beta O_{1-42}$;

- Avaliar o imunoconteúdo das proteínas sinaptofisina, Mfn1 e Drp1 em hipocampo, córtex pré-frontal e cerebelo da prole de ratas submetidas ao exercício materno antes e durante a gestação, na idade adulta após administração icv do peptídeo $A\beta O_{1-42}$;

- Determinar o estado redox no cerebelo da prole de ratas submetidas ao exercício materno antes e durante a gestação, na idade adulta após administração icv do peptídeo $A\beta O_{1-42}$: atividade das enzimas antioxidantes SOD, CAT e GPx, bem como o conteúdo de GSH, tióis e carbonilas;

- Avaliar o imunoconteúdo das proteínas PSD95, iNOS, GSK-3β, tau e fosfo-tau no cerebelo da prole de ratas submetidas ao exercício materno antes e durante a gestação, na idade adulta após administração icv do peptídeo $A\beta O_{1-42}$.

PARTE II

Capítulo I

Capítulo II

Capítulo III

Capítulo IV

CAPÍTULO I

Swimming exercise before and during pregnancy: promising preventive approach to impact offspring´s health

O capítulo I apresenta o artigo intitulado *Swimming exercise before and during pregnancy: promising preventive approach to impact offspring´s health*, o qual está submetido ao periódico *International Journal of Developmental Neuroscience*.

Swimming exercise before and during pregnancy: promising preventive approach to impact offspring´s health

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Highlights

A consistent maternal behavior is displayed by exercised dams.

Maternal exercise does not change litter parameters.

Maternal exercise does not affect reflex maturation of female and male pups.

Maternal exercise increases pups' exploratory behavior.

Abstract

Several environmental factors affect child development, such as the intrauterine environment during the embryonic and fetal development and early postnatal environment provided by maternal behavior. Although mechanistic effects of maternal exercise on offspring health improvement are not yet completely understood, the number of reports published demonstrating the positive influence of maternal exercise have increase. Herein, we addressed issues related to early postnatal environment provided by maternal behavior and early developmental physical landmarks, sensorimotor reflexes, and motor movements ontogeny. In brief, adult female rats underwent involuntary swimming exercise, in a moderated intensity, one week before mating and throughout pregnancy, 30 minutes a day, 5 days a week. Maternal exercised dams have unchanged gestational outcomes compared to sedentary dams. We found no differences concerning the frequency of pup-directed behavior displayed by dams. However, sedentary dams displayed a poorer pattern of maternal care quality during dark cycle than exercised dams. Physical landmarks and sensorimotor reflexes development of female and male littermates did not differ between maternal groups. Developmental motor parameters such as immobility, lateral head movements, head elevation, pivoting, rearing with forelimb support and crawling frequencies did not differ between groups. Pups born to exercised dams presented higher frequency of walking and rearing on the hind legs. These data suggest that female and male littermates of exercised group present a high frequency of exploratory behavior over sedentary littermates. Taken together, the present findings reinforce that maternal exercise throughout pregnancy represent a window of opportunity to improve offspring's postnatal health.

Key words: maternal exercise, metabolic programming, maternal care, motor development

1. Introduction

Pregnancy is a crucial period for the development of the fetus. It will be throughout pregnancy and during early postnatal period that environmental factors will affect the fate of development, through programming the fetal metabolism and epigenome, without affecting the genome. The fetal programming occurs during normal development or even in the presence of a stimuli at different stages during intrauterine development [1]. Therefore, the pregnancy and lactation periods are recognized as critical windows of developmental susceptibility.

Maternal lifestyle during pregnancy exert profound effects on offspring's development and also has the ability to program the offspring's healthy state and/or susceptibility to diseases early and later in life [2]. The ability of fetal programming is supported by the paradigm of "developmental origins of health and disease" (DOHaD) concept, which was initially described by Hales, Barker [3] as the "thrifty phenotype hypothesis".The DOHaD paradigm says that the intrauterine development and growth are adapted to the predicted postnatal environment [4]. Barker, Osmond [5] showed that the mismatch between intrauterine nutrient restriction and abundance of nutrients in the postnatal life leads to development of adult disease [5]. The capacity of the fetus to respond adaptively to environment occurs due the high developmental plasticity allowing the fetus to change its phenotype [6,7].

The health benefits elicited by physical activity are evidenced by numerous researchers [8-12]. Sedentary lifestyle is a modifiable risk factor for several chronic metabolic diseases, and the adoption of an active lifestyle leads to additional improvement in the health status throughout life [12]. Regular physical activity is highly indicated in all phases of life, including the pregnancy [13,14]. The updated American Congress of Obstetricians and Gynecologists (ACOG) guideline for exercise during pregnancy recommends an exercise program, lasting 20-30 minutes per day, for pregnant women without medical contraindications [13]. Pregnant women are encouraged to continue or to initiate safe activities, such as walking, swimming, stationary cycling, Pilates, Yoga,

and low-impact aerobics [13]. The exercise during pregnancy offers no harm to the fetus. The exercise reduces uterine blood flow up to 20 % and fetal oxygen uptake (PO2) remains unaltered because the compensatory increase in hematocrit and uterine oxygen extraction [15].

It has been shown that maternal exercise elicits benefits in several tissues, such as muscle [16], adipose tissue [17], brain [18] and liver [19]. The effects of maternal exercise to the fetus are highlighted by benefits such as improved glucose tolerance [17,19,20], enhanced insulin sensitivity [21,22], enhanced brain antioxidant defenses [18] and improved memory [23-25], which persist in adulthood. In a previous work we demonstrated that swimming during pregnancy enhances mitochondrial function in the brain of 7-day-old pups [18] and that recognition memory is enhanced in 60-day-old male offspring [26]. Lee et al. [27] demonstrated that swimming during pregnancy enhances aversive memory in 28-day old rats' offspring by increasing neurogenesis. Moreover, it has also been demonstrated that maternal environment is able to change the fate of the pathology despite genetic factors [28], which are known to be highly relevant in the development of chronic diseases. Interestingly, experimental studies showed that male and female littermates present distinct pattern of modifications and differences in the phenotype [29].

Advances in the metabolic programming area have evolved and several factors, such as physical activity, diet, stress, infection and gestational diabetes, have been shown to influence fetal development, providing either deleterious or beneficial effects on fetal metabolism. There is a lack of data describing the effect of exercise during pregnancy on maternal behavior and the ontogeny of motor development in the offspring born to exercised dams. In the present study, we aimed to analyze and to describe the effects of maternal swimming before and during pregnancy on pregnancy outcomes, maternal care, offspring's physical and sensorimotor reflexes development, and ontogeny of motor behavior in rats. Herein, we demonstrated for the first time that swimming before and during pregnancy improves maternal care quality and accelerates pups' motor development. The results obtained with the present study highlight swimming exercise during

pregnancy as safe approach to impact offsprings' health. Studying such approach during developmental period is important and represents a valuable tool for future researches on DOHaD focusing in public health, to encourage women to begin exercising aiming to improve the health status and to prevent the development of diseases in the offsprings' postnatal life.

2. Material and methods

2.1. Animals and Ethics

Adult male and female Wistar rats (80 days old) were used. Animals were maintained under a standard 12h dark/light cycle (lights on between 7:00h and 19:00h), controlled temperature (22 ± 2°C) and humidity (50-60%), with ad libitum access to a 20% (w/w) protein commercial chow (CR1 lab chow, Nuvilab Ltda., Curitiba, Brazil) and water. Four animals were housed per home cage up to mating. All experimental procedures and animals care were conducted in accordance with the National Institutes for Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1996) and were approved by the local Ethics Commission of Universidade Federal do Rio Grande do Sul (CEUA/UFRGS; protocol number 27349). All efforts were made to minimize animal suffering, and to keep the number of animals at the minimum to demonstrate consistent effects. The number of animals used in each experiment is indicated in the Figures Captions.

2.2. Experimental design

Female rats were divided into two groups: sedentary (control) and exercised (swimming). The swimming protocol was previously described by Marcelino et al. [18]. Female rats from exercised group swam one week, prior the mating, to habituate to aquatic environment, and swam throughout pregnancy in a schedule of 5 days/week for four weeks lasting 30 min/day in a pool (30 cm wide x 30 cm long x 90 cm deep) filled with 32 ± 1°C water. The animals were left free to swim and were gently stimulated to swim when necessary. Control rats were immersed in water,

carefully dried, and returned to the housing boxes. During the mating two females were housed with one male. The pregnancy was confirmed by the presence of a vaginal plug and the conception day was noted as gestational day (GD) 0. Pregnant females were housed individually on GD20 and allowed to normal spontaneous vaginal delivery. We checked for birth twice a day (at 8 and 18 h) to annotate the postnatal day (PD) 0. The offsprings was weaned on PD21 and four rats, of the same gender, were housed per cage, according maternal intervention group. For maternal care examination experiments, the pups were maintained undisturbed with their mothers, except for cleaning. To examine physical and neurobehavioral development of pups, from PD2 to PD18, different dams were used.

2.3. Gestational and litter parameters

Female rats were weighed weekly, on habituation week and gestational period. Starting on GD1, the chow intake was measured daily. The consumption of food was calculated subtracting the quantity of chow supplied to rats of the residual quantity weighed 24 h after. Litter size and weight, number of living pups born, and sex ratio were checked on PD1. On PD1, the litter was culled in order to maintain 8 pups per dam (four male and four female whenever possible) ensuring equal nutrition, as the size of litter may affect significantly the development of pups [30].

2.4. Offspring parameters

Two pups per litter, one male and one female, were weighed daily up to PD22, and weekly from PD22 to PD60. Beginning on PD21, the chow intake was measured from PD22 to PD60. Four littermates were used for neurobehavioral (physical and motor) development, a pair of pups for each parameter. Pups were uniquely marked with a nontoxic marker. All observations were conducted between 18:00 and 20:00 h. In order to eliminate the litter effect, only a pair of pups from each dam (one male and one female) was assigned to assessment of each set of neurodevelopmental parameters.

2.5. Maternal care

Maternal behavior observation was scored for five 72 min periods daily from PD2 to PD10 at regular times (06:00, 09:00, 12:00, 16:30, and 20:00 h) as described by Champagne et al. [31]. Within each observation period, the dams were monitored in sequence every 3 minutes, and the observer recorded the ongoing behavior at the instant of the observation. The schedule is resumed as follows: 25 observations/period x periods per day, resulting in 125 observations/dam/day yielding 1,125 observations/dam during the experiment (n= 8 to 11 litters/group). The following behaviors were scored by trained observers: dam in or out of the nest, dam licking any pup, dam nursing pups in arched back, blanket (in which the dam lays over the pups) or supine (in which the dam is lying either on her back or side while the pups nurse) posture, nest building, retrieving pups, and dam drinking/eating. Quantitative measure of maternal behavior was analyzed as the frequency (in percentage) of observations in which animals engaged in the target behavior.

Reduced duration of nurturing bouts results in a more fragmented pattern of care and in an increased behavioral inconsistency. Herein, we used the behavioral inconsistency score as a qualitative measure of maternal behavior as described by Ivy et al. [32] and Couto-Pereira et al. [33]. Maternal behavior was analyzed for each period to provide a behavioral inconsistency score, which varies between 0 and 1. The transition between one to other behavior in two sequential observations was scored as 1, and if there was no transition, the score was 0. The sum of scores was divided by 24 (the possible number of behavioral transitions per period). The higher the score, the more fragmented and inconsistent the maternal care. Transitions behaviors considered: nursing, licking, retrieving pups, nest building, away from pups, and dam drinking/eating.

2.6. Reflex development

A pair of pups from each dam was randomly selected for observation of appearance and/or disappearance of several developmental reflexes. The observations were conducted according

detailed description published by Heyser [30]. The following parameters were tested: cliff avoidance, surface righting, negative geotaxis and bar holding.

For the cliff avoidance test, the pup was placed with its nose and foreleg over the edge of a wooden platform (30 cm in height). The time that the pup spent to move away from the "cliff", by backing up or by turning sideways, was recorded from PD2 to PD15.

For the surface righting test, the pup was placed onto its back in a wood surface. The latency, in sec, to the pup to turn over onto its belly was recorded from PD2 to PD15.

For the negative geotaxis test, the pup was placed on an inclined wood plane (30°) with its head facing downwards. The latency to the pup changes its orientation, so that its head faces up, in a maximum of 60 sec, was recorded from PD3 to PD10.

For the bar holding, the pup was lifted by the trunk and allowed to grab hold with its front paws a thin metal bar (4 mm in diameter), such that when the animal is released, it is hanging only by its front paws. The time of pups hold on a metal bar, up to a maximum of 10 sec, was recorded from PD10 to PD13.

2.7. Motor development

The ontogeny of motor behavior encompasses at least two main processes: development of quadruped stance and quadruped locomotion [34]. The former refers to postural parameters, such as contact with the surface, position of the head, torso, limbs, and pelvis; and the later refers to motor movement, which develops gradually over postnatal days: pivoting, crawling, and walking [34]. Daily observations on the development of locomotor behavior, such as quadruped stance and locomotion, were conducted for a period of 5 min on the PD2 to PD18 [30]. For this experiment, a pair of pups from each dam was randomly selected. The following movements and stances were monitored and recorded every 10 sec: immobility (I), lateral movements with head (L), elevation of the head (H), pivoting (P), crawling (C), walking (W), grooming (G), rearing with forelimb

support (S; vertical movement with torso and pelvis), and rearing (R; vertical movement, in which the rat stands on hind legs). In each test day, the frequency of each behavior was recorded for 30 observations.

2.8. Statistical analysis

All statistical analyzes were performed using GraphPad 6.0 software. Initially, the data were tested for normality. Comparisons between two groups were analyzed by independent Student's t test. Comparisons between two groups involving factorial designs (body weight, maternal care, and neurodevelopmental behavior) were analyzed using two-way ANOVA (repeated measures) with significant main effects and interactions analyzed by Tukey's post-hoc test. Differences were considered significant if $p<0.05$.

3. Results

3.1. Dams and pups outcomes

Swimming exercise before conception and throughout pregnancy did not affect pregnancy rate, delivery and abortion index, number of living born pups or sex ratio of pups (Table 1). Along pregnancy days, dams' weight (Fig. 1A) and chow intake (Fig. 1B) did not differ between sedentary and maternal exercise groups [$F(1,41)<0.001$; $p=0.993$ and $F(1,17)=0.630$; $p=0.438$, respectively]. In addition, the exercised dams' weight gain (G20 - G1 weight) did not differ significantly from control group [$t(41)=0.639$; $p=0.527$](Fig. 1A insert).

Maternal swimming before and during pregnancy did not affect female or male pups weight across PD2 to PD60 (Fig. 2A). Male and female body weights up to PD21 were not different between groups [$F(3,33)=0.274$; $p=0.843$]. However, there was an interaction between gender and PD [$F(3,57)=14.9$; $p<0.0001$]; females and males had different daily weight over time from PD28 to PD60 [$F(3,33)=73.47$; $p<0.0001$]. Similarly, maternal swimming did not affect weight gain of the female and male pups on PD21 [$F(1,34)=1.194$; $p=0.282$] or on PD60 [$F(1,34)=0.429$;

p=0.517] (Fig. 2B); however, weight gain on PD60 differed from male and female [F(1,34)=287.8; p<0.0001]. Average weight of pups born to sedentary or exercised dams at birth (weighed within 24 h after delivery) did not differ significantly (t(36)=1.474; p=0.149) (Fig. 2C), and also, the average of chow intake (PD22 to PD60) (Fig. 2D) did not differ significantly between the groups [F(1,34)=0.191; p=0.665] and gender [F(1,34)=0.410; p=0.526].

3.2. Maternal behavior

To assess whether swimming during pregnancy induces differential maternal care of dams toward its pups, we observed maternal behavior on the PD2 to PD10 (Fig. 3). Cumulative frequency of each target behavior spent by dams throughout the experiment (1,125 observations per dam) is represented in Figure 3A; data were analyzed as percentage of the total number of observations. Eating, drinking and immobility with no contact with pups, self-grooming, or digging the sawdust are represented within dam's behavioral category 'no contact with pups'; retrieving pups to the nest and passive contact with pups were categorized into 'in contact with pups'. Data analysis showed that no behavior in the cumulative frequency analysis differed between the two groups (p>0.05). Two-way ANOVA repeated measures indicated the time that dams spent in contact with their pups decreased significantly along postnatal days (Figure 3B) [F(8,136)=19.95; p<0.0001], and there was no statistical difference between sedentary and swimmers behavior [F(8,136)=0.861; p=0.366]. In addition, quantitative analysis of maternal behavior, such as licking, arched-back nursing, nursing (including blanket and supine positions), and the time that dams spent off their pups, were conducted according each regular period of observation (06:00, 09:00, 12:00, 16:30, and 20:00 h) (Fig. 3C-F). Pup licking frequency, nursing frequency in arched-back and other positions, and frequency of time off pups did not differ significantly between maternal groups [(F(1,16)=0.057; p=0.813); (F(1,16)=3.653; p=0.074); (F(1,16)=0.980; p=0.337); and (F(1,16)=0.685; p=0.420), respectively]. In addition, a marked variation of maternal behavior was observed among the different periods for all these parameters

[(F(4,64)=27.38; p<0.0001); (F(4,64)=15.49; p<0.0001); (F(4,64)=129.5; p<0.0001); and (F(4,64)=115.0; p<0.0001), respectively]. The higher frequency for licking behavior (Fig. 3C) occurred for both groups at 06:00 h and 20:00 h in dark cycle (12.5 and 10.6 % in sedentary group, and 13.6 and 9.2 % in exercised group), while the lowest frequency occurred at 13:00 h (4.4 % in sedentary group and 4.8 % in exercised group). The higher frequency of nursing behavior (Fig. 3E) occurred for both groups at 10:00 h, 13:00 and 16:30 h in light cycle (43.2, 51.6 and 38.5 %, respectively, in sedentary group, and 49.5, 59.8 and 44 %, respectively, in exercised group), while the lowest frequency occurred at 20:00 h (5.9 % in sedentary group and 6.2 % in exercised group). The higher frequency of dam with no contact with pups (Figure 3F) occurred for both groups at 06:00 h and 20:00 h in dark cycle (42.2 and 62.5 % in sedentary group, and 34.7 and 64.5 % in exercised group), while the lowest frequency occurred at 10:00 h (22.4 % in sedentary group and 19.9 % in exercised group).

To assess qualitative maternal care through the calculation of the behavioral inconsistency score, which reflects the fragmentation of pup-oriented behaviors in dams, we did not consider changes in nursing positions to calculate the score, because dams frequently exchange nursing positions [35]. Behavioral inconsistency scores varied according to different periods of observations within both groups of dams [F(4,68)=39.89; p<0.0001]. The behavioral inconsistency scores did not differ significantly between the groups during four periods of observations (06:00, 09:00, 12:00 and 16:30 h), indicating that exercised dams altered their pups-directed behavior at the same frequency that sedentary dams. However, at 20:00 h a statistically significant difference was observed between the groups (Fig. 3G). Exercised dams presented a reduced inconsistency score at 20:00 h compared to sedentary dams [F(1,17)=5.127; p=0.037], indicating that exercised dams altered their pups-directed behavior in a lesser frequency than sedentary dams, thus presenting a more consistent pattern of maternal care.

3.3. Effects of maternal swimming during pregnancy on offspring´s sensorimotor reflexes development

Sensorimotor tests were performed to assess whether maternal swimming during pregnancy delays or accelerates the appearance of pups' reflexes. The time spent time in the righting reflex test by female and male pups from PD2 to PD14 did not differ significantly between maternal exercise and sedentary groups [$F(3,36)=0.393$; $p=0.758$], and as expected, the time to turn over to restore their normal prone position reduced across postnatal days in both groups [$F(12,432)=18.45$; $p<0.0001$] (Fig. 4A). No statistical differences in the development of negative geotaxis from PD2 to PD12 were observed between pups born to sedentary and exercised mothers [$F(3,34)=1.332$; $p=0.280$]; however, differences were observed across postnatal days in both groups [$F(10,340)=35.77$; $p<0.0001$] (Fig. 4B). As observed in righting reflex and negative geotaxis, the latency of male and female pups in the cliff aversion test did not differ significantly between the groups from PD2 to PD14 [$F(3,34)=0.200$; $p=0.895$], and the latency of pups to move away from the cliff reduced across postnatal days in both groups [$F(12,408)=33.71$; $p<0.0001$] (Fig. 4C). In the bar holding test is expected an increase in the time that the animal holds on a metal bar. The test was performed until the pups open their eyes, because after this event the animals jump off the bar (Fig. 4D). No statistical differences in the bar holding from PD10 to PD13 were observed between pups born to sedentary and exercised mothers [$F(3,36)=0.719$; $p=0.547$]; however, there were observed differences in the time of holding the bar across postnatal days [$F(3,108)=9.78$; $p<0.0001$].

3.4. Effects of maternal swimming during pregnancy on offspring´s motor development

To investigate the effects of maternal swimming during pregnancy on offspring's motor maturation, we analyzed movements related to ontogeny of locomotor development. Figure 5 depicts the frequency of each movement over the days. Specific movements display a pattern of appearance or disappearance over postnatal days.

Immobility behavior frequency decreased progressively across postnatal days for female and male littermates of sedentary and maternal exercise groups [$F(16,576)=229.5$; $p<0.0001$]. Two-way ANOVA repeated measures indicated a difference between groups [$F(3,36)=6.530$; $p=0.0002$] (Fig. 6A), with Tukey post-hoc test indicating differences of gender on PD2, in which the immobility frequency of male in both sedentary and maternal exercise groups was reduced in comparison to female littermates of both groups. In addition, on PD4 two-way ANOVA indicated that male and female littermates born to exercised dams have reduced immobility frequency compared to male and female littermates of sedentary group.

Lateral head movements frequency decreased progressively across postnatal days for female and male littermates of sedentary and maternal exercise groups [$F(16,576)=71.84$; $p<0.0001$]. Two-way ANOVA indicated no differences in the frequency of lateral movements between groups [$F(3,36)=0.109$; $p=0.955$] (Fig. 6B).

Head elevation movement frequency increased progressively across postnatal days for female and male littermates of sedentary and maternal exercise groups [$F(16,576)=113.3$; $p<0.0001$]. Two-way ANOVA indicated no differences in the frequency of head elevation movements between groups [$F(3,36)=0.150$; $p=0.329$] (Fig. 6C). Starting on PD8, 100% of female and male littermates of sedentary and maternal exercise groups performed at least once the movement of head elevation.

Grooming behavior frequency increased progressively across postnatal days for female and male littermates of sedentary and groups [$F(16,576)=77.52$; $p<0.0001$]. The first grooming behavior performed by female and male pups occurred between PD8 and PD13 in both groups. Grooming behavior frequency differed between groups [$F(3,36)=0.370$; $p=0.032$] (two-way ANOVA) (Fig. 6D). Tukey post-hoc test indicated that maternal exercised female pups differed from male and female littermates born to sedentary dams on PD13. Male pups of exercised group differed from female of exercised group and male of sedentary on PD15.

Pivoting movement frequency changed across postnatal days for female and male littermates of sedentary and maternal exercise groups [$F(16,576)=101.7$; $p<0.0001$]. Pivoting movement reaches the peak on PD8-9 and starts declining along PD10 up to PD18. Two way ANOVA indicated that pivoting frequency did not differ between female and male born to sedentary or maternal exercise groups in any day of observation [$F(3,36)=0.370$; $p=0.775$] (Fig.7A). From PD8 up to PD11, 100% of animals in both groups performed pivoting movement.

Rearing with forelimb support frequency increased progressively across postnatal days for female and male littermates of sedentary and maternal exercise groups [$F(16,576)=61.82$; $p<0.0001$]. Two-way ANOVA indicated no differences between the groups [$F(3,36)=0.722$; $p=0.539$] (Fig. 6E). The first appearance of rearing with support was observed on PD7 for female pups of sedentary and exercised groups and PD8 for male pups of both groups; however, no difference was observed concerning the mean of first day of rearing movement appearance between groups ($p>0.05$).

Rearing on the hind legs (vertical) movement frequency increased progressively across postnatal days for female and male littermates of sedentary and maternal exercise groups [$F(16,576)=57.42$; $p<0.0001$]. The first appearance of rearing on hind legs movement was observed on PD14 for pups born to exercised dams and PD15 for pups born to sedentary dams; however, no difference was observed concerning the mean of first day of rearing movement appearance between groups. Two-way ANOVA indicated differences between the groups [$F(3,36)=7.760$; $p<0.0001$] (Fig. 6F). On PD17 and 18 maternal exercised female and male littermates showed increased frequency of rearing that was significantly different from female and male littermates of sedentary groups (Tukey post-hoc test).

Crawling movement frequency changed across postnatal days [$F(16,576)=54.91$; $p<0.0001$] and did not differ between the groups in any day of observation [$F(3,36)=0.398$; $p=0.755$] (two-way ANOVA repeated measures) (Fig. 7B). The first appearance of crawling

movement occurred on PD4, and two-way ANOVA not repeated measures indicated that the mean of first appearance of crawling movement differed between sedentary and exercised groups [$F(1,36)=4.799$; $p=0.035$] with no effect of gender [$F(1,36)=0.065$; $p=0.799$]. Crawling movement reached the peak on PD11, with 100% of animals in both groups performing such movement.

The shift of crawling to walking movements occurred for all animals on PD13. Two-way ANOVA repeated measures indicated that walking movement changed across postnatal days [$F(16,576)=141.5$; $p<0.0001$] and indicated a main effect for maternal intervention [$F(3,36)=4.332$; $p=0.005$] (Fig. 7C). To verify which groups differed significantly Tukey post-hoc test was performed and showed that female and male pups born to exercised dams differed significantly from female and male littermates born to sedentary dams on PD13, indicating increased frequency of walking for maternal exercised offspring. In addition, Tukey post-hoc test indicated that male of maternal exercise group differed significantly from female pups of sedentary maternal group on PD14; and female pups born to exercised dams differed significantly from all other pups on PD15.

4. Discussion

A milieu of factors influence pup's development; among these, maternal environment during embryonic and fetal periods as well as the environment provided by maternal care during early postnatal period play important role in the offspring's health. Maternal exercise has long-lasting effects on offspring's health but mechanistic questions have not yet been elucidated; therefore, issues in this context deserve emphasis.

Some clinical investigations have addressed maternal exercise strategy to enhance metabolic health in the offspring [36-39]. Albeit human studies offer public health relevance, the results are associative [40]. In view of human studies, concerning metabolic programming and its effects on adult health are difficult, studies with animal models that aim to examine the effects of

maternal exercise on offspring's health span and its ability to offsets detrimental effects of insults during adulthood are important.

Gestational outcomes obtained from exercised dams in the present study did not differ to that obtained from sedentary dams. Despite most studies show that gestational outcomes did not differ between maternal groups, some differences obtained among studies examining the effect of maternal exercise are thought to be due to different rodent models used (distinct mice and rat strains), exercise modality employed (voluntary or involuntary), timing (before and during pregnancy, only before or only during pregnancy) and duration (days or weeks) [18,41-43]. Low and moderate intensity exercise improves offsprings' outcomes, such as improved glucose tolerance [19,22] and memory [26,43]. In contrast, submaximal intensity exercise during pregnancy has been associated with worsened glucose tolerance in adult offspring [41]. Female mice performing voluntary running wheel before and during pregnancy have unchanged body weight, litter size or sex distribution compared to sedentary dams [19,22]. Involuntary treadmill or swimming exercise, at low and moderated intensity, performed by pregnant Wistar rats have also unchanged body weight, chow consumption and number of pups in comparison to sedentary dams [18,41,43].

Variations in maternal care are known to influence pups development [31]. Beyond dams' endocrine signals to influence maternal behavior, stimuli provided by the offspring are also needed, thus establishing mother-pup interaction [35]. Herein, we investigated whether exercise during pregnancy affects maternal behavior towards its pups through analyzing quantitative and qualitative parameters of maternal care. As expected, mother-pup contact displayed a circadian variation of maternal behavior. We observed that maternal behavior did not differ between sedentary and exercised groups regarding licking, nursing, and time off. Licking/grooming and arched-back nursing are thought to be the main factors of maternal behavior influencing pups development, and they are mechanistically distinct of mother-pup contact behavior [31].

Analyzing maternal care through behavioral inconsistency score allowed us to determine that maternal care quality did not differ between sedentary and exercised dams in the most phases (6:00, 9:00, 13:00, and 16:30 h) with exception of the dark phase (at 20:00 h), in which exercised dams showed a more consistent behavior compared to sedentary dams. In the dark phase of cycle, activity levels in the rats increase, therefore dams spent more time off the nest [32]. Reduced quality of maternal care are commonly observed during the active phase (dark), which is characterized by a fragmented/disrupted behavior with short and unpredictable duration of mother-pup contact [32]. Data concerning the possibility of maternal exercise to elicit variation in maternal behavior have not been studied so far, thus the outcomes presented here add to existing literature the evidence that maternal swimming exercise before and during pregnancy have impact on maternal care by improving its quality in the dark phase.

Another important issue that has not yet been studied is the effect of maternal exercise on neurodevelopmental parameters. The offspring impel their mother to nurture them, and sensory stimuli from pups, such as vocalizing and odor, evokes maternal responsiveness in addition to tactile stimuli [35]. Tactile stimulation from pups to mother elicits maternal behavior such as retrieval, licking, and nursing [35]. This concept of nature of nurturing together with our findings obtained with maternal care analyses lead us to investigate neurodevelopmental parameters of pups. Pups born to sedentary or exercised dams did not differ at birth. Along the first 60 days of age, sedentary and exercised groups of pups did not differ concerning weight gain and chow consumption, with differences between female and male littermates weight starting on PD28 up to PD60. Developmental ontogeny of physical landmarks (supplemental table 1) and reflexes of rat pups were unchanged by maternal swimming exercise. Ethological studies have been commonly conducted when investigating new substances in order to define its effects on behavior [44]. Opposite effects have been observed when testing different substances of diet; caffeine consumption during pregnancy have been associated to delayed neuromotor development in rats

[45], while a goat milk lipid diet during pregnancy have been associated to accelerated physical development and reflex maturation in rats [46]. It is worthy to state that the present study is the first to observe the effects of maternal exercise before and during pregnancy on developmental behavior pattern.

We further examined the effects of maternal exercise on the ontogeny of exploratory behavior in the first postnatal days of pups. When removed from it nest and placed in a new environment, the pups assume a "warm-up" behavior, which involves a build-up in the movement amplitude along two dimensions, lateral and longitudinal (forward) movements [47]. During early development pups remains relatively immobile, showing small lateral head movements, minimal or no longitudinal and vertical movements [47,48]. In accordance, during early postnatal days we observed that rat pups born to either sedentary or exercised dams spent most of the time immobile showing increased frequency of lateral head movements, and at less extent, increased frequency of head elevation from the ground over the days. The pivoting movement is the first motor behavior to become apparent, followed by crawling [34]. We observed that the occurrence of these two movements overlap, and both are featured by the immaturity of hind limbs. Until quadruped stance to become mature, the hind limbs are not able to provide support to the pelvis that remains anchored to the ground [34]. Herein, rat pups initiated to move in a walking fashion on PD13, which is in accordance to existing literature that states the quadruped stance reaches maturity by PD10-14, thus displaying a motor movement in a walking fashion [for details of locomotor ontogeny see Altman & Sudarshan (1975) and Golani [49]]. Some differences observed in the present study seem to be reached by chance, such as grooming frequency that occurred sparsely on PD13 and PD15. The walking frequency on PD14 and PD15 seem to follow the same sparse pattern.

The most strikingly findings observed in the present study concerning the effect of maternal swimming on ontogeny of motor movements was related to rearing data. Rearing behavior was

scored as rearing with forelimb support and rearing on hind legs without support in order to differentiate immature of the most mature movement. We observed that female and male pups born to exercised dams showed the rearing on hind legs movement earlier than female and male littermates born to sedentary dams. In addition, female and male littermates of exercised groups had increased frequency of rearing without support in the last two days of observation. Rearing on hind legs presupposes functional maturation of the hind limbs, and the frequency of rearing with support tends to be higher than rearing on hind legs [34]. Rearing on the hind legs represents a form of exploratory behavior commonly observed in rats with mature motor skills that tends to increase over later development [34]. In view of existing data on ontogeny of motor development, these data suggest a slight acceleration of motor development elicited by maternal swimming (Table 2). In a previous study, we have demonstrated that maternal exercised adult (PD60) female and male littermates' performance in the locomotor and exploratory parameters observed in the open field test did not differ from controls [26]. Moreover, in the same study we have demonstrated that male offspring born to exercised dams displayed an improved recognition memory over male offspring born to sedentary dams, and no difference was observed between female pups [26].

Recently, increasing evidences have showed that maternal insults may represent a crucial factor to development of diseases during adulthood, and conversely, a favorable intrauterine environment is able to modulate the course of development improving offspring health [20]. A recent review highlights maternal exercise before and during pregnancy as adult offspring's metabolic health improver and shows compelling evidences that maternal exercise has the ability to negate the detrimental effects of an impaired maternal diet on the offspring's metabolic health in rodents [20]. Maternal exercise before and during pregnancy is necessary to confer maximal beneficial effects to offspring, and this timing address the likelihood of epigenetic changes represent the underlying mechanism by which maternal exercise improves offspring's metabolic health [20]. Several data, including from our laboratory, indicates that maternal intervention during

pregnancy affects differentially male and female littermates [26,50-52]. On reason of this, in the present study we analyzed female and male parameters separately.

In accordance with others [19,22], we have already observed that exercise conferred beneficial effects to offspring's brain redox state and mitochondrial function if applied before and during pregnancy [18]. Mechanistic effects of maternal exercise on offspring's health are slightly known. Akhavan et al. [43] revealed that maternal voluntary wheel running selectively enhanced the acquisition phase of spatial memory, while involuntary swimming enhanced both acquisition and retention phases of spatial memory. These differences were assigned to the significant increase of corticosterone levels in the serum of dams, measured at the end of gestation, after the last session of involuntary swimming compared to voluntary running or sedentary, indicating that maternal corticosterone hormone might modulate the litter metabolism [43]. Conversely, Bustamante et al. [53] showed that maternal restraint stress increased dams' plasma corticosterone levels, which was related to the neuronal impairment in the offsprings. When dams were submitted to a maternal exercise schedule, the effects induced by maternal restraint were abolished and there was no change in the maternal corticosterone levels [53]. These contradictory assignments to maternal corticosterone levels on the offsprings' metabolic changes raise the need to unveil the exact contribution of this hormone. Other factors, such as redox status and epigenetic modifications, have been emerged as underlying mechanism responsible by maternal exercise effects on offspring's health [20,40].

In summary, maternal swimming exercise before and during pregnancy was associated with unchanged pregnancy outcomes, frequency of pup-directed behavior during maternal care, pups' physical developmental landmarks, and pups' sensorimotor reflexes development. Exercised dams exhibited a better pattern of maternal care quality during the dark cycle. Moreover, maternal swimming was associated with a higher frequency of exploratory behavior in rat offsprings. Therefore, the present study reinforces that maternal exercise before and throughout pregnancy

represents a window of opportunity to improve offspring's health. Future investigations are essential to unveil the potential role of maternal exercise to confer benefits to long-term offspring's health and which are its mechanistic effects.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1. Maternal gestational outcomes.

	Sedentary	Swimmers	P value
Mated female	36	36	
Pregnancy rate (%)	58.3±3.93 (21)	61.1±15.7 (22)	0.831
Delivery index (%)	85.4±7.71 (18)	90.6±2.42 (20)	0.463
Abortion index (%)	14.5±7.71 (3)	9.40±2.42 (2)	0.463
Living born pups	9.13±2.26	9.12±2.19	0.920
Pups sex ratio			
Female (%)	51.0±16.3	45.1±17.8	0.338
Male (%)	49.0±16.3	54.4±17.7	0.381

Table 2. Summary of significant differences between maternal sedentary and exercised groups.

Sedentary	Exercised
Poorer pattern of maternal care quality on dark phase	Improved pattern of maternal care quality on dark phase
Lower frequency of walking on postnatal day 13	Higher frequency of walking on postnatal day 13
First appearance of rearing on the hind legs on postnatal day 15	First appearance of rearing on the hind legs on postnatal day 14
Lower frequency of rearing on the hind legs on postnatal days 17 and 18	Higher frequency of rearing on the hind legs on postnatal days 17 and 18

Figure captions

Figure 1. Maternal parameters. Daily weight (A), weight gain (A, insert), and daily chow intake (C). Data are presented as mean ± standard deviation.

Figure 2. Litter parameters. Pups daily weight (A), pups weight gain (B), and mean chow intake (C). Pups weight data were analyzed through two-way ANOVA repeated measures; pups weight gain and mean chow intake were analyzed through two-way ANOVA not repeated measures. *** $p<0.001$ indicating difference between female and male pups along postnatal days. Data are presented as mean ± standard deviation.

Figure 3. Maternal behavior. Cumulative frequency of each maternal behavior (A), frequency of maternal contact with pups during postnatal days (B), frequency of licking behavior (C), frequency of arched-back nursing behavior (D), frequency of nursing (blanchet and supine) behavior (E), time off from pups (F), and maternal behavior inconsistency score (G). Data were analyzed through two-way ANOVA repeated measures. * $p<0.05$ indicating difference between sedentary and maternal exercise groups. Data are presented as mean ± standard deviation.

Figure 4. Sensorimotor reflexes. Righting reflex (A), cliff avoidance (B), negative geotaxis (C), and bar holding (D). Data were analyzed through two-way ANOVA repeated measures. No difference was observed between sedentary and maternal exercise groups. Data are presented as mean ± standard deviation.

Figure 5. Frequency of each motor movement over postnatal days. Female pups born to sedentary dams (A), female pups born to exercised dams (B), male pups born to sedentary dams (C), and male pups born to exercised dams (D). Data are presented as mean.

Figure 6. Frequency of quadruped stance over postnatal days. Immobility (A), lateral head movement (B), head elevation (C), grooming (D), rearing with forelimb support (E), and rearing on hind legs (F). Immobility and rearing on hind legs frequency: *** $p<0.001$ sedentary pups

differed from exercised pups group; ### $p<0.001$ maternal sedentary and exercised female pups differed from male of both groups. Grooming frequency: * $p<0.05$ maternal exercised male pups differed from sedentary maternal male pups and maternal exercised female pups; # $p<0.05$ maternal exercised female pups differed from both female and male littermates from maternal sedentary groups. Data are presented as mean ± standard deviation.

Figure 7. Frequency of quadruped movement over postnatal days. Pivoting (A), crawling (B), and walking (C). ** $p<0.005$ indicating difference between sedentary and maternal exercise groups. ## $p<0.005$ indicating difference between maternal exercised male pups and maternal sedentary female pups on PD14; and indicating differences between maternal exercised female pups and all other groups on PD15. Data are presented as mean ± standard deviation.

Figure 1

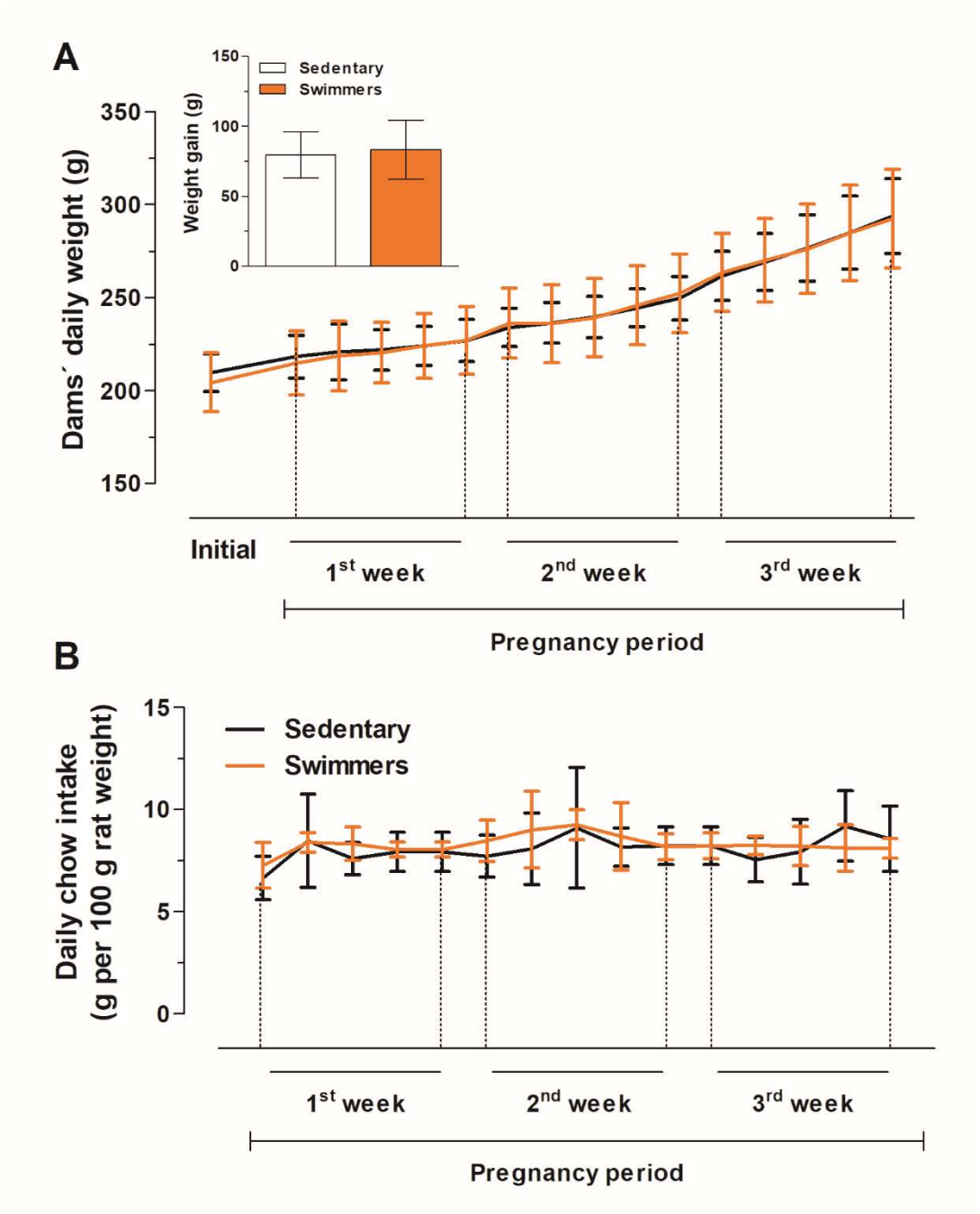

A
Sedentary
Swimmers
Weight gain (g)
150
100
50
0
Dams´ daily weight (g)
350
300
250
200
150
Initial
1st week
2nd week
3rd week
Pregnancy period
B
Daily chow intake
(g per 100 g rat weight)
15
10
5
0
Sedentary
Swimmers
1st week
2nd week
3rd week
Pregnancy period

Figure 2

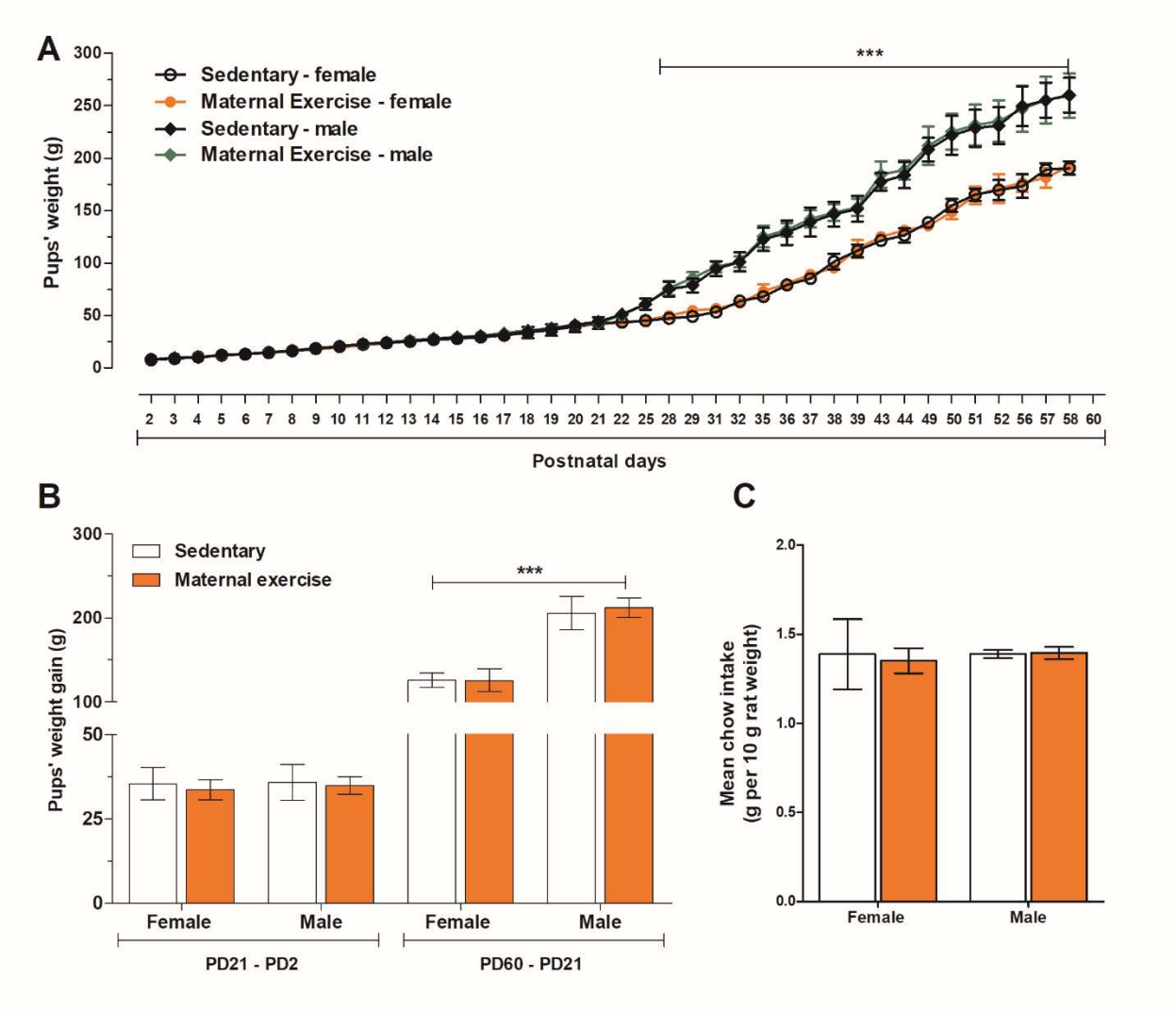

A
Sedentary - female
Maternal Exercise - female
Sedentary - male
Maternal Exercise - male
***
Pups' weight (g)
300
250
200
150
100
50
0
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 25 28 29 31 32 35 36 37 38 39 43 44 49 50 51 52 56 57 58 60
Postnatal days
B
Sedentary
Maternal exercise
***
Pups' weight gain (g)
300
200
100
50
25
0
Female
Male
Female
Male
PD21 - PD2
PD60 - PD21
C
Mean chow intake
(g per 10 g rat weight)
2.0
1.5
1.0
0.5
0.0
Female
Male

Figure 3

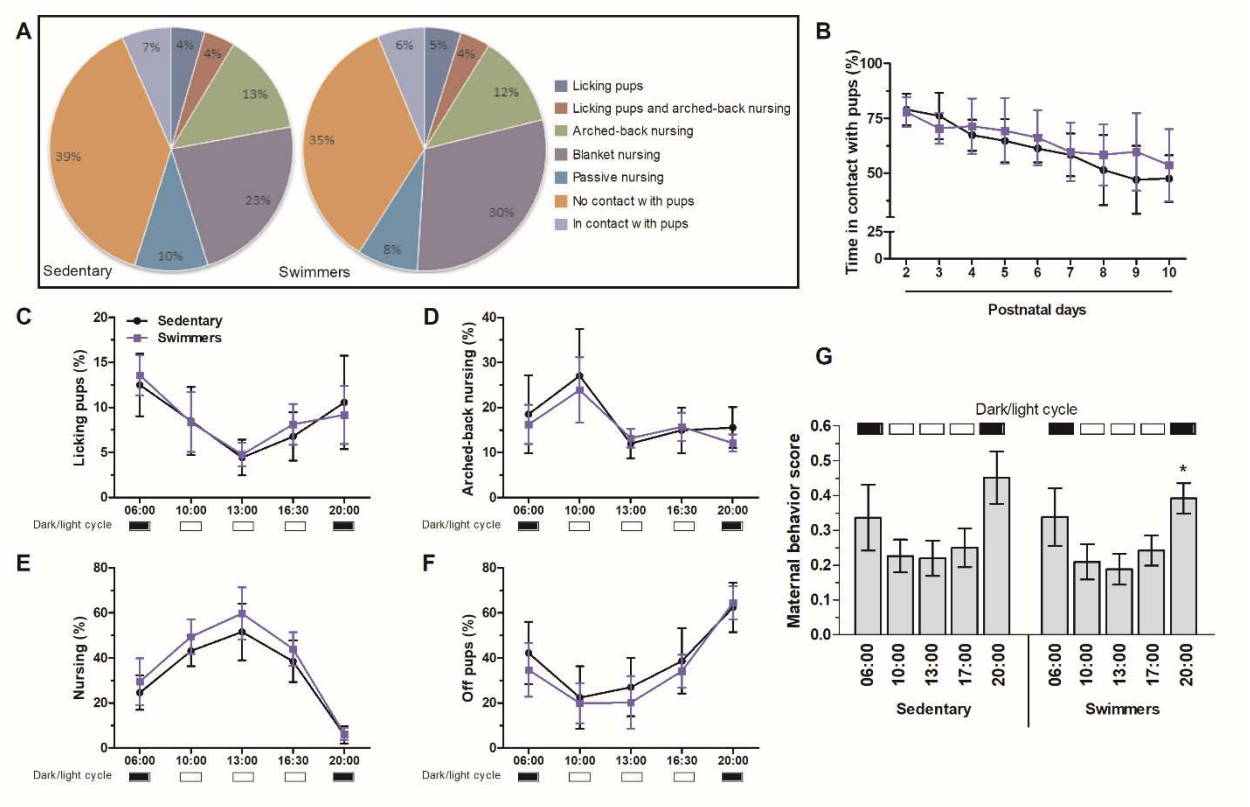

A
7%
4%
4%
13%
39%
23%
10%
Sedentary
6%
5%
4%
12%
35%
30%
8%
Swimmers
Licking pups
Licking pups and arched-back nursing
Arched-back nursing
Blanket nursing
Passive nursing
No contact w ith pups
In contact w ith pups
B
Time in contact with pups (%)
Postnatal days
C
Sedentary
Swimmers
Licking pups (%)
Dark/light cycle
D
Arched-back nursing (%)
E
Nursing (%)
F
Off pups (%)
G
Maternal behavior score
Sedentary
Swimmers
06:00
10:00
13:00
16:30
17:00
20:00

Figure 4

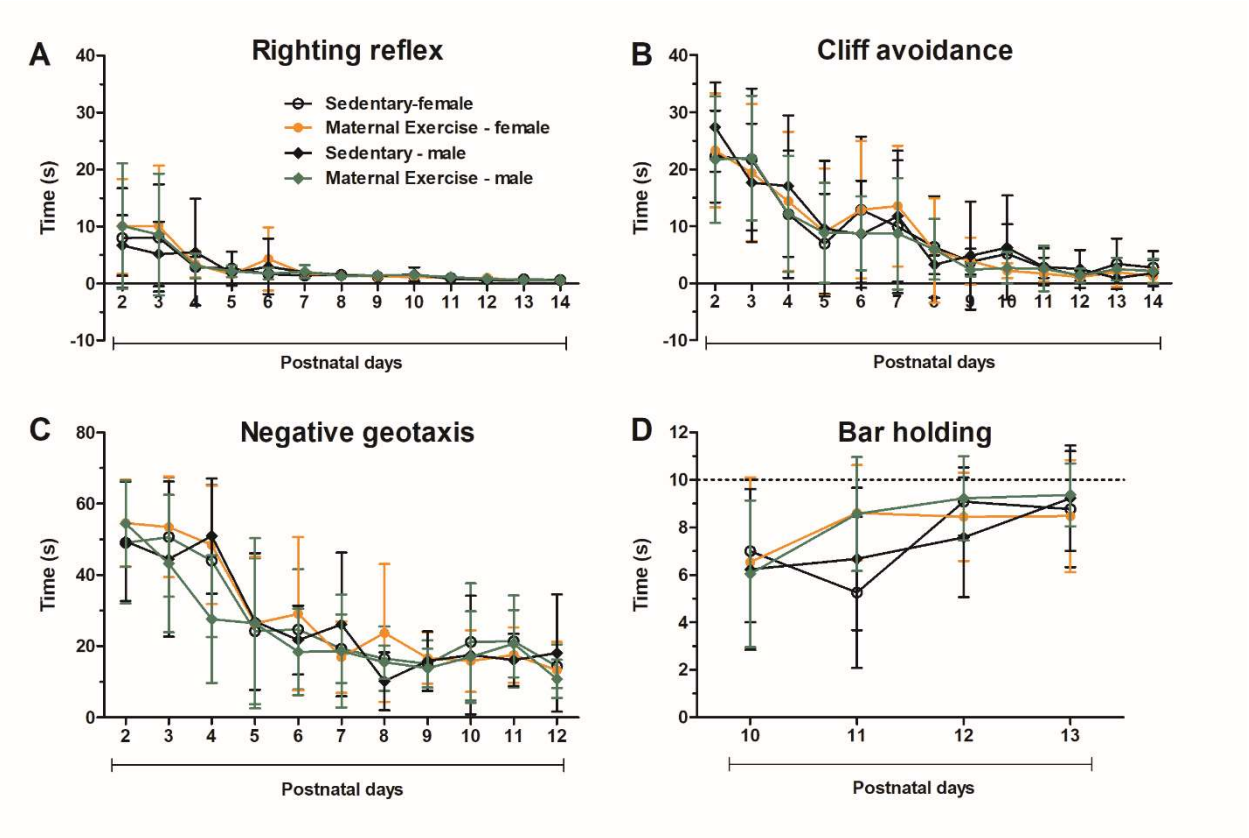

A
Righting reflex
Sedentary-female
Maternal Exercise - female
Sedentary - male
Maternal Exercise - male
Time (s)
Postnatal days
B
Cliff avoidance
Time (s)
Postnatal days
C
Negative geotaxis
Time (s)
Postnatal days
D
Bar holding
Time (s)
Postnatal days

Figure 5

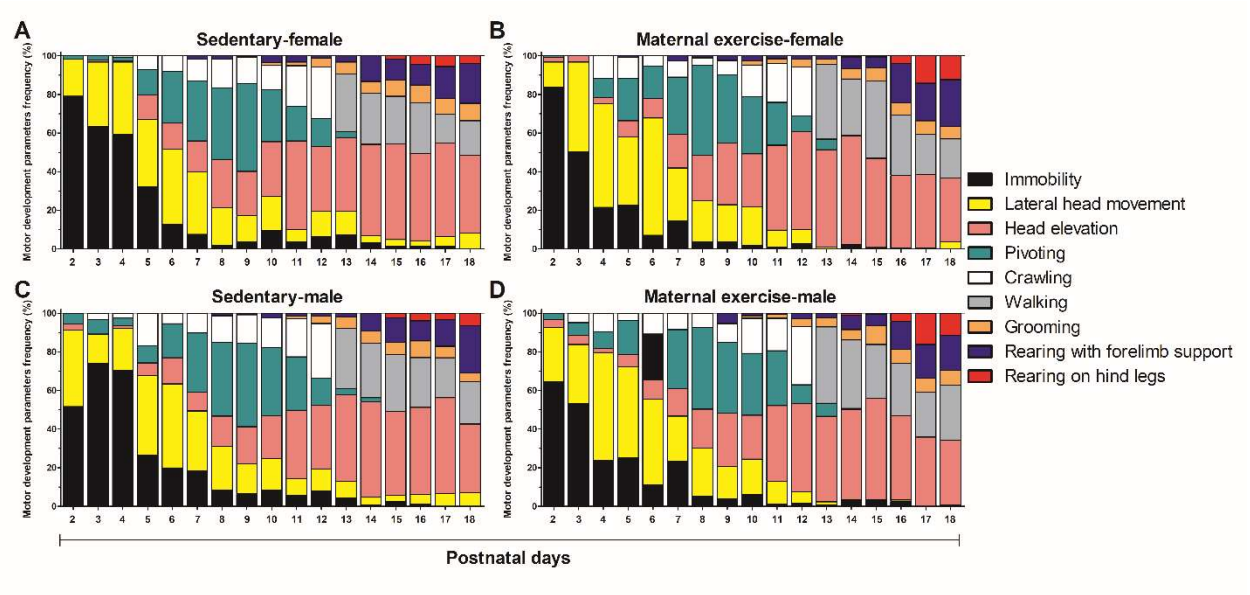

A
Sedentary-female
B
Maternal exercise-female
C
Sedentary-male
D
Maternal exercise-male
Motor development parameters frequency (%)
Postnatal days
Immobility
Lateral head movement
Head elevation
Pivoting
Crawling
Walking
Grooming
Rearing with forelimb support
Rearing on hind legs

Figure 6

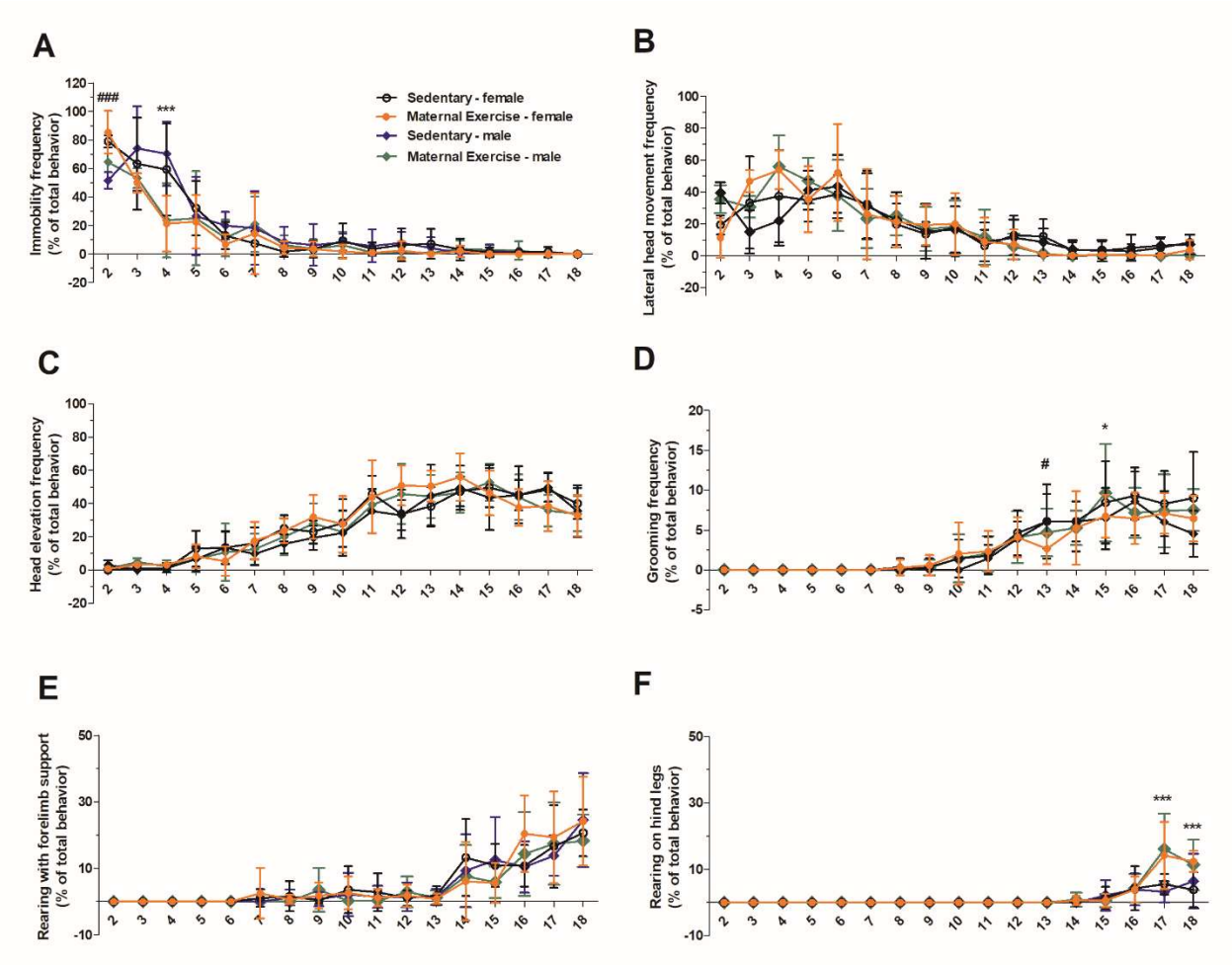
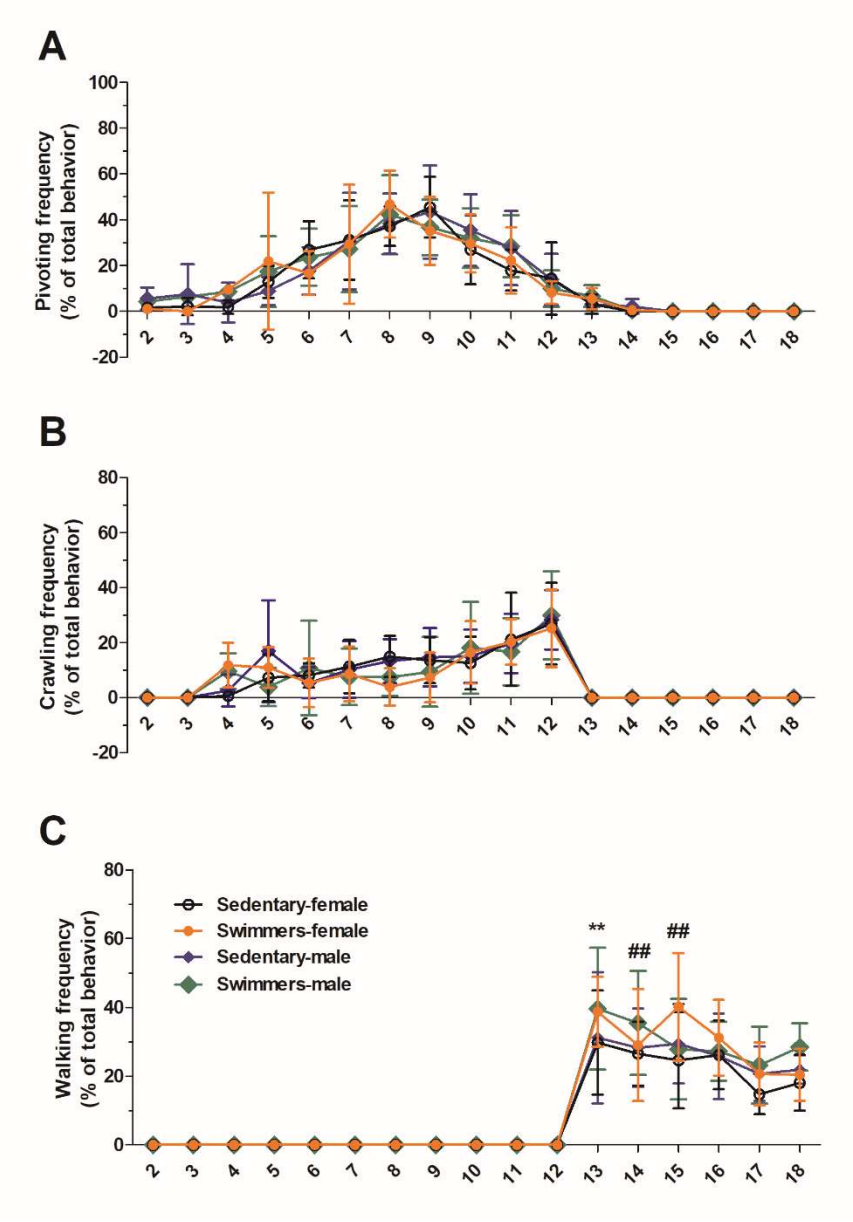

A
B
C
D
E
F
Sedentary - female
Maternal Exercise - female
Sedentary - male
Maternal Exercise - male
Immobility frequency (% of total behavior)
Lateral head movement frequency (% of total behavior)
Head elevation frequency (% of total behavior)
Grooming frequency (% of total behavior)
Rearing with forelimb support (% of total behavior)
Rearing on hind legs (% of total behavior)
###
***
#
*

Figure 7


A
Pivoting frequency (% of total behavior)
100
80
60
40
20
0
-20
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
Crawling frequency (% of total behavior)
80
60
40
20
0
-20
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
C
Walking frequency (% of total behavior)
80
60
40
20
0
Sedentary-female
Swimmers-female
Sedentary-male
Swimmers-male
**
##
##
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

CAPÍTULO II

Exercise in Pregnancy Activates GSK-3β-downstream Akt and upregulates SIRT1 and 3 signaling in pups' cerebellum

O capítulo II apresenta o artigo intitulado *Exercise in Pregnancy Activates GSK-3β-downstream Akt and upregulates SIRT1 and 3 signaling in pups' cerebellum*, o qual está submetido ao periódico *FEBS Letters*.

Exercise in Pregnancy Activates GSK-3β-downstream Akt and upregulates SIRT1 and 3 signaling in Wistar rats pups' cerebellum

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Running title

Maternal exercise alters offspring's cerebellum signaling

Abbreviations

AMPK, AMP-activated protein kinase; BDNF, brain-derived neurotropic factor; DOHaD, Developmental Origin of Health and Disease; DRP1, dynamin-related protein 1; ETC, electron transport chain; ED, embryonic day; FOXO, Forkhead box O; GD, gestational day; GSK-3, glycogen synthase kinase 3; TFAM, mitochondrial transcription factor A; MFN1, mitofusin 1; PGC-1α, peroxisome proliferator-activated receptor gamma co-activator 1α; PMSF, phenylmethanesulfonyl fluoride; PD, postnatal day; ROS, reactive oxygen species; SIRT, sirtuin; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TCA, tricarboxylic acid.

Keywords

Metabolic programming, maternal exercise, mitochondria, sirtuin 1, sirtuin 3, GSK-3

Compliance with Ethical Standards

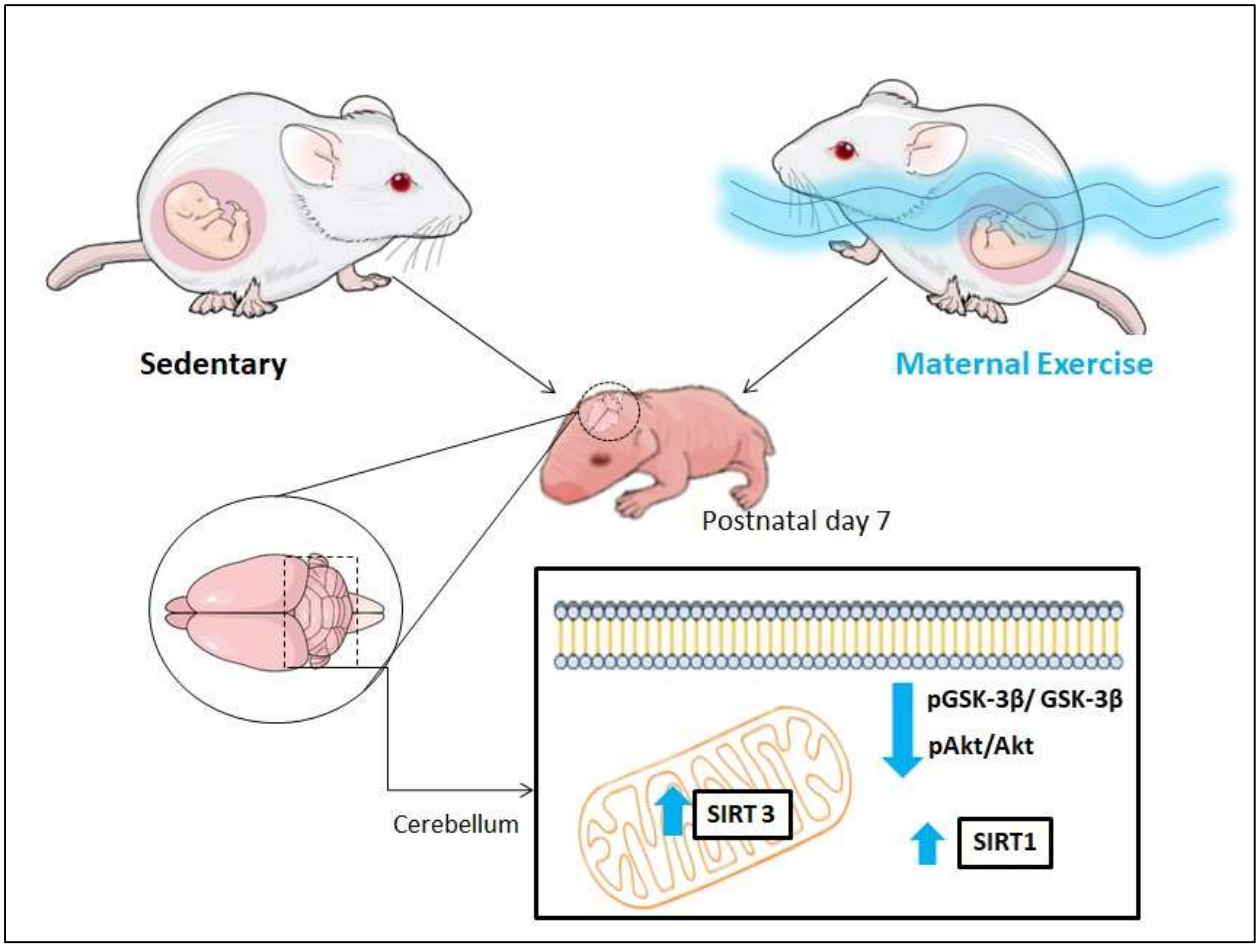
Conflict of Interest

The authors declare that they have no conflict of interest.

Abstract

The intrauterine milieu offered by maternal lifestyle influences the fetal development by programming the metabolism. Maternal exercise affects positively offspring's brain metabolism conferring resistance to adverse conditions in postnatal life through adaptive mechanisms that must be determined. We investigated the involvement of some signaling molecules underlying the effects of maternal swimming on the offspring's cerebellum. Our analyses revealed unchanged pGSK-3β/GSK-3β and pAkt/Akt ratios by maternal exercise in the fetal cerebellum on embryonic day 20. On postnatal day 7, both pGSK-3β/GSK-3β and pAkt/Akt ratios reduced significantly in the cerebellum, while the immunocontent of energy metabolism-sensing proteins SIRT1 and 3 was increased by maternal exercise without affecting Mfn1, Drp1, and TFAM levels. Our findings show that offspring's cerebellar metabolism is programmed adaptively in postnatal life by maternal exercise environment.

Graphical abstract



Introduction

Brain development encompasses sequential processes of proliferation, migration, differentiation, synaptogenesis, and apoptosis to reach mature functional neural circuitry [1-3]. Each one of these processes occurs differentially across various brain regions depending on regional and temporal emergence of developmental processes [2]. In both humans and rodents, the brain initiates to develop during embryonic and fetal periods and continues postnatally [2-5]. The activation of glycogen synthase kinase 3 (GSK-3) signaling pathway is crucial during brain development [6]. GSK-3 controls brain developmental processes such as neurogenesis, neuronal polarization, and axon growth; therefore, abnormal GSK-3 signaling can be associated with neurodevelopmental disorders [7].

Environmental cues during critical periods of development can produce long-lasting consequences [2] on brain function by programming cellular functions [4]. Metabolic and epigenomic adaptive modifications occurs due the plastic ability whereby the organism respond adaptively to environmental stimuli [8]. The Developmental Origin of Health and Disease (DOHaD) explains the long-term influence of developmental programming [9]. Initial evidences of developmental brain vulnerability have been described after the exposure of animals or humans to neurotoxic compounds. Such compounds interfere with developmental processes and influence the susceptibility to diseases [10-12]. Lately, evidences have demonstrated that healthy maternal lifestyle, across the vulnerable brain developmental windows, emerged as beneficial stimuli, which can have long-term benefits to maternal and offspring's health conferring resistance to diseases [8]. Thinking on developmental processes of central nervous system development as opportunity instead susceptibility window, several studies demonstrated that maternal exercise before and during pregnancy promotes metabolic adaptations in the offspring protecting against hazardous stimuli [13-15]. These adaptations include modulation of antioxidant system in several brain regions [16], increased mitochondrial function [16], induction of hippocampal neurogenesis [17], increased brain-derived neurotrophic factor (BDNF) [18], and enhanced learning and memory [17, 19].

It is known that mitochondria are the main players to optimize metabolic adaptation in response to exercise [20]. Dynamic properties of mitochondria are essential to support high energy demand at long distances in neurons, thus controlling its distribution and function [21]. Therefore, in response to cellular energy requirement mitochondrial morphology dynamically change to ensure the proper energy support to different compartments in cellular processes [22]. Mitochondrial dynamic is determined by a balance between fusion and fission, which are orchestrated mainly by the GTPases mitofusin (Mfn) and dynamin-related protein (Drp), respectively [21]. Furthermore, energy metabolism is improved by inducing mitochondrial biogenesis, which is regulated by peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) and mitochondrial transcription factor A (TFAM) [23, 24]; the latter drives the initiation of transcription and replication of mitochondrial DNA (mtDNA) [24, 25]. Mitochondrial biogenesis and energy metabolism are under control of sirtuins (SIRTs), SIRT1 and SIRT3, respectively [26]. SIRT1 promotes mitochondria proliferation and oxidative phosphorylation by activating PGC-1α Conversely, activates proteins engaged in the oxidative phosphorylation, tricarboxylic acid (TCA) cycle, and fatty-acid oxidation [26].

It is largely known that physical exercise induces mitochondrial biogenesis and improves mitochondrial bioenergetics in the brain [27-30]. However, little is known with regard to maternal exercise and its underlying mechanisms to promote benefits in offspring's brain metabolism. Park [31] showed that maternal running during pregnancy improves mitochondrial function in the hippocampus of neonates. In addition, our group demonstrated that maternal swimming improves mitochondrial function and antioxidant defenses in the cerebellum, parietal cortex, hippocampus, and striatum of 7-day-old rats [16]. However, the molecular mechanisms underlying these benefits remain to be elucidated. Therefore, aiming to decipher the underlying adaptive processes responsible by offspring's metabolic benefits elicited by maternal exercise our goal was to

investigate the effects of maternal exercise during pregnancy on some signaling molecules levels in the cerebellum of pups on embryonic day 20 and on postnatal day 7.

Materials and Methods

Animals

Adult male and female Wistar rats (80 days old) were housed with 12h dark/light cycle (lights on between 7:00h and 19:00h), controlled temperature (22 ± 2°C) and humidity (50-60%), and had free access to food and water. All experimental procedures and animals care were conducted in accordance with the National Institutes for Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1996) and were approved by the local Ethics Commission of Universidade Federal do Rio Grande do Sul (CEUA/UFRGS; protocol number 32852). All efforts were made to minimize animal suffering, and to keep the number of animals at the minimum to demonstrate consistent effects.

Maternal swimming protocol

Female rats were randomly assigned to sedentary (control) or exercised (involuntary swimming) groups. Rats swam one week prior the mating, to habituate to aquatic environment, and throughout pregnancy in a schedule of 5 days/week for 4 weeks lasting 30 min/day in a pool filled with 32 ± 1°C water, as previously described [16]. Control rats were immersed in water, carefully dried, and returned to the housing boxes in the same schedule of exercise group.

Experimental design

During the mating two females were housed with one male. Pregnancy was confirmed by the presence of a vaginal plug and the embryonic day 0 (E0)/gestational day 0 (GD0) was established. Up to GD20, all pregnant females were housed individually. One subset of pregnant females from sedentary and exercise group was euthanized by rapid decapitation without anesthesia and the

fetuses on E20 were removed from the uteri of dams. Fetuses brain was dissected, and fetal cerebellum was isolated. Although all fetuses were collected, only one per litter, randomly selected, was used for each analysis. Another subset of pregnant females was allowed to normal spontaneous vaginal delivery, and they were checked for birth twice a day (at 8 and 18 h) to allow the assignment of postnatal day (PD) 0. Littermates were housed up to PD7 with their mothers. The pups were euthanized on PD7 by decapitation; the brains were removed, and pups' cerebellum was dissected. Encephalic samples were frozen at -80°C immediately after their dissection and it were stored until use.

Mature BDNF measurement

Mature BDNF protein content was measured in the cerebellar homogenate of pups at age of 7 days. Mature BDNF was measured through the E-Max ELISA kit (Promega) according to the manufacturer's recommendations. Briefly, cerebellum were individually homogenized (1:10 w:v) in lysis buffer containing: 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), Igepal (1%), glycerol (10%), 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM sodium vanadate, 0.1 mM EDTA, and 0.1 mM EGTA, and centrifuged for 3 min at 14,000 rpm at 4°C. Supernatant was diluted (1:5 v/v) in sample buffer and incubated on a 96-well flat-bottom plates previously coated with anti-BDNF monoclonal antibody and blocked with block and sample buffer. After sample incubation, plates were incubated with polyclonal anti-human antibody for 2 h and horseradish peroxidase for 1 h. Colorimetric reaction with tetramethylbenzidine was quantified in a plate reader at 450 nm. The standard BDNF curve, ranging from 0 to 500 pg/mL was assayed in each plate in parallel with the samples.

Western blot assay

Cerebellar samples from fetuses on E20 and from pups on PD7 were homogenized in ice-cold lysis buffer containing 4% sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mM Tris, and 1% protease

inhibitor cocktail. The homogenates were denatured 100°C for 5 min, and then centrifuged at 10,000 *g* for 30 min. After this, the supernatant containing the cytosolic fraction was collected, β-mercaptoethanol was added to a final concentration of 5%, and then, the samples were stored at -80°C until use. Equal concentration of protein (50 μg) was loaded and immunodetected as previously described [32]. Membranes were incubated for 60 min at 4°C in blocking solution (Tris-buffered saline containing 5% non-fat milk and 0.1% Tween-20, pH 7.4) prior the incubation with the primary antibody. Membranes were incubated overnight at 4°C in blocking solution containing one of the following primary antibodies: rabbit monoclonal anti-phospo(S473)-Akt (1:1000, Cell Signaling, catalog number #4058), anti-Akt (1:1000, Cell Signaling, catalog number #9272), anti-phospho(S9)-GSK-3β (1:1000, Cell Signaling, catalog number #9336), anti-GSK-3β (1:1000, Cell Signaling, catalog number #9315), anti-Mfn-1 (1:500, Abcam, catalog number #ab104274), anti-Drp-1 (1:500, Abcam, catalog number #ab154879), anti-SIRT1 (1:500, Santa Cruz Technologies, catalog number #sc-15404), anti-SIRT3 (1:500, Abcam, catalog number #ab189860), and rabbit monoclonal anti-β-actin (1:2000, Cell Signaling, catalog number #4967). After washing, the membranes were incubated with secondary antibody containing peroxidase-conjugated anti-rabbit IgG (1:2000, GE Healthcare Life Sciences, catalog number #NA934V) for 1 h. The chemiluminescence was detected using a digital imaging system (Image Quant LAS 4000, GE Healthcare Life Sciences) and analyzed using the Image J Software. The average optical density for the control group was designated as 100%.

Statistical analyses

Statistical analyses were performed using GraphPad Prism, version 6 (La Jolla, CA, USA). Data were analyzed using two-tailed t test. Differences was considered when P value was <0.05.

Results

Effects of maternal swimming on Akt/GSK-3β signaling pathway in the fetus's cerebellum on embryonic day 20 and postnatal day 7

GSK-3β is a downstream target of Akt, which phosphorylates GSK-3 on Ser9 inhibiting its activity [33]. Data analyses of Akt/GSK-3β pathway in the cerebellum obtained from fetuses on E20 indicated that Akt and GSK-3β activities remained unchanged in response to maternal swimming in comparison to control group (Fig. 1A and B). The values of p-Akt/Akt ratio in the cerebellum removed from fetuses of exercised group were similar to fetuses of control group [$t(10) = 0.459$, $p = 0.656$]. Similarly, the values of the ratio between p(Ser-9)-GSK-3β/GSK-3β in the cerebellum of fetuses of exercised group were similar to fetuses of control group [$t(10) = 0.778$, $p = 0.454$].

Interestingly, on postnatal day 7, we found a totally different profile in cerebellum. Akt phosphorylation was found significantly reduced in the cerebellum of 7-day-old pups born to exercised dams compared to control pups (Fig. 1C). Akt phosphorylation was found significantly reduced in the cerebellum of 7-day-old pups born to exercised dams compared to control pups (Fig. 1C) [$t(14) = 2.993$, $p = 0.010$], without modifying total Akt levels [$t(14) = 0.718$, $p = 0.484$]. As shown in Fig. 1D, GSK-3β phosphorylation at Ser-9 was found reduced in the cerebellum of 7-day-old pups born to exercised dams compared to control pups [$t(14) = 2.549$, $p = 0.023$], without modifying total GSK-3β levels [$t(14) = 0.0002$, $p = 1.0$]. These data indicate that maternal swimming reduces the kinase activity of Akt leading to increased levels of dephosphorylated GSK-3β, which becomes more active in the 7-day-old pup's cerebellum.

Maternal swimming does not alter mature BDNF levels in the offspring's cerebellum on postnatal day 7

The role of the neurotrophin BDNF as intracellular signaling inducer is important for neuronal survival, morphology, and plasticity in developing and mature central nervous system [34]. Mature BDNF levels were measured through ELISA in the offspring's cerebellum. It was observed that

maternal exercise during pregnancy exerted no effect on this parameter, as demonstrated by similar values in the pups' cerebellum of exercised and control groups [$t(14) = 0.881$, $p = 0.393$] (Fig. 2).

Maternal swimming elicits augmentation of Sirtuin 1 and 3 immunocontent in the offspring's cerebellum on postnatal day 7

Next, we measured the immunocontent of SIRTs 1 and 3 through Western blotting in pups' cerebellum. The SIRTs are deacetylases proteins playing essential role in energetic metabolism [35]. Sirt1 (Fig. 3A) and Sirt3 (Fig. 3B) immunocontent was increased in the pups cerebellum born to exercised dams in comparison to control [$t(18) = 2.282$, $p = 0.035$; and $t(12) = 2.290$, $p = 0.041$, respectively].

Effects of maternal swimming on mitochondrial parameters in the offspring's cerebellum on postnatal day 7

Mitochondria proliferation and distribution in addition to its function of energy exchange are regulated by mitochondrial fusion and fission processes [21]. Mitochondria continuously undergo fusion and fission cycles [21], and this requires the transcription factor TFAM to ensure the stability of mitochondrial genome [36]. We assessed mitochondrial parameters such as the immunocontent of fusion protein, Mfn1, fission protein, Drp1, and mitochondrial transcription factor, TFAM (Fig. 4A-C). Mitochondrial dynamics proteins, Mfn1 and Drp1, were found unaltered in the cerebellum of pups [$t(14) = 1.165$, $p = 0.263$; and $t(14) = 0.235$, $p = 0.817$, respectively], indicating no effect of maternal exercise on this parameters on PD7. Similarly, it was found unchanged TFAM levels in the cerebellum of maternal exercised pups [$t(14) = 0.284$, $p = 0.780$] compared to control pups.

Discussion

It has been widely recognized that physical exercise during pregnancy promotes benefits to mother and fetus [37]. Exercising during pregnancy prevents maternal hypertension and excessive weight gain, prevents offspring's macrosomia at birth [38], and enhances cerebral maturation in newborn [39]. Despite available data from animal studies help to clarify whole body metabolic changes elicited by maternal exercise on offspring, the molecular basis on brain changes remains to be elucidated. Herein, we assessed some key molecules involved in brain signaling that might be responsive to maternal exercise.

Setting up neural circuits during development relies on several morphogenetic steps, including neuronal migration and polarization, axon outgrowth and branching [40, 41]. These steps are orchestrated under influence of the signaling mediator GSK-3. GSK-3 transduces upstream signaling to reorganization of the axonal microtubules of cytoskeleton directed by extracellular cues [40]. As seen that GSK-3 is an important molecular regulator of neurogenesis, we initially sought to evaluate GSK-3β downstream Akt pathway. Our analyses of Akt/GSK-3β signaling demonstrated that increased activity of GSK-3β was present on 7-day-old pups' cerebellum, which seems to be a result of intrauterine metabolic programming by maternal exercise. Interestingly, this signaling pathway was not affected on embryonic day 20, despite the maternal exercise was performed up to delivery. The mechanism behind the switch effect must be investigated in future works, and could be related to synergic effects of maternal exercise stimulus and postnatal environment. Here, the increased GSK-3β activity on 7-day-old pups' cerebellum was a result of decreased Akt activity. It has also been demonstrated that the control of GSK-3 activity during development occurs through activation of the ubiquitin–proteasome system [40]. Phosphorylated Akt is known to phosphorylate GSK-3β at serine 9, reducing GSK-3β activity [33]. During development, phosphorylated Akt has been shown to be target of local protein degradation to ensure neuronal polarity [42]. Among the brain regions assessed in the present study, upregulation of GSK-3β was observed only in the cerebellum of 7-day-old pups. These findings might be

explained by distinct temporal and regional maturation of brain regions, in which neurogenesis vary temporally within different regions [2]. Cerebellar neurogenesis is delayed in comparison to other brain developing regions, initiating early during embryonic stages and extending at postnatal days [2].

BDNF is known to play a role in neuron survival and differentiation during brain development [43]. In addition, physical exercise has long been shown to induce the expression of neurotrophic factors such as BDNF [44]. Several works have demonstrated that exercise increases BDNF mRNA expression and/or protein levels in the brain of adolescent [45, 46], adult [47], and aging rats [48, 49]. Furthermore, some studies have demonstrated that maternal exercise during pregnancy increases BDNF levels [18, 50, 51], while others studies have demonstrated that BDNF levels are transiently increased at early postnatal age [52] or even unchanged by maternal exercise during pregnancy [19]. Exercise modality employed, the time-point evaluated and rodent strains used can account for these differences. Moreover, methodological variations are found among these studies, which measure mRNA expression or protein levels of BDNF. BDNF is synthesized as a precursor, pro-BDNF, which is converted to mature BDNF. Pro- and mature BDNF play distinct functions by interacting with different receptors and activating different intracellular pathways [53]. In the present study, mature BDNF levels were found unchanged by maternal exercise in the cerebellum of pups.

SIRTs are NAD+-dependent deacetylases with key roles not only in histones deacetylation but also in transcriptional regulators deacetylation [54]. The role played by SIRTs controlling transcription factors activities allows the communication between metabolism and epigenetic regulation [55], highlighting the importance to study SIRTs during development in the metabolic programming field. SIRT1 and SIRT3 are known to control mitochondrial biogenesis and energy metabolism [26]. SIRT1 is known to deacetylate and activate PGC-1α and Forkhead box O (FOXO) transcription factor [56]. When activated, PGC-1α mediates transcription of nuclear and

mitochondrial genes involved in mitochondrial biogenesis and function [57], while FOXO mediates transcription of genes related to redox balance, such as antioxidant superoxide dismutase 2 (SOD2) and catalase genes, to increase the oxidative stress resistance [56]. The mitochondrial SIRT3 deacetylates several mitochondrial proteins, thus regulating mitochondrial functions [58]. SIRT3 acts as direct activator of oxidative phosphorylation, TCA cycle and fatty-acid oxidation proteins, and indirect activator of PGC-1α and AMP-activated protein kinase (AMPK) [26, 59] demonstrated that SOD2 deacetylation by SIRT3 is essential for the enzyme activity, evidencing the role of SIRT3 in sensing redox imbalance. Interestingly, Cheng, et al. [60] demonstrated that SIRT3 mediates adaptive responses of neurons to exercise. Here, we observed that maternal exercise induced an increase in SIRT1 and SIRT3 levels, indicating the exercise performed by mother promotes adaptive changes in the offspring`s cerebellar metabolism that remain evident at least on the first postnatal days. The present findings are complementary to our previous work [16]. In Marcelino, et al. [16] we demonstrated significant increased activity of antioxidant enzymes, increased non-enzymatic antioxidant potential and reactivity, and increased mitochondrial mass and membrane potential in the cerebellum of pups. It seems that the enhanced mitochondrial function and antioxidant status underlying molecular mechanism is assigned to SIRT signaling in the cerebellum of 7-day-old pups. Our findings suggest that the increased levels of SIRT1 and SIRT3 mediates the beneficial adaptive effects of maternal environment promoted by physical exercise in the offspring's cerebellum.

The central nervous system is highly plastic as it undergoes continuous remodeling not restricted to fetal and early postnatal development but also during adulthood [2]. Neuronal plasticity enables the adaptation to the environment by changing the structure and function of cells [61]. Mitochondria are fundamental organelles in the plasticity of neuronal circuits to supply the high metabolic requirement and to sustain vesicular neurotransmitter release [61, 62]. In addition, mitochondrial biogenesis, turnover, distribution, and morphogenesis are essential for brain

development [63]. A balance between fusion and fission must be maintained for optimal mitochondrial function [64], and the nuclear encoded-TFAM is necessary for mitochondrial biogenesis. TFAM is translated in the cytosol and transported into the mitochondria to induce the transcription of essential mitochondrial enzymes encoded by mitochondrial genome [20]. The important role of mitochondrial dynamic in the development of central nervous system and synaptic plasticity is described by several authors [65-67]. Ishihara et al. [65] demonstrated that mitochondrial fission protein Drp1 is essential for embryonic development and synapse formation in mice. Kageyama et al. [66] demonstrated that mitochondrial division orchestrated by Drp1 is a mechanism of quality control to suppress oxidative damage and to promote neuronal survival. Here, we examined the Mfn1 and Drp1 levels, and we demonstrated that maternal exercise maintains the protein levels at control values. In addition, we observed that TFAM protein levels also remained unchanged by maternal exercise in 7-day-old pups' cerebellum.

Taken together, the data obtained in the present study demonstrates that exercise during pregnancy programs the metabolism of offspring's cerebellum by modulating activation of GSK-3β-downstream Akt pathway and SIRT1/3 signaling. So far, the present work offers insights to DOHaD and opens new avenues to promote preventive strategies to disease development. As temporal ontogeny of brain development extends beyond early postnatal days, it remains to be determined whether maternal exercise further induces time-dependent changes on brain metabolism at cellular and molecular levels that goes beyond postnatal day 7. In summary, in the present study we found that maternal swimming during pregnancy differentially activated GSK-3β downstream Akt pathway in the cerebellum. Moreover, further analyses in the 7-day-old pups' cerebellum indicated an upregulation of energy metabolism-sensing proteins, SIRT1 and SIRT3, associated to unchanged levels of TFAM, Mfn1, and Drp1.

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Author Contributions

CPK and ABS designed research, conducted experiments, analyzed the results, formatted the graphs, and wrote the paper, JBH, conducted experiments and assisted the writing of the manuscript, PMA, TBM, conducted the experiments, PNL and CGS assisted the research design, CM designed research, conducted the experiments, obtained financial support, and assisted the manuscript preparation. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Effect of maternal swimming on Akt/GSK-3β signaling pathway in the offspring's cerebellum. Ratio of p-Akt/Akt and p-GSK-3β/GSK-3β immunocontent in the cerebellum of fetuses on embryonic day 20 (A-B). Ratio of p-Akt/Akt and p-GSK-3β/GSK-3β immunocontent in the cerebellum of pups on postnatal day 7 (C-D). Data are presented as mean ± standard mean error (n=6-8). Student t test showed a significant difference in the cerebellar Akt/GSK-3β activities between maternal swimming and control group. Representative immunoblot is shown in the bottom of each graph. *$p<0.05$ and **$p<0.01$ compared to control.

Figure 2. Effect of maternal swimming on mature BDNF levels in the pup's cerebellum on postnatal day 7. Data are presented as mean ± standard mean error (n=8), and data were analyzed through Student t test.

Figure 3. Effect of maternal swimming on sirtuins 1 and 3 immunocontent in the pup's cerebellum on postnatal day 7. Immunocontent of Sirtuin 1 (A) and Sirtuin 3 (B). Sirtuin 1 and Sirtuin 3 levels are expressed as the average percentage of control. Representative immunoblot (normalized to b-actin protein) is shown in the bottom of each graph. Data are presented as mean ± standard mean error (n=8). Student t test showed a significant difference in the cerebellar sirtuin 1 and 3 immunocontent between maternal swimming and control group. *$p<0.05$ compared to control.

Figure 4. Effect of maternal swimming on mitochondrial parameters in the pup's cerebellum on postnatal day 7. Immunocontent of TFAM (A), mitofusin (B), and dynamin-related protein (C). TFAM, mitofusin, and dynamin-related protein levels are expressed as the average percentage of control. Representative immunoblot (normalized to b-actin protein) is shown in the bottom of each graph. Data are presented as mean ± standard mean error (n=8). Student t test showed no significant difference between maternal swimming and control group.

Figure 1

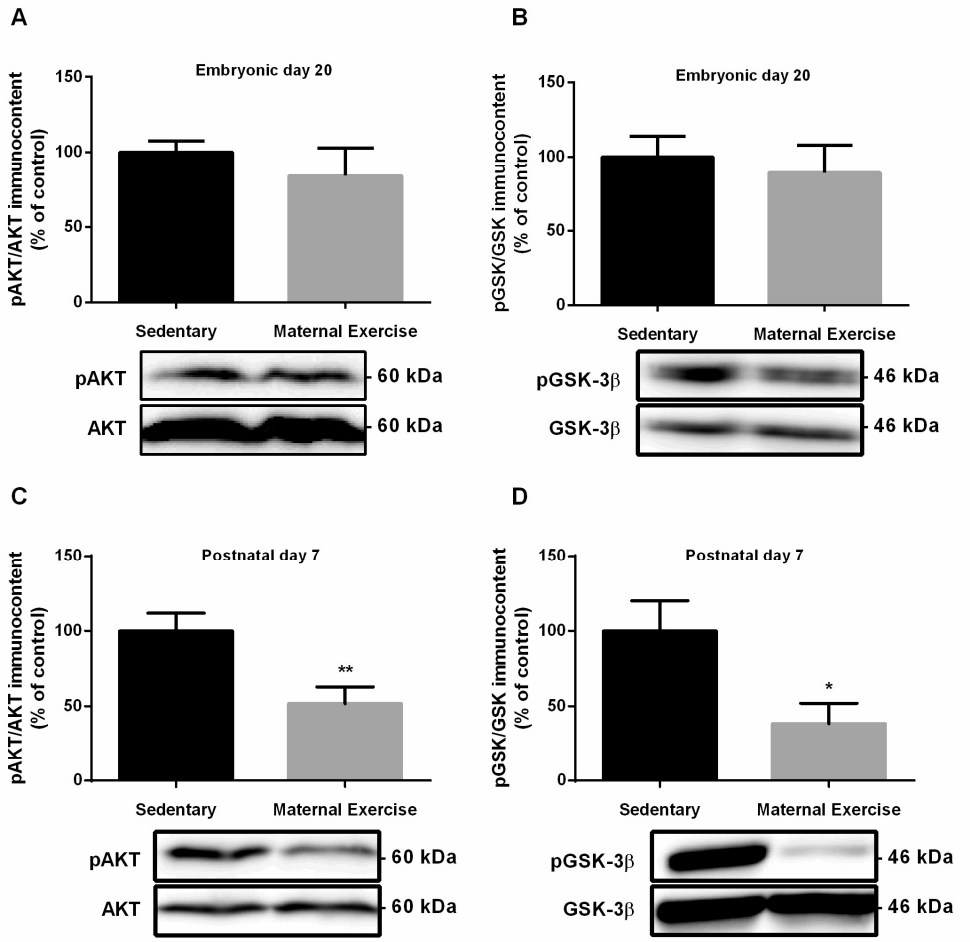

A
Embryonic day 20
pAKT/AKT immunocontent (% of control)
150
100
50
0
Sedentary
Maternal Exercise
pAKT
60 kDa
AKT
60 kDa
B
Embryonic day 20
pGSK/GSK immunocontent (% of control)
150
100
50
0
Sedentary
Maternal Exercise
pGSK-3β
46 kDa
GSK-3β
46 kDa
C
Postnatal day 7
pAKT/AKT immunocontent (% of control)
150
100
50
0
**
Sedentary
Maternal Exercise
pAKT
60 kDa
AKT
60 kDa
D
Postnatal day 7
pGSK/GSK immunocontent (% of control)
150
100
50
0
*
Sedentary
Maternal Exercise
pGSK-3β
46 kDa
GSK-3β
46 kDa

Figure 2

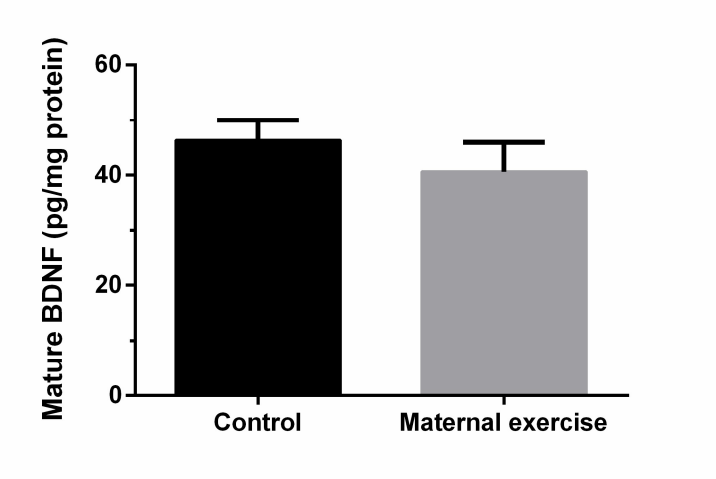

Mature BDNF (pg/mg protein)
60
40
20
0
Control
Maternal exercise

Figure 3

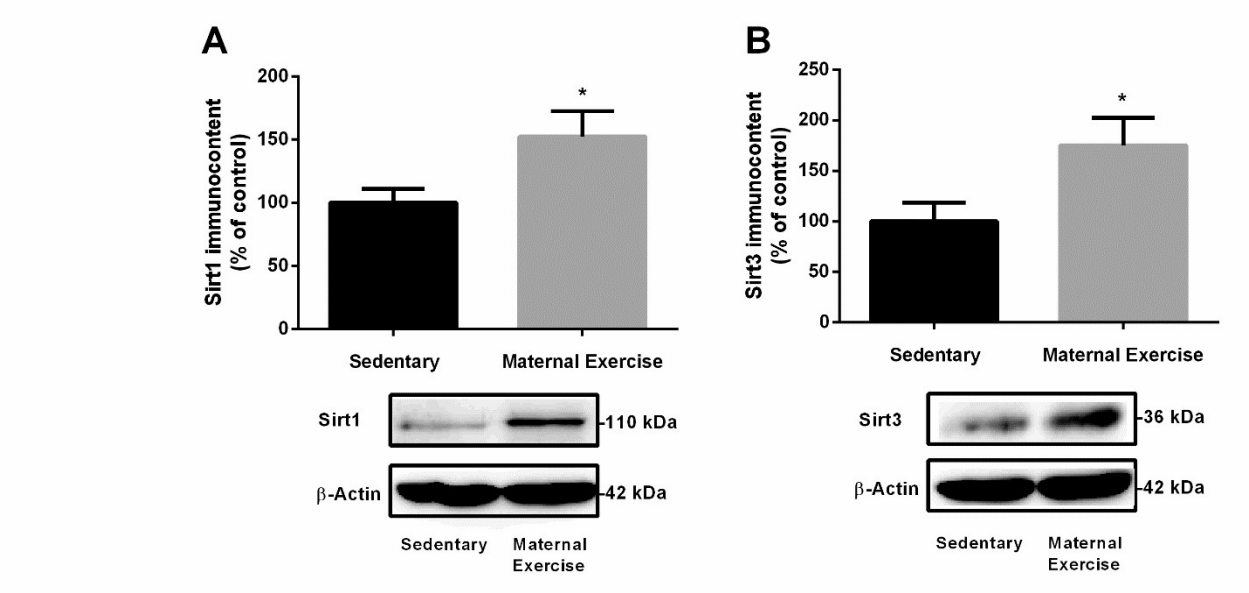
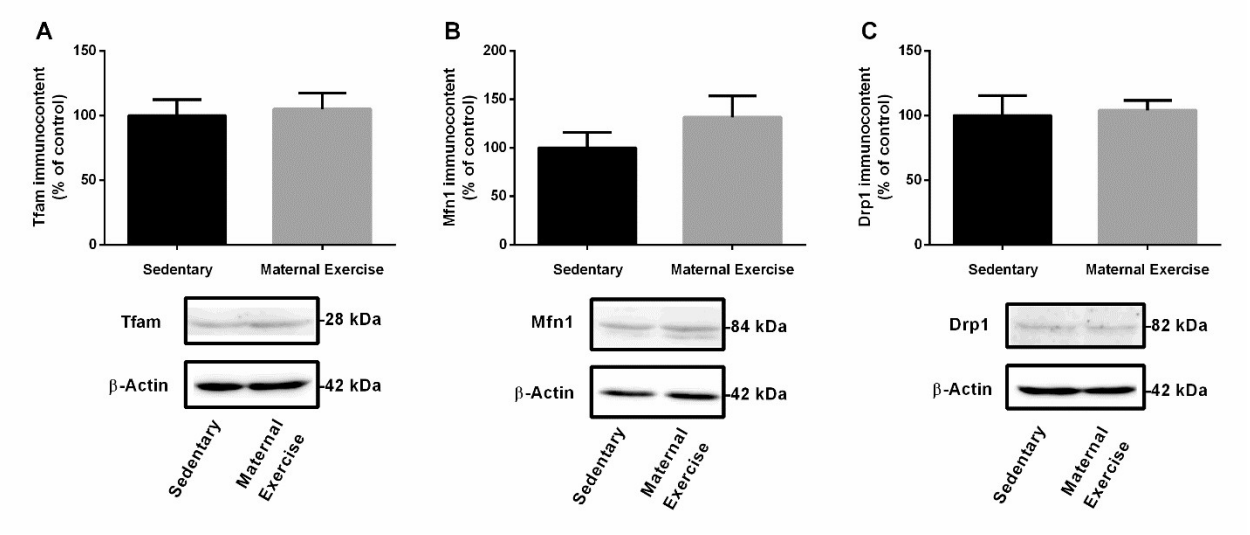

A
Sirt1 immunocontent (% of control)
200
150
100
50
0
*
Sedentary
Maternal Exercise
Sirt1
110 kDa
β-Actin
42 kDa
Sedentary
Maternal Exercise
B
Sirt3 immunocontent (% of control)
250
200
150
100
50
0
*
Sedentary
Maternal Exercise
Sirt3
36 kDa
β-Actin
42 kDa
Sedentary
Maternal Exercise

Figure 4


A
Tfam immunocontent (% of control)
150
100
50
0
Sedentary
Maternal Exercise
Tfam
28 kDa
β-Actin
42 kDa
Sedentary
Maternal Exercise
B
Mfn1 immunocontent (% of control)
200
150
100
50
0
Sedentary
Maternal Exercise
Mfn1
84 kDa
β-Actin
42 kDa
Sedentary
Maternal Exercise
C
Drp1 immunocontent (% of control)
150
100
50
0
Sedentary
Maternal Exercise
Drp1
82 kDa
β-Actin
42 kDa
Sedentary
Maternal Exercise

CAPÍTULO III

Physical Exercise during Pregnancy Prevents Cognitive Impairment induced by Amyloid-β in Adult Offspring Rats

O capítulo III apresenta o artigo intitulado *Physical Exercise during Pregnancy Prevents Cognitive Impairment induced by Amyloid-β in Adult Offspring Rats*, o qual está publicado no periódico *Molecular Neurobiology*, disponível em: https://doi.org/10.1007/s12035-018-1210-x

Physical Exercise during Pregnancy Prevents Cognitive Impairment induced by Amyloid-β in Adult Offspring Rats

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Abstract

Alzheimer's disease (AD) is the main aging-associated neurodegenerative disorder, and is characterized by mitochondrial dysfunction, oxidative stress, synaptic failure, and cognitive decline. It has been a challenge to find disease course-modifying treatments. However, several studies demonstrated that regular physical activity and exercise are capable of promoting brain health by improving the cognitive function. Maternal lifestyle, including regular exercise during pregnancy, has also been shown to influence fetal development and disease susceptibility in adulthood through fetal metabolism programming. Here, we investigated the potential neuroprotective role of regular maternal swimming, before and during pregnancy, against amyloid-β neurotoxicity in the adult offspring. Behavioral and neurochemical analyses were performed fourteen days after male offspring received a single, bilateral, intracerebroventricular (icv) injection of amyloid-β oligomers (AβOs). AβOs-injected rats of the sedentary maternal group exhibited learning and memory deficits, along with reduced synaptophysin, brain-derived neurotrophic factor (BDNF) levels, and alterations of mitochondrial function. Strikingly, the offspring of the sedentary maternal group had AβOs-induced behavioral alterations that were prevented by maternal exercise. This effect was accompanied by preventing the alteration of synaptophysin levels in the offspring of exercised dams. Additionally, offspring of the maternal exercise group exhibited an augmentation of functional mitochondria, as indicated by increases in mitochondrial mass and membrane potential, α-ketoglutarate dehydrogenase, and cytochrome c oxidase enzymes activities. Moreover, maternal exercise during pregnancy induced long-lasting modulation of fusion and fission proteins, Mfn1 and Drp1, respectively. Overall, our data demonstrates a potential protective effect of exercise during pregnancy against AβOs-induced neurotoxicity in the adult offspring brain, by mitigating the neurodegenerative process triggered by Alzheimer-associated AβOs through programming the brain metabolism.

Keywords

Maternal swimming, Metabolic programming, Neuroprotection, Alzheimer's disease, Mitochondrial function

Introduction

Late-onset Alzheimer's disease (AD) is the main aging-associated neurodegenerative disorder, and is the leading cause of dementia with progressive cognitive decline [1,2]. The neuropathological hallmarks of AD are amyloid-β (Aβ) accumulation in the brain and intracellular neurofibrillary tangles that leads to neuronal damage [2]. Soluble Aβ oligomers (AβOs) are thought to exert the major neurotoxic effects found in AD [3,4], due its ability to interact with central nervous system (CNS) cell surface receptors and intracellular proteins, and, thereby, trigger alterations in signaling pathways and loss of protein function [3,5,6]. As AβOs accumulate in mitochondria, they interact with key mitochondrial enzymes of the tricarboxylic acid (TCA) cycle and the electron transport system (ETS), induce reactive species formation, and consequently disrupt mitochondrial function and dynamics [6-8]. Mitochondria play an essential role in neuron energy metabolism and neurotransmission [9] because synapses have high energy requirements [10]. Mitochondrial dysfunction, bioenergetics failure, and synaptic failure are displayed during the course of AD [11]. In fact, synaptic dysfunction and the loss of synapses are the best correlates of cognitive decline in AD [12]. To date, finding disease-modifying treatments have been challenging, and there is a lack of effective treatments to halt or reverse the AD progress [13,14]. Thus, several studies have suggested that therapeutic approaches aiming counteract AβO-induced neurotoxicity and enhance synaptic function by improving of mitochondrial function may be effective [15-18].

Health-promoting approaches, such as regular exercise, are generally recommended to improve the lifestyle of population, and reduce the risk of developing brain-related diseases. Nearly two decades ago, it was proposed that physical activity and exercise are capable of enhancing brain function and plasticity, and may improve cognition in patients with AD [19-23] by promoting neuroprotection through metabolic adaptation in the CNS [24]. However, benefits arising from exercise during mid-to-late life do not extinguish the risks factors accumulated by a sedentary lifestyle during an individual's youth [25].

Investigating the effects of maternal exercise, during pregnancy, on the metabolic programming of offspring throughout intrauterine and early postnatal development is a new, attractive research area. The updated guidelines from the American Congress of Obstetricians and Gynecologists (ACOG) recommends a moderate-intensity exercise program, lasting 20-30 minutes per day, for pregnant women without medical contraindications [26]. Clinical studies have shown that physical exercise during pregnancy enhances cerebral maturation in newborns [27] and childhood language development [28]. Similarly, in rodents, we, and others, have demonstrated that maternal exercise during pregnancy modulates the intrauterine environment to favor the offspring's health, and it can enhance brain function and cognition throughout life [29-35]. Moreover, the health-promoting effects of maternal exercise have emerged as an approach to reduce disease susceptibility in the offspring's adult life. This concept is also encompassed by the Developmental Origins of Health and Disease (DOHaD) paradigm, in which the intrauterine and early postnatal environments influence the offspring`s development and induce permanent changes that dictate their health status later in life [36,37]. This phenotype shaping is established during development, due the plastic processes that enables adaptation to the environment [38,39]. Incipient studies have addressed the potential protective effect of maternal exercise during pregnancy against AD-like pathology in TgCRND8 mice [40], maternal high-fat-diet-induced glucose metabolism disturbance [41], obesity [42,43], hepatic steatosis [44], and tumorigenesis [45]. Herein, we sought to investigate whether maternal swimming during pregnancy is able to prevent memory impairment, and to unveil some of the cellular mechanisms involved in the processes of brain damage in a rat model of Aβ-neurotoxicity. Our findings demonstrated that maternal exercise during pregnancy has long-lasting metabolic effects on the offspring's brain, specifically on mitochondrial function, which are able to protect the offspring from Aβ-neurotoxicity and cognitive impairment in adulthood. These data highlight maternal exercise as a promising approach to delay, or even to prevent the development, of AD. Moreover, it opens new avenues to investigate the prevention of other ageing-related diseases through metabolic programming.

Experimental procedures

Animals

Adult female (220 - 260 g, n = 54) and male (300 - 350 g, n = 27) Wistar rats (Rattus norvegicus albinus) were obtained from in-house breeding colonies at the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil. The animal facility was under controlled light (12:12 hour light/dark cycle), temperature (22 ± 1°C), and humidity conditions (50–60%). Four adult animals were housed per cage, while the litters were maintained with their mothers until weaning. All animals had ad libitum access to a 20% (w/w) protein commercial chow and water.

Ethics

All experimental procedures were approved by the local Ethics Commission on the Use of Animals (Comissão de Ética no Uso de Animais - CEUA/UFRGS) under the protocol number 27341, and were performed in accordance with the National Animal Rights Regulations (Law 11.794/2008), the American National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1996), and the Directive 2010/63/EU. All efforts were made to minimize the number of animals used and their suffering.

Experimental design

A timeline of experiments is depicted in Fig. 1. Adult female rats were randomly selected and divided into two groups: (1) sedentary control, rats exposed to aquatic environment stress, without exercising; and (2) maternal exercise, rats subjected to the swimming protocol. The animals in the maternal exercise group underwent involuntary swimming for four weeks (without extra weight); with one week of involuntary swimming, so that the rats could adapt to the aquatic environment, prior to mating and swimming throughout the entire pregnancy period. At the end of the first week, two females were mated with one male, and the pregnancy was confirmed by the presence of a

vaginal plug or sperm in the vaginal fluid. The pregnant rats underwent the exercise protocol during the entire pregnancy. Beginning on the 20th gestational day, the dams were housed individually and observed, twice a day (8 a.m. and 6 p.m.), to verify the litter's birth. The offspring's birth date was considered postnatal day (PND) 0. Within 24 h after delivery, randomly selected pups were culled to maintain litters of eight pups per dam. From delivery (PND 0) until weaning (PND 21), each dam was housed with its litter. On PND 21, male and female offspring from each dam were separated by sex; female littermates were euthanized, and male littermates were housed, four per cage, until the PND 60. On PND 60, male offspring were subjected to a surgical procedure in order to bilaterally microinject Aβ peptide oligomers (AβO) or vehicle into the brain ventricles. Male offspring was selected in order to avoid interference of the hormonal and estrous cycle on the results. Thus, the offspring of maternal groups was subdivided into two groups, yielding four offspring groups: 1) sedentary control + vehicle, 2) maternal exercise + vehicle, 3) sedentary + AβOs, and 4) maternal exercise + AβOs. On the 14th day post-surgery, vehicle- and AβOs-injected adult male offspring born to sedentary or exercised rats were randomly designated for behavioral testing or euthanized by decapitation without anesthesia, to collect samples for analysis of biochemical parameters.

Swimming protocol

The exercise type, duration, frequency and intensity are important components of exercise that influence the conferred benefits from mother and fetus [46]; swimming exercise is a highly recommended form of physical exercise for pregnant females [47]. According to the protocol initially described by Lee et al. [48], and modified by Marcelino et al. [34], adult female rats underwent individual swimming in a pool (30 cm wide x 30 cm long x 90 cm deep) filled with water at 32 ± 1°C, without additional weight. Swimming sessions were performed from 9 to 12 a.m., 5 days/week and lasted 30 min, daily, for four weeks. The animals were left free to swim, and were gently stimulated to swim when necessary. Following the same schedule of swimming

group, the sedentary/control rats were exposed to aquatic environment, without exercising, in order to avoid any bias of water contact. Control rats were immersed in water, carefully dried, and returned to the housing boxes.

Aβ1-42 peptide oligomers preparation

Soluble AβOs were prepared according to the protocol published by Klein [49]. The Aβ (sequence 1-42) peptide (American Peptide Co., Sunnyvale, CA, USA) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma Chemical Co., St. Louis, MO, USA) to stabilize monomers of the Aβ peptide. Then the monomers were incubated for 1 h at room temperature, followed by a 10 min incubation on ice. After being aliquoted, the tubes containing the Aβ peptide were maintained in the hood overnight to allow the complete removal of HFIP. Following this step, samples were centrifuged in a SpeedVac system for 10 min to completely remove the HFIP and result in a clear film of monomeric Aβ peptide at the bottom of the tubes. The tubes were stored at -80°C. At the time of use, aliquots were solubilized in DMSO, diluted in phosphate saline buffer pH 7.4 (PBS), and incubated at 4°C for 24 h. After incubation, the tubes were centrifuged at 14,000 g for 10 min at 4°C, and the AβO-containing supernatants were transferred to a new tube. Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fischer), and structural characterization was performed through western blot analysis using the specific Aβ 1-16 antibody, 6E10 (Covance).

Surgical procedure for AβO1-42 infusion

On the PND 60, male offspring were subjected to surgical procedures previously described by Hoppe et al. [50]. Animals were anesthetized with ketamine and xylazine (100 mg/Kg and 15 mg/Kg, respectively) through the intraperitoneal (i.p.) route, and then placed in a stereotaxic frame. Using sterile surgical instruments, a middle sagittal incision was made in the scalp and bilateral holes were drilled in the skull, using a dental drill over the lateral ventricles, according to Paxinos and Watson atlas coordinates: 0.8 mm posterior to the bregma and 1.5 mm lateral to the sagittal

suture [51]. The depth of the microinjection, 3.5 mm beneath the surface of brain, was also chosen according to Paxinos and Watson coordinates. Rats received a single, bilateral, icv injection of 5 μL Aβ1-42 peptide oligomers (500 pmol/rat), and control rats received injections of an equal volume of PBS and 2% DMSO into each lateral ventricle. The dose of AβO1-42 for icv injection was chosen according previous works [5,52]. Microinjections were performed using a 10 μL Hamilton syringe fitted with a 26-gauge needle, and an injection rate of 1 μL/min over a period of 5 min. At the end of infusion, the needle was left in place for an additional 3 to 5 min before being slowly withdrawn, to allow diffusion from the tip and prevent reflux of the solution. After the injection, the scalp was sutured, and the animals were allowed to recover from the anesthesia on a heating pad, to maintain body temperature at 37.5 ± 0.5°C.

Behavioral analyses

Behavioral tests were performed from PND 74 to 96 (Fig. 1c). The offspring underwent the open field test on PND 74, the object recognition test from PND 75 to 76, and the Morris water maze tests from PND 80 to 85 (reference memory) and from PND 93 to 96 (working memory). All behavioral tasks were conducted from 8 a.m. to 1 p.m. in a room with low light intensity (up to 60 lux) and attenuated sound. Before starting each test, animals were allowed to habituate to the testing room for 1 h. Behavior data were collected and analyzed automatically using a video-tracking system (Any-maze, Stoelting, Woods Dale, IL), with the camera positioned above the center of the apparatus.

Open field task

The open field task was used to assess spontaneous locomotor behavior in a novel environment [53]. The open field arena consisted of a 60 x 60 x 60 cm (length x width x height) black wood square chamber. Rats were placed individually in the left corner of the arena facing the wall and their behavior was tracked for 5 min. Through the automated video-tracking software, the ground floor of the arena was segmented into a grid with equal sized squares, and then further divided into

two zones, the periphery and the center. For analysis of locomotion, total distance traveled, average speed, and number of squares crossed were measured. For analysis of anxiety-like behavior, the excretion of fecal bolus, and the overall time spent in the central and peripheral zones were measured. The arena was cleaned with 10% (v/v) ethanol and dried after each animal had completed the task. The apparatus was also used for the novel object recognition task, so the open field task was considered as the habituation phase.

Novel object recognition task

The novel object recognition task is based on the tendency of rodents to interact with a novel object over a familiar object in order to study learning and memory. The task was conducted according the method initially described by Ennaceur and Delacour [54], with modifications. The novel object recognition task consisted of three phases to assess the effect of AβOs on recognition memory. Fourteen days after AβOs (500 pmol/rat) animals underwent a habituation phase in the open field arena. On the next day, the training phase, the rats were placed in the arena with two identical objects (A and A') and allowed to freely explore them for 5 min. On a 24 h retention interval, the test phase to evaluate long-term memory, the rats were placed in the arena with a familiar (A') and a novel object (B) and allowed to freely explore them for 5 min. Object exploration was defined when the animal directed the nose to the object at a maximal distance of 2 cm, sniffed or touched the object. Climbing onto the object, unless the rat sniffed it, was not considered exploration. The total interaction time with both objects in training and testing phases was recorded. The discrimination ratio, a memory index, was calculated according the following formula: (exploration of novel object)/(exploration of novel + exploration of familiar object), where a higher time exploring the novel object was assigned to an enhanced cognitive performance [50]. After performing the task, the rats returned to their home cage. The arena and the objects were cleaned with 10% (v/v) ethanol and dried to minimize olfactory cues to the next use.

Morris water maze task

The Morris water maze task was used to assess spatial learning, long-term and working memories [55]. Using distal cues to escape the water, the rats must learn to navigate toward to a hidden platform, since they start from random locations in the water tank. The water maze consisted of a black circular tank, 200 cm in diameter and 100 cm in height. The tank was filled with a 50 cm depth of water (22 ± 1°C) and the transparent acrylic platform was submerged 2 cm beneath the water surface at the center of a quadrant. The tank was conceptually segmented by two perpendicular lines (+) demarcating North (N), South (S), East (E) and West (W) points, creating four equal sized quadrants designated as Southeast (SE), Northeast (NE), Southwest (SW), and Northwest (NW). On the walls of the testing room, distal visual cues were available. A random start position for each trial was established and no sequence was repeated, and the rat was placed in water facing the tank wall. The Morris water maze task consisted in three phases: acquisition (learning), retention (reference memory) and working memory [56].

Learning and reference memory. The acquisition phase is required as training prior the retention test [55]. To this end, learning trials were conducted across five days and consisted of 4 trials/day. Inter-trial intervals lasted 20 min. Rats were allowed 60 s to search for the platform, which was located in the SE quadrant during all acquisition phases. The escape latency was measured in each trial; and if the rat failed to find the platform, it was gently guided to it. At the end of each trial, the rats were allowed to remain on the platform for 15 s, and then they were removed, dried, and returned to their home cages. The retention phase refers to the probe trial and assesses spatial reference memory. To this end, on the sixth day, each rat was placed into the water in the opposite quadrant, and the platform was removed to measure the latency to the first target-site crossover, the number of platform-site crossovers, and the time spent in each quadrant.

Working memory. The working memory task was conducted one week after the reference memory probe trial to assess trial-dependent learning and memory [55]. The platform was reallocated daily and the rats were subjected to four consecutive trials/day, with the inter-trial interval lasting 30 s,

during four testing days. Mean latencies to find the platform in each trial was calculated for all testing days [57].

Mature BDNF assay

Mature BDNF protein content was measured in the hippocampi and prefrontal cortices of rats that underwent behavioral tasks. Rats were euthanized 24 h after the last working memory test session. Mature BDNF was measured through the E-Max ELISA kit (Promega), according to the manufacturer's recommendations. Briefly, the hippocampus and prefrontal cortex of each rat was individually homogenized (1:10 w:v) in lysis buffer containing: 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), Igepal (1%), glycerol (10%), 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM sodium vanadate, 0.1 mM EDTA, and 0.1 mM EGTA, and lysates were centrifuged for 3 min at 14,000 rpm at 4°C. Supernatant was diluted (1:5 v/v) in sample buffer and incubated in 96-well flat-bottom plates previously coated with anti-BDNF monoclonal antibody, and blocked with Block & Sample buffer. After sample incubation, plates were incubated with a polyclonal anti-human antibody for 2 h and horseradish peroxidase for 1 h. Colorimetric reaction with tetramethylbenzidine was quantified using a plate reader at 450 nm. The standard BDNF curve, ranging from 0 to 500 pg/mL was assayed in each plate in parallel with the samples.

Western blot assay

Prefrontal cortices and hippocampi were homogenized in ice-cold lysis buffer containing 4% sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mM Tris, and 1% protease inhibitor cocktail. The homogenates were denatured at 100°C for 5 min, and then centrifuged at 10,000 g for 30 min. After this, the supernatant containing the cytosolic fraction was collected, β-mercaptoethanol was added to a final concentration of 5%, and the samples were stored at -80°C until use. Equal concentration of protein (50 μg) was loaded and immunodetected, as previously described [50]. Membranes were incubated for 60 min at 4°C in blocking solution (Tris-buffered saline containing 5% non-fat milk and 0.1% Tween-20, pH 7.4) prior to the incubation with the primary antibody.

Membranes were incubated, overnight at 4°C, in blocking solution containing one of the following primary antibodies: rabbit monoclonal anti-synaptophysin (1:2000, Millipore, catalog number #AB9272), anti-mitofusin 1 (1:1000, Abcam, catalog number # ab104274), anti-dinamin-related protein 1 (1:1000, Abcam, catalog number #ab154879), or rabbit monoclonal anti-β-actin (1:2000, Cell Signaling, catalog number #4967). After washing, the membranes were incubated with a secondary antibody containing peroxidase-conjugated anti-rabbit IgG (1:2000, GE Healthcare Life Sciences, catalog number #NA934V) for 1 h. The chemiluminescence was detected using a digital imaging system (Image Quant LAS 4000, GE Healthcare Life Sciences) and analyzed using the Image J Software. The average optical density for the control group was designated as 100%.

Tricarboxylic acid cycle (TCA) α-ketoglutarate dehydrogenase (α-KGDH) enzyme activity

For the tricarboxylic acid cycle enzymes, the hippocampus and prefrontal cortex were dissected, weighed, and put into ice-cold isolation buffer for the mitochondria-enriched fraction. Mitochondrial fraction was isolated as described by Rosenthal et al. [58], with slight modifications [59] using a buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin (free of fatty acids), and 10 mM HEPES (pH 7.2). The homogenate was centrifuged at 2,000 g for 3 min at 4°C. After centrifugation, the supernatant was again centrifuged at 12,000 g for 8 min at 4°C. The pellet was suspended in isolation buffer containing 4 μL of 10% digitonin, and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was discarded, and the final pellet was gently washed and suspended in isolation buffer devoid of EGTA, at an approximate protein concentration of 2.5 mg·mL−1. The activity of α-KGDH (EC 1.2.4.2) complex was assayed according to Lai, Cooper [60] and Tretter, Adam-Vizi [61], with some modifications. The incubation medium contained mitochondrial preparations, 1 mM MgCl2, 0.2 mM thiamine pyrophosphate, 0.4 mM ADP, 10 μM rotenone, 0.2 mM EGTA, 0.12 mM coenzyme A-SH, 1 mM α-ketoglutarate, 2 mM NAD+, 0.1% Triton X-100 and 50 mM potassium phosphate, pH 7.4. The reduction of NAD+ was recorded at wavelengths of excitation and emission of 366

and 450 nm, respectively. α-KGDH activity was calculated and expressed as nmol NADH.H+.min−1.mg protein−1.

Respiratory Chain Complex IV Activity

Animals were euthanized 14 days after the AβOs injection by decapitation, without anesthesia, in order to avoid tissue chemical contamination. Regarding the respiratory chain complex IV (cytochrome c oxidase) activity assessment, the prefrontal cortex and hippocampus were rapidly dissected, weighed and immediately frozen (-80°C) until homogenization. The encephalic areas were homogenized (1:20 w/v) in SETH buffer, pH 7.4, that contained 250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base, and 50 IU.mL−1 heparin, and were centrifuged at 800 g for 10 min at 4°C. The pellet was discarded, and the supernatant was collected and subjected to three subsequent freeze-thaw procedures before performing the experiments [59]. Mitochondrial respiratory chain enzyme activity was measured in the homogenates with a protein concentration varying from 1.5 to 5.0 mg protein.mL-1. The activity of cytochrome c oxidase (EC 1.9.3.1) was measured according to Rustin et al. [62]. The incubation medium consisted in 10 mM potassium phosphate buffer, pH 7.0, 125 mM n-dodecil-β-D-maltoside, and 1% cytochrome c. Complex IV activity was determined, following the decrease in absorbance, due to reduced cytochrome c oxidation at 550 nm at 550 nm. The activity of complex IV was calculated as nmol.min-1.mg protein-1.

Protein determination

Protein concentration was measured according the method described by Lowry et al. [63], which was adapted for the microplate, using bovine serum albumin as standard. The absorbance was measured at 750 nm. The results were presented as mg of protein/mL.

Flow cytometry assay

Flow cytometric analysis was conducted according to the protocol described by Marcelino et al. [34]. Briefly, the tissue samples (approximately 100 mg) were dissociated in PBS containing 1 mg% of colagenase IV and 0.5 mg% of DNase, filtered using a 40 μm pore cell strainer (SPL Lifesciences Co., Naechon-Myeon Pocheon, South Korea), and were then incubated for 45 min at 37°C with the molecular probes. Mitochondrial mass and membrane potential were measured using 100 nM MitoTracker® Green and 100 nM MitoTracker® Red (Invitrogen, Molecular Probes, Eugene, OR - USA), respectively. Cells were gated based on the FSC and SSC pattern of the sample cells and 30,000 events were acquired per sample in a FACSCalibur flow cytometer (BD Biosciences); a non-labeled sample was used as negative fluorescent control. Data were analyzed using the FlowJo software.

Statistical analyses

Data are expressed as mean + standard error of the mean (SEM), and statistical analyses were performed using the GraphPad Prism 6.0 software. All data were tested for normality. Two-way ANOVA was used to analyze the effect of the two independent variables, maternal exercise and AβOs injection. All tests involving multiples observations per group were analyzed with repeated measures. Differences between the groups concerning repeated measures analysis were demonstrated by Tukey post-hoc test. Data were considered statistically significant when $p < 0.05$.

Results

AβOs injection did not alter motor and exploratory activities in adult offspring born to sedentary or exercised rats

The adult offspring rats were tested in the open field for 5 min to habituate to the new space and to assess both exploratory activity and spontaneous locomotor behavior (Table 1). AβOs injection (500 pmol/rat) did not cause any change in the exploratory and motor activities in the offspring born to exercised or sedentary rats, as measured by total distance travelled [$F(1,53) = 0.009$, $p =$

0.924], average speed [F(1,53) = 0.123, p = 0.727], and crossings [F(1,52) = 0.269, p = 0.605]. Moreover, signs of anxiety-like behavior such as number of excreted fecal bolus [F(1,52) = 0.009, p = 0.924], time spent in the periphery [F(1,53) = 0.309, p = 0.580] or in the center [F(1,53) = 0.446, p = 0.507] of the arena were not altered. These results showed that neither maternal exercise during pregnancy nor AβOs injection altered exploratory, locomotor and anxiety-like behavior in the adult offspring.

AβOs impaired object recognition memory in the adult offspring, which was prevented by maternal exercise during pregnancy

Recognition memory was assessed through the novel object recognition test, a non-spatial memory task that relies mainly on hippocampus processing [64]. The more time spent exploring the novel object depicts intact object recognition memory. In the training phase, all animals explored both objects equally (Fig. 2a), showing no preference for any object [F(1,47) = 0.138, p = 0.711]. In the test phase, matching analysis for objects showed that AβOs-injected rats spent similar time exploring the novel object and the familiar object, whereas the other groups showed preference for the novel object (Fig. 2b; $p < 0.001$ Tukey post-hoc test). In addition, discrimination ratio in sedentary + AβO group was significantly reduced compared to control (Fig. 2c) [F(1,47) = 4.446, p = 0.040], suggesting an impaired recognition memory in these animals. Strikingly, maternal exercise prevents the recognition memory deficit in the AβOs-injected adult offspring in comparison to AβOs-injected sedentary offspring (Fig. 2c) [F(1,47) = 8.950, p = 0.004]. These data suggest that maternal exercise during pregnancy is able to prevent recognition memory impairment elicited by AβOs injection into cerebral ventricles.

Maternal exercise benefits on learning and reference memory in the adult offspring prevailed in the AβOs-injection detrimental effects

Spatial learning in the Morris water maze task depends on distal cues for rats to navigate and to locate a hidden platform [55]. The escape latency during the acquisition phase decreased from

days one to five for all groups (Fig. 3a) [$F(4,167) = 16.59$, $p < 0.0001$]. On the fifth day, the sedentary + AβO adult offspring group spent more time to find the platform (higher mean escape latency) compared to the sedentary/control group [$F(3,42) = 2.995$, $p = 0.032$]. Further analysis showed an interaction between maternal exercise and AβOs injection on day 5 [$F(1,42) = 4.122$, $p = 0.048$]. Assessing reference memory on the retention phase is a measure of long-term memory. Latency to first target-site crossover, number of platform-site crossovers (platform entries), and time spent in the target quadrant compared with the other quadrants (Fig. 3b-d, respectively) were used to assess reference memory. On the sixth day, an interaction between maternal exercise and AβOs injection was observed in the escape latency to first target-site crossover [$F(1,46) = 4.883$, $p = 0.032$]. In addition, sedentary + AβO animals took more time to first target-site crossover compared to the sedentary/control group (Fig. 3b) [$F(1,46) = 8.556$, $p = 0.005$]. No difference was observed in the number of platform-site crossovers among the experimental groups (Fig. 3c); two-way ANOVA showed no effect of maternal exercise [$F(1,46) = 0.033$, $p = 0.857$] and AβOs injection [$F(1,46) = 1.949$, $p = 0.169$]. Analysis of the time spent in each quadrant (Fig. 3d) in the water maze showed that the sedentary + AβO group spent less time in the target quadrant (SE) compared to sedentary/control group, as indicated by significant interaction between the factors [$F(1,46) = 4.738$, $p = 0.035$]. These results suggest that maternal exercise during pregnancy abrogates the impairment in learning and long-term spatial memory elicited by AβOs injection on adulthood, pointing to a neuroprotective role of maternal exercise on the hippocampal-dependent memory acquisition and consolidation in the offspring.

Intact working memory was observed in AβOs-injected adult offspring born to exercised rats

The working memory task in the Morris water maze is also called the reversal phase; therefore, it depends on the rat's knowledge of the location of the hidden platform before this phase begins [55]. The platform is relocated daily, and the animals should learn the new location as they perform

the successive trials (4 trials/day) in order to travel the shortest path to find the hidden platform. AβOs injection significantly affected the working memory in the adult offspring of sedentary rats (Fig. 3e), which was demonstrated by the high escape latency, to find the platform new location at the fourth trial compared to sedentary/control group [$F(3,46) = 3.634$, $p = 0.019$]. Strikingly, rats from the maternal exercise + AβO group displayed a high performance in the escape latency, observed by reduced escape latency that differed significantly from sedentary + AβO group [$F(3,46) = 3.634$, $p = 0.019$], and similar to that observed in the control group. These results indicate the spatial working memory that depends on the hippocampal-prefrontal interaction is disrupted by AβOs injection in the offspring of sedentary rats but remains unaffected in maternally exercised offspring, supporting a preventive potential of maternal exercise against the challenged-promoted cognitive decline in adulthood.

AβOs-injection induced a reduction in hippocampal but not in prefrontal cortical BDNF levels

Mature BDNF levels were measured in the offsprings' hippocampus and prefrontal cortex 24 h after the end of behavioral tests. In accordance with other authors [50,65,66], mature hippocampal BDNF levels were reduced by AβOs injection (Fig. 4, right) [$F(1,27) = 6.261$, $p = 0.019$]; however, maternal exercise during pregnancy does not prevented such reduction [$F(1,27) = 0.017$, $p = 0.897$]. Conversely, mature BDNF levels in the prefrontal cortex (Fig. 4, left) were not altered by either maternal exercise or AβOs injection [$F(1,26) = 0.822$, $p = 0.373$; $F(1,26) = 0.130$, $p = 0.722$, respectively].

Reduced hippocampal synaptophysin by AβO1-42 injection in adult offspring was prevented by maternal exercise during pregnancy

Synaptophysin is a presynaptic protein that is considered to be a synaptic marker, and it is known to be downregulated in AD [67,68]. Here, we measure the immunocontent of the synaptophysin through western blot analysis (Fig. 5). There was no statistical difference in the synaptophysin

content measured in the offsprings' prefrontal cortex after AβOs injection [$F(1,26) = 0.539$, $p = 0.469$] and maternal exercise during pregnancy [$F(1,26) = 0.606$, $p = 0.443$]. Furthermore, there was a significant reduction in the hippocampal synaptophysin content after AβOs injection, in comparison to control/sedentary group [$F(1,27) = 4.636$, $p = 0.040$]. Strikingly, maternal exercise during pregnancy was able to prevent such effect on the offsprings' hippocampus [$F(1,27) = 4.811$, $p = 0.037$].

Increased number of functional mitochondria, induced by maternal exercise during pregnancy, was not abolished by AβOs injection in the adult offsprings' prefrontal cortex and hippocampus

Mitochondrial dysfunction is an event that accompanies AD pathology, underlying the major metabolic changes [7]. We examined the number of functional mitochondria through flow cytometric analysis of simultaneous Mitotracker Green and Mitotracker Red labeled events, which indicate mitochondrial mass and membrane potential, respectively. Double positive labeling indicates functional respiring mitochondria, and an increase in fluorescence is indicative of mitochondrial biogenesis [69]. It was observed that maternal exercise during pregnancy induces an increase in the number of functional mitochondria in the offsprings' prefrontal cortex [$F(1,26) = 4.375$, $p = 0.046$] and hippocampus [$F(1,37) = 18.15$, $p < 0.0001$], evidenced by the increased percentage of Mitotracker Green and Mitotracker Red double positive events compared to sedentary/control group (Fig. 6a and b). Furthermore, AβOs injection did not alter the beneficial role elicited by maternal exercise on mitochondrial functionality in both brain areas, the prefrontal cortex [$F(1,26) = 0.348$, $p = 0.560$] and hippocampus [$F(1,37) = 0.674$, $p = 0.417$].

AβOs-induced reduction in the adult offspring's hippocampal α-KGDH enzyme activity was prevented by maternal exercise during pregnancy

Long-term reduced brain energy metabolism is observed in AD and is highly correlated to cognitive decline. This shift in energy metabolism involves alterations in mitochondrial enzymes

[70]. To examine the mitochondrial metabolism, we measured the activity of an important enzyme of the tricarboxylic acid cycle that is involved in mitochondrial bioenergetics in the prefrontal cortex and hippocampus of offspring born to exercised and sedentary rats. The activity of α-KGDH in the prefrontal cortex (Fig. 7a, left panel) showed that maternal exercise during pregnancy increased α-KGDH activity [$F(1,19) = 6.861$, $p = 0.017$], while AβOs injection did not exert any effect on enzyme activity [$F(1,19) = 0.013$, $p = 0.908$]. Similarly, α-KGDH activity in the hippocampus (Fig. 7a, right panel) was significantly reduced by AβOs injection compared to control/sedentary [$F(1,21) = 6.928$, $p = 0.015$] offspring. Strikingly, maternal exercise during pregnancy prevented the decrease of α-KGDH activity [$F(1,21) = 8.100$, $p = 0.001$] elicited by AβOs infusion, which was evidenced by a statistical difference in hippocampal α-KGDH activity of offspring born to sedentary + AβO and maternal exercise + AβO groups. These data indicate that AβOs promote the impairment of TCA function and that maternal exercise during pregnancy increases TCA α-KGDH enzyme activity, preventing AβOs effects.

Increased respiratory chain complex IV activity induced by maternal exercise during pregnancy was not abolished by AβOs injection in the adult offspring's prefrontal cortex and hippocampus

Aβ peptide has been shown to impair mitochondrial respiration by affecting the activity of the electron transport chain [71]. To investigate whether Aβ peptide could alter the transfer of electrons, we then assessed the effect of maternal exercise and AβOs injection on respiratory chain complex IV. Strikingly, as shown in Fig. 7b, maternal exercise increased cytochrome c oxidase activity (CIV) in the prefrontal cortex [$F(1,24) = 5.042$, $p = 0.034$] and hippocampus [$F(1,25) = 5.635$, $p = 0.026$]. AβOs injection did not significantly effect CIV in the prefrontal cortex [$F(1,24) = 0.691$, $p = 0.414$] or hippocampus [$F(1,25) = 2.634$, $p = 0.118$]. These data indicate that maternal exercise augments activity of the mitochondrial electric transport chain, and that AβOs injection did not affect mitochondrial respiration.

Maternal exercise during pregnancy upregulated mitochondrial fusion protein in the offspring´s prefrontal cortex

Mitochondria continuously undergo fusion and fission cycles to allow proliferation, distribution and cellular adaptation to energy shifts [9]. To assess mitochondrial dynamics, we measured the immunocontent of the fusion protein mitofusin 1 (Mfn1) and the fission protein dynamin-related protein (Drp1) (Fig. 8). Maternal exercise during pregnancy increased Mfn1 immunocontent in the offspring's prefrontal cortex [$F(1,36) = 5.347$, $p = 0.027$], which was not abolished by AβOs injection [$F(1,36) = 0.026$, $p = 0.871$] (Fig. 8a, left panel). Maternal exercise during pregnancy did not affect Drp1 immunocontent in the prefrontal cortex [$F(1,31) = 1.453$, $p = 0.237$] and neither did the AβOs injection [$F(1,31) = 0.003$, $p = 0.955$] (Fig. 8b, left panel).

Altered immunocontent of mitochondrial fission protein in the hippocampus of AβOs-injected offspring is prevented by maternal exercise during pregnancy

To assess mitochondrial dynamics in the hippocampus, s we measured the immunocontent of the Mfn1 and Drp1 (Fig. 8). Maternal exercise during pregnancy did not affect hippocampal Mfn1 immunocontent [$F(1,20) = 0.017$, $p = 0.897$] and neither did the AβOs injection [$F(1,20) = 0.059$, $p = 0.811$]. However AβOs injection did increase Drp1 levels [$F(1,29) = 6.908$, $p = 0.013$]. In contrast, Drp1 immunocontent in the hippocampi of offspring from the maternal exercised group injected with AβOs was similar to the control group [$F(1,29) = 4.226$, $p = 0.049$]. These results indicate that AβOs injection increased the mitochondrial fission protein, which was prevented by maternal exercise during pregnancy.

Discussion

This study examined the potential role of maternal exercise during pregnancy on the in utero programming of the offsprings' brain metabolism, and if this programming could modify the course of diseases later in life. Here, we report that a single, bilateral injection of AβOs into the

brain ventricles can elicit memory deficits and dysregulation of energy metabolism in specific brain regions of the offspring, when assessed 14 days after the induction of AD-associated AβOs pathology. In addition, the intrauterine environment, triggered by maternal exercise, can influence the long-term health of the offspring by modifying the response to hazardous stimulus of AβOs infusion. This effect occurred through the modulation of cellular metabolism, particularly mitochondrial function, and prevented the mnemonic deficits induced by AβOs.

Intracerebroventricular administration of the Aβ peptide is a well-described model of the early phase of AD-like pathology, and it has been used extensively to characterize Aβ neurotoxicity in rodents [3,5,72]. Aβ peptides accumulate primarily in the prefrontal cortex and hippocampus [73], triggering molecular and cellular alterations that lead to neurodegeneration and, finally, culminate in cognitive deficits characteristically observed in AD patients [5,6]. In AD pathology, episodic and spatial memory impairments are common features [74,75]; the former is widely recognized to be assessed in rodents through the novel object recognition task [35,54,64], and the later through the spatial navigation in the mazes tasks [76-78]. Here, we demonstrated that icv injection of AβOs caused a marked decline in nonspatial object recognition memory, and it impaired the acquisition of new information for spatial reference and the working memories of adult male offspring born to sedentary rats. Cognitive deficits caused by AβO are well correlated with synaptic dysfunction and loss [11]. We demonstrated that AβOs injection significantly reduced the synaptic marker, synaptophysin, in the hippocampus. Decreased levels of synaptophysin have long been associated with Aβ toxicity in human post-mortem brains [67,68], in animal models [79], and in in vitro studies [80]. In view of AβOs toxicity on synaptic function, therapeutic approaches aiming to prevent synaptic deficits, with potential to prevent or decelerate cognitive decline, are desired [10]. In addition, the benefits on cognition promoted by maternal exercise seem to be modulated by mitochondrial function and are not dependent on BDNF, because mature BDNF levels in offspring were not affected by maternal swimming exercise. Accordingly, our previous publication

demonstrated that maternal swimming before and during pregnancy improved object recognition memory in adult male offspring in a BDNF-independent manner [32]. Moreover, the high performance observed in the object recognition test by offspring of the maternal exercise group might be explained by c-FOS expression-associated enhanced object recognition memory and increased neural activity in these brain regions, as demonstrated by Robinson and Bucci [35].

The regulation of synaptic density and plasticity rely on proper mitochondrial energy metabolism [81]. The distribution of mitochondria in the dendrites is critical to meet the high metabolic requirement of neurons for vesicular neurotransmitter release [82], and to support learning and memory [83], which are impaired in AD [84]. Several studies have already shown that physical exercise promotes metabolic adaptation in the CNS, such as an increase of glucose uptake [85], activity of the electron transport chain [86], endogenous antioxidant defense [87], and induction of mitochondrial biogenesis [17]. Interestingly, there has been an increase in the publication of clinical papers [27,28] and experimental studies [29-34] that have evaluated the benefits of maternal exercise on offspring metabolism. Among the neurometabolic benefits promoted by maternal exercise during pregnancy, there is an increased antioxidant defense system, induction of mitochondriogenesis in different encephalic areas [34], neurogenesis [29,48], and memory improvement [29,32]. Moreover, maternal exercise during pregnancy has the potential to protect the offspring against disease susceptibility to either peripheral or CNS disorders [40-42,44,45]. Based on these data, maternal exercise during pregnancy may represent a remarkable way to mitigate amyloid-β-induced synaptic loss and mitochondrial dysfunction. Thus, we employed the maternal swimming exercise protocol, before and during pregnancy, aiming to show that metabolic adaptations can confer neuroprotection to the offspring against Aβ-induced neurotoxicity in adulthood. We demonstrate here that maternal swimming during pregnancy protected the AβOs-induced cognitive impairment of adult offspring in the novel object recognition and water maze tests. These observations might be due to an adaptive response of fetal brain metabolism to the

maternal environment that is triggered by swimming exercise during pregnancy. As already suggested in a previous work, Herring et al. [40] have demonstrated that running exercise during pregnancy mitigates Alzheimer-like pathology in the offspring of TgCRND8 mice, through promotion of long-lasting protection, i.e., reduced Aβ plaque burden, inflammation and oxidative stress, against neurodegeneration [34].

One remarkable feature of AβOs injection in experimental models of AD is their accumulation in mitochondria. Thereby, AβOs target essential metabolic enzymes, impairing their function and altering mitochondrial dynamics [6-8]. The disruption of these processes promotes the dysfunction of mitochondrial bioenergetics [3,70], leading to reduced energy metabolism that negatively affects the axonal transport, and contributes to cognitive deficits [88]. Our findings of reduced α-KGDH activity in the hippocampus of offspring from the sedentary maternal group injected with AβOs are in accord with previous works, in which α-KGDH enzyme activity is found to be decreased in the brain of AD patients [89]. The neuroenergetic failure hypothesis posits that a compensatory increase in oxidative phosphorylation, in healthy neurons, occurs to maintain adequate energy production and to ensure neuronal viability and counterbalance energy failure of damaged neurons [90]. The lack of an AβOs injection-induced alteration of the activity of ETS enzymes might agree with the neuroenergetic failure hypothesis, and, therefore, there was no difference in enzyme activities between groups. As proposed by Klupp et al. [91], these processes are thought to be due to the AβO toxicity-induced decrease in functional connectivity between brain regions. Strikingly, AβOs-induced downregulation of α-KGDH was prevented by maternal exercise during pregnancy.

Mitochondrial dynamics is important to maintain bioenergetic functionality of each mitochondrion [9]. Continuous mitochondrial fusion and fission cycles controls the morphology, number and bioenergetics functionality of the mitochondria, and both play important role in physiology and development of CNS and synapses [92]. Exercise has been associated with improved

mitochondrial function and it can modulate the expression of fusion and fission proteins. We demonstrated that maternal exercise during pregnancy was able to increase the immunocontent of the Mfn1, an important protein for mitochondrial fusion, in the prefrontal cortex of the adult offspring, while this long-lasting effect was not observed at the Drp1 levels. It has been demonstrated that the protein Drp1, which is related to mitochondrial fission, is altered in AD brain in response to metabolic changes [93]. Persistent fission negatively affects mitochondrial function, leading to deleterious effects on synaptic function and bioenergetics, and, consequently, contributes to neurodegeneration [9]. Together with the other findings observed in the present study, increased Drp1 levels elicited by AβOs injection suggest that a bioenergetics failure stimulates mitochondrial fission in the sedentary group, which seems to be prevented in the maternal exercise group. Moreover, these results support the hypothesis for a mitochondrial compensatory mechanism. As described elsewhere, increased Drp1 levels are responsible for excessive fragmentation of mitochondria, which in turn are unable to move to synapses and supply the necessary ATP at nerve terminals [80]. Further, by attempting to deal with the energy failure, the defective mitochondria undergo excessive fission; thus, they may not be able to support the energy demand necessary to sustain neurotransmission, leading to synaptic damage and neurodegeneration [94].

Mechanisms underlying cellular and metabolic adaptive responses are influenced by intrauterine and early postnatal environment and relies on epigenetic modifications that modulate gene expression and the redox state throughout offsprings' life [95]. Exploiting the benefits of maternal swimming on the brains of adult offspring, we highlight some points: 1) the ability to prevent the AβO-induced loss of synaptophysin in the hippocampus; 2) the increased number of functional mitochondria in the prefrontal cortex and hippocampus, as indicated by increases in the mitochondrial mass and membrane potential; 3) the increased activity of α-KGDH enzyme of tricarboxylic acid cycle in the prefrontal cortex and hippocampus; 4) the increased activity of the

ETS enzyme cytochrome c oxidase in the prefrontal cortex and hippocampus; 5) the increased Mfn1, in the prefrontal cortex; and 6) the capacity to prevent the increase of Drp1 immunocontent in the hippocampus, which was induced by icv AβO injection. Moreover, our group previously reported that maternal swimming induced mitochondrial biogenesis in the hippocampus, parietal cortex, and cerebellum of seven-day-old pups [34]. Taken together, these data suggest that maternal swimming exercise before and during pregnancy might induce long-lasting mitochondriogenesis in the prefrontal cortex and hippocampus of offspring. The present work highlights the important role of maternal exercise in determining long-term health, as stated by the concept of DOHaD. To the best of our knowledge this is the first report to demonstrate that moderate intensity exercise during pregnancy, involuntary swimming, promotes long-lasting neurometabolic adaptations in the offspring, and protects against Aβ peptide-induced cognitive deficits.

In summary, our data reinforce and extend the notion that intrauterine environment, provided by maternal exercise to the fetus, improve the offsprings' brain metabolism and confers resistance against metabolic changes triggered by AD-associated AβO. Therefore, successfully extrapolating the health promoting findings of maternal exercise to the clinic might open new insights into biomedical area; this lifestyle-based approach, allied to low costs to public health, has the advantage of preventing the development of chronic diseases and may reduce the global burden of dementia-causing neurodegeneration, such as AD.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1 Open filed test parameters assessed in adult offspring injected with vehicle or $A\beta O_{1-42}$

	Sedentary	**Maternal exercise**	**Sedentary + $A\beta O_{1-42}$**	**Maternal exercise + $A\beta O_{1-42}$**		
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	F value	p value*
Distance travelled (m)	15.4± ±.28	15.8 ± 0.62	15.9 ± 0.92	15.2 ± 1.08	(1,53) = 0.009	0.924
Average speed (cm/s)	4.90 ± 0.41	5.24 ± 0.30	5.18 ± 0.20	5.02 ± 0.36	(1,53) = 0.123	0.727
Crossing	276 ± 13.0	293 ± 11.0	286 ± 17.0	267 ± 17.0	(1,52) = 0.269	0.605
Fecal bolus	5.55 ± 0.44	6.06 ± 0.84	5.64 ± 0.91	5.81 ± 0.71	(1,52) = 0.009	0.924
Time in periphery (s)	281 ± 2.68	282 ± 2.87	280 ± 3.14	283 ± 2.75	(1,53) = 0.309	0.580
Time in center (s)	18.6 ± 2.68	17.5 ± 3.09	20.2 ± 3.14	17.2 ± 2.75	(1,53) = 0.446	0.507

*A two-way ANOVA showed no significant differences ($p > 0.05$; n = 10-17/group)

Figure captions

Fig. 1 Experimental design. **a** Maternal swimming protocol, **b** offspring timeline, and **c** offspring's behavioral testing schedule

Fig. 2 Maternal exercise prevents $A\beta O_{1-42}$-elicited object recognition memory impairment in the adult offspring. **a-b** Time spent exploring each object in the training and test sessions, respectively, in the novel object recognition task in the adult offspring, after injection of vehicle or $A\beta O_{1-42}$ (500 pmol/rat), born to exercised or sedentary dams. **c** Discrimination ratio between familiar and novel object. In the bars of graphs, A and A' denotes the familiar object, and B denotes the novel object. A two-way ANOVA showed an effect of maternal exercise (n=10-16/group). Data are expressed as mean + SEM. * $p<0.05$ compared to sedentary/control; *** $p<0.001$ object B compared to object A (Tukey post hoc test repeated measures); ## $p<0.01$ compared to maternal exercise + $A\beta O_{1-42}$ (Tukey post hoc test)

Fig. 3 Maternal exercise prevents memory deficits caused by $A\beta O_{1-42}$ injection in the adult offspring in the Morris water maze task. **a** Escape latency to find the platform during learning phase across the days 1 to 5; two-way ANOVA showed an interaction between maternal exercise and $A\beta O_{1-42}$ injection ($p<0.05$). **b** Platform latency during reference memory on day 6; two-way ANOVA showed an interaction between maternal exercise and $A\beta O_{1-42}$ injection. **c** Platform entries during reference memory on day 6; two-way ANOVA showed no significance. **d** Time spent in each quadrant during reference memory on day 6; two-way ANOVA matching by group showed an effect of $A\beta O_{1-42}$ injection on the time spent in the target quadrant (SE). **e** Escape latency to find the platform in a new location during the working memory task on Trials 1 to 4 in each day; two-way ANOVA showed an effect for maternal exercise and for $A\beta O_{1-42}$ injection. Data are expressed as mean + SEM; n=10-14/group. * $p<0.05$ compared to sedentary/control group; # $p<0.05$ compared to maternal exercise + $A\beta O_{1-42}$ group

Fig. 4 Effect of maternal exercise during pregnancy on mature BDNF levels in the hippocampus and prefrontal cortex of the adult offspring. Mature BDNF levels in the prefrontal cortex (left panel) and hippocampus (right panel) of adult offspring, after injection of vehicle or $A\beta O_{1-42}$ (500 pmol/rat), born to exercised or sedentary dams. A two-way ANOVA showed an effect of $A\beta O_{1-42}$ on hippocampal mature BDNF levels ($p<0.05$; n=6-9/group). Data are expressed as mean + SEM

Fig. 5 Maternal exercise prevents hippocampal synaptophysin reduction induced by $A\beta O_{1-42}$ injection. Synaptophysin immunocontent in the prefrontal cortex (left panel) and hippocampus (right panel) of adult offspring, after injection of vehicle or $A\beta O_{1-42}$ (500 pmol/rat), born to exercised or sedentary dams. Synaptophysin level is expressed as the average percentage of control. Representative quantification of synaptophysin immunocontent normalized to b-actin protein (loading control) is shown below the graphs. A two-way ANOVA showed an effect of both, $A\beta O_{1-42}$ and maternal exercise, on hippocampal synaptophysin levels ($p<0.05$; n=7-9/group). Data are expressed as mean + SEM

Fig. 6 Maternal exercise induces an increment in mitochondrial functionality in the adult offspring's prefrontal cortex and hippocampus. Percentage of double positive Mitotracker Green and Mitotracker Red labeled cells were measured in the **a** prefrontal cortex and **b** hippocampus of adult offspring, after injection of vehicle or $A\beta O_{1-42}$ (500 pmol/rat), born to exercised or sedentary dams. Representative dot plot analyses of Mitotracker Green and Mitotracker Red by flow cytometer are depicted in right panels (**a** and **b**). Two-way ANOVA showed an effect of maternal exercise on mitochondrial mass and membrane potential double positive cells number ($p<0.05$; n=9-11/group). Data are expressed as mean + SEM. * $p<0.05$ compared to sedentary/control group; *** $p<0.0001$ compared to sedentary/control

Fig. 7 Maternal exercise increases mitochondrial function of the tricarboxylic acid cycle enzymes in the adult offspring's prefrontal cortex and hippocampus. The activity of the enzymes **a** α-ketoglutarate dehydrogenase (α-KGHD), and **b** cytochrome c oxidase (CIV) were measured in the prefrontal cortex (left panels) and hippocampus (right panels) of adult offspring born to exercised or sedentary dams, after injection of vehicle or $A\beta O_{1-42}$ (500 pmol/rat). Two-way ANOVA showed an effect of maternal exercise and $A\beta O_{1-42}$ injection on α-KGHD enzyme activity in the prefrontal cortex, and an effect of maternal exercise on hippocampal α-KGHD enzyme activity ($p<0.05$; n=5-7/group). Two-way ANOVA showed an effect of maternal exercise on CIV enzyme activity in the prefrontal cortex and hippocampus ($p<0.05$; n=6-9/group). Data are expressed as mean + SEM. * $p<0.05$ compared to sedentary/control group; # $p<0.05$ compared to maternal exercise + $A\beta O_{1-42}$ group

Fig. 8 Effects of $A\beta O_{1-42}$ injection and maternal exercise during pregnancy on mitochondrial dynamic-related proteins levels. **a** Immunocontent of mitofusin and **b** dynamin-related protein in the prefrontal cortex (left panel) and hippocampus (right panel) of adult offspring born to exercised or sedentary dams, after injection of vehicle or $A\beta O_{1-42}$ (500 pmol/rat). Two-way ANOVA showed

an effect of maternal exercise on mitofusin immunocontent in the prefrontal cortex, and an effect of maternal exercise and $A\beta O_{1\text{-}42}$ injection on dynamin-related protein immunocontent in the hippocampus ($p<0.05$; n=8-10/group). Data are expressed as mean + SEM. * $p<0.05$ compared to sedentary/control group

Figure 1

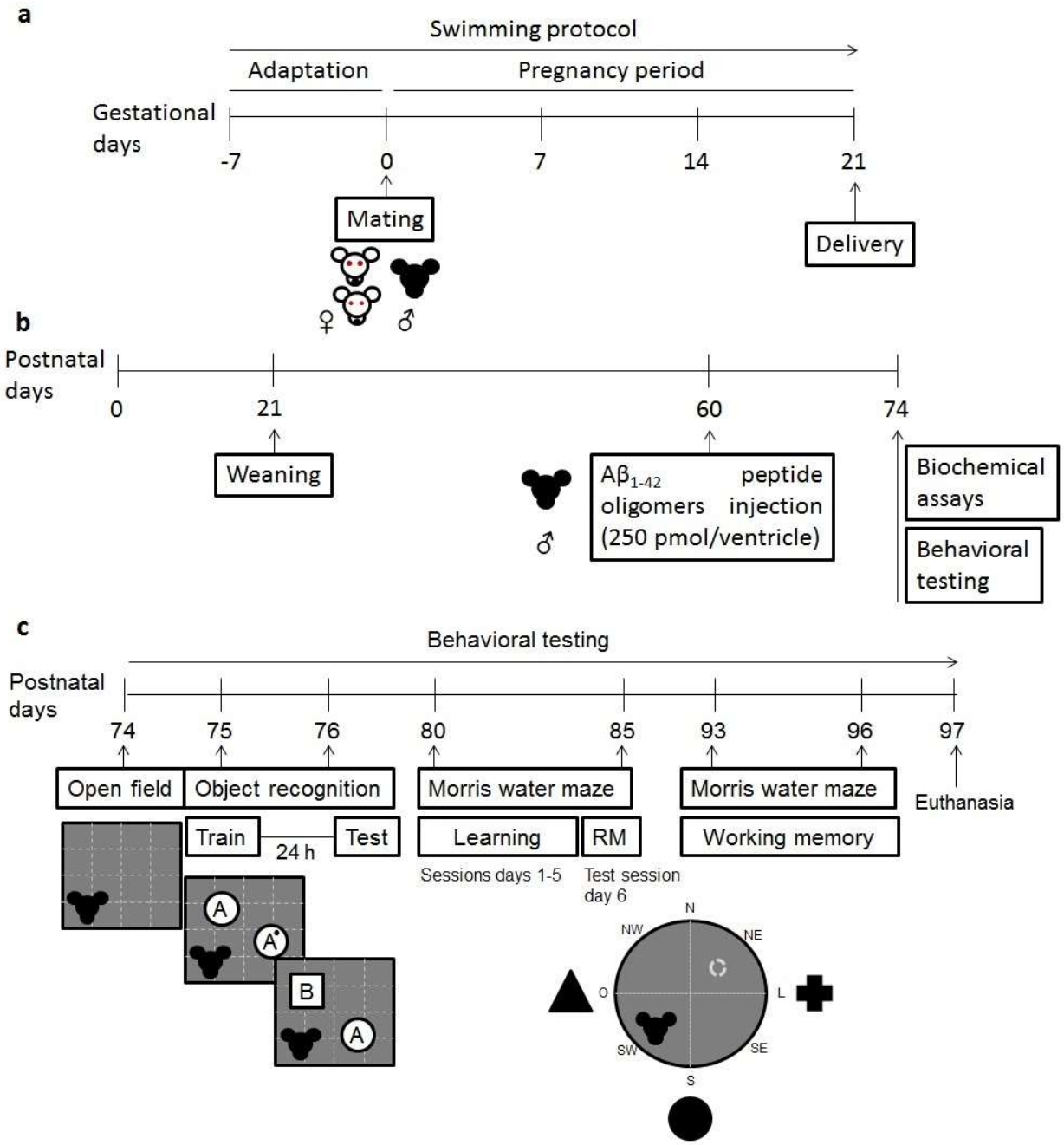

a
Swimming protocol
Adaptation
Pregnancy period
Gestational days
-7
0
7
14
21
Mating
Delivery
♀
♂
b
Postnatal days
0
21
60
74
Weaning
Aβ1-42 peptide oligomers injection (250 pmol/ventricle)
♂
Biochemical assays
Behavioral testing
c
Behavioral testing
Postnatal days
74
75
76
80
85
93
96
97
Open field
Object recognition
Morris water maze
Morris water maze
Euthanasia
Train
24 h
Test
Learning
RM
Working memory
Sessions days 1-5
Test session day 6
A
A'
B
A
N
NW
NE
O
L
SW
SE
S

Figure 2

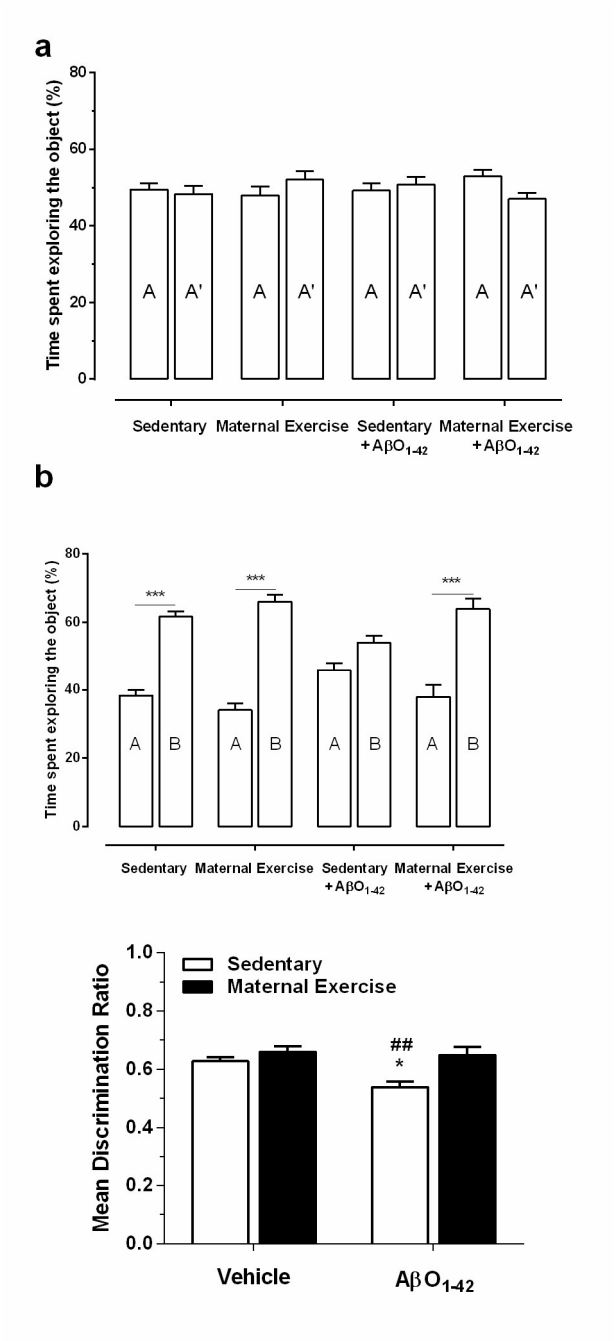

a
Time spent exploring the object (%)
0
20
40
60
80
A
A'
Sedentary
Maternal Exercise
Sedentary +AβO1-42
Maternal Exercise +AβO1-42
b
Time spent exploring the object (%)
***
A
B
Sedentary
Maternal Exercise
Sedentary +AβO1-42
Maternal Exercise +AβO1-42
Mean Discrimination Ratio
Sedentary
Maternal Exercise
##
*
Vehicle
AβO1-42

Figure 3

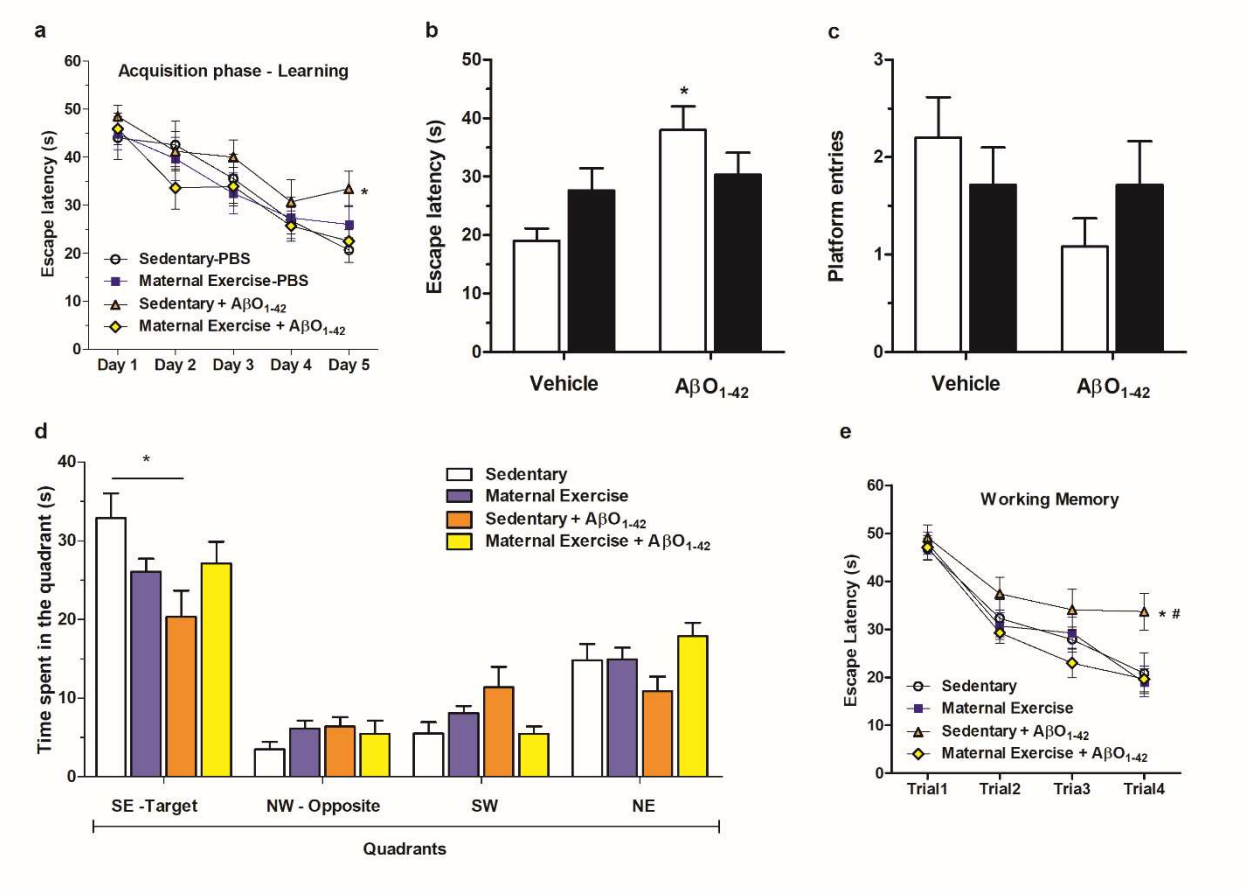

a
Acquisition phase - Learning
Escape latency (s)
Sedentary-PBS
Maternal Exercise-PBS
Sedentary + AβO1-42
Maternal Exercise + AβO1-42
Day 1
Day 2
Day 3
Day 4
Day 5
b
Escape latency (s)
Vehicle
AβO1-42
c
Platform entries
Vehicle
AβO1-42
d
Time spent in the quadrant (s)
Sedentary
Maternal Exercise
Sedentary + AβO1-42
Maternal Exercise + AβO1-42
SE -Target
NW - Opposite
SW
NE
Quadrants
e
Working Memory
Escape Latency (s)
Sedentary
Maternal Exercise
Sedentary + AβO1-42
Maternal Exercise + AβO1-42
Trial1
Trial2
Tria3
Trial4

Figure 4

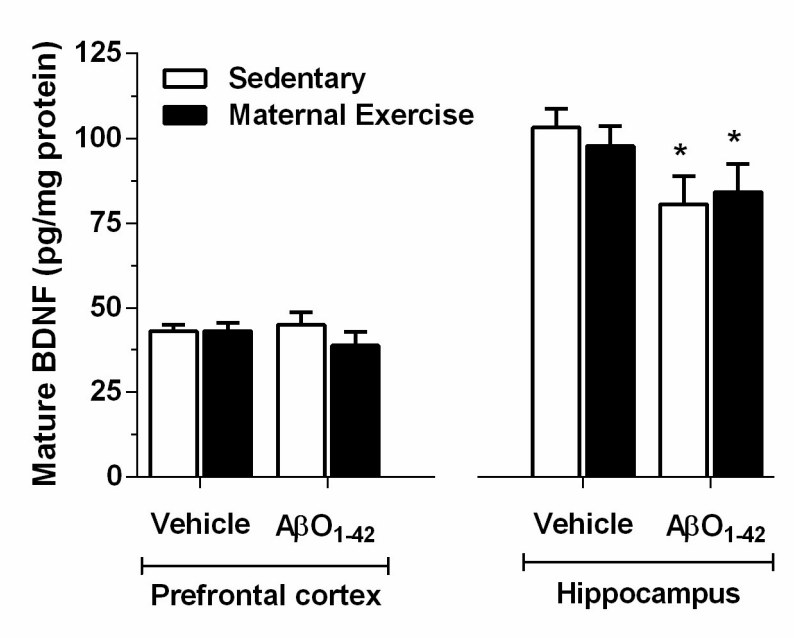

Mature BDNF (pg/mg protein)
125
100
75
50
25
0
Sedentary
Maternal Exercise
*
*
Vehicle
AβO1-42
Vehicle
AβO1-42
Prefrontal cortex
Hippocampus

Figure 5

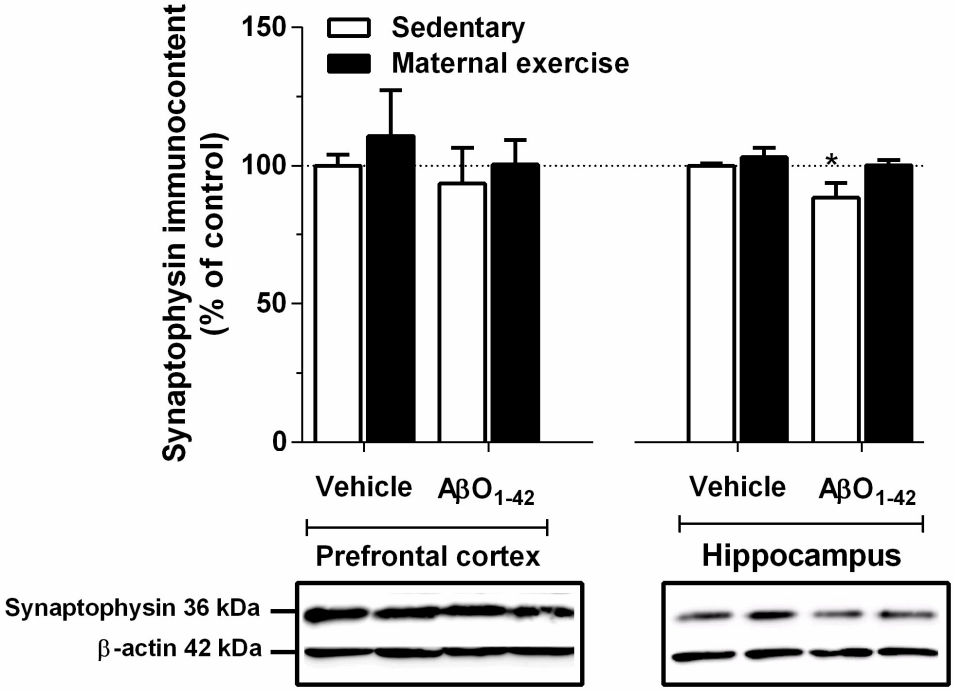

Synaptophysin immunocontent
(% of control)
150
100
50
0
Sedentary
Maternal exercise
*
Vehicle
AβO1-42
Vehicle
AβO1-42
Prefrontal cortex
Hippocampus
Synaptophysin 36 kDa
β-actin 42 kDa

Figure 6

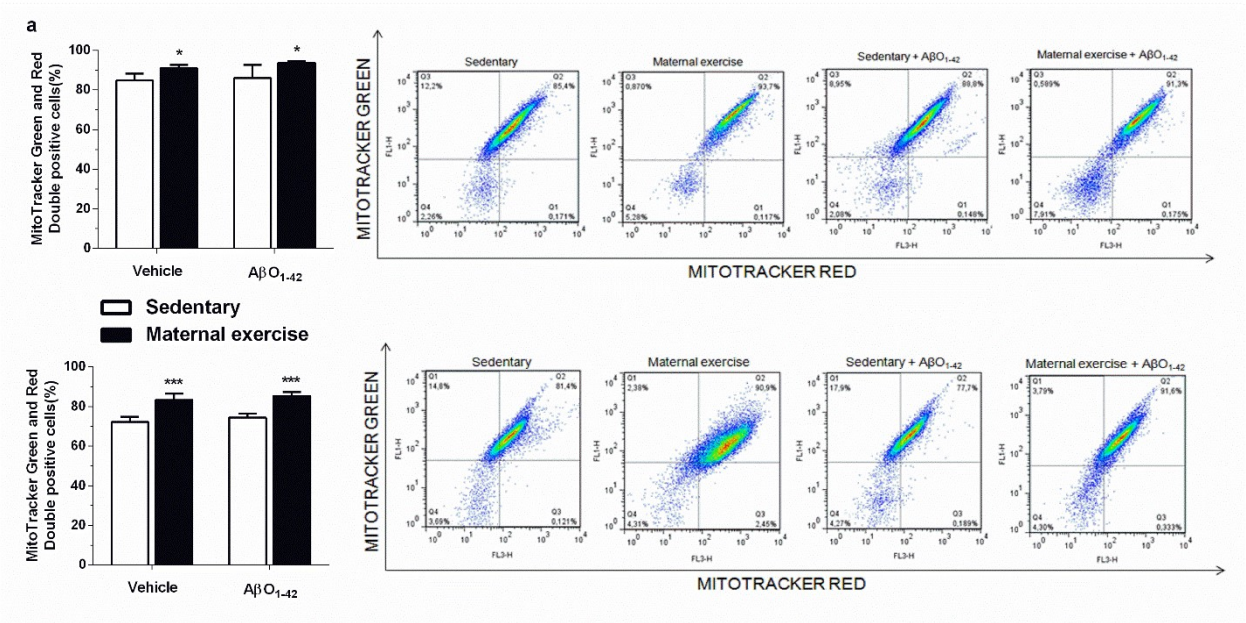

a
MitoTracker Green and Red Double positive cells(%)
Vehicle
AβO1-42
Sedentary
Maternal exercise
MITOTRACKER GREEN
MITOTRACKER RED
Sedentary
Maternal exercise
Sedentary + AβO1-42
Maternal exercise + AβO1-42

Figure 7

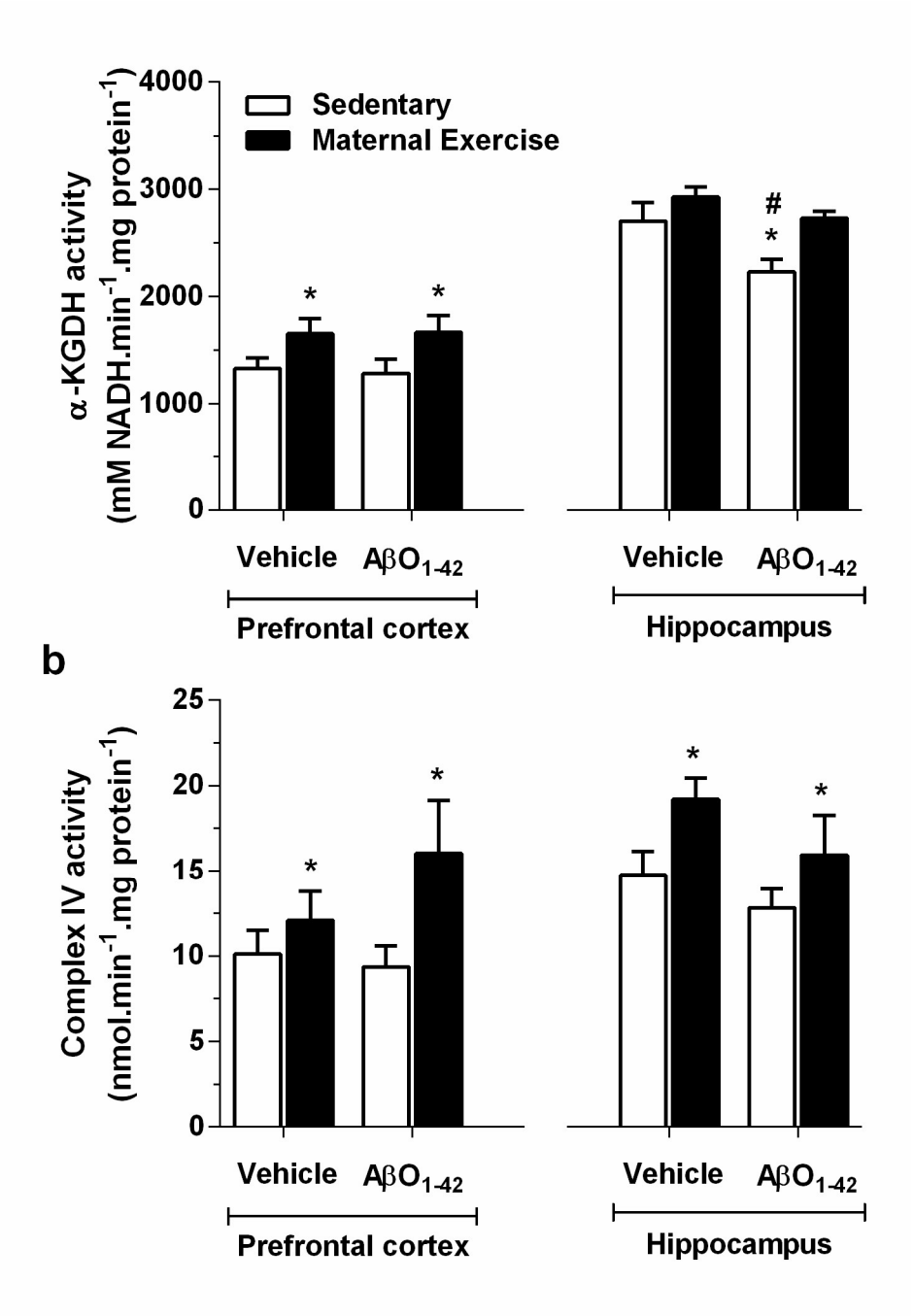
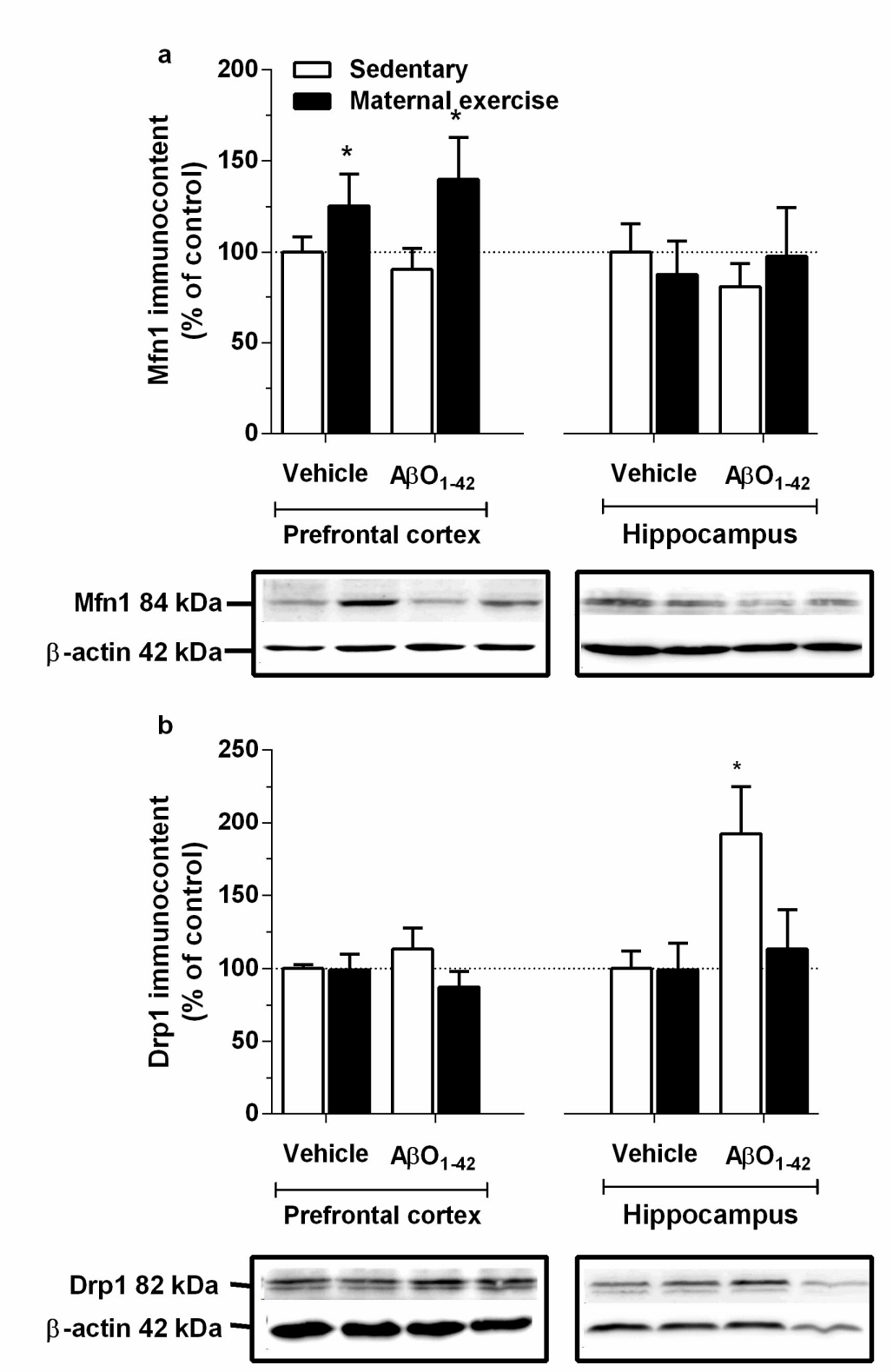

Sedentary
Maternal Exercise
α-KGDH activity
(mM NADH.min-1.mg protein-1)
4000
3000
2000
1000
0
*
*
#
*
Vehicle
AβO1-42
Vehicle
AβO1-42
Prefrontal cortex
Hippocampus
b
Complex IV activity
(nmol.min-1.mg protein-1)
25
20
15
10
5
0
*
*
*
*
Vehicle
AβO1-42
Vehicle
AβO1-42
Prefrontal cortex
Hippocampus

Figure 8


a
Sedentary
Maternal exercise
Mfn1 immunocontent (% of control)
200
150
100
50
0
*
*
Vehicle
AβO1-42
Vehicle
AβO1-42
Prefrontal cortex
Hippocampus
Mfn1 84 kDa
β-actin 42 kDa
b
Drp1 immunocontent (% of control)
250
200
150
100
50
0
*
Vehicle
AβO1-42
Vehicle
AβO1-42
Prefrontal cortex
Hippocampus
Drp1 82 kDa
β-actin 42 kDa

CAPÍTULO IV

Exercise in Pregnancy Programs Adult Male Offspring's Cerebellum Metabolism: Protective Effect against Amyloid-β Neurotoxicity

O capítulo IV apresenta o artigo intitulado *Exercise in Pregnancy Programs Adult Male Offspring's Cerebellum Metabolism: Protective Effect against Amyloid-β Neurotoxicity*, o qual está submetido ao periódico *Journal of Neurochemistry*.

Exercise in Pregnancy Programs Adult Male Offspring's Cerebellar Metabolism: Protective Effect against Amyloid-β Neurotoxicity

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Running Title

Programming protection against Aβ Neurotoxicity

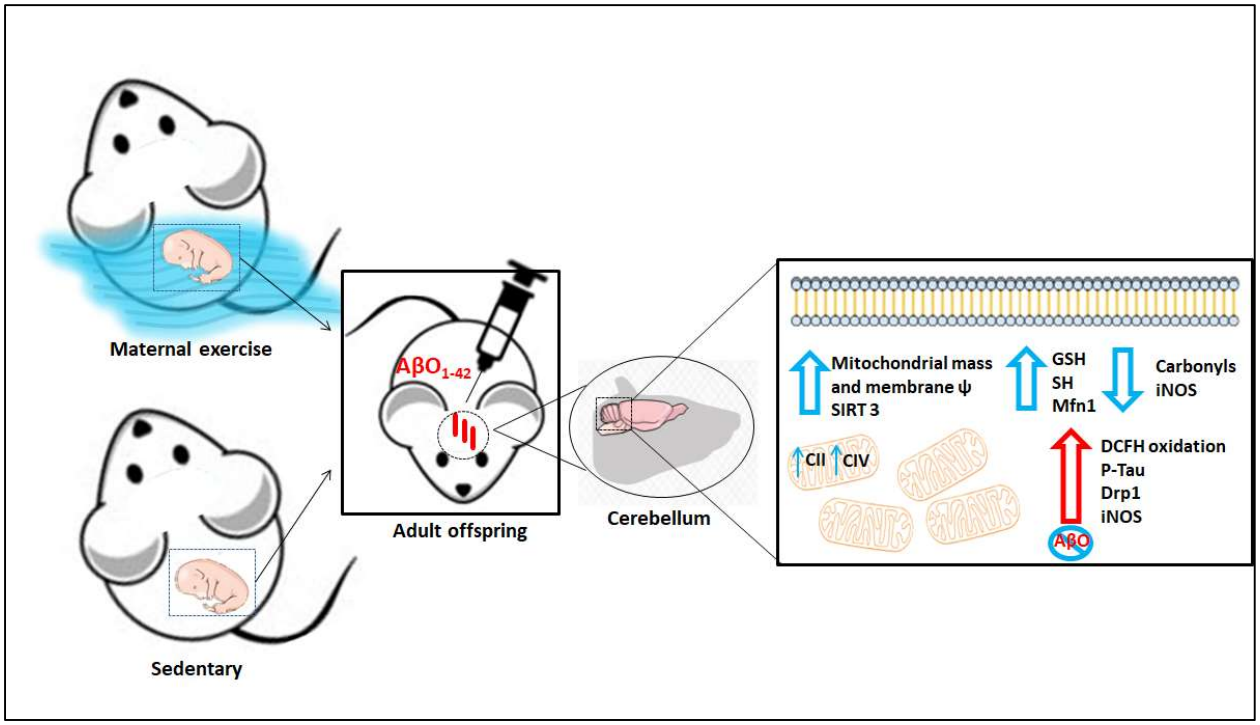
Keywords

Metabolic programming; exercise; bioenergetics; Alzheimer's disease

Abbreviations

AD, Alzheimer's disease; Aβ, amyloid β; AβO, amyloid β oligomers; BDNF, brain-derived neurotrophic factor; CII, complex II; CIV, complex IV; CAT, catalase; cGMP, cyclic guanosine monophosphate; DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate; DOHaD, Developmental Origins of Health and Disease; Drp1, dynamin-related protein 1; eNOS, endothelial nitric oxide sintase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSK-3β, glycogen synthase kinase 3β; H_2DCF-DA, 2′,7′-dichlorofluorescin diacetate; Icv, intracerebroventricular; iNOS, inducible nitric oxide sintase; MFI, mean fluorescence intensity; Mfn1, mitofusin 1; $NO^{\bullet}$, nitric oxide; PD, postnatal day; RS, reactive species; SIRT3, sirtuin 3; SH, thiol; SOD, superoxide dismutase

Graphical abstract



Exercise in Pregnancy Programs Adult Male Offspring's Cerebellar Metabolism: Protective Effect against Amyloid-β Neurotoxicity

Abstract

The developmental origins of health and disease states that intrauterine maternal environment influences postnatal life through programming the metabolism. Intrauterine milieu offered by exercise during pregnancy promotes long-lasting benefits to the offspring's health and seems to confer resistance against chronic diseases in adult life. Alzheimer's disease is a public health concern and the treatment effectivity is limited. In the present study, we assessed the ability of maternal exercise in programming the offspring's cerebellar metabolism to confer neuroprotection against amyloid-β neurotoxicity in adult life. Herein, we demonstrate that maternal exercise during pregnancy attenuated the reactive species rise, the increase of inducible nitric oxide synthase immunocontent and tau phosphorylation induced by Aβ oligomers, as well as the increase of protein carbonylation and dynamin-related protein 1 (Drp1). Strikingly, we evidenced that maternal exercise promotes changes in the offspring's cerebellum that are still evident in adult life. In summary, it is possible that favorable molecular changes in offspring's cerebellum induced by maternal exercise contributed to a more robust phenotype against Aβ-induced neurotoxicity in adult offspring.

Introduction

Intrauterine and early postnatal development provides a therapeutic window for metabolic programming, aiming to reach long-term offspring's health. Over recent years, it has been demonstrated the maternal environment impacts in the offspring's metabolism, modulating the susceptibility of disease development (Hanson & Gluckman 2014, Harris et al. 2018). The conceptual basis of metabolic programming is encompassed by the Developmental Origins of Health and Disease (DOHAD) theory (Gluckman et al. 2010). The optimization of maternal environment influences the early embryonic and fetal development, modulating offspring's early and late life health and conferring resistance to incoming diseases (Hanson & Gluckman 2014). The phenotype shaping of developing metabolism is established through plastic processes that enables an adaptation to environment (Bateson et al. 2014) by metabolic programming (Bale 2015), which rely on epigenetic alterations that modulates gene expression, and oxidative challenge, which in turn modulates the redox state (Barnes & Ozanne 2011).

We and others have demonstrated that maternal exercise during pregnancy improves antioxidant defense system (Marcelino et al. 2013), glucose homeostasis and insulin sensitivity (Carter et al. 2013), induces mitochondrial biogenesis (Marcelino et al. 2013, Klein et al. 2018) and neurogenesis (Akhavan et al. 2008, Bick-Sander et al. 2006), as well as increases brain-derived neurotrophic factor (BDNF) levels (Gomes da Silva et al. 2016) in young and adult offspring's brain. These findings highlight the maternal exercise as a modulator of intrauterine environment favoring offspring's health state and enhancing brain function. On basis of these wide ranges of metabolic programming effects, different groups have showed that maternal exercise during pregnancy is able to protect adult offspring against chronic metabolic alterations (Herring et al. 2012, Stanford et al. 2015, Wasinski et al. 2015, Sheldon et al. 2016, Camarillo et al. 2014, Robinson & Bucci 2014).

Since two decades, the physical exercise has been proposed to improve brain function in healthy and unhealthy individuals, including those diagnosed with neurodegenerative diseases, such as Alzheimer´s disease (AD) (Kim et al. 2016, Maliszewska-Cyna et al. 2016, Palleschi et al. 1996, Vidoni et al. 2015). Although human and animal studies have shown that physical exercise during pregnancy improves several brain functions and cognitive performance (Labonte-Lemoyne et al. 2016, Jukic et al. 2013, Akhavan et al. 2008, Marcelino et al. 2016, Akhavan et al. 2012, Kim et al. 2007), scarce studies have addressed the beneficial effects of maternal exercise during pregnancy regarding neuroprotective role against neurodegeneration (Herring et al. 2012, Klein et al. 2018). The hallmarks of AD are the amyloid-β (Aβ) peptide accumulation and intracellular neurofibrillary tangles that leads to neuronal damage (Reitz & Mayeux 2014). In addition, events including oxidative stress, mitochondrial dysfunction, energetic metabolism failure, and neuroinflammation are suggested to be part of the underlying molecular mechanisms of the disease (Dumont et al. 2010, Querfurth & LaFerla 2010). To date, there is no available treatment to counteract the establishment of the disease once its pathophysiological mechanisms are not fully known. In this context, the cerebellum events in Alzheimer's disease present as a new frontier of study. Non-motor functions have been recently assigned to cerebellum, such as emotional and cognitive associative learning control (Timmann et al. 2010). Additionally, several changes related to exercise adaptive responses in central nervous system such as enhance synaptic plasticity, neurogenesis, and cognition have also been observed in the cerebellum (Mattson 2012).

Therefore, we set out to investigate whether maternal swimming before and during pregnancy promotes long-lasting metabolic effects on offspring's cerebellum unveiling the cellular and molecular underlying mechanisms. Moreover, we set out to investigate the ability of maternal swimming before and during pregnancy to confer neuroprotection to the rat offspring against amyloid-β-induced neurotoxicity.

Material and methods

Reagents

All reagents for biochemical assays were obtained from Sigma-Aldrich (RRID:SCR_008988). Probes for flow cytometric analyses were obtained from Thermo Fischer (RRID:SCR_008452). Antibodies for Western blotting were obtained from Millipore (RRID:SCR 008983), Cell Signaling (RRID:SCR 004431), Abcam (RRID:SCR 012931), Santa Cruz Technologies (RRID:SCR 008987), and GE Healthcare (RRID:SCR 000004).

Animals and Ethical Approval

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

All experimental procedures were approved by the local Ethics Commission on the Use of Animals (Comissão de Ética no Uso de Animais - CEUA/UFRGS) under the protocol number 27349, and were performed in accordance with the National Animal Rights Regulations (Law 11.794/2008), the American National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1996), and the Directive 2010/63/EU. ARRIVE guidelines were followed in the preparation of the manuscript. The study was not pre-registered for preclinical study. All efforts were made to minimize the number of animals used and their suffering. The number of animals used in each experiment is indicated in the figures captions.

Experimental design

The study design is depicted in the Figure 1. The animals were assigned to experimental groups randomly, by chance, and identified by marking on the tail using nontoxic marker. Adult female rats were initially divided into two groups: (1) sedentary control dams, group in which the rats were exposed to aquatic environment stress, without exercising; and (2) maternal exercise dams, group in which the rats were subjected to a swimming protocol in a pool (30 width x 30 length x 90 depth cm) filled with water at 32 ± 1oC as described in (Marcelino et al. 2013). The animals underwent involuntary swimming, from 9 to 12 a.m., 5 days/week lasting 30 min daily, during 4 weeks, one week for adaptation to aquatic environment prior to mating, and during entire pregnancy period. At the end of first week, two females were mated with one male, and the pregnancy was confirmed by the presence of a vaginal plug or sperm in the vaginal fluid. The pregnant rats underwent the exercise protocol during the entire pregnancy. The animals were left free to swim and were gently stimulated to swim when necessary. After all rats swam, control rats were immersed in water, carefully dried, and returned to the housing boxes. Beginning on the 20th gestational day, the dams were housed individually and observed twice a day (8 a.m. and 6 p.m.), to verify litter's birth. The offspring's birth was considered postnatal day (PD) 0. Within 24 h after delivery, the litter was adjusted to a number of 8 pups per dam. From delivery (PD 0) until weaning (PD 21), each dam was housed together with its litter. On PD 21, female littermates were euthanized, and male littermates were housed in four per cage until the PD 60. On 60 days of age, male offspring were subjected to a surgical procedure for bilateral microinfusion of Aβ peptide oligomers (AβO) or vehicle into the brain ventricles. Thus, the offspring was subdivided, into the following groups: 1) sedentary control + vehicle, 2) maternal exercise + vehicle, 3) sedentary + AβO, and 4) maternal exercise + AβO. On the 14th day post-surgery, vehicle- and AβO-infused adult male offspring born to sedentary or exercised rats were euthanized by decapitation in order to evaluate biochemical parameters. The samples were identified as consecutive numbers, and the corresponding group was tabulated, known to only one experimenter. The experimenters were blinded at the time of sample preparation, data analysis, and calculation.

Aβ1-42 peptide oligomers preparation

Soluble AβO were prepared according Klein (2002). The Aβ (sequence 1-42) peptide (American Peptide Co., Sunnyvale, CA, USA, RRID:SCR_012606) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma-Aldrich, St. Louis, MO, USA) in order to form monomers of Aβ peptide. After incubation lasting 1 h at room temperature and 10 min on ice, the solution was aliquoted, and the tubes containing the Aβ peptide were maintained overnight in the hood to removal of HFIP followed by centrifugation in a SpeedVac system to obtain a clear film of monomeric Aβ peptide at the bottom of the tubes, which were stored at -80oC. Twenty-four hours before in vivo infusion, aliquots were solubilized in DMSO, diluted in phosphate saline buffer pH 7.4 (PBS) and incubated at 5oC for 24 h. After incubation, the tubes were centrifuged at 14,000 *g* for 10 min at 4oC, and the AβO-containing supernatants were transferred to a new tube. Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fischer) and the structural characterization were performed using the specific Aβ 1-16 antibody 6E10 (Covance) through western blotting.

Surgical procedure for AβO infusion

On the PD 60, male offspring were anesthetized (100 mg/Kg ketamine and 15 mg/Kg xylazine, i.p.), and then placed in a stereotaxic frame to the following surgical procedure previously described by Hoppe et al. (2013). Two trained experimenters performed surgical procedures. Using sterile surgical instruments, a middle sagittal incision was made in the scalp and bilateral holes were drilled in the skull using a dental drill over the lateral ventricles, according to Paxinos and Watson atlas coordinates (RRID:SCR_006369): 0.8 mm posterior to bregma and 1.5 mm lateral to the sagittal suture; and the depth of the microinfusion consisted in 3.5 mm beneath the surface of brain (Paxinos & Watson 2005). Rats received a single icv infusion of 5 µL bilaterally of Aβ peptide oligomers (500 pmol/rat), and control rats received bilateral infusions into each lateral ventricle of equal volume of PBS and 2% DMSO. The microinfusions were

performed using a 10 µL Hamilton syringe fitted with a 26-gauge needle in an infusion rate of 1 µL/min over a period of 5 min. At the end of infusion, the needle was left in place for an additional 3 to 5 min before being slowly withdrawn to allow diffusion from the tip and prevent reflux of the solution. After the infusion, the scalp was sutured, and the animals were allowed to recover from the anesthesia on a heating pad to maintain body temperature at 37.5 ± 0.5°C.

Sample preparation for biochemical assays

Animals were euthanized 14 days after the AβO infusion by decapitation, without anesthesia in order to avoid any interference of anesthetic on biochemical assays. For redox state parameters assessment, the cerebellum was rapidly dissected, weighed and immediately frozen (-80°C) until homogenization (1:10 w/v in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl). After homogenized, the samples were and centrifuged at 750 g for 10 min at 4°C to obtain the supernatant containing cytosol, mitochondria and other organelles. The assays were performed in duplicate using SpectraMax microplate reader (Molecular Devices, Multi-Mode Analysis Software, RRID:SCR_014789).

Superoxide dismutase (SOD) activity

SOD enzyme (EC 1.15.1.1) activity was assayed according to Misra and Fridovich (1972). It was measured the total SOD activity by quantifying the inhibition of superoxide-dependent epinephrine autoxidation at 480 nm.. SOD activity is expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit. Data are expressed as Units/mg protein.

Catalase (CAT) activity

CAT enzyme (EC 1.11.1.6) activity was assayed according to Aebi (1984). The decrease in the absorbance at 240 nm was measured in a reaction medium containing 20 mM H2O2, 0.1%

Triton X-100 and 10 mM potassium phosphate buffer, pH 7.0.. The CAT unit is defined as 1 μmol of H2O2 consumed per minute. Data of specific activity are expressed as Units/mg protein.

Glutathione peroxidase (GPx) activity

GPx enzyme (EC 1.11.1.9) activity was assayed according to Wendel (1981). NADPH disappearance was monitored at 340 nm. The reaction medium contained 100 mM potassium phosphate buffer, pH 7.7, containing 1mM EDTA, 2 mM reduced glutathione (GSH), 0.15 U/mL glutathione reductase (EC 1.8.1.7), 0.4 mM azide, 0.1 mM NADPH, and 0.5 mM tert-butyl hydroperoxide as enzyme substrate. The GPx unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as Units/mg protein.

Reduced glutathione content (GSH)

GSH concentration was measured according to Browne and Armstrong (1998). Initially, the proteins in supernatant were precipitated with meta-phosphoric acid (1:1, v/v), and centrifuged at 5,000 *g* for 10 min at 25°C. GSH present in the supernatant reacts with the fluorophore o-phtaldialdehyde present in the reaction medium at a concentration of 7.5 mM in addition to 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA. The fluorescence was measured at excitation and emission wavelengths of 350 nm and 420 nm, respectively. Standard GSH curve ranging from 0.001 to 1 mM was prepared and a blank sample was performed in parallel. Data are expressed as nmol/mg protein.

Thiol content

Thiol content was measured according to Aksenov and Markesbery (2001) with slight modifications. The assay is based on the reduction of 50-dithiobis-(2-nitrobenzoic acid) (DTNB) by thiols, which become oxidized (disulfide), generating a yellow derivative (TNB). The absorbance was measured at 412 nm in a reaction medium containing 20 mM sodium phosphate

buffer, pH 7.4, and 10 mM DTNB prepared in a 0.2 M potassium phosphate solution, pH 8.0. Data are expressed as nmol TNB/mg protein.

Carbonyl content

Protein carbonyl content was measured according to Reznick and Packer (Reznick & Packer 1994) and adapted by Stone et al. (2016). Protein carbonyls react with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound that was measured at 370 nm. Data are expressed as nmol/mg protein.

Respiratory System Complexes Activities

Animals were euthanized 14 days after the AβO injection by decapitation, without anesthesia in order to avoid tissue chemical contamination. The cerebellum was rapidly dissected, weighed and immediately frozen (-80°C) until homogenization. The cerebellum was homogenized (1:20 w/v) in SETH buffer, pH 7.4, that contains 250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 IU/mL heparin, and centrifuged at 800 g for 10 min at 4°C. The pellet was discarded, and the supernatant was collected and submitted to three subsequent freeze-thawing procedures before performing the experiments [54]. Mitochondrial respiratory chain enzyme activities were measured in homogenates with a protein concentration varying from 1.5 to 5.0 mg protein/mL. The assays were performed in duplicate using SpectraMax microplate reader (Molecular Devices, Multi-Mode Analysis Software, RRID:SCR_014789).

Succinate:phenazine oxireductase activity

The activity of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) was determined according to the method of Fischer et al. [59], slightly modified as described previously by da Silva et al. [60]. The incubation medium consisted in 62.5 mM potassium phosphate buffer, pH 7.4, 250 mM succinate, and 0.5 mM DCIP, which was pre-incubated with 10 µL of homogenized sample at 30°C for 10 min. Complex II activity was determined following the

decrease in absorbance due to reduction of DCIP at 600 nm. The activity of complex II was calculated as nmol/min/mg protein.

Cytochrome c oxidase (Complex IV) activity

The activity of cytochrome c oxidase was measured according to Rustin et al. [61], slightly modified, as described previously [60]. The incubation medium consisted in 10 mM potassium phosphate buffer, pH 7.0, 125 mM n-dodecil-β-D-maltoside, and 1% cytochrome c. Complex IV activity was determined following the decrease in absorbance due to reduced cytochrome c oxidation at 550 nm at 550 nm. The activity of complex IV was calculated as nmol/min/mg protein.

Protein determination

Protein concentration was measured according the method described by Lowry et al. (Lowry et al. 1951), which was adapted for microplate, using bovine serum albumin as standard. The absorbance was measured at 750 nm. The results were presented as mg of protein/mL.

Flow cytometry

Flow cytometric analysis was conducted according to Marcelino et al. (2013). Briefly, the tissue samples (approximately 100 mg) were dissociated in PBS containing 1 mg% of colagenase IV and 0.5 mg% of DNase, filtered using the 40 μm pore size cell strainer (SPL Lifesciences Co., Naechon-Myeon Pocheon, South Korea, RRID not registered), and then incubated at 37°C with the molecular probes. Reactive oxygen and nitrogen species levels were measured using 10 μM 2′,7′-dichlorofluorescin diacetate (H2DCF-DA; Sigma-Aldrich, St. Louis, MO, USA, catalog number D6883), nitric oxide (NO•) levels were measured using 10 μM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM; Invitrogen, Molecular Probes, Eugene, OR, USA, catalog number D23841), mitochondrial superoxide were measured using 1 μM MitoSOX® Red (Invitrogen, Molecular Probes, Eugene, OR, USA, catalog number M36008), mitochondrial mass and membrane potential were measured using 100 nM MitoTracker® Green and 100 nM

MitoTracker® Red (Invitrogen, Molecular Probes, Eugene, OR, USA, catalog number M7514 and M7513), respectively. Cells were gated based on the FSC and SSC pattern of the sample cells and 30,000 events were acquired per sample in a BD FACSCalibur Flow Cytometry System (RRID:SCR_000401); a non-labeled sample was used as negative fluorescent control. DCFH, DAF-FM and MitoSOX data are expressed as mean fluorescence intensity (MFI), and MitoTracker® Green and MitoTracker® Red data are expressed as percentage of double positive cells. Data were analyzed using the software FlowJo (Tree Star, Ashland, OR, USA, RRID:SCR_008520).

Western blot assay

Cerebellum were homogenized in ice-cold lysis buffer containing 4% sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mM Tris, and 1% protease inhibitor cocktail. The homogenates were denatured 100°C for 5 min, and then centrifuged at 10,000 g for 30 min. After this, the supernatant containing the cytosolic fraction was collected, β-mercaptoethanol was added to a final concentration of 5%, and then, the samples were stored at -80°C until use. Equal concentration of protein (50 μg) was loaded and immunodetected as previously described (Hoppe et al. 2013). Membranes were incubated for 60 min at 4°C in blocking solution (Tris-buffered saline containing 5% non-fat milk and 0.1% Tween-20, pH 7.4) prior the incubation with the primary antibody. Membranes were incubated overnight at 4°C in blocking solution containing one of the following primary antibodies: rabbit monoclonal anti-synaptophysin (1:2000 dilution, Millipore, catalog number #AB9272), anti-PSD-95 (1:1000 dilution, Cell Signaling, catalog number #2507), anti-SIRT3 (1:1000 dilution, Abcam, catalog number #ab189860), anti-mitofusin 1 (1:1000 dilution, Abcam, catalog number # ab104274), anti-dinamin-related protein 1 (1:1000 dilution, Abcam, catalog number #ab154879), anti-phospho(S9)-GSK-3β (1:1000 dilution, Cell Signaling, catalog number #9336), anti-GSK-3β (1:1000 dilution, Cell Signaling, catalog number #9315), anti-NOS2 (1:250 dilution, Santa Cruz Technologies, catalog number #sc-7271), anti-phospho(S396)-Tau

(1:1000 dilution, Cell Signaling, catalog number #9632) anti-Tau (1:1000 dilution, Cell Signaling, catalog number #4019), and rabbit monoclonal anti-β-actin (1:2000 dilution, Cell Signaling, catalog number #4967). After washing, the membranes were incubated with secondary antibody containing peroxidase-conjugated anti-rabbit IgG (1:1000 dilution, GE Healthcare Life Sciences, catalog number #NA934V), and anti-mouse IgG (1:1000 dilution, GE Healthcare Life Sciences, catalog number #NA931V) for 1 h. The chemiluminescence was detected using the digital imaging system Image Quant LAS 4000 (GE Healthcare Life Sciences, RRID:SCR 014246) and analyzed using the Image J Software (RRID:SCR 003070). The average optical density for the control group was designated as 100%.

Statistical analyses

The sample size of the study was calculated to n = 6-7 per group for flow cytometric analysis and n = 7-9 for biochemical assays and Western blotting, according to previous works performed in the lab (with $\alpha = 0.05$, $\beta = 0.2$) using the Minitab 16 software (Minitab Inc., State College, PA, USA, RRID:SCR_014483). Data are expressed as mean + standard error of the mean (SEM), and statistical analyses were performed using the GraphPad Prism 6.0 software (RRID:SCR_002798). All data were tested for normality. Data points outside the 95% confidence interval were treated as outliers and excluded from the data analysis. Two-way ANOVA was used to analyze the effect of the two independent variables, maternal exercise and AβO infusion. Post hoc analysis was carried out using Tukey's test. Main effects were considered significant if $p \leq 0.05$.

Results

Maternal exercise prevents the increase of AβO-induced RS levels

At first, we assessed the effect of AβO on neurochemical parameters and the ability of maternal exercise during pregnancy to counteract possible alterations triggered by AβO in the adult

offspring´s cerebellum. Initially, we measured RS levels in the adult offspring´s cerebellum through flow cytometry. Concerning overall RS measured by DCFH oxidation, two-way ANOVA indicated an interaction between maternal exercise and AβO infusion [$F(1,17) = 5.862$, $p = 0.027$], indicating that AβO infusion induced an increase of RS levels and that maternal exercise was able to prevent completely such rise (Fig. 1A). To examine the contribution of specific RS on the difference observed in DCFH oxidation we measured NO• levels. NO• levels were not altered by any treatment in the cerebellum of adult offspring (Fig. 1B). Neither maternal exercise [$F(1,17) = 0.538$, $p = 0.473$] nor AβO infusion [$F(1,17) = 0.208$, $p = 0.653$] altered cerebellar NO• levels. Furthermore, we examined superoxide levels arising from mitochondria. Mitochondrial superoxide levels were not altered by any factor in the cerebellum of adult offspring (Fig. 1C). Neither maternal exercise [$F(1,16) = 1.125$, $p = 0.304$] nor AβO infusion [$F(1,16) = 0.825$, $p = 0.377$] altered cerebellar mitochondrial superoxide levels. These results indicated maternal exercise during pregnancy is able to prevent overall RS levels increment induced by AβO infusion, also that neither NO• nor mitochondrial superoxide contributes to the increased overall cellular RS observed in the adult offspring's cerebellum of sedentary + AβO group.

Maternal exercise increases GSH levels in adult offspring's cerebellum

To examine the antioxidant system, we measured the content of the most important non-enzymatic antioxidant in the brain, GSH (Fig. 2A), as well as the activity of antioxidant enzymes SOD, GPx, and CAT (Fig. 2B, C and D). Two-way ANOVA indicated a significant effect of maternal exercise on GSH levels [$F(1,37) = 14.63$, $p = 0.0005$], which was found increased in the adult offspring´s cerebellum, and no effect of AβO infusion [$F(1,37) = 0.123$, $p = 0.727$]. Two-way ANOVA indicated that both factors, maternal exercise and AβO infusion, exerted no effect on activities of the enzymes SOD [$F(1,33)=0.533$, $p=0.470$ and $F(1,33) = 0.559$, $p = 0.459$, respectively], GPx [$F(1,33) = 1.096$, $p = 0.303$ and $F(1,33) = 2.751$, $p = 0.107$, respectively], and CAT [$F(1,31) = 1.972$, $p = 0.170$ and $F(1,31) = 0.691$, $p = 0.412$].

Increased protein carbonylation elicited by AβO is prevented by maternal exercise

Oxidative damage occurs in the brain in the presence of AβO (Sultana et al. 2006). To verify whether AβO elicits oxidative damage in our model, we measured thiol and carbonyl content in the cerebellum of adult offspring. Thiol groups react with RS to maintain the redox state, being considered a redox buffer. Two-way ANOVA indicated an effect of maternal exercise on thiol content [$F(1,35) = 5.170$, $p = 0.029$], which was found increased in the offspring's cerebellum (Fig. 2E); AβO infusion exerted no effect on thiol content [$F(1,35) = 0.398$, $p = 0.532$]. Protein carbonylation is an oxidative protein modification that is present as indicative of oxidative stress in the cell. Concerning carbonyl levels, two-way ANOVA indicated an effect of both maternal exercise [$F(1,26) = 12.15$, $p = 0.002$] and AβO infusion [$F(1,26) = 5.396$, $p = 0.028$]. AβO infusion increased carbonyl levels, while maternal exercise reduced carbonyl levels (Fig. 2F). These data indicated that AβO infusion promoted protein oxidation in cerebellum, and maternal exercise prevented the oxidative effect of AβO in the cerebellum of adult offspring.

Effects of maternal exercise and AβO infusion on mitochondrial parameters

It has been shown that AβO accumulates in mitochondria (Manczak et al. 2006); thus AβO can target essential metabolic enzymes impairing its function and altering mitochondrial dynamics (Kandimalla & Reddy 2016). To assess whether maternal exercise and AβO infusion modify the function of mitochondrial enzymes we measured the activity of CII and CIV (Fig. 3A and B). Two-way ANOVA indicated a main effect of maternal exercise on CII [$F(1,23) = 12.92$, $p = 0.001$] and CIV [$F(1,22) = 13.58$, $p = 0.001$], evidenced by increased activities, and no effect of AβO infusion on both enzymes activities, CII [$F(1,22) = 1.774$, $p = 0.197$] and CIV [$F(1,23) = 0.676$, $p = 0.419$].

Furthermore, we measured mitochondrial mass and membrane potential using MitoTracker Green and Red molecular probes, respectively. Double positive labeling was assumed as functional respiring mitochondria and an increasing of fluorescence is indicative of mitochondrial biogenesis

(Tal et al. 2009). In agreement with the effect of maternal swimming, which activated the mitochondrial complexes, two-way ANOVA indicated a main effect of maternal exercise on MitoTracker Green and Red double positive cells [$F(1,18) = 4.846$, $p = 0.041$], indicating increased mitochondrial mass and membrane potential. In addition, mitochondrial mass and membrane potential were found unchanged by AβO infusion [$F(1,18) = 2.369$, $p = 0.141$]. It is important to verify that AβO infusion did not abolish the effect promoted by maternal exercise on mitochondrial function (Fig. 3C and D).

In order to examine whether AβO infusion alter mitochondrial dynamics we measured mitochondrial fusion protein Mfn1 and mitochondrial fission protein Drp1 in the adult offspring's cerebellum through Western blot. Two-way ANOVA indicated an interaction between maternal exercise and AβO infusion on Mfn1 immunocontent in the adult offspring's cerebellum (Fig.4A) [$F(1,29) = 4.799$, $p = 0.037$]. Maternal exercise increased offspring's cerebellar Mfn1 levels; however, AβO infusion was able to abolish the effect promoted by maternal exercise. Concerning cerebellar Drp1 immunocontent (Fig.4B), two-way ANOVA indicated a main effect of AβO infusion [$F(1,31) = 4.517$, $p = 0.042$], and a borderline significant effect of maternal exercise on Drp1 levels [$F(1,31) = 3.985$, $p = 0.055$]. These data indicate that AβO infusion increases Drp1 levels in the offspring's cerebellum, and, strikingly, such rise of Drp1 levels in maternal exercised + AβO group reached values similar to control group.

We further measured mitochondrial SIRT3 immunocontent (Fig. 4C). Two-way ANOVA indicated a main effect of maternal exercise on SIRT3 levels [$F(1,23) = 4.775$, $p = 0.039$], increasing SIRT3 immunocontent in the cerebellum of adult offspring. No effect of AβO infusion was found [$F(1,23) = 0.114$, $p = 0.739$]. Interestingly, it was observed that AβO infusion did not abolish the SIRT3-mediated adaptive ability of mitochondria to maternal exercise.

Maternal exercise prevented the increment of inducible nitric oxide sintase (iNOS) immunocontent elicited by AβO infusion in the cerebellum of adult offspring

AβO are known to induce alteration on synaptic proteins levels (Liu et al. 2010a, Sebollela et al. 2012). To investigate the effect of AβO infusion in the cerebellum of offspring born to sedentary or exercised dams on pre- and post-synaptic proteins levels, we measured the immunocontent of synaptophysin and PSD-95 (Fig. 5). Two-way ANOVA indicated that neither maternal exercise nor AβO infusion exerted any effect on synaptophysin [$F(1,28) = 2.233$, $p = 0.146$ and $F(1,28) = 0.232$, $p = 0.634$, respectively] and PSD-95 levels [$F(1,25) = 1.211$, $p = 0.281$ and $F(1,25) = 1.009$, $p = 0.325$, respectively] in the cerebellum of adult offspring. Concerning the immunocontent of iNOS in the offspring's cerebellum, two-way ANOVA indicated an effect of both maternal exercise and AβO infusion. While maternal exercise reduced iNOS levels [$F(1,28) = 6.741$, $p = 0.015$], AβO infusion increased iNOS levels [$F(1,28) = 8.173$, $p = 0.008$]. Strikingly, the rise of iNOS levels in maternal exercised + AβO group reached values similar to control group, indicating a neuroprotective effect.

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

Lastly, we assessed the phosphorylated status of two proteins that present altered in AD (Reddy 2013). The ratio between phosphorylated and total content of GSK-3β was found unaltered in the cerebellum of all groups (Fig. 5D), in which neither maternal exercise nor AβO infusion exert effect on p-GSK-3β/GSK-3β ratio [$F(1,20) = 1.156$, $p = 0.295$ and $F(1,20) = 0.003$, $p = 0.958$]. Concerning tau phosphorylation status, two-way ANOVA indicated an interaction between both maternal exercise and AβO infusion on p(Ser-396)-tau/tau ratio [$F(1,31) = 4.541$, $p = 0.041$]. These data indicated that AβO infusion increases tau phosphorylation while maternal exercise prevented such increase (Fig. 5E).

Discussion

In the present study, we observed that maternal exercise during pregnancy promotes adaptive response in developing fetuses through programming cerebellar neurochemistry that

remains evident in adulthood. Nevertheless, the noxious stimuli in adulthood by AβO-peptide infusion into offspring's bilateral cerebral ventricles elicited changes in the cerebellum. We have demonstrated here that maternal exercise was able to prevent several alterations induced by neurotoxic AβO into the adult offspring's cerebellum.

The developmental processes of brain, such as growth and maturation, extend from gestational period beyond early postnatal days (Rice & Barone 2000). The majority of literature data focus on cortical and hippocampal modulatory effects promoted by maternal exercise in the offspring's brain (Akhavan et al. 2008, Bick-Sander et al. 2006, Gomes da Silva et al. 2016, Park et al. 2013), and little is known about these effects on cerebellum. The available research of adaptive changes in the cerebellum induced by maternal exercise was recently published by our group (Marcelino et al. 2016, Marcelino et al. 2013). We have shown that swimming during pregnancy enhances mitochondrial function and antioxidant defenses in the cerebellum, parietal cortex, hippocampus, and striatum of 7-day-old pups (Marcelino et al. 2013). Increased GSH and SH content, and reduced protein carbonylation in adult offspring's cerebellum presented herein reinforce our previous findings, evidencing long-term positive influence of maternal exercise on brain development. In addition, similar levels of oxidants in the cerebellum of adult offspring contrasted to increased oxidants levels in the cerebellum of 7-day-old pups (Marcelino et al. 2013). Taken together, these findings suggest that increased oxidant levels on PD7 precede the metabolic adaptation improving antioxidant system efficiency that persists in adulthood.

Exercise-induced metabolic adaptation in individuals occurs to regulate bioenergetics demand (Marques-Aleixo et al. 2012). Likewise fetal adaptive responses to maternal exercise are engaged to deal with energetic challenge by modulating mitochondrial function (Marcelino et al. 2013, Park et al. 2013). Mitochondria are crucial to maintain the constant supply of energy required by brain and other organs (Knott et al. 2008). The cerebellum suffers markedly metabolic adaptive response to exercise (Chalimoniuk et al. 2015, Marques-Aleixo et al. 2015), even during

pregnancy (Marcelino et al. 2013). Herein, we found that maternal exercise increased CII and cytochrome c oxidase activities, which were associated with increased Mfn1 levels and unchanged Drp1 and SIRT3 immunocontent, as well as with mitochondrial mass and membrane potential in adult offspring's cerebellum. Currently, there are only two reports in the literature that investigated the effect of maternal exercise on mitochondrial enzymes activities and biogenesis in offspring's brain (Park et al. 2013, Klein et al. 2018). Park et al. (2013) reported that pregnant mice undergoing treadmill exercise affects positively hippocampal mitochondrial function and biogenesis of 3-days-old pups. In addition, our group recently demonstrated that maternal swimming enhances mitochondrial function in the 60-days-old offspring's prefrontal cortex and hippocampus by increasing mitochondrial mass and membrane potential, and the activity of cytochrome c oxidase (Klein et al. 2018). It has been shown that mitochondrial SIRT3 increases in response to different exercise modalities (Marques-Aleixo et al. 2015). SIRT3 expression modulates adaptive responses of hippocampal neurons to exercise and strengths resistance to oxidative stress and apoptosis (Cheng et al. 2016). To the best of our knowledge, this is the first study to demonstrate that mitochondrial SIRT3 and dynamic proteins in adult offspring's cerebellum are modulated by maternal exercise.

NO• in the brain acts as a signaling molecule that regulates cerebral blood flow (Garry et al. 2015), and as a neuromodulator that regulates synaptic transmission through NO•/cyclic guanosine monophosphate (NO•/cGMP) pathway (Chalimoniuk et al. 2015, Furini et al. 2010, Llansola et al. 2009). Brain adaptive response to exercise seems to be mediated by NO• through upregulation of endothelial nitric oxide synthase (eNOS) (Chalimoniuk et al. 2015, Gertz et al. 2006) and downregulation of calcium-independent inducible NOS (iNOS) (Liu et al. 2010b), whose effects can be neurotoxic (Garry et al. 2015). In the present study, we reported unaltered NO• levels and reduced iNOS immunocontent in the cerebellum of adult offspring delivered to exercised dams. On basis of these results, it seems that increased NO• levels are only necessary to

induce metabolic adaptation in early postnatal brain of pups born to exercised dams, as previously demonstrated by our group (Marcelino et al. 2013).

Different exercise regimens have been shown to increase proteins related with neuroplasticity. Treadmill exercise in a schedule of 40 min/day, 3 days/week for 4 weeks or 30 consecutive days increased synaptophysin immunocontent in the cerebellum of rats (Real et al. 2015). Liu et al. (2018) demonstrated that swimming exercise (60 min/day, 5 days/week for 4 weeks) is able to reverse the reduction of synaptophysin levels in the hippocampus of mice previously submitted to a chronic unpredictable mild stress. Here, we observed for the first time that maternal exercise does not modify pre-and post-synaptic protein levels in the cerebellum of adult offspring.

Scarce studies have examined the effect of AD-associated Aβ neurotoxicity in the cerebellum (Kuwabara et al. 2014, Lee et al. 2014, Kozuki et al. 2011). Interestingly, we found an increase in overall cerebellar RS levels and tau phosphorylation following AβOs infusion, which were markedly prevented by maternal exercise. The positive augmentation of several metabolic processes induced by maternal exercise in the offspring's cerebellum was not abolished by AβOs infusion. AβOs infusion increased protein carbonylation, Drp1, and iNOS levels. While these parameters were significantly higher in the offspring's cerebellum born to sedentary dams than control offspring, protein carbonylation, Drp1, and iNOS levels reached control values in the offspring born to exercised dams. These findings suggest a neuroprotective potential of maternal exercise against AD-associated AβOs neurotoxicity. Accordingly, Herring et al. (2012) reported that running during pregnancy alleviated cerebral oxidative stress and inflammation associated with decreased Aβ plaque in 5-months-old offspring carrying APP transgene.

Immunoprecipitation analyses of mitochondrial dynamics showed that AβOs interact with Drp1 leading to abnormal mitochondrial dynamics and enhanced mitochondrial fragmentation, thus culminating in neuronal damage (Manczak et al. 2011). We could observe that Mfn1 and

Drp1 levels in maternal exercised offspring infused with AβOs remained at control values, indicating a balance between fusion and fission processes despite the extinction of maternal exercise-induced Mfn1 increase. In our previous work, we demonstrated that maternal exercise increased Mfn1 levels in the offspring's prefrontal cortex and prevented the increase of Drp1 levels induced by AβOs in the hippocampus (Klein et al. 2018). Moreover, the main regulator of mitochondrial energy homeostasis, SIRT3 (Giralt & Villarroya 2012), was found increased together with enhanced mitochondrial enzymes activities, mass and membrane potential. On the other hand, an unbalance of mitochondrial dynamic seems to occur in the maternal sedentary offspring, once Drp1 levels were increased by AβOs and exerted no effect on Mfn1 levels. It is possible that there is a compensatory mechanism being orchestrated by mitochondria to maintain its function and to deal with toxic effects of AβOs in maternal sedentary offspring. A compensatory mechanism was postulated by Demetrius et al. (2014), in which occurs an up-regulation of oxidative phosphorylation in some cells to compensate energy production and to maintain impaired cells viable. As increased phosphorylated tau is also involved in AD pathology (Reitz & Mayeux 2014), the rise in pTAU/TAU ratio in the cerebellum of maternal sedentary offspring, which was prevented by maternal exercise, is another indicative of neuronal damage following AβOs infusion.

The work conducted by Thal et al. (2002) demonstrates that cerebellar pathological features of Aβ deposition in the cerebellum and related clinical manifestation are evident in later stages of AD. In accordance, cerebellar neuropathology and accumulation of Aβ appears with disease progression in APPswe/PS1dE9 double transgenic mice (Kuwabara et al. 2014). Furthermore, this report revealed that Aβ accumulation in the cerebellum was associated with impaired long-term depression but unaffected synaptic transmission (Kuwabara et al. 2014), which might help to explain why AβOs did not induce reduction of synaptic proteins in the offspring's cerebellum. We previously demonstrated that AβOs reduced significantly synaptophysin levels in the hippocampus

of 60-days-old male rats, and that maternal exercise was able to mitigate this Aβ-induced synaptophysin reduction (Klein et al. 2018). In this way, it is possible that we could not observe deeper alterations following AβO infusion as function of the time we assessed the parameters, but we cannot exclude the possibility of later appearance of AD-associated pathological features in the cerebellum. So far, one suggested molecular mechanism responsible by maternal exercise-promoted protective effects observed against biochemical changes caused by AβOs could be the improved mitochondrial bioenergetics.

Conclusion

In summary, here we report for the first time that exercise during pregnancy exert long-lasting effects on the offspring's cerebellar metabolism by inducing adaptive changes in utero. Strikingly, these metabolic programming effects were still evident during adulthood. Our findings also reveal that AβOs affect the cerebellum and that the long-term influence of maternal exercise on offspring metabolism was able to modify the cellular and molecular response to AD-associated neurotoxic AβO. In addition, our findings highlight the exercise practice during pregnancy as a promising neuroprotective approach to modify the course of disease later in life.

Declaration of interest

The authors declare no conflicts of interest.

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Figure captions

Fig. 1 Effects of maternal exercise and AβO infusion on offspring's cerebellar reactive species levels. (A) Representative histograms of DCFH, DAF-FM, and MitoSox mean fluorescence intensities. Bars show mean + standard error of **(B)** DCFH oxidation, **(C)** DAF-FM, and **(D)** MitoSOX Red mean fluorescence intensity. Two-way ANOVA showed an interaction between maternal exercise and AβO (n=6). *P<0.05 sedentary + AβO compared to other groups (two-way ANOVA followed by Tukey post hoc test).

Fig. 2 Effects of maternal exercise and AβO infusion on offspring's cerebellar antioxidant system and oxidative damage. (A) Reduced glutathione (GSH) content; **(B)** activity of superoxide dismutase (SOD), **(C)** glutathione peroxidase (GPx) and **(D)** catalase (CAT) enzymes; and **(E)** sulfhydryl and **(F)** carbonyl contents. Values are expressed as mean + standard error (n=8-11). Two-way ANOVA showed an effect of maternal exercise on GSH, sulfhydryl and carbonyl contents, and an effect of AβO infusion on carbonyl content. *P<0.05, **P<0.01, and ***P<0.001 different from sedentary groups (two-way ANOVA), #P<0.05 different from vehicle-treated groups (two-way ANOVA).

Fig. 3 Effects of maternal exercise and AβO infusion on mitochondrial function in the cerebellum of adult offspring. (A) Complex II and **(B)** Complex IV enzymes activities, **(C)** percentage of double positive Mitotracker Green and Mitotracker Red labeled cells (n=6), and **(D)** Representative dot plots of Mitotracker Green and Red-immunolabeled cells. Values are expressed as mean + standard error. A two-way ANOVA showed an effect of maternal exercise on these parameters. **P<0.01 different from sedentary groups.

Fig. 4 Effects of maternal exercise and AβO infusion on mitochondria-related proteins levels in the cerebellum of adult offspring. Immunocontent of **(A)** mitofusin (n=8-9), **(B)** dynamin-related protein (n=8-9), and **(C)** Sirt3 (n=6) expressed as average percentage of control. Representative quantification of proteins immunocontent normalized to b-actin protein (loading

control) is shown below the graphs. Values are expressed as mean + standard error. A two-way ANOVA showed interaction between maternal exercise and AβO infusion for mitofusin (°p<0.05). *P<0.05 effect of maternal exercise (two-way ANOVA), #P<0.05 effect of AβO infusion (two-way ANOVA).

Fig. 5 Effects of maternal exercise and AβO infusion on synaptic proteins, and inducible nitric oxide sintase (iNOS) in the cerebellum of adult offspring. Immunocontent of synaptic proteins **(A)** synaptophysin (n=7-9), and **(B)** post-synaptic density-95 protein (PSD95) (n=7-8). Immunocontent of **(C)** iNOS (n=7-9), **(D)** phospho-GSK-3β/GSK-3β ratio (n=6), and **(E)** phospho-Tau/Tau ratio (n=8-9). Data are expressed as the average percentage of control. Representative quantification of proteins immunocontent normalized to b-actin protein (loading control) is shown below the graphs. A two-way ANOVA showed interaction between maternal exercise and AβO infusion for phospho-Tau/Tau ratio (°p<0.05). *P<0.05 effect of maternal exercise (two-way ANOVA), ##P<0.01 effect of AβO infusion (two-way ANOVA).

Figure 1

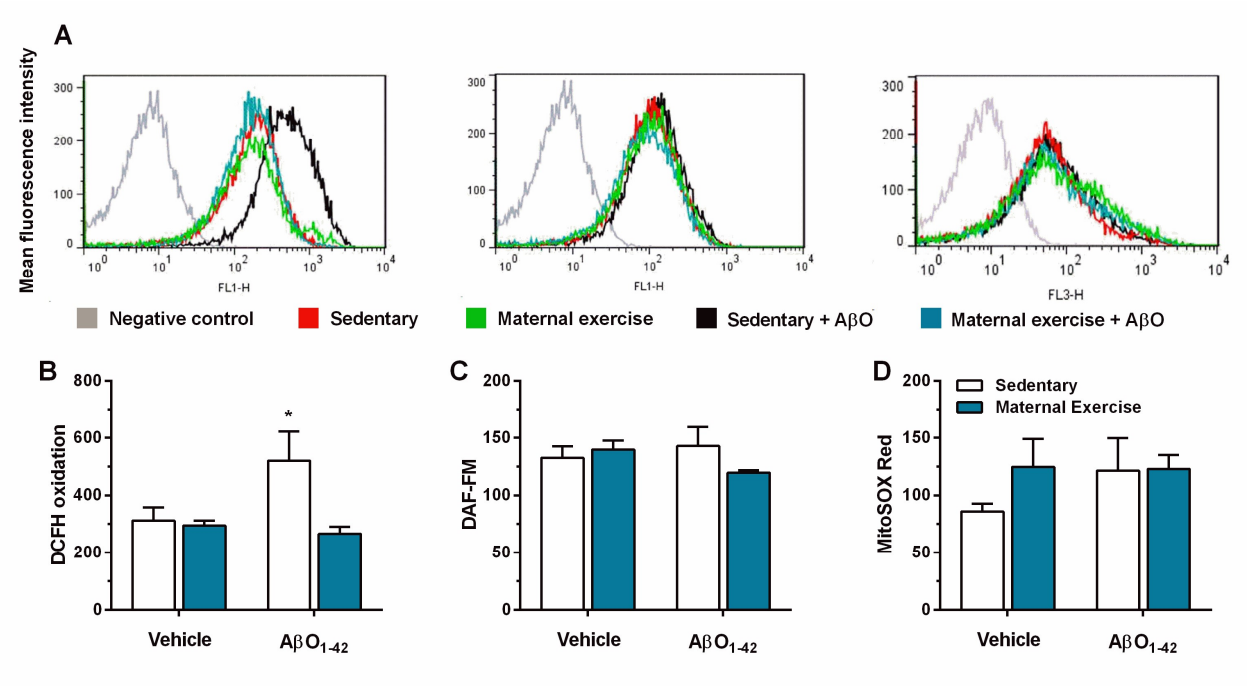

A
Mean fluorescence intensity
FL1-H
FL1-H
FL3-H
Negative control
Sedentary
Maternal exercise
Sedentary + AβO
Maternal exercise + AβO
B
DCFH oxidation
Vehicle
AβO1-42
C
DAF-FM
Vehicle
AβO1-42
D
MitoSOX Red
Sedentary
Maternal Exercise
Vehicle
AβO1-42

Figure 2

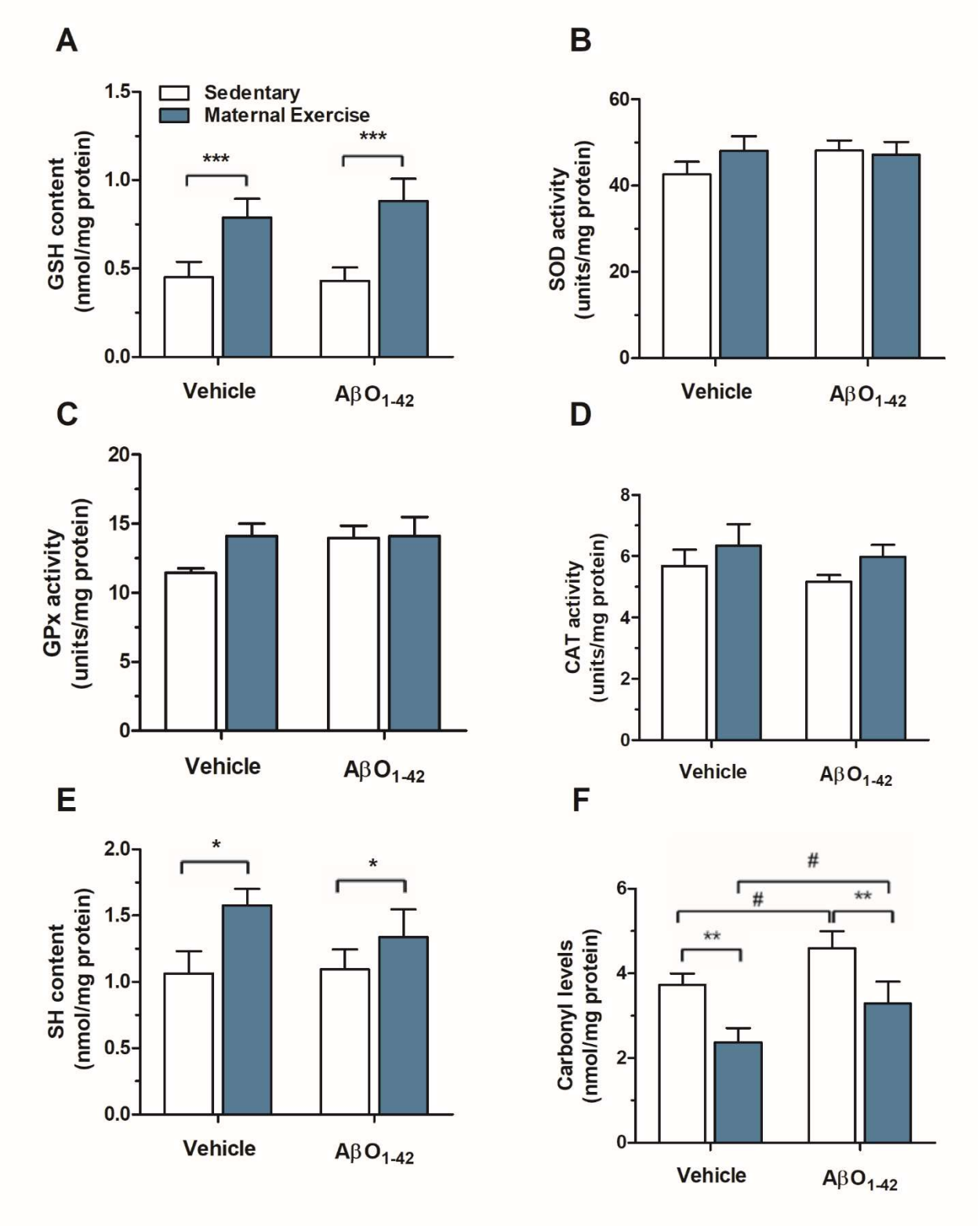

A
Sedentary
Maternal Exercise
GSH content (nmol/mg protein)
1.5
1.0
0.5
0.0
***
***
Vehicle
AβO1-42
B
SOD activity (units/mg protein)
60
40
20
0
Vehicle
AβO1-42
C
GPx activity (units/mg protein)
20
15
10
5
0
Vehicle
AβO1-42
D
CAT activity (units/mg protein)
8
6
4
2
0
Vehicle
AβO1-42
E
SH content (nmol/mg protein)
2.0
1.5
1.0
0.5
0.0
*
*
Vehicle
AβO1-42
F
Carbonyl levels (nmol/mg protein)
6
4
2
0
#
#
**
**
Vehicle
AβO1-42

Figure 3

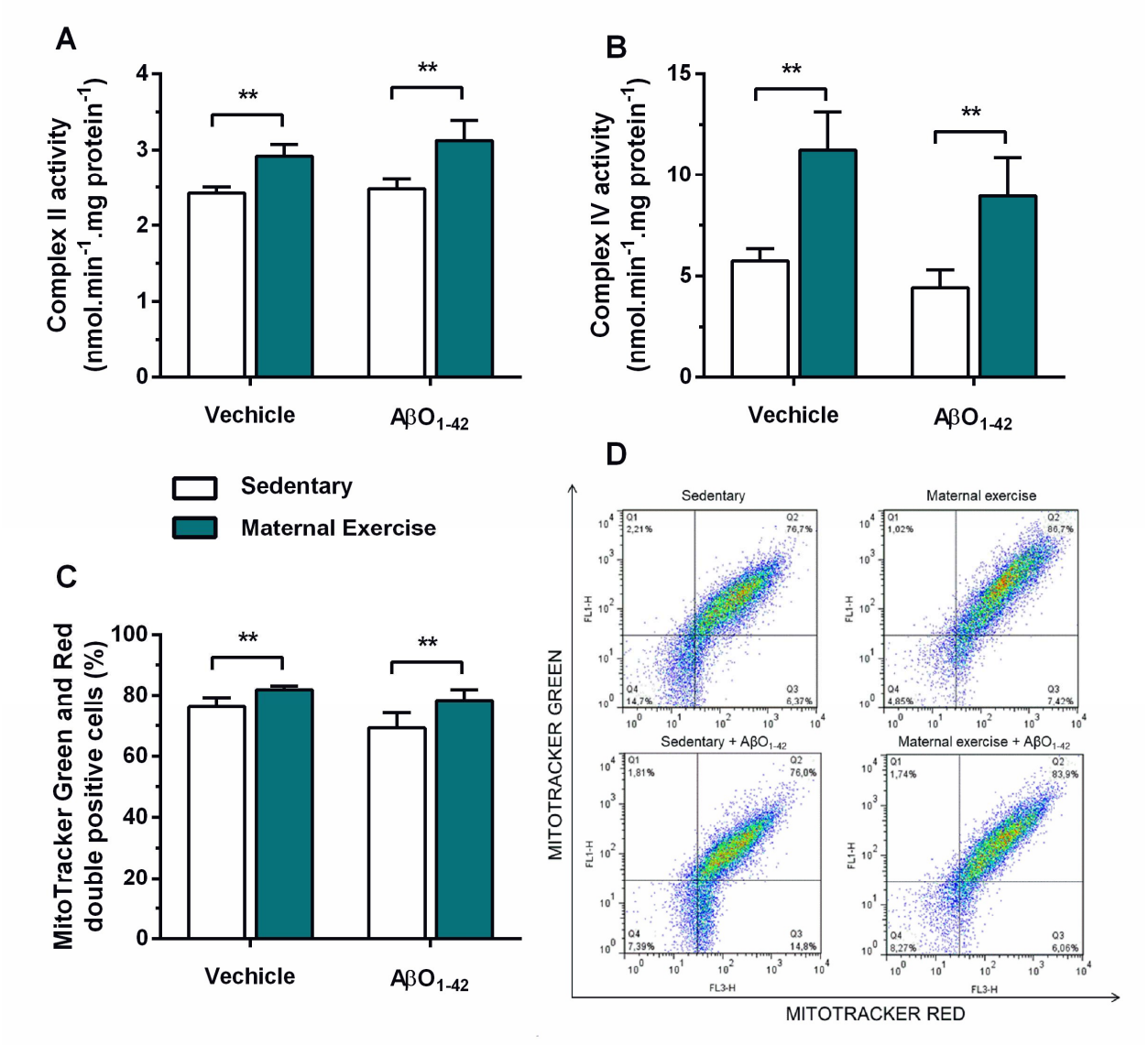

A
Complex II activity (nmol.min-1.mg protein-1)
**
**
Vechicle
AβO1-42
B
Complex IV activity (nmol.min-1.mg protein-1)
**
**
Vechicle
AβO1-42
Sedentary
Maternal Exercise
C
MitoTracker Green and Red double positive cells (%)
**
**
Vechicle
AβO1-42
D
Sedentary
Maternal exercise
Sedentary + AβO1-42
Maternal exercise + AβO1-42
MITOTRACKER GREEN
MITOTRACKER RED
FL1-H
FL3-H

Figure 4

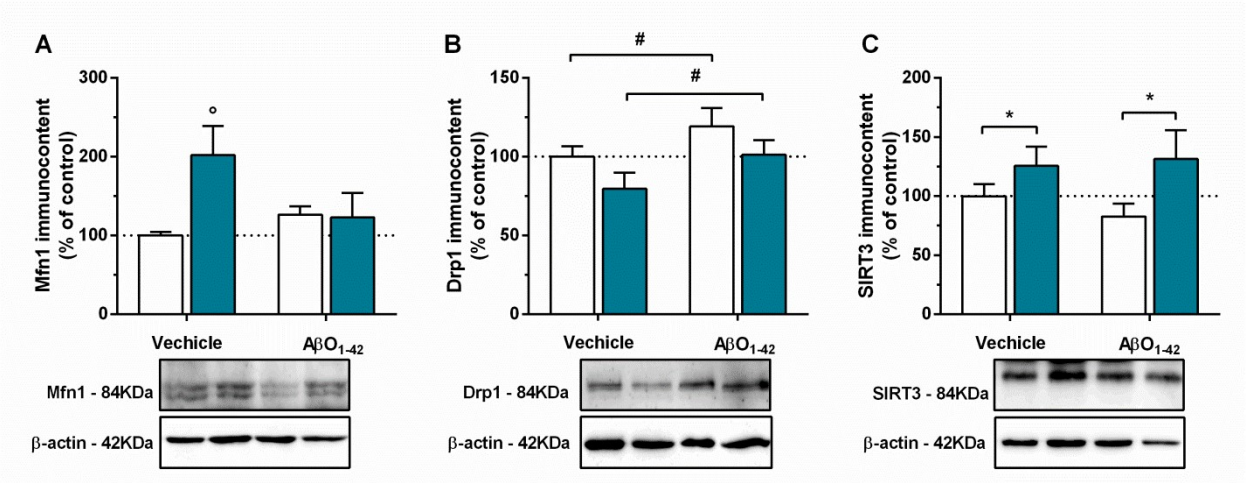
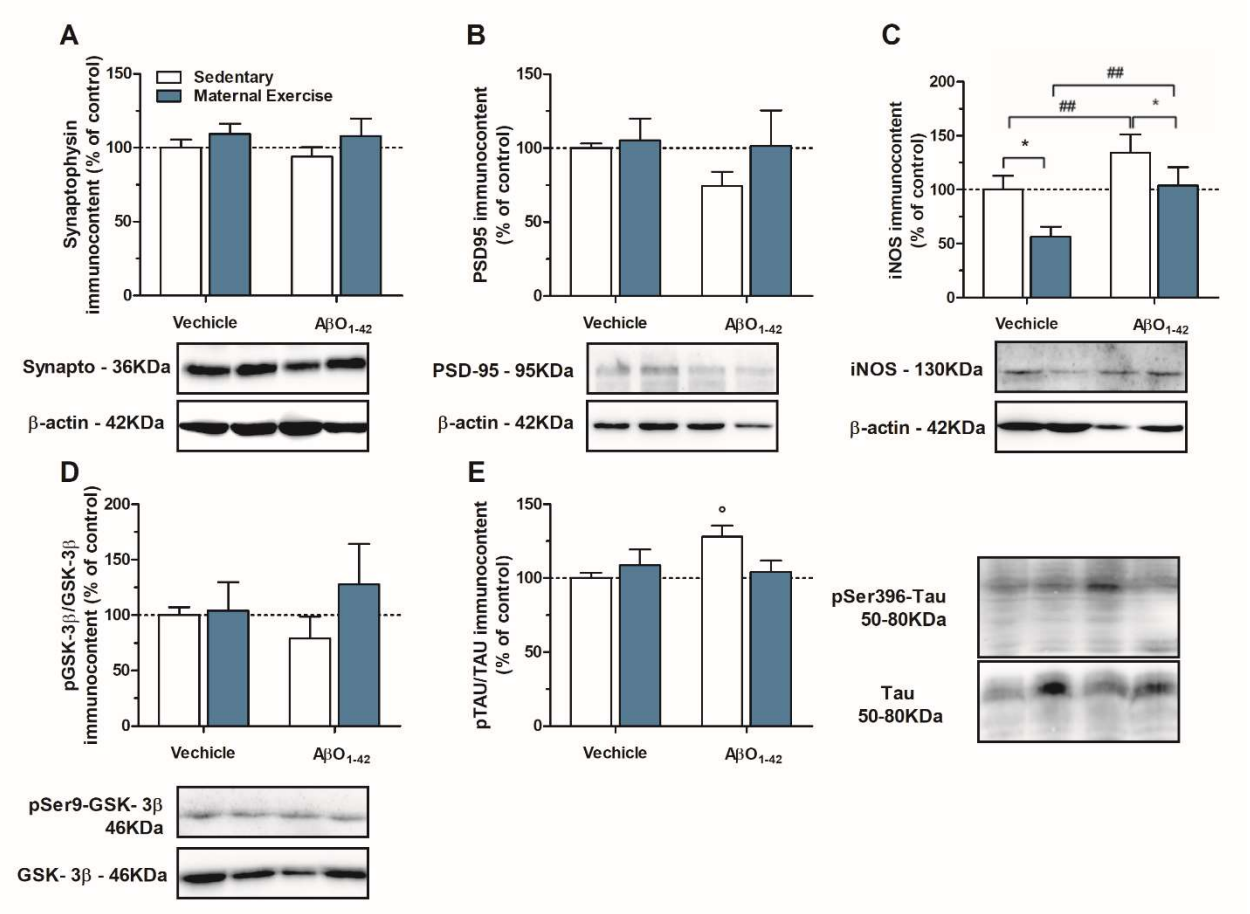

A
Mfn1 immunocontent (% of control)
300
200
100
0
°
Vechicle
AβO1-42
Mfn1 - 84KDa
β-actin - 42KDa
B
Drp1 immunocontent (% of control)
150
100
50
0
#
#
Vechicle
AβO1-42
Drp1 - 84KDa
β-actin - 42KDa
C
SIRT3 immunocontent (% of control)
200
150
100
50
0
*
*
Vechicle
AβO1-42
SIRT3 - 84KDa
β-actin - 42KDa

Figure 5


A
Synaptophysin immunocontent (% of control)
Sedentary
Maternal Exercise
Vechicle
AβO1-42
Synapto - 36KDa
β-actin - 42KDa
B
PSD95 immunocontent (% of control)
PSD-95 - 95KDa
β-actin - 42KDa
C
iNOS immunocontent (% of control)
iNOS - 130KDa
β-actin - 42KDa
D
pGSK-3β/GSK-3β immunocontent (% of control)
pSer9-GSK- 3β 46KDa
GSK- 3β - 46KDa
E
pTAU/TAU immunocontent (% of control)
pSer396-Tau 50-80KDa
Tau 50-80KDa

PARTE III

Discussão

DISCUSSÃO

No contexto atual de saúde pública é imprescindível o desenvolvimento e implementação de estratégias preventivas e terapêuticas para a ampla gama de doenças que acometem a população global. Ao longo das últimas décadas, o avanço da medicina juntamente com melhorias sanitárias permitiu um aumento significativo na expectativa de vida dos indivíduos pelo mundo todo. No entanto, o estilo de vida atual que vem acompanhando os avanços médicos, tecnológicos e socioeconômicos é caracterizado, principalmente, por hábitos como a inatividade física e o consumo de alimentos industrializados, com alto teor de açúcares, gorduras saturadas e conservantes, que, em conjunto, são fatores de risco para o desenvolvimento de diversas doenças crônicas não transmissíveis (Schmidt *et al.*, 2011; Hunter e Reddy, 2013; Hui, 2017). Dessa forma, a população vivencia um processo de envelhecimento que está associado à redução da qualidade de vida devido à alta prevalência de doenças e à falta de tratamentos eficientes para algumas delas.

Desde recentemente, o interesse pelo entendimento das bases desenvolvimentistas da saúde e da doença tem crescido entre a comunidade científica. A ideia por trás do estabelecimento do conceito da *Developmental Origins of Health and Disease* (DOHaD) se baseia na influência que o ambiente materno exerce sobre o desenvolvimento do organismo, durante os períodos intrauterino e pós-natal, ao promover modificações permanentes no metabolismo, as quais podem alterar a susceptibilidade ao desenvolvimento de doenças ao longo da vida (Hanson e Gluckman, 2014). Sendo assim, a programação metabólica do organismo em desenvolvimento em prol da promoção da resistência contra doenças ao modificar o estilo de vida materno surge como uma promissora estratégia preventiva ao desenvolvimento de doenças ao longo da vida da prole. Stephenson *et al.* (2018) ressaltam a importância de intervenções pré-concepcionais que focam na melhora da saúde da mãe e da prole como medidas necessárias para reduzir a incidência de doenças crônicas não transmissíveis.

Diante do exposto acima, o objetivo proposto nesta tese foi investigar o potencial neuroprotetor do exercício físico materno sobre alterações comportamentais e neuroquímicas encontradas em um modelo *in vivo* da doença de Alzheimer na prole de ratas Wistar. Nesta perspectiva, a presente tese tem como meta acrescentar dados relevantes à literatura e promover o avanço científico contribuindo para o conhecimento dos efeitos do exercício físico durante a gestação, especificamente relativo às adaptações metabólicas geradas, que podem ser capazes de afetar a vida da progênie logo após o nascimento e em longo prazo, inclusive prevenindo o desenvolvimento de doenças crônicas, como as doenças neurodegenerativas. Além disso, espera-se com esta tese elucidar os possíveis mecanismos envolvidos no potencial papel neuroprotetor do exercício materno, avaliando algumas das vias de sinalização que podem mediar esses efeitos na prole.

Nessa pesquisa, utilizamos ratos Wistar (*Rattus norvegicus*) fêmeas adultas (80 dias de idade) que foram submetidas a um protocolo de exercício físico, a natação, antes e durante a gestação. O protocolo de natação materna, estabelecido pelo nosso laboratório de pesquisa, consiste em um período de 4 semanas, com frequência de 5 vezes por semana, duração de 30 minutos por dia, em água com temperatura controlada para o conforto térmico dos animais (Marcelino *et al.*, 2013). As 4 semanas de exercício compreendem dois períodos: pré-concepcional e gestacional. O período pré-concepcional se refere à semana anterior ao acasalamento com machos adultos (80 dias de idade), para adaptação dos animais ao ambiente aquático, enquanto o período gestacional se refere às três semanas de gestação das ratas. As proles das ratas exercitadas e das ratas que não foram exercitadas foram utilizadas para as investigações objetivadas nessa pesquisa.

Primeiramente, no capítulo 1 desta tese, nos propusemos a investigar se o comportamento materno, a relação mãe-filhote e parâmetros gestacionais são modificados pelo protocolo de exercício ao qual as ratas foram submetidas. Além disso, investigamos se o exercício materno é capaz de alterar o aparecimento das características físicas dos filhotes de ambos os sexos, bem

como de alterar o desenvolvimento de reflexos neuromotores e a ontogenia do comportamento motor dos filhotes. O estudo que originou o capítulo 1 foi realizado com a proposta de demonstrar a validade do modelo de natação materna em ratas como ferramenta experimental para investigar os efeitos benéficos à saúde da prole promovidos pelo exercício materno.

Em humanos, a prática de exercício durante a gestação é associada à melhora da saúde da mãe e da prole, desde a vida *in utero* até a vida adulta, como resultado de adaptações fisiológicas. Extensivos trabalhos conduzidos por James F. Clapp e colaboradores testaram a hipótese de que a prática continuada de exercício durante a gestação pode alterar parâmetros gestacionais e os perfis morfométrico e neurocomportamental da prole (Clapp, 1996a; b; Clapp *et al.*, 1998; Clapp *et al.*, 2000). O exercício materno melhora a capacidade funcional da placenta estando relacionado ao aumento da entrega de nutrientes ao feto, enquanto que o ganho de peso gestacional, a idade gestacional e a média do consumo calórico durante a gestação foi similar entre mulheres exercitadas e não exercitadas (Clapp *et al.*, 2000). Em adição, mulheres do grupo exercício materno apresentam taxa de aborto espontâneo (15% *vs* 17% no grupo controle) e nascimento prematuro (7% *vs* 8% no grupo controle) similar ao grupo controle (Clapp, 1996a). Muitos dos resultados relacionados aos parâmetros gestacionais apresentados nesta tese estão em concordância com os resultados supracitados encontrados em humanos. Os resultados apresentados no capítulo 1 demonstram que o exercício involuntário de ratas durante a gestação não altera parâmetros gestacionais como a taxa de prenhez, peso gestacional, ganho de peso, consumo de ração, número de filhotes nascidos vivos e número de filhotes machos e fêmeas (Capítulo 1/*Figure 1 and Table 1*) e que o comportamento da mãe em relação aos seus filhotes em comparação às ratas que não foram exercitadas não diferiram entre os grupos (Capítulo 1/*Figure 3*). Outros estudos em animais demonstram que o exercício materno não altera o ganho de peso das ratas gestantes (Sheldon *et al.*, 2016; Ribeiro *et al.*, 2017; Ferrari *et al.*, 2018) nem mesmo afeta o tamanho da ninhada ou a distribuição dos sexos (Marcelino *et al.*, 2013; Santana Muniz *et al.*, 2014; Stanford *et al.*, 2015; Sheldon *et al.*, 2016).

A literatura demonstra dados controversos quanto à influência do exercício materno sobre o peso da prole. Essas diferenças podem ser decorrentes das diferenças entre intensidade, duração, frequência e modalidade do exercício realizado nos estudos. Enquanto que alguns pesquisadores demonstraram que o exercício materno altera o peso da prole ao nascer em humanos (Hatch *et al.*, 1993; Clapp, 1996a; b; Clapp *et al.*, 2000; Hopkins *et al.*, 2010) e animais (Pinto e Shetty, 1995; Wasinski *et al.*, 2015; Eclarinal *et al.*, 2016), outros demonstraram que o exercício materno não altera esse parâmetro em humanos (Nieuwenhuijsen *et al.*, 2002; Leet e Flick, 2003; Barakat *et al.*, 2009; Haakstad e Bo, 2011; Da Silva, Hallal*, et al.*, 2017; Da Silva, Ricardo*, et al.*, 2017; Watson *et al.*, 2018) ou animais (Akhavan *et al.*, 2008; Fidalgo *et al.*, 2013; Ribeiro *et al.*, 2017). É importante mencionar que, nos estudos onde o exercício materno alterou o peso ao nascerem, os valores permaneceram dentro da área de variação de peso considerado normal (Clapp, 1996a; Barakat *et al.*, 2009). Na presente tese demonstramos que a natação materna, em regime de frequência e duração regular, não alterou o peso corporal da prole tanto ao nascer como ao longo dos dias pós-natal (Capítulo 1/*Figure 2*). De forma similar aos nossos resultados de peso ao longo dos dias pós-natais, os resultados de outros estudos com animais concordam quanto à ausência de diferença de peso corporal da prole em diferentes idades, embora diferentes modalidades de exercício tenham sido utilizadas (Fidalgo *et al.*, 2013; Eclarinal *et al.*, 2016; Ribeiro *et al.*, 2017; Ferrari *et al.*, 2018). A diferença de peso ao nascer observada por Eclarinal *et al.* (2016) não foi mantida ao longo dos dias pós-natais 21, 60, 120, 160 e 300 entre a prole de ratas exercitadas e não exercitadas.

O desenvolvimento neuromotor está intimamente relacionado com a maturação do SNC e o curso normal do desenvolvimento neurológico do neonato pode ser avaliado através dos reflexos (Smart e Dobbing, 1971; Nguyen *et al.*, 2017). Em roedores, essa abordagem é amplamente empregada para avaliar a toxicidade durante o desenvolvimento da prole pela exposição a determinadas substâncias em modelos gestacionais (Slamberova *et al.*, 2006; Khalki *et al.*, 2012; Li *et al.*, 2014; Cheng *et al.*, 2015) e, recentemente, tem sido empregada para demonstrar a

segurança da mudança do estilo de vida materno sobre o desenvolvimento da prole (Falcao-Tebas *et al.*, 2012; Soares *et al.*, 2014; Cardenas *et al.*, 2015). A maturação do cérebro é acompanhada pelo aparecimento dos reflexos posturais, avaliados em roedores por tarefas como o endireitamento de superfície e a geotaxia negativa (Altman e Sudarshan, 1975). No capítulo 1, demonstramos ainda que o aparecimento/maturação das características físicas da prole ao longo dos dias pós-natais (Capítulo 1/*Supp. Table 1*), como a erupção dos incisivos superiores, a cobertura total do corpo por pelos, a abertura dos ouvidos e dos olhos, abertura vaginal, em fêmeas, e deiscência dos testículos, em machos, não diferiram entre os grupos. Além disso, não foi observada diferença entre os grupos quanto à redução do tempo para executar as tarefas de avaliação dos reflexos de desenvolvimento neuromotor (Capítulo 1/*Figure 4*), como o endireitamento de superfície, a geotaxia negativa, a aversão à queda e a suspensão em barra. Pouquíssimos estudos investigaram os efeitos do exercício materno sobre o aparecimento das características físicas e o desenvolvimento dos reflexos neuromotores da prole (Falcao-Tebas *et al.*, 2012; Santana Muniz *et al.*, 2014; Fragoso *et al.*, 2017). Os resultados apresentados no capítulo 1 corroboram os achados demonstrados por Falcao-Tebas *et al.* (2012), que demonstraram que o exercício materno involuntário em esteira, antes e durante a gestação, não alterou nenhum dos parâmetros de maturação das características físicas e de desenvolvimento neuromotor da prole e que foi capaz de prevenir atraso de desenvolvimento provocado por dieta materna hipoproteica. Da mesma forma, Fragoso *et al.* (2017) demonstraram que o exercício materno voluntário em rodas de correr, antes e durante a gestação, não alterou nenhum dos parâmetros de maturação das características físicas e de desenvolvimento neuromotor. Por outro lado, o estudo publicado por Santana Muniz *et al.* (2014) demonstrou que diferentes níveis de atividade física materna, categorizados com base no tempo gasto pelas ratas em rodas de correr [inativas ($\leq$ 20 min/dia), ativas (> 20 e $\leq$ 120 min/dia) e muito ativas (> 120 min/dia)], atrasaram o desenvolvimento dos reflexos nos testes de endireitamento de superfície e aversão à queda, enquanto que características

físicas como o aparecimento dos incisivos inferiores e a abertura dos ouvidos maturaram antes na prole de ratas muito ativas (Santana Muniz *et al.*, 2014).

Visto a grande quantidade de dados gerados e a dificuldade de interpretação com a avaliação da ontogenia do desenvolvimento locomotor, a maioria dos estudos utiliza apenas os testes reflexos para avaliar o desenvolvimento neuromotor. No entanto, ótimas descrições da ontogenia locomotora estão presentes na literatura e, por isso, decidimos avaliar parâmetros relacionados com a ontogenia do desenvolvimento locomotor e descrevê-los (Capítulo 1/*Figures 5 and 6*). Demonstramos que o exercício materno não alterou a frequência do movimento lateral da cabeça, de elevação da cabeça, movimento de rastejar e girar em torno do próprio eixo, movimento de esticar o corpo com o suporte das patas dianteiras ao longo dos dias pós-natais. Por outro lado, algumas diferenças, possivelmente, ocorreram ao acaso entre os grupos e os sexos quanto à frequência de imobilidade, *grooming* e movimento de caminhar. Interessantemente, o movimento de esticar o corpo com suporte apenas das patas traseiras foi significativamente mais frequente entre a prole de ratas exercitadas durante a gestação em comparação com a prole de não exercitadas nos dias pós-natais 17 e 18. Tendo em vista que o movimento de esticar o corpo com suporte apenas das patas traseiras está relacionado à maturação do comportamento exploratório em roedores, cuja frequência tende a aumentar à medida que o animal alcança a idade adulta (Altman e Sudarshan, 1975), podemos concluir que o exercício materno foi capaz de melhorar o desenvolvimento neuromotor da prole, evidenciado pelo aumento da frequência do comportamento exploratório. De certa forma, nossos achados podem ser comparados com aqueles obtidos a parir de diferentes coortes, que avaliaram o desenvolvimento de crianças de 5 dias de idade, 1 e 5 anos de idade, cujas mães se exercitaram antes e durante a gestação (Clapp, 1996b; Clapp *et al.*, 1998; Clapp *et al.*, 1999). Em Clapp *et al.* (1999), neonatos apresentaram melhores escores de orientação e auto regulação na escala de *Brazelton Neonatal Behavioral Assessment*, 5 dias após o nascimento. Clapp *et al.* (1998) demonstraram que crianças de 1 ano de idade apresentaram melhor desenvolvimento neuromotor especialmente quanto às habilidades de

ambulação, porém desenvolvimento mental similar às crianças de mães controle, com base na escala de *Bayley Scale of Infnat Development*. Clapp (1996b) demonstrou que, aos 5 anos de idade, a crianças apresentaram melhores resultados para inteligência geral e melhor habilidade de linguagem oral, com base na escala de *Wechsler*. Adicionalmente, Jukic *et al.* (2013) demonstraram que, aos 8 anos de idade, crianças nascidas de mães exercitadas durante a gestação apresentaram melhor desenvolvimento da linguagem e vocabulário.

Em conjunto, os resultados apresentados no Capítulo 1 ressaltam que o exercício físico realizado pela mãe durante a gestação não causa efeitos negativos ao desenvolvimento físico e neurocomportamental da prole, reforçando os dados apresentados em outros estudos de que o exercício durante a gestação deve ser recomendado devido aos seus efeitos benéficos à saúde da mãe e da prole, e possíveis efeitos protetores. Além disso, nossos resultados claramente suportam o fato de que o modelo de exercício materno utilizado na presente tese é uma ferramenta útil para a investigação dos efeitos do exercício materno sobre a prole e, ainda, que os resultados obtidos a partir da utilização desse modelo podem ser extrapolados, cautelosamente, para os humanos.

O cerebelo é uma região do encéfalo particularmente susceptível a insultos ambientais, visto que o início do seu período de desenvolvimento é precoce e se estende até o período pós-natal (Rice e Barone, 2000; Koning *et al.*, 2017). O desenvolvimento do cerebelo inicia por volta da quinta semana após a concepção, em humanos, e por volta do dia gestacional 12, em ratos (Rice e Barone, 2000). Alterações no desenvolvimento do cerebelo estão relacionadas a prejuízos da função motora, cognitiva e neurocomportamental (Limperopoulos *et al.*, 2007). Trabalhos anteriores, em humanos e roedores, demonstram evidências de que o cerebelo é altamente susceptível à exposição materna a substâncias como cigarro e álcool (Handmaker *et al.*, 2006; De Zeeuw *et al.*, 2012; Sharma e Hill, 2017), bisfenol A (Mathisen *et al.*, 2013), metil-mercúrio (Heimfarth *et al.*, 2018), ácido valproico (Dai *et al.*, 2017), e piretroides (Syed *et al.*, 2016), bem como à dieta materna hipoproteica (Ranade *et al.*, 2012). Além disso, estudos anteriores realizados

pelo nosso grupo de pesquisa indicam que o cerebelo parece ser a região cerebral da prole mais fortemente modulada pelo exercício materno (Marcelino *et al.*, 2013; Marcelino *et al.*, 2015).

Portanto, no capítulo 2, decidimos investigar algumas vias de sinalização que podem ser as responsáveis pelos efeitos adaptativos do exercício materno no cerebelo da prole em dois períodos do desenvolvimento, no dia embrionário 20 e no dia pós-natal 7. Durante o período de desenvolvimento, a GSK está envolvida em múltiplos aspectos do desenvolvimento do sistema neural, como autorrenovação das progenitoras neurais, neurogênese, migração, diferenciação, morfogênese, crescimento dos neuritos e desenvolvimento sináptico (Kim e Snider, 2011). Em concordância, camundongos heterozigotos para a GSK-3β apresentam anormalidades no desenvolvimento (Hoeflich et al., 2000). A GSK regula várias vias de sinalização que estão envolvidas na regulação temporal e espacial do término da proliferação e início da diferenciação, de forma a controlar o número correto de neurônios a serem formados (Kim et al., 2009). A GSK-3β fosforila a β-catenina, o que leva a sua degradação e, dessa forma, promove a diminuição da proliferação e gera o estímulo para a diferenciação neuronal (Kim et al., 2009). Além disso, a GSK coordena eventos como a polaridade neuronal, crescimento e ramificação axonal ao mediar a reorganização dos microtúbulos do citoesqueleto (Hur e Zhou, 2010; Kim et al., 2011). Nós demonstramos que o exercício materno ativa a via da Akt/GSK-3β no cerebelo da prole aos 7 dias pós-natal, enquanto nenhuma alteração foi observada no dia embrionário 20 (Capítulo 2/Figure 1). A atividade reduzida da Akt e o consequente aumento da atividade da GSK-3β no cerebelo pode resultar em diferenciação neuronal, o que é considerado benéfico durante o período de desenvolvimento, podendo conferir uma futura neuroproteção. Nós ainda demonstramos, no capitulo 2, que o exercício materno regula positivamente os níveis das proteínas SIRT1 e SIRT3 no cerebelo dos filhotes (Capítulo 2/Figure 2), sem alterar os níveis de BDNF maduro e o imunoconteúdo de Mfn1, Drp1 ou TFAM. Os níveis aumentados de SIRT1 e 3 explicam os resultados publicados previamente pelo nosso grupo de pesquisa, onde foi observado um aumento da rede antioxidante e estímulo da biogênese mitocondrial (Marcelino *et al.*, 2013). A SIRT1 ativa

o PGC-1α, que é o principal regulador da biogênese mitocondrial, e as proteínas FOXO, que podem estar funcionalmente mais ativas, regulando o aumento da expressão de enzimas antioxidantes. A SIRT3 regula a expressão da enzima SOD2 e pode resultar no aumento da sua atividade. Além disso, a SIRT3 regula indiretamente a ativação de PGC-1α (Brenmoehl e Hoeflich, 2013).

Os resultados apresentados nos dois capítulos anteriores reforçam a ideia de que o exercício materno não oferece risco adverso ao desenvolvimento da prole, e inclusive melhora o desenvolvimento neuromotor quanto ao comportamento exploratório da prole, bem como indicam que a modificação do ambiente intrauterino ocasionada pela natação materna promove adaptações metabólicas positivas relacionadas ao metabolismo energético.

Diferentes estudos na literatura indicam o potencial que o exercício materno apresenta na prevenção de diversas patologias. Estudos em modelos animais têm demonstrado que o exercício materno melhora a saúde da prole ao promover adaptações metabólicas que conferem resistência ao organismo frente a um estímulo danoso (Harris *et al.*, 2018).

Os benefícios do exercício na população geral já são bem estabelecidos. Além de promover o bem-estar, a prática de exercício tem a capacidade de prevenir doenças, e diminuir os sintomas e a progressão de doenças quando as mesmas já estão estabelecidas. Esses efeitos observados são atribuídos à capacidade adaptativa do organismo, especialmente do sistema neurobiológico, já que o SNC exerce controle sobre os sistemas mental, fisiológico e comportamental (Dishman, 2006). O risco para a perda das habilidades cognitivas e para o desenvolvimento de doenças cardiovasculares, diabetes, e obesidade são negativamente correlacionados com a prática de exercício (Kramer *et al.*, 2005; Laskowski e Lexell, 2012). Por sua vez, a neurobiologia do exercício tem sido extensivamente estudada nas últimas décadas e o exercício tem despontado como uma estratégia neuroprotetora capaz de reverter o curso da progressão de doenças neurodegenerativas em humanos e animais.

Tendo como base: 1) os indícios de que o SNC responde, de forma adaptativa, aos desafios energéticos provocados pelo exercício, melhorando a capacidade cognitiva e prevenindo alterações metabólicas presentes da DA, e 2) os fundamentos da DOHaD associados a neuromodulação promovida pelo exercício materno em beneficio à saúde da prole, como a melhora da memória de reconhecimento e a modulação positiva do estado redox e da função mitocondrial; decidimos ampliar a nossa pesquisa na área de programação metabólica com o intuito de avaliar o potencial neuroprotetor do exercício materno no encéfalo da prole, frente a um estímulo sabidamente neurotóxico. Dessa forma, escolhemos o modelo de neurotoxicidade induzida pelo peptídeo AβO, cujo acúmulo no SNC é uma característica patológica determinante na DA. O efeito neuroprotetor do exercício materno contra a neurotoxicidade induzida pela infusão icv de oligômeros de peptídeo Aβ (500 pmol/rat) na prole adulta foi investigado em diferentes estruturas encefálicas, como o córtex pré-frontal e o hipocampo, cujos resultados estão apresentados no capítulo 3, e o cerebelo, cujos resultados estão apresentados no capítulo 4. A caracterização estrutural do peptídeo Aβ foi realizada utilizando o anticorpo específico Aβ 1-16 6E10 (Covance) através da técnica de Western blotting (Figura 4).

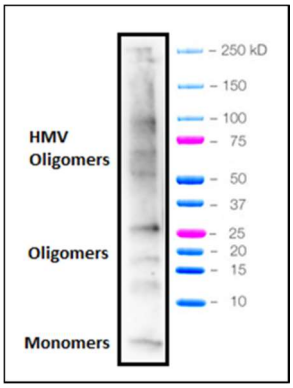


Figura 4. Caracterização estrutural do peptídeo Aβ.

A forma esporádica da DA, no contexto epidemiológico, é a desordem neurodegenerativa associada ao envelhecimento, com uma taxa de incidência crescente. Clinicamente, um indivíduo diagnosticado com a DA apresenta sinais de demência, que iniciam com perda da memória, déficit cognitivo progressivo, relações sociais e funções intelectuais prejudicadas, mudança de

personalidade e, por fim, perda das habilidades físico-motoras (www.alz.org). Essa mudança de comportamento está relacionada ao grau de progressão da doença e ao grau de degeneração do cérebro, especialmente nas regiões responsáveis pelo aprendizado e memória. Tem sido amplamente sugerido que o peptídeo AβO se acumula tanto no compartimento extracelular como no intracelular. Intracelularmente, os AβOs podem interagir com moléculas-chave do metabolismo celular e da transmissão sináptica e, portanto, causa modificações na sinalização celular e na neurotransmissão. Oligômeros solúveis de Aβ são substâncias neurotóxicas que se acumulam no cérebro de pacientes com DA em decorrência do processamento exacerbado da APP pelas enzimas β- e γ-secretase, ou são utilizados para mimetizar a patologia em modelos animais (Selkoe, 2008; Querfurth e Laferla, 2010). Uma vez que os AβO se acumulam, eles causam modificações celulares e moleculares que culminam na manifestação dos déficits cognitivos e que progressivamente causa neurodegeneração (Hu *et al.*, 2008; Reddy *et al.*, 2010), primariamente no hipocampo e córtex pré-frontal (Venkateshappa *et al.*, 2012). O córtex pré-frontal e o hipocampo foram estudados no capítulo 3, e essa escolha se deve ao fato de que eles são as estruturas encefálicas mais afetadas na DA com considerável perda da massa cerebral, e as suas funções estão relacionadas aos processos de aprendizado e memória. A injeção icv de AβO no cérebro de roedores é um modelo reconhecido para mimetizar a DA. No presente estudo, nós injetamos AβO1-42 nos ventrículos cerebrais de ratos nascidos de ratas exercitadas ou sedentárias a fim de obter um modelo de patologia tipo Alzheimer com os achados fisiopatológicos da DA.

No capítulo 3, nós inicialmente avaliamos os efeitos comportamentais da prole, nascida de ratas exercitadas ou sedentárias durante a gestação, em um modelo similar a DA, induzido pela infusão icv de AβOs, nos testes de campo aberto, reconhecimento de objetos e labirinto aquático de Morris. Nós demonstramos que o exercício materno ou a infusão de AβOs não alterou o comportamento de locomoção, exploração e do tipo ansioso dos animais, avaliados no teste de campo aberto (Capítulo 3/*Table 1*). Outros trabalhos também observaram que a infusão icv de peptídeo Aβs, oligomérico ou fibrilar, não altera o comportamento locomotor dos animais (Lu *et*

al., 2009; Li *et al.*, 2010; Prado Lima *et al.*, 2018; Rosa *et al.*, 2018; Souza *et al.*, 2018). Em contrapartida, a infusão de AβOs causou um prejuízo tanto na memória de reconhecimento quanto no aprendizado de novas informações na fase de aquisição do teste do labirinto aquático de Morris e na memória espacial de curto e longo prazo (memória de referência e de trabalho) dos animais (Capítulo 3/*Figures 2 and 3*), testados no labirinto aquático de Morris, o que está de acordo com outros estudos em camundongos (Jiang *et al.*, 2016) e ratos (Mclarnon e Ryu, 2008; Rammes *et al.*, 2015). De forma interessante, o exercício materno durante a gestação foi capaz de prevenir os prejuízos causados pelo peptídeo nos testes de comportamento. Além disso, os efeitos positivos do exercício materno sobre a memória de reconhecimento já foram descritos anteriormente (Robinson e Bucci, 2014; Marcelino *et al.*, 2016), bem como sobre o aprendizado e memória no teste do labirinto aquático de Morris (Akhavan *et al.*, 2008), no teste da esquiva inibitória (Lee *et al.*, 2006; Kim *et al.*, 2007), e no teste do labirinto em T (Parnpiansil *et al.*, 2003). O estudo conduzido por Akhavan *et al.* (2008) avaliou o efeito do exercício materno voluntário, em rodas de correr, e involuntário, natação, na prole aos 29 dias de idade e demonstrou que ambas as modalidades empregadas de exercício materno foram capazes de melhorar significativamente o aprendizado, mas somente a natação materna melhorou a retenção da memória no labirinto aquático de Morris.

O hipocampo e o córtex pré-frontal estão funcionalmente conectados durante as tarefas de memória episódica através da interface de informação fornecida pelo núcleo medial do tálamo (Vertes, 2006). Sendo assim, a memória de reconhecimento depende da integridade da interação bidirecional entre o hipocampo e o córtex pré-frontal (Place *et al.*, 2016) e, por isso, determinamos os níveis de BDNF nessas regiões. O BDNF maduro é uma neurotrofina importante para os processos de aprendizado e memória, e encontra-se reduzido na DA (Bekinschtein *et al.*, 2008). Nós demonstramos que a infusão com ABOs induziu uma redução nos níveis de BDNF no hipocampo da prole de ratas sedentárias e exercitadas, indicando que o exercício materno não foi capaz de prevenir essa alteração (Capítulo 3/*Figure 4*). Embora alguns experimentos com

exercício físico demonstrem um aumento no níveis de BDNF e seu RNAm no hipocampo da prole de ratas exercitadas durante a gestação (Parnpiansil *et al.*, 2003; Kim *et al.*, 2007; Aksu *et al.*, 2012; Gomes Da Silva *et al.*, 2016), nossos resultados indicaram que os níveis de BDNF maduro no córtex pré-frontal e no hipocampo permaneceram similares aos níveis do grupo de ratas não exercitadas. Os dados apresentados na presente tese estão de acordo com um trabalho previamente publicado pelo nosso grupo de pesquisa, onde foi demonstrado que a natação materna antes e durante a gestação não exerce influência sobre os níveis de BDNF maduro no cerebelo, estriado e córtex parietal da prole adulta (PN 60) (Marcelino *et al.*, 2016). Parnpiansil *et al.* (2003) demonstraram que a prole de ratas exercitadas em esteira, apenas durante a gestação, apresentou um aumento da expressão hipocampal do RNAm de BDNF no PN 0, os quais retornaram aos níveis controle no PN 14 e no PN 47, e diminuíram no PN 28, enquanto que Kim *et al.* (2007) demonstraram um aumento no RNAm de BDNF na prole, no PN 29, de ratas que foram exercitadas em esteira na última semana de gestação. Gomes Da Silva *et al.* (2016) demonstraram que os níveis aumentados de BDNF no hipocampo, mas não no córtex, foi associado a melhora do desempenho da prole adulta (PN 60), cujas mães foram exercitadas em esteira apenas durante a gestação, após a realização das tarefas comportamentais. É importante considerar que o BDNF é inicialmente sintetizado como uma proteína precursora, pro-BDNF, que, quando clivada, libera a proteína madura, BDNF maduro, pois as funções da proteína precursora e proteína madura são diferentes e antagonistas. O BDNF maduro regula a plasticidade sináptica e sinaptogênese, e é importante para a sobrevivência, diferenciação e proliferação neuronal, enquanto o pro-BDNF parece estar envolvido em processos de apoptose neuronal e neurodegeneração (Lim, Y. *et al.*, 2015). Dessa forma, a medida do RNAm de BDNF não é proporcional a quantidade de BDNF maduro. A discordância entre os dados apesentados pode ser devido a abordagem metodológica aplicada e, portanto, às bases bioquímicas e moleculares para explicar essas diferenças encontradas entre os estudos ainda precisam ser elucidadas. Os resultados apresentados nesta tese quanto aos dados

obtidos a partir dos testes comportamentais e da medida dos níveis de BDNF indicam que a prevenção do prejuízo da memória induzido pelos AβOs é BDNF-independente.

A bioenergética do SNC é dependente da função mitocondrial e, por isso, os terminais sinápticos encontram-se enriquecidos dessas organelas para fornecer ATP e sustentar a liberação vesicular de neurotransmissores (Mergenthaler *et al.*, 2013; Cai e Tammineni, 2017). A distribuição neuronal de mitocôndrias nos dendritos é crítica para suprir a alta demanda metabólica. À medida que os dendritos crescem, o número de mitocôndrias aumenta no local e, dessa forma, a distribuição mitocondrial é essencial para a regulação da plasticidade e densidade sináptica (Li *et al.*, 2004). As alterações iniciais da DA estão relacionadas ao metabolismo energético, afetando o metabolismo da glicose, a sinalização pela insulina e a função mitocondrial, e, em paralelo, ocorre a ativação de vias inflamatórias e desequilíbrio do estado redox (Querfurth e Laferla, 2010; Abolhassani *et al.*, 2017). Ao se acumular na mitocôndria, os AβOs são capazes de interagir com enzimas metabólicas essenciais prejudicando suas funções e alterando a dinâmica mitocondrial (Manczak *et al.*, 2006; Kandimalla e Reddy, 2016). A redução da atividade de enzimas chave do TCA e do STE tem sido demonstrada em cérebro com patologia de Alzheimer (Bubber *et al.*, 2005; Abolhassani *et al.*, 2017). Essas alterações afetam o transporte axonal e a função sináptica que culminam em neurodegeneração e déficits cognitivos (Kapogiannis e Mattson, 2011). A redução dos níveis das proteínas de densidade pré- sináptica, como a sinaptofisina, e das proteínas de densidade pós-sináptica 95 (PSD 95) no cérebro de pacientes com a DA está diretamente correlacionada com a cognição (Terry *et al.*, 1991; Sze *et al.*, 1997; Selkoe, 2002). Na presente tese, foi demonstrado que a infusão com AβOs na dose de 500 pmol foi capaz de reduzir os níveis de sinaptofisina no hipocampo da prole de ratas sedentárias, enquanto que os níveis permaneceram inalterados no hipocampo da prole de ratas exercitadas, ressaltando o efeito neuroprotetor do exercício materno e explicando a performance cognitiva dos animais nas tarefas comportamentais (Capítulo 3/*Figure 5*).

A modulação do metabolismo bioenergético mitocondrial pelo exercício materno já foi evidenciada em vários estudos (Marcelino *et al.*, 2013; Park *et al.*, 2013; Chung *et al.*, 2017; Xu *et al.*, 2017), por isso o ponto chave do nosso trabalho, para desvendar os mecanismos responsáveis pelos achados descritos acima, foi investigar parâmetros de função mitocondrial. No capítulo 3, foi também demonstrado que o exercício materno induziu aumento do número de mitocôndrias funcionais no córtex pré-frontal e no hipocampo da prole adulta, indicado pelo aumento da massa e do potencial de membrana mitocondriais (Capítulo 3/*Figure 6*), o que, segundo Tal *et al.* (2009), sugere um aumento da função mitocondrial. O fato de termos encontrado um efeito significativamente positivo sobre a função mitocondrial no córtex pré-frontal e no hipocampo da prole adulta (PN 74) de ratas exercitadas durante a gestação indica que o efeito do exercício materno sobre o metabolismo cerebral da prole se estende até a idade adulta, já que, em um estudo prévio, nosso grupo de pesquisa demostrou que a natação materna durante a gestação induziu biogênese mitocondrial em várias regiões encefálicas da prole no PN 7 (Marcelino *et al.*, 2013). No presente estudo, os AβOs não induziram modificações na massa e no potencial de membrana mitocondriais. No entanto, ao avaliarmos a atividade das principais enzimas do TCA, observamos que os AβOs induziram uma redução significativa na atividade da enzima α-KGDH no hipocampo da prole de ratas sedentárias. Interessantemente, a redução da atividade hipocampal da enzima α-KGDH induzida pela injeção icv com AβOs foi prevenida pelo exercício materno durante a gestação (Capítulo 3/*Figure 7*). Por outro lado, o exercício materno durante a gestação induziu um aumento na atividade da α-KGDH no córtex pré-frontal da prole adulta, a qual não foi afetada pela injeção icv com AβOs. Além disso, a injeção com AβOs não alterou a atividade das enzimas IDH e MDH no hipocampo e no córtex pré-frontal da prole de ratas sedentárias ou exercitadas (dados não mostrados). Esses dados são consistentes com outros trabalhos, que demonstram uma redução significativa da atividade da α-KGDH em várias regiões do cérebro (Gibson *et al.*, 1999; Gibson *et al.*, 2005; Gibson *et al.*, 2012). Estudos também demonstram que os AβOs interagem com a enzima citocromo c oxidase do STE e alteram a sua atividade enzimática (Hernandez-Zimbron *et*

al., 2012; Shi *et al.*, 2016; Reddy *et al.*, 2018). No presente estudo, nós demonstramos que a atividade do complexo IV do STE permaneceu inalterada após a infusão com os AβOs e que o exercício materno promoveu um aumento significativo na atividade do complexo IV no hipocampo e córtex pré-frontal da prole, a qual não foi abolida pela administração de AβOs.

A disfunção mitocondrial promovida pelos AβOs e a consequente falha bioenergética pode ser acompanhada de fragmentação excessiva da mitocôndria observada pelo aumento dos níveis de Drp1 (Reddy *et al.*, 2018). Manczak *et al.* (2011) demonstraram que monômeros e oligômeros do peptídeo Aβ interagem com a Drp1; essa observação foi realizada por técnicas de imunocolocalização em amostra de cérebro *post-mortem* de pacientes com a DA e em camundongos transgênicos AβPP/PS1 que apresentam características patológicas da DA. Dentre os achados reportados pelos autores também estão a redução da expressão gênica de proteínas relacionadas com a fusão, como Mfn1 e 2 e a proteína atrofia ótica 1 (Opa1) (Manczak *et al.*, 2011). O desequilíbrio entre os processos de fusão e fissão mitocondrial favorece a fragmentação mitocondrial e, assim, ocorre o prejuízo do transporte mitocondrial e da função neuronal (Reddy *et al.*, 2011; Park *et al.*, 2015; Kandimalla e Reddy, 2016). Nós demonstramos no capítulo 3 que o exercício materno induziu um aumento de Mfn1 no córtex pré-frontal da prole e que os AβOs não alteraram o imunoconteúdo de Mfn1. Por outro lado, o exercício materno não alterou os níveis de Drp1 nem no córtex pré-frontal nem no hipocampo. No entanto, os AβOs induziram um aumento significativo no imunoconteúdo de Drp1, que foi prevenido pelo exercício materno (Capítulo 3/*Figure 8*). Em conjunto, os resultados apresentados no capítulo 3, análises neuroquímicas e comportamentais, indicam que a prevenção do prejuízo de memória pelo exercício materno durante a gestação na prole injetada com AβO pode ser decorrente da regulação positiva da função mitocondrial, a qual mantém o metabolismo oxidativo da glicose no cérebro e a função sináptica. Por outro lado, os déficits comportamentais provocados pelos AβO na prole de ratas sedentárias foram acompanhados por algumas alterações metabólicas, mas não pela massa e potencial de membrana mitocondrial. Esses dados sugerem que um mecanismo compensatório está

sendo operado pela mitocôndria para manter o suprimento energético do metabolismo cerebral. O mecanismo de falha energética, que ocorre por causa da desregulação metabólica na DA, já descrito anteriormente, indica que ocorre um aumento da fosforilação oxidativa, acompanhada pelo aumento de ER como mecanismo compensatório para manter a viabilidade celular (Demetrius e Simon, 2012; Demetrius e Driver, 2013; Demetrius *et al.*, 2014).

Na DA, a atrofia cerebelar está associada com o desempenho cognitivo (Thomann *et al.*, 2008) e alguns autores apontam para funções distintas do cerebelo (Noroozian, 2014), incluindo processos como memória de trabalho (Luis *et al.*, 2015) e memória motora (Lee *et al.*, 2015). Como descrito anteriormente, o cerebelo é uma região bastante susceptível às variações ambientais durante os períodos críticos de desenvolvimento. No capítulo 2 nós demonstramos que o cerebelo é programado diferencialmente pelo exercício materno. Com base nisso e nos achados apresentados no capítulo 3, nós decidimos investigar, no capítulo 4, se os efeitos benéficos do exercício materno se entendem até a idade adulta da prole, se a neurotoxicidade induzida pela infusão de AβOs afeta o metabolismo cerebelar e, por fim, se na presença de alterações, o exercício materno é capaz de prevenir contra possíveis alterações no cerebelo.

Embora pouca atenção tenha sido dada para o envolvimento do cerebelo na DA, características como a presença de placas amiloides, redução do metabolismo e densidade celular já foram observadas no córtex cerebelar de pacientes (Braak *et al.*, 1989; Fukutani *et al.*, 1997; Ishii *et al.*, 1997; Sjobeck e Englund, 2001; Thomann *et al.*, 2008; Guo *et al.*, 2016). Recentemente, uma rede de conexão cerebral cortical-cerebelar foi identificada em pacientes com demência, onde as regiões cerebelares estruturalmente e funcionalmente conectadas às regiões corticais com atrofia no cérebro de pacientes com DA também apresentam atrofia (Guo *et al.*, 2016). Esses achados evidenciam a vulnerabilidade do cerebelo à neurodegeneração e ressaltam o envolvimento do mesmo na patologia da DA. No capítulo 4, foram avaliados alguns parâmetros bioquímicos no cerebelo da prole de ratas sedentárias ou exercitadas com base na hipótese de que um desequilíbrio

no estado redox pode ser provocado pela infusão icv com AβOs nessa região como resultado da dispersão da patologia. Inicialmente, nós demonstramos que a programação do metabolismo cerebelar em resposta ao exercício materno permanece presente na idade adulta da prole. As proles de ratas exercitadas apresentaram conteúdo aumentado de GSH e de grupamentos tióis (SH), diminuição da carbonilação de proteínas, aumento da atividade das enzimas dos complexos II e IV do STE, bem como aumento do número de mitocôndrias funcionais representado pelo aumento da massa e potencial de membrana mitocondrial (Capítulo 4/*Figures 3 and 4*). A infusão de AβOs induziu um aumento dos níveis de ER derivadas de oxigênio e nitrogênio, embora não tenha induzido alterações nos níveis das espécies óxido nítrico ($NO^{\bullet}$) e de superóxido mitocondrial ($mO_2^{\bullet-}$), e esse aumento foi significativamente prevenido pelo exercício materno (Capítulo 4/*Figure 2*). Tanto o exercício materno quanto a infusão de AβOs não alteraram os parâmetros antioxidantes avaliados, ou seja, a atividade das enzimas SOD, CAT e GPx, e o conteúdo de GSH (Capítulo 4/*Figure 3*). Embora a administração dos AβOs tenha induzido um aumento na carbonilação de proteínas em ambos os grupos, os níveis de carbonilas no cerebelo da prole de ratas exercitadas permaneceu similar aos níveis do grupo controle, indicando que o exercício materno é capaz de atenuar o dano proteico frente ao estímulo neurotóxico já que os níveis basais de carbonilas no cerebelo da prole de ratas exercitadas estão diminuídos em relação ao grupo controle (Capítulo 4/*Figure 3*). Além disso, os AβOs não aboliram os efeitos benéficos promovidos pelo exercício materno sobre qualquer parâmetro descrito acima. Esses resultados indicam que a ausência dos efeitos neurotóxicos provocados pelos AβOs sobre os parâmetros redox no cerebelo da prole em comparação ao grupo controle pode ser atribuído ao período de tempo em que os parâmetros foram avaliados, uma vez que as características patológicas e as manifestações clínicas associadas ao cerebelo se tornam evidentes nos estágios mais avançados da DA (Thal *et al.*, 2002), e mesmo no córtex pré-frontal e no hipocampo de pacientes o sistema antioxidante está diminuído nos estágios avançado da DA (Venkateshappa *et al.*, 2012). Ao avaliar o imunoconteúdo de Mfn1 e Drp1, nós demonstramos que a Mfn1 aumentou no cerebelo da prole

de ratas exercitadas e que os AβOs aboliram esse efeito; por outro lado, os AβOs induziram um aumento de Drp1 em ambos os grupos (Capítulo 4/*Figure 5*). Nós verificamos que o exercício materno aumenta o imunoconteúdo de SIRT3 mitocondrial no cerebelo da prole no PN 7 (capítulo 2) e no capitulo 4 demonstramos que esse efeito permanece na prole adulta. Além disso, os AβOs não aboliram o efeito do exercício materno sobre os níveis de SIRT3.

As placas amiloides e os emaranhados neurofibrilares estão presentes no cerebelo de pacientes com DA (Braak *et al.*, 1989), e o acúmulo de placas amiloides já foram demonstradas no cerebelo de camundongos transgênicos APPswe/PS1dE9 de forma a prejudicar parâmetros eletrofisiológicos no córtex cerebelar desse animais (Kuwabara *et al.*, 2014). Esses achados dão suporte ao fato de que o cerebelo é também uma região cerebral susceptível a neurodegeneração na DA. As moléculas de sinalização podem ser mais sensíveis aos efeitos tóxicos dos AβOs em modelos animais (Bubber *et al.*, 2005; Shields *et al.*, 2015). Embora nós não tenhamos observado um aumento nos níveis de $NO^{\bullet}$, nós observamos que o exercício materno reduziu os níveis da enzima óxido-nítrico-sintase induzível (iNOS), enquanto que os AβOs induziram um aumento do imunoconteúdo da iNOS em ambos os grupos. No entanto, os níveis de iNOS no cerebelo da prole de ratas exercitadas permaneceu aos níveis do grupo controle, indicando que o exercício materno é capaz de atenuar o efeito dos AβOs já que os níveis basais de iNOS no cerebelo da prole de ratas exercitadas estão diminuídos em relação ao grupo controle (Capítulo 4/*Figure 6*). A enzima iNOS está envolvida em processos inflamatórios e já foi demonstrada estar aumentada na DA (Heneka *et al.*, 2005; Kummer *et al.*, 2012; Heneka *et al.*, 2013). O fato de não termos encontrado um aumento dos níveis de $NO^{\bullet}$ após a infusão de AβOs pode ser explicada pelo fato de que o $NO^{\bullet}$ pode ser convertido em produtos secundários, como o peroxinitrito ($ONOO^{-}$) e o dióxido de nitrogênio (NO_2). Além disso, na DA ocorre um aumento da nitração de moléculas (Kummer *et al.*, 2011), inclusiva do peptídeo Aβ, impedindo a sua degradação pela enzima de degradação da insulina (IDE) (Kummer *et al.*, 2012).

Diversas proteínas sinápticas estão reduzidas na DA, contudo, no nosso trabalho, o imunoconteúdo de sinaptofisina e PSD95 no cerebelo não foram alterados nem pelo exercício materno, nem pelos AβOs. O aumento da atividade da enzima glicogênio-sintase-cinase 3β (GSK-3β) está relacionado à DA, pois a GSK-3β é a principal enzima responsável por fosforilar a proteína tau. Por sua vez, a hiperfosforilação da proteína tau caracteriza um dos achados predominantes no cérebro de paciente com a DA, os emaranhados neurofibrilares (Querfurth e Laferla, 2010). Nós demonstramos um aumento no imunoconteúdo da proteína tau fosforilada no cerebelo da prole de ratas sedentárias, enquanto que nenhuma alteração foi observada no cerebelo da prole de ratas exercitadas, indicando o efeito protetor do exercício materno. No entanto, a atividade da enzima GSK-3β permaneceu inalterada pela infusão com AβOs (Capítulo 4/*Figure 6*). Coletivamente, os resultados apresentados no capítulo 4 indicam que a programação do metabolismo cerebelar pelo exercício materno permanece evidente na idade adulta da prole conferindo proteção contra algumas alterações induzidas pelos AβOs, já que a regulação positiva dos processos metabólicos pelo exercício materno não foi afetada pela patogenicidade dos AβOs.

Em resumo, como esquematizado na Figura 5, os resultados obtidos com a presente tese contribuem para o avanço científico no contexto da DOHaD, onde o estilo de vida materno saudável programa o fenótipo da prole, por mecanismos adaptativos, conferindo resistência ao desenvolvimento de doenças crônicas na vida adulta.

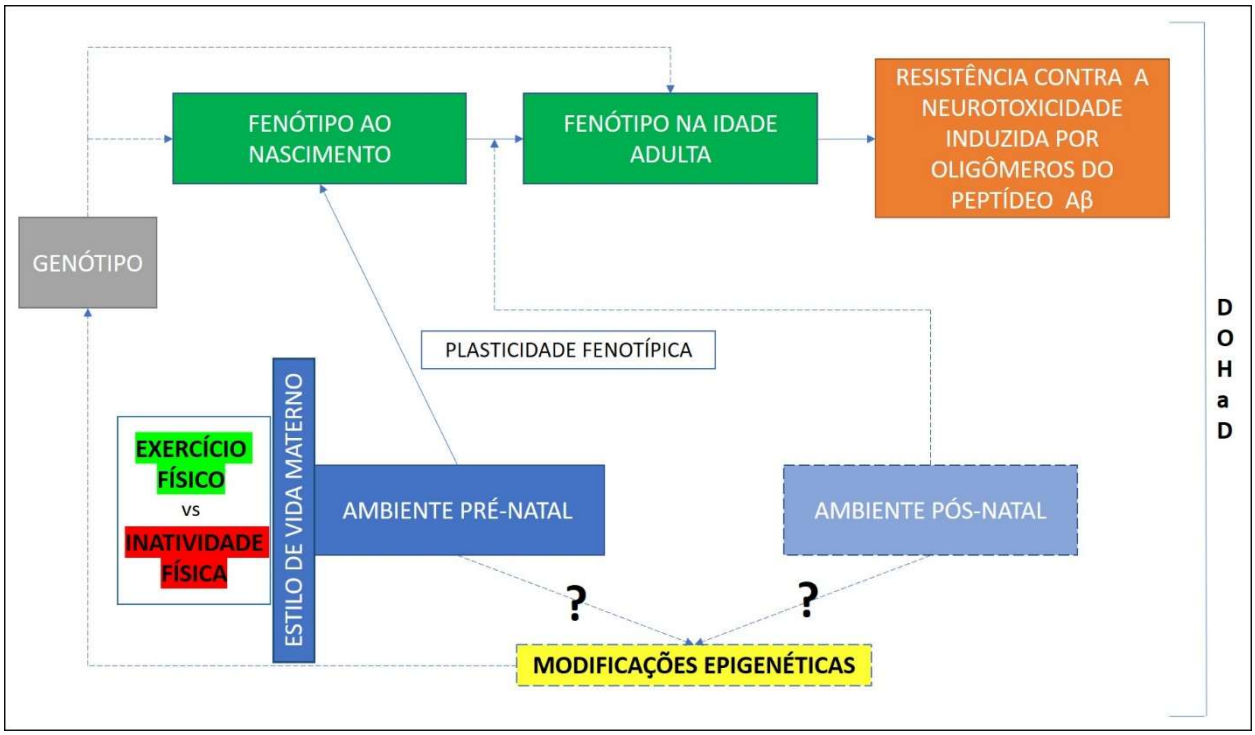


Figura 5. Resumo esquemático da contribuição científica da presente tese no contexto da Origem Desenvolvimentista da Saúde e da Doença. Linhas contínuas, demonstrados no presente trabalho; linhas descontínuas, perspectivas de investigação futura.

CONCLUSÕES

Conclusão geral

A partir dos resultados obtidos com a presente tese, é possível concluir que o ambiente vivenciado pelo feto *in utero* pode definir a resposta ao insulto resultante do desenvolvimento de doenças neurodegenerativas na vida adulta e que o exercício materno pode ser empregado como estratégia neuroprotetora frente às alterações metabólicas presentes na DA, visto que as adaptações neurometabólicas relacionadas à atividade mitocondrial, resultante do exercício aeróbico materno, são capazes de contribuir para a prevenção dos danos causados pela deposição do peptídeo Aβ em diversas regiões do SNC.

Conclusões específicas

- O nado involuntário realizado por ratas antes e durante a gestação não altera o comportamento das mães em relação aos seus filhotes;

- A natação materna gestacional não altera o desenvolvimento das características físicas e os reflexos neuromotores da prole logo após o nascimento;

- A natação materna melhora o comportamento de exploração da prole ao longo dos dias pós-natais, sem alterar a ontogenia do comportamento de locomoção;

- A natação materna ativa as vias de sinalização mediada por AKT/GSK-3β, SIRT1 e SIRT3 no cerebelo da prole aos 7 dias pós-natal, sem causar alterações nos níveis de BDNF maduro ou no imunoconteúdo de TFAM, Mfn1 e Drp1;

- A natação materna promove adaptações neurometabólicas duradouras no córtex pré-frontal, hipocampo e cerebelo da prole, sendo capaz de proteger contra os déficits comportamentais e alterações neuroquímicas induzidos pela infusão icv de AβOs em um modelo similar à DA, através da modulação do metabolismo energético mitocondrial e independentemente dos níveis de BDNF maduro.

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ANEXOS

Anexo I

Carta de Aprovação da Comissão de Ética no Uso de Animais

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 27349
Título: EXERCÍCIO FÍSICO MATERNO COMO ESTRATÉGIA NEUROPROTETORA EM UM MODELO DA DOENÇA DE ALZHEIMER

Pesquisadores:

Equipe UFRGS:

CRISTIANE MATTE - coordenador desde 01/06/2014
CHRISTIANNE GAZZANA SALBEGO - pesquisador desde 01/06/2014
Caroline Peres Klein - Aluno de Doutorado desde 01/06/2014

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 09/06/2014 - Sala Multiuso da Biblioteca Central - Prédio da Reitoria - Porto Alegre - RS, em seus aspectos éticos e metodológicos, para a utilização de ratos Wistar (200 fêmeas e 100 machos adultos, e 800 filhotes machos) de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Sexta-Feira, 27 de Junho de 2014

STELA MARIS KUZE RATES
Coordenador da comissão de ética