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JÉSSICA PRISCILA FRAGOSO DE MOURA

EFEITO DA ATIVIDADE FÍSICA VOLUNTÁRIA E DA DIETA HIPOPROTEICA MATERNA SOBRE A EXPRESSÃO DE GENES RELACIONADOS COM O CRESCIMENTO E DESENVOLVIMENTO DO FETO:

estudo de fatores tróficos neurais e placentários

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Tese apresentada ao Programa de Pós-Graduação em Nutrição do Centro de Ciências da Saúde, da Universidade Federal de Pernambuco como parte dos requisitos parciais para obtenção do Título de Doutora em Nutrição.

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Orientadora: Profª Drª Carol Góis Leandro

Coorientadora: Profª Drª Raquel da Silva Aragão

Coorientador: Drº Luciano Pirola

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BANCA EXAMINADORA

_____

Profª. Drª. Carol Virginia Góis Leandro (Orientadora)

Universidade Federal de Pernambuco

_____

Profº. Drº. João Henrique da Costa Silva (Examinador Interno)

Universidade Federal de Pernambuco

_____

Profº. Drº. Marcos David da Silva Cavalcanti (Examinador Externo)

Universidade Federal de Pernambuco

_____

Profº. Drº. Rubem Carlos Araújo Guedes (Examinador Interno)

Universidade Federal de Pernambuco

_____

Profª. Drª. Wylla Tatiana Ferreira e Silva (Examinador Externo)

Universidade Federal de Pernambuco

A minha Mãe “Eneide Fragoso” e a minha Tia “Eliane Fragoso”, que são meu alicerce. Grande parte do que sou, devo a essas grandiosas mulheres,

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RESUMO

No presente estudo, investigamos o efeito da atividade física e da dieta hipoproteica materna sobre o nível de expressão gênica de BDNF, NTF4 (NT-4), NTRK2 (TrkB), IGF-1 e IGF-1r nas diferentes áreas do cérebro materno (hipotálamo, hipocampo e córtex), placenta e do cérebro dos fetos. Ratas da linhagem *Wistar* (*n*=20) foram alojadas individualmente em gaiolas de atividade física voluntária, contendo roda de corrida. Nessas gaiolas foram acoplados ciclocomputadores que permitiram o registro da distância percorrida, estimativa do gasto calórico e tempo de atividade. As ratas passaram por um período de adaptação (30 dias), recebendo neste período dieta de manutenção (AIN-93M). Posteriormente, foram classificadas de acordo com o nível diário de atividade física em: Inativas (*n*=11) e Ativas (*n*=9). Após detecção da prenhez, metade de cada grupo experimental recebeu dieta normoproteica (18% proteína) e a outra metade recebeu dieta hipoproteica (8% proteína) durante o período de gestação. No 20º dia de gestação, foram coletados: córtex, hipotálamo e hipocampo das mães, placenta e cérebro dos filhotes para análise de expressão gênica utilizando a técnica de PCR quantitativo. Nossos resultados demonstraram que a dieta hipoproteica durante a gestação provocou: Aumento na expressão de IGF-1r e BDNF no hipotálamo, aumento de IGF-1r e NTRK2 (TrkB) no hipocampo, aumento de BDNF, NTRK2 (TrkB), IGF-1 e IGF-1r no córtex. Em adição, houve redução na expressão de IGF-1 na placenta e redução na expressão de IGF-1r e NTRK2 (TrkB) no cérebro dos filhotes. Vimos que a prática de atividade física materna foi capaz de atenuar os efeitos da desnutrição proteica materna sobre a expressão de IGF-1r no hipotálamo, IGF-1r e NTRK2 (TrkB) no hipocampo, IGF-1 na placenta e NTRK2 (TrkB) no cérebro dos filhotes. Esses resultados indicam que: i) a dieta hipoproteica e a atividade física materna podem influenciar a expressão gênica de alguns fatores neurotróficos no cérebro materno, na placenta e no cérebro do feto; ii) a prática de atividade física antes e durante a gestação é capaz de atenuar os efeitos da dieta hipoproteica materna em relação a expressão de alguns genes relacionados com o crescimento da prole; iii) existe uma interação importante entre o organismo materno e desenvolvimento placentário no controle da expressão de genes relacionados com o crescimento fetal.

Palavras-chave: Atividade física. Desnutrição proteica. Gestação. Neuroplasticidade. Ratos. Fatores neurotróficos.

ABSTRACT

In the present study, we investigated the effect of maternal physical activity and lowprotein diet on gene expression of BDNF, NTF4 (NT-4), NTRK2 (TrkB), IGF-1 and IGF-1r in different areas of mother's brain (hypothalamus, hippocampus and cortex), placenta and fetus brain of rats. Female Wistar rats (n=20) were housed in voluntary physical activity cages, containing a running wheel. In these cages were coupled cyclocomputers that allowed the recording of the traveled distance, time and daily estimated calorie burned. During adaptation (30 days before gestation), rats received maintenance diet (AIN-93M). Rats were classified as inactive (n=11) or active (n=9) according to the level of voluntary physical activity. During gestation, dams remained to have access to the running wheel and half of each group received normoprotein diet (18% protein) and the other half received low-protein diet (8% protein). At the 20th day of gestation, gene expression of neurotrophic factors was analysed by quantitative PCR. Dams submitted to a low-protein diet during gestation showed up-regulation of IGF-1r and BDNF mRNA in the hypothalamus, IGF-1r and NTRK2 in the hippocampus, BDNF, NTRK2, IGF- 1 and IGF-1r in the cortex. In the placenta, there was a down regulation of IGF-1. In fetus brain, there was a down regulation in IGF-1r and NTRK2. Voluntary physical activity attenuated the effects of low-protein diet on IGF-1r in the hypothalamus, IGF-1r and NTRK2 in the hippocampus, IGF-1 in the placenta and NTRK2 in the fetus brain. These results indicate: i) maternal lowprotein diet and physical activity can influence the gene expression of some neurotrophic factors in the mother's brain, placenta and brain of the fetus; ii) maternal physical activity is able to attenuate the effects of the maternal lowprotein diet on expression of genes related to offspring growth; iii) There is an important interaction between mother and placental development in the control of fetal growth factors gene expression.

Keywords: Physical exercise. Low-protein diet. Pregnancy. Neuroplasticity. Rats. Neurotrophic factors.

SUMÁRIO

1 INTRODUÇÃO

A nutrição materna tem um papel fundamental sobre a trajetória de crescimento e desenvolvimento feto-placentário e pós-natal (RISNES *et al.*, 2011; MARTIN-GRONERT e OZANNE, 2012). Evidências epidemiológicas e experimentais têm demonstrado que o desequilíbrio nutricional nos períodos de gestação e/ou lactação, está associado com adaptações fisiológicas que podem repercutir no aparecimento de doenças ao longo da vida, como diabetes, hipertensão e obesidade (BARKER, 2007; HARDER *et al.*, 2009; RISNES *et al.*, 2011; MARTIN-GRONERT e OZANNE, 2012; ALHEIROS-LIRA *et al.*, 2015). Essa associação entre a influência ambiental, como a nutrição, durante o desenvolvimento inicial e o maior risco de doenças na vida adulta está inserida no modelo teórico conhecido como “Origem desenvolvimentista da saúde e doença” (DOHaD) (HANSON e GLUCKMAN, 2014).

A escassez de nutrientes durante os períodos iniciais da vida representa um desafio para o desenvolvimento dos diversos sistemas orgânicos, como o sistema nervoso (FALCAO-TEBAS *et al.*, 2012; AKITAKE *et al.*, 2015; BELLUSCIO *et al.*, 2016). Em humanos, estudos recentes demonstraram que restrição de crescimento fetal (que pode ocorrer devido à escassez de nutrientes no período gestacional) resultou em baixos *scores* neurocognitivos, como em habilidades de memória, durante a infância (CHEN *et al.*, 2016; SWAMY *et al.*, 2018). Em animais, dieta baixa em proteína materna ocasionou retardo na ontogenia dos reflexos e diminuição do eixo laterolateral e anteroposterior da cabeça, redução na quantidade de neurônios e do nível de sinaptogênese no hipocampo, aumento do comportamento depressivo, aumento da ansiedade e baixa função cognitiva da prole (FALCAO-TEBAS *et al.*, 2012; AKITAKE *et al.*, 2015; BELLUSCIO *et al.*, 2016)

Os mecanismos subjacentes podem estar relacionados com alterações na expressão de fatores neurotróficos e de crescimento, como: fator neurotrófico derivado do cérebro (BDNF), neurotrofina-4 (NTF4/NT-4), receptor de tropomiosina quinase B (TrkB ou NTRK2), fator de crescimento semelhante a insulina 1 (IGF-1) e receptor do fator de crescimento semelhante a insulina 1 (IGF-1r) (MAYEUR *et al.*, 2010; SOLVSTEN *et al.*, 2018). Esses fatores estão envolvidos no controle do crescimento, sobrevivência e diferenciação de neurônios, regulam o desenvolvimento sináptico, da plasticidade e da mielinização (DHOBALE, 2014; DYER *et al.*, 2016;

SKAPER, 2018). Além de atuarem em células neuronais, esses fatores regulam o processo de crescimento, diferenciação, proliferação e sobrevivência de células placentárias (KAWAMURA *et al.*, 2009).

A placenta é um órgão formado durante o período gestacional, que permite a interação do organismo materno com o organismo fetal, tendo diversas funções, como produção de hormônios, transporte de oxigênio e de nutrientes (SANDOVICI *et al.*, 2012; VAUGHAN *et al.*, 2017). Condições adversas (como desnutrição materna) podem repercutir em adaptações placentárias que alteram a capacidade de transferência de nutrientes para o feto (SANDOVICI *et al.*, 2012; VAUGHAN *et al.*, 2017). Estudos em animais demonstraram que dieta hipoproteica (6% de proteína) no período gestacional ocasionou alterações morfológicas na placenta (redução de células gigantes trofoblásticas e de células de glicogênio) e prejudicou a diferenciação de células espongiotrofoblásticas e células gigantes trofoblásticas na zona juncional placentária (GAO *et al.*, 2013; REBELATO *et al.*, 2013; REBELATO *et al.*, 2016). Cada tipo de célula placentária possui funções específicas, como produção de hormônios ou fonte energética. Por isso, redução de células placentárias pode contribuir para danos no crescimento fetal.

Além da nutrição materna, tem sido demonstrado que um estilo de vida ativo durante a gestação pode beneficiar a saúde materna e do feto (CLAPP, 2003; ROSA *et al.*, 2011; FERRARI *et al.*, 2018). Um estudo recente em humanos demonstrou que mulheres que realizaram um programa de exercício (intensidade moderada) durante a gestação, apresentaram menor percentual de gordura corporal e aumento nos níveis de BDNF (FERRARI *et al.*, 2018). Essas alterações auxiliam no controle do peso corporal e melhorias nas funções cerebrais da mãe. Foi demonstrado que bebês de mulheres que realizaram exercício físico durante a gestação (intensidade moderada) apresentaram maior peso e comprimento corporal ao nascer (CLAPP *et al.*, 2000). Outro estudo demonstrou que o exercício regular durante a gestação melhorou a função placentária, aumentando a transferência de nutrientes para o feto em desenvolvimento (CLAPP, 2003). Em animais, os filhotes e as placentas de mães ativas durante a gestação foram mais pesados no 19º dia de gestação em relação ao grupo controle (ROSA *et al.*, 2011).

Estudos têm relatado a ideia de que um estilo de vida materno ativo causa alterações no desenvolvimento intrauterino, mesmo em caso de aporte inadequado de proteína (AMORIM *et al.*, 2009; FALCÃO-TEBAS *et al.*, 2012). Em animais, os

filhotes de ratas treinadas em esteira durante a gestação apresentaram aumento nos valores de indicadores de crescimento somático e antecipação na maturação de alguns reflexos (FALCAO-TEBAS *et al.*, 2012). Utilizando esse mesmo desenho experimental, foi demonstrado que filhotes de mães que realizaram treinamento físico apresentaram diminuição nos níveis de colesterolemia, glicemia e menor percentual de ganho de peso quando comparado com o grupo de filhotes provindos de mães que receberam dieta hipoproteica e que não realizaram treinamento físico (AMORIM *et al.*, 2009; FIDALGO *et al.*, 2010; FALCAO-TEBAS *et al.*, 2012).

Em nossos estudos anteriores, utilizamos um modelo de atividade física voluntária materna em roda de corrida e vimos que este modelo foi capaz de alterar a trajetória de crescimento e desenvolvimento da prole (SANTANA MUNIZ *et al.*, 2014; FRAGOSO *et al.*, 2017a). A atividade física materna voluntária aumentou indicadores de crescimento somático da prole durante a lactação (SANTANA MUNIZ *et al.*, 2014). Além disso, houve uma ocorrência mais precoce do dia da abertura da orelha, abertura da conduta auditiva interna, erupção dos incisivos inferiores e do reflexo de preensão palmar nos filhotes das mães muito ativas durante a lactação (SANTANA MUNIZ *et al.*, 2014). Em outro estudo, demonstramos que a atividade física voluntária materna atenuou os efeitos da dieta materna pobre em proteínas (8% de proteína) nos padrões de atividade locomotora da prole aos 60 dias de idade (FRAGOSO *et al.*, 2017a). Os mecanismos subjacentes podem estar relacionados ao maior fluxo sanguíneo uterino e à maior transferência placentária de oxigênio e nutrientes (CLAPP, 2003). No presente estudo, utilizamos o mesmo modelo experimental de atividade física voluntária materna para investigar a neuroplasticidade induzida pela atividade física na expressão gênica transcricional de fatores neurotróficos.

Neste estudo, testamos a hipótese de que a atividade física e a dieta hipoproteica materna são estímulos ambientais que alteram a neuroplasticidade materna e assim influenciam a expressão de fatores neurotróficos e de crescimento placentário com consequências no desenvolvimento fetal. Para isso, nosso objetivo foi avaliar o efeito da atividade física e da dieta hipoproteica materna sobre a expressão gênica de BDNF, NTF4/NT-4, NTRK2/TrkB, IGF-1 e IGF-1r nas diferentes áreas do cérebro materno (hipotálamo, hipocampo e córtex), placenta e do cérebro dos fetos.

2 REVISÃO DA LITERATURA

2.1 Nutrição materna: efeitos sobre o crescimento e desenvolvimento fetal

A nutrição materna tem um papel fundamental sobre a trajetória de crescimento e desenvolvimento feto-placentário e pós-natal (RISNES *et al.*, 2011; MARTIN-GRONERT e OZANNE, 2012). Evidências epidemiológicas e experimentais têm demonstrado que o desequilíbrio nutricional nos períodos de gestação e/ou lactação, está associado com adaptações fisiológicas que podem repercutir em desordens metabólicas na vida adulta (BARKER, 2007; HARDER *et al.*, 2009; RISNES *et al.*, 2011; MARTIN-GRONERT e OZANNE, 2012; ALHEIROS-LIRA *et al.*, 2015). Em humanos, um estudo de revisão sistemática com meta-análise demonstrou que o baixo peso ao nascer (um dos indicadores de desnutrição materna) está associado com maior taxa de mortalidade por doenças cardiovasculares (RISNES *et al.*, 2011).

Em animais, a redução do consumo de proteína materna (8% de caseína) ocasionou resistência central e periférica à insulina nos filhotes, e contribuiu para o desenvolvimento de diabetes mellitus tipo 2 (BERENDS *et al.*, 2018). Estudos prévios demonstraram que o aporte inadequado de proteína materna (8% e 6% de proteína) está relacionado com alterações na morfologia e fisiologia renal, como redução no número de néfrons e disfunção do sistema renina-angiotensina-aldosterona, podendo contribuir para o desenvolvimento de hipertensão na prole ao longo da vida (21 e 60 dias de vida) (CHEN *et al.*, 2010; SIDDIQUE *et al.*, 2014).

Modelos experimentais com dieta hipocalórica ou hiperlipídica também tem sido utilizado em estudos prévios (ALHEIROS-LIRA *et al.*, 2015; ALHEIROS-LIRA *et al.*, 2017). Filhotes de mães que receberam dieta com menor valor energético total (contendo 2,3 kcal/g de dieta, enquanto o grupo controle recebeu dieta contendo 3,5 kcal/g de dieta) durante a lactação, apresentaram aumento das concentrações séricas de triglicerídeos aos 150 dias de vida (ALHEIROS-LIRA *et al.*, 2015). Outro estudo demonstrou que filhotes que receberam dieta hiperlipídica (45,08% do valor energético total de lipídeos) após o desmame apresentaram aumento das concentrações séricas de triglicerídeos aos 60 dias de vida. Esse aumento nas concentrações de triglicerídeos foi ainda maior quando os filhotes que receberam dieta hiperlipídica eram advindos de mães que receberam dieta com redução de proteína

(8% de proteína) durante a gestação e a lactação (ALHEIROS-LIRA *et al.*, 2017). Essa associação entre a influência ambiental, como a nutrição, durante o desenvolvimento inicial e o maior risco de doenças na vida adulta está inserida no modelo teórico conhecido como “Origem desenvolvimentista da saúde e doença” (DOHaD) (HANSON e GLUCKMAN, 2014).

O DOHaD pode ser compreendido num conceito mais amplo conhecido como “plasticidade fenotípica”. Plasticidade fenotípica refere a capacidade de um organismo em reagir aos desafios impostos pelo ambiente, alterando a sua morfologia, funcionamento e/ou comportamento (WEST-EBERHARD, 2005). O organismo em desenvolvimento apresenta respostas fenotípicas que visam promover a sobrevivência imediata frente a um ambiente desfavorável (HANSON e GLUCKMAN, 2014). Um estudo experimental demonstrou que filhotes de mães que receberam dieta hipoproteica (8% de proteína) durante a gestação e a lactação, apresentaram alteração de alguns parâmetros ventilatórios, como aumento da frequência respiratória e da ventilação, aos 30 dias de vida (DE BRITO ALVES *et al.*, 2014). Essa alteração no padrão ventilatório pode ser considerada uma resposta adaptativa imediata frente a uma situação ambiental adversa, como menor disponibilidade de proteína. Apesar de garantir a sobrevivência, essa adaptação pode resultar em maiores riscos de doenças crônicas na vida adulta, caso haja incompatibilidade ambiental no decorrer da vida (HANSON e GLUCKMAN, 2014).

A escassez de nutrientes durante os períodos iniciais da vida representa um desafio para o desenvolvimento dos diversos sistemas orgânicos, como o sistema nervoso (FALCAO-TEBAS *et al.*, 2012; AKITAKE *et al.*, 2015; BELLUSCIO *et al.*, 2016). Em humanos, estudos recentes demonstraram que restrição de crescimento fetal (que pode ocorrer devido à escassez de nutrientes no período gestacional) resultou em baixos *scores* neurocognitivos, como em habilidades de memória, durante a infância (CHEN *et al.*, 2016; SWAMY *et al.*, 2018). Em animais, um estudo demonstrou que redução de proteína (8% de proteína) durante os períodos de gestação e lactação, promoveu aumento no consumo de dieta rica em gordura e carboidratos simples na idade adulta, devido a mecanismos de ativação neuronal em regiões cerebrais relacionadas com o sistema de recompensa (DA SILVA *et al.*, 2016).

Em modelos experimentais, estudos têm demonstrado que o aporte inadequado de nutrientes durante o período perinatal repercute no desenvolvimento do sistema nervoso. Por exemplo, em resposta a uma dieta baixa em proteína, houve retardo na

ontogenia dos reflexos (geotaxia negativa, queda livre, aversão ao precipício e colocação das vibrissas) e diminuição do eixo laterolateral e anteroposterior da cabeça (durante o período de lactação), redução na quantidade de neurônios e do nível de sinaptogênese no hipocampo, aumento do comportamento depressivo, aumento da ansiedade e baixa função cognitiva (FALCAO-TEBAS *et al.*, 2012; AKITAKE *et al.*, 2015; BELLUSCIO *et al.*, 2016). Um estudo com ratos Wistar observou que filhotes de mães que receberam dieta hipoproteica (10% proteína) apenas durante a gestação ou durante toda a gestação e lactação, apresentaram maior comportamento de ansiedade (no teste de labirinto elevado) e menor exploração da zona central (no teste de campo aberto) indicando maior estresse, aos 90-93 dias de vida (REYES-CASTRO *et al.*, 2012).

Os mecanismos subjacentes podem estar relacionados com alterações na expressão de fatores neurotróficos e de crescimento. Recentes estudos têm relatado o papel de moléculas como: fator neurotrófico derivado do cérebro (BDNF), neurotrofina-4 (NTF4/NT-4), receptor de tropomiosina quinase B (TrkB ou NTRK2), fator de crescimento semelhante à insulina 1 (IGF-1) e receptor do fator de crescimento semelhante à insulina 1 (IGF-1r) (MAYEUR *et al.*, 2010; SOLVSTEN *et al.*, 2018).

2.2 Desnutrição perinatal e fatores neurotróficos

Os fatores neurotróficos, como BDNF e NTF4 (NT-4), são uma família de peptídeos que interagem principalmente como o receptor TrkB (NTRK2) (DHOBALE, 2014; SKAPER, 2018). Os fatores neurotróficos estão envolvidos no controle do crescimento, sobrevivência e diferenciação de neurônios, regulam o desenvolvimento sináptico, da plasticidade e da mielinização, atuando sobre diversas funções como formação da memória, aprendizagem, habilidade cognitiva e plasticidade sináptica (DHOBALE, 2014; SKAPER, 2018). O IGF-1 pertence à família dos hormônios polipeptídeos (conhecido também como hormônio neurotrófico), sendo estruturalmente e funcionalmente similar a insulina e liga-se ao seu receptor IGF-1r para desempenhar suas funções (DYER *et al.*, 2016). O sistema IGF-1/IGF-1r é crucial para o processo de diferenciação, proliferação e desenvolvimento das células neurais, bem como sua integração estrutural e funcional em circuitos neurais preexistentes,

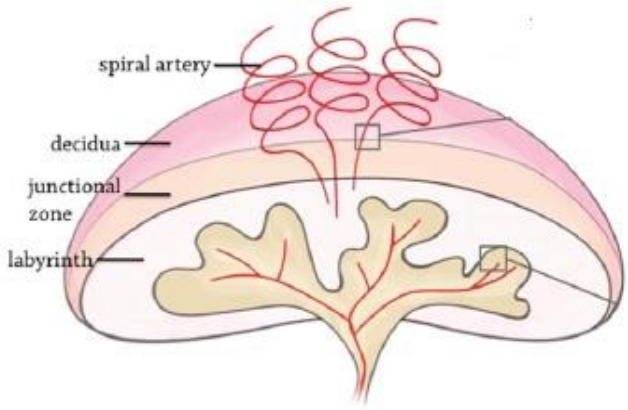
mediando mudanças na morfologia, eficácia sináptica e organização celular no sistema nervoso central (DYER *et al.*, 2016). Tendo ação primordial em funções cognitivas, como memória, aprendizagem e função executiva (ALVINO *et al.*, 2011; DYER *et al.*, 2016; MAJORCZYK e SMOLAG, 2016).

Wang e Xu (2007), realizaram um estudo com ratos utilizando modelo de dieta com redução de proteína (6% de proteína) durante a gestação (um grupo iniciou a dieta a partir do 8º dia e outro a partir do 15º dia de gestação) até 4 semanas de vida pós-natal. Foi visto que aos 28 dias de vida, os filhotes de mães que receberam dieta hipoproteica (ambos os grupos que receberam dieta com redução de proteína) apresentaram menor concentração de BDNF no hipocampo que resultou em déficit em testes de memória e aprendizagem quando comparado com o grupo controle (que recebeu dieta com 20% de proteína) (WANG e XU, 2007). Outro estudo demonstrou que filhotes de mães que receberam aporte inadequado de proteína (8% de proteína) durante todo o período de gestação e lactação tiveram danos na consolidação da memória (GEORGINA PÉREZ-GARCÍA *et al.*, 2016). Este déficit ocorreu devido à redução na neurogênese e na expressão de RNAm de BDNF no hipocampo aos 90 dias de vida (GEORGINA PÉREZ-GARCÍA *et al.*, 2016). Um estudo recente demonstrou que filhotes de mães que receberam dieta com redução de proteína (8% de proteína) antes (3 semanas) e durante toda a gestação, apresentaram diminuição na expressão e nos níveis de BDNF no cérebro ao nascimento (MARWARHA *et al.*, 2017).

Em estudo prévio, foi demonstrado que restrição de crescimento intrauterino (que pode ser um indicador de aporte inadequado de nutrientes) repercutiu em assimetria em diferentes órgãos avaliados como diminuição do tamanho dos rins, pulmão e fígado, porém com preservação do tamanho do cérebro do feto no 18º dia gestacional (NOVITSKAYA *et al.*, 2011). Nesse mesmo estudo foi demonstrado que os órgãos que tiveram seu tamanho reduzido também apresentaram menor expressão de IGF-1 e redução da ativação do receptor de IGF do tipo 1 (IGF-Ir). Em contrapartida, foi visto um aumento na expressão de IGF-1 no cérebro, permitindo a manutenção do seu tamanho (NOVITSKAYA *et al.*, 2011). Essas alterações nos sistemas IGF-1/IGF-1r e BDNF/TrkB no feto em resposta a uma dieta inadequada no início da vida, tem sido relacionada com prejuízos no desenvolvimento placentário.

2.3 Nutrição e interação feto-placentária

A placenta é um órgão formado durante o período gestacional, que permite a interação do organismo materno com o organismo fetal, tendo diversas funções, como imunológica, excretora, produção de hormônios, transporte de oxigênio e de nutrientes (glicose, ácido graxos, aminoácidos, vitaminas e sais minerais) (SANDOVICI *et al.*, 2012; VAUGHAN *et al.*, 2017). A placenta, de ratos, possui duas principais zonas: 1) juncional (interface entre o tecido materno e o feto); 2) labiríntica (interface fetal) (REBELATO *et al.*, 2013). Na zona juncional, há três tipos de células distintas morfologicamente: células gigantes trofoblásticas, células espongiotrofoblásticas e células de glicogênio. Na zona labiríntica, têm-se dois tipos de células distintas morfologicamente: células gigantes trofoblásticas e células gigantes trofoblásticas sincitrial (REBELATO *et al.*, 2013). O sangue materno chega à placenta através das artérias espirais deciduais e o sangue fetal chega à placenta pelas artérias umbilicais (HSIAO e PATTERSON, 2012).



Fonte: Artigo de Hsiao e Patterson, 2012

O adequado desenvolvimento placentário permite um maior suporte para o crescimento intrauterino (GRISSOM e REYES, 2013; REBELATO *et al.*, 2016). No período crítico do desenvolvimento, uma nutrição equilibrada é essencial, visto que condições adversas (como desnutrição materna) podem repercutir em adaptações placentárias que alteram a capacidade de transferência de nutrientes para o feto (SANDOVICI *et al.*, 2012; VAUGHAN *et al.*, 2017). Tais adaptações compreendem

alterações morfológicas (modificação na densidade e disposição da vascularização, composição de células e/ou espessura das membranas das células placentárias) e alterações funcionais (modificação na quantidade de proteínas transportadoras de glicose, ácidos graxos, cálcio, ferro, ácido fólico e aminoácidos) (SANDOVICI *et al.*, 2012).

Estudos em animais demonstraram que dieta hipoproteica (6% de proteína) no período gestacional ocasionou alterações morfológicas na placenta, como redução de células gigantes trofoblásticas (no 15º, 19º e 21º dia gestacional) e redução de células de glicogênio (no 17º e 19º dia gestacional) (REBELATO *et al.*, 2013; REBELATO *et al.*, 2016). Gao *et al*., (2013) realizaram um estudo em animais e verificaram que uma dieta hipoproteica (6% de proteína) durante a gestação, prejudicou a diferenciação de células espongiotrofoblásticas e células gigantes trofoblásticas na zona juncional da placenta no 18º dia de gestação (GAO *et al.*, 2013). As células gigantes trofoblásticas e as células espongiotrofoblásticas secretam hormônios esteroides e peptídeos que, no final do período gestacional, tem como função o remodelamento da vascularização. Já as células de glicogênio funcionam como fonte energética devido ao acúmulo de glicogênio (GAO *et al.*, 2013). Essa redução de células placentárias pode contribuir para danos no crescimento fetal.

O desenvolvimento e a funcionalidade da placenta são regulados por diversos fatores de crescimento (como o sistema IGF-1/IGF-1r) e neurotróficos (como BDNF e NT-4) (KAWAMURA *et al.*, 2009; MAYEUR *et al.*, 2010; KAWAMURA *et al.*, 2011; JONES *et al.*, 2013). Foi visto que o aumento na expressão de IGF-1 na placenta pode ocorrer como um mecanismo adaptativo para atenuar o déficit no peso fetal (resultante de ambiente intrauterino inadequado) através de mecanismos que aumentam a expressão de transportadores de glicose na placenta, como o GLUT-3 (JONES *et al.*, 2013). Um estudo em animais, demonstrou que há expressão de BDNF e NT-4 em células placentárias (trofoblásticas), sendo responsáveis pelo crescimento, proliferação e sobrevivência das mesmas (KAWAMURA *et al.*, 2009). Foi visto que o BDNF é capaz de aumentar o crescimento dos blastocistos (conjunto de células embrionárias), no período pré-implantação (KAWAMURA *et al.*, 2009). Outro estudo demonstrou que restrição alimentar (50%) durante a gestação foi capaz de reduzir os níveis de RNAm e de proteína BDNF na placenta à termo, podendo contribuir para um desenvolvimento fetal inadequado (MAYEUR *et al.*, 2010).

Estudo prévio em humanos no qual foi coletado tecido placentário de mulheres no primeiro trimestre de gestação (entre 6 e 11 semanas), demonstrou que o sistema BDNF/TrkB é expresso em diferentes células placentárias e participam da diferenciação, proliferação e sobrevivência celular (KAWAMURA *et al.*, 2011). Outro estudo demonstrou aumento na expressão de RNAm de BDNF e TrkB (NTRK2) à termo em placentas e esse aumento foi associado com restrição de crescimento intrauterino (MAYEUR *et al.*, 2010). Alterações na expressão de fatores neurotróficos e de crescimento podem contribuir com prejuízos no desenvolvimento e função placentária e consequentemente no crescimento fetal (KAWAMURA *et al.*, 2009; MAYEUR *et al.*, 2010; KAWAMURA *et al.*, 2011).

Recentemente, tem sido sugerido que o transcriptoma materno (conjunto completo de transcritos, como RNAs mensageiros, RNAs ribossômicos, RNAs transportadores e os microRNAs) pode ser modulado durante a gestação provavelmente em resposta à placenta e ao desenvolvimento fetal (BEHURA *et al.*, 2018). Porém, a compreensão sobre a comunicação entre placenta, desenvolvimento do cérebro fetal e funcionalidade do cérebro materno é limitada. Behura *et al*., (2018) realizaram um estudo no qual foi feito um mapeamento sobre o padrão de correlação de genes entre cérebro materno, cérebro fetal e placenta para tentar elucidar possíveis genes que fazem essa interação (BEHURA *et al.*, 2018). Dessa forma, se faz necessário estudos que avaliem como fatores ambientais (como a nutrição) durante o período de gestação podem influenciar a expressão de genes que estão relacionados com o funcionamento cerebral materno, estrutura e funcionalidade placentária e desenvolvimento do sistema nervoso fetal. Tal compreensão pode contribuir para o estabelecimento de estratégias para prevenção de futuros problemas de saúde.

2.4 Atividade física materna: Implicações sobre o crescimento e desenvolvimento fetal

Atividade física refere-se a qualquer movimento do músculo esquelético que demande gasto energético maior que o repouso (LEANDRO *et al.*, 2009). Quanto ao nível de atividade física, o indivíduo pode ser classificado como inativo, ativo (com diferentes estratificações) ou muito ativo. De acordo com o questionário internacional de atividade física (IPAQ), essa classificação pode ser feita levando em consideração

alguns parâmetros como frequência (quantidade de dias na semana que o indivíduo pratica atividade física) e duração (tempo de atividade) (MATSUDO *et al.*, 2001). Assim como em humanos, os ratos podem ser classificados de acordo com o nível diário de atividade física em inativo, ativo ou muito ativo levando em consideração alguns parâmetros, como distância percorrida, tempo de atividade e estimativa do gasto calórico (SANTANA MUNIZ *et al.*, 2014).

De acordo com o *American College of Obstetricians and Gynecologists* (ACOG), mulheres gestantes (sem complicações médicas ou obstétricas) podem realizar exercícios de forma regular com intensidade moderada e duração entre 20-30 minutos diários ou até 150 minutos semanais (ACOG, 2015). Essa prática de atividade física durante a gestação pode promover diversos benefícios para a saúde materna (CLAPP, 2003; FERRARI *et al.*, 2018). Estudo recente em humanos demonstrou que mulheres que realizaram um programa de exercício (frequência de duas vezes por semana, duração de 60 minutos e intensidade moderada) a partir da 14ª semana até a 30ª semana de gestação, apresentaram menor percentual de gordura corporal e aumento nos níveis de BDNF (FERRARI *et al.*, 2018). Essas alterações auxiliam no controle do peso corporal e melhorias nas funções cerebrais da mãe.

Além dos benefícios para a mãe, a prática regular de atividade física materna pode repercutir em alterações no crescimento feto-placentário (CLAPP *et al.*, 2000; CLAPP, 2003; ROSA *et al.*, 2011). Foi demonstrado que bebês de mulheres que realizaram exercício físico durante a gestação (intensidade entre 55% e 60% da capacidade aeróbia máxima) apresentaram maior peso e comprimento corporal ao nascer em comparação aos filhos de mulheres sedentárias (CLAPP *et al.*, 2000). Outro estudo demonstrou que o exercício regular durante a gestação melhorou a função placentária, aumentando a transferência de nutrientes para o feto em desenvolvimento (CLAPP, 2003). Em animais, os filhotes e as placentas de mães ativas durante a gestação foram mais pesados no 19º dia de gestação em relação ao grupo controle (ROSA *et al.*, 2011).

Alterações placentárias em resposta a prática de atividade física materna podem modular o desenvolvimento do sistema nervoso da prole, devido a prováveis mudanças adaptativas na expressão de fatores neurotróficos e de crescimento (PARNPIANSIL *et al.*, 2003; LEMOYNE *et al.*, 2012; SOLVSTEN *et al.*, 2018). Em humanos, bebês de mães ativas durante a gestação (três vezes por semana, pelo menos 20 min/dia a 55% da capacidade aeróbica máxima) apresentaram uma melhor

resposta à discriminação sonora e memória auditiva, conforme medido pela eletroencefalografia (LABONTE-LEMOYNE *et al.*, 2017). Em animais, filhotes nascidos de mães ativas durante a gestação mostraram aumento na quantidade de células neuronais e não neuronais no hipocampo, melhoraram as funções cognitivas (comportamento de habituação e aprendizado espacial) e melhoraram a memória num paradigma de reconhecimento de objetos, sendo essas alterações associadas com o aumento na expressão de RNAm de BDNF no hipocampo (ROBINSON e BUCCI, 2014; GOMES DA SILVA *et al.*, 2016). Aksu *et al.* (2012) demonstraram que a prática de atividade física materna durante a gestação foi capaz de aumentar os níveis de BDNF no córtex pré-frontal dos filhotes aos 26 e aos 120 dias de vida (AKSU *et al.*, 2012). Estes filhotes apresentaram menor ansiedade e maior atividade locomotora (aumento na mobilidade) nas duas idades avaliadas.

Estudos têm suportado a ideia de que um estilo de vida materno ativo causa alterações no desenvolvimento intrauterino, mesmo em caso de aporte inadequado de proteína (AMORIM *et al.*, 2009; FIDALGO *et al.*, 2010; FALCÃO-TEBAS *et al.*, 2012). Em animais, os filhotes de ratas treinadas em esteira com intensidade moderada (65% $VO_{2máx}$) antes da gestação e intensidade leve (40% $VO_{2máx}$) durante a gestação apresentaram aumento nos valores de indicadores de crescimento somático (taxa de crescimento, comprimento da cauda, eixo laterolateral e anteroposterior da cabeça) e antecipação na maturação de alguns reflexos quando comparado com o grupo de filhotes provindos de mães que receberam dieta hipoproteica e que não realizaram treinamento físico (FALCAO-TEBAS *et al.*, 2012). Utilizando esse mesmo desenho experimental, foi demonstrado que filhotes de mães que realizaram treinamento físico apresentaram diminuição nos níveis de colesterolemia, glicemia e menor percentual de ganho de peso quando comparado com o grupo de filhotes provindos de mães que receberam dieta hipoproteica e que não realizaram treinamento físico (AMORIM *et al.*, 2009; FIDALGO *et al.*, 2010; FALCAO-TEBAS *et al.*, 2012; FALCÃO-TEBAS *et al.*, 2012).

Em nossos estudos anteriores, utilizamos um modelo de atividade física voluntária materna em roda de corrida e vimos que este modelo foi capaz de alterar a trajetória de crescimento e desenvolvimento da prole (SANTANA MUNIZ *et al.*, 2014; FRAGOSO *et al.*, 2017a). A atividade física materna voluntária aumentou os indicadores de crescimento somático (eixo laterolateral do crânio, eixo longitudinal e comprimento da cauda) da prole durante a lactação (SANTANA MUNIZ *et al.*, 2014).

Além disso, houve uma ocorrência mais precoce do dia da abertura da orelha, abertura da conduta auditiva interna, erupção dos incisivos inferiores e do reflexo de preensão palmar nos filhotes das mães muito ativas durante a lactação (SANTANA MUNIZ *et al.*, 2014). Em outro estudo, demonstramos que a atividade física voluntária materna atenuou os efeitos da dieta materna pobre em proteínas (8% de proteína) nos padrões de atividade locomotora (distância percorrida, potência média, energia total e tempo de imobilidade) da prole aos 60 dias de idade (FRAGOSO *et al.*, 2017a). Os mecanismos subjacentes podem estar relacionados ao maior fluxo sanguíneo uterino e à maior transferência placentária de oxigênio e nutrientes (CLAPP, 2003). Neste trabalho, utilizamos o mesmo modelo experimental de atividade física voluntária materna para investigar a neuroplasticidade induzida pela atividade física na expressão gênica transcricional de fatores neurotróficos.

3 HIPÓTESE

A atividade física e a dieta hipoproteica materna são estímulos ambientais que alteram a neuroplasticidade materna e assim influenciam a expressão de fatores neurotróficos e de crescimento placentário com consequências no desenvolvimento fetal.

4 OBJETIVOS

4.1 Geral

Avaliar o efeito da atividade física e da dieta hipoproteica materna sobre o nível de expressão gênica de BDNF, NTF4/NT-4, NTRK2/TrkB, IGF-1 e IGF-1r nas diferentes áreas do cérebro materno (hipotálamo, hipocampo e córtex), placenta e do cérebro dos fetos.

4.2 Específicos

Durante o período de adaptação (30 dias antes da gestação):

- Caracterizar as ratas (mães) quanto ao nível de atividade física voluntária com avaliação diária dos parâmetros: distância percorrida, estimativa do gasto calórico e tempo de atividade física;
- Avaliar o peso corporal, o consumo alimentar e a glicemia de jejum das ratas (mães);

Durante o período de gestação:

- Acompanhar o nível de atividade física voluntária, com avaliação diária dos parâmetros: distância percorrida, estimativa do gasto calórico e tempo de atividade física das ratas (mães);
- Avaliar o peso corporal, o consumo alimentar e a glicemia de jejum das ratas (mães);
- Descrever os parâmetros no 20º dia gestacional: Número de filhotes, peso corporal e peso da placenta;
- Avaliar a expressão de RNAm IGF-1, IGF-1r, BDNF e NTRK2 (TrkB) no hipotálamo, hipocampo e córtex das mães;
- Avaliar a expressão de RNAm IGF-1, IGF-1r, BDNF e NTF4 na placenta;
- Avaliar a expressão de RNAm IGF-1, IGF-1r, BDNF e NTRK2 (TrkB) no cérebro dos fetos.

5 MÉTODOS

5.1 Animais

Foram utilizadas 20 ratas albinas da linhagem *Wistar*, com idade entre 85-95 dias, foram provenientes da colônia do Departamento de Nutrição da UFPE. Os animais foram mantidos em biotério de experimentação, em condições padronizadas e livre acesso à água e alimentação. O manejo e os cuidados para com os animais seguiram as recomendações do Colégio Brasileiro de Experimentação Animal (COBEA). O projeto foi aprovado pela Comissão de Ética no uso de Animal do Centro de Ciência Biológicas da UFPE (processo 23076.015984/2015-30).

5.2 Protocolo de atividade física voluntária e dieta experimental

A gaiola de atividade física voluntária (GAFV) é feita de acrílico com as seguintes dimensões: 27 cm de largura, 34 cm de altura e 61 cm de comprimento (Figura 1A). Em uma das extremidades foi posicionado um cicloergômetro com 27 cm de diâmetro, composto por acrílico e raios em aço inoxidável (Figura 1A-C). Acoplado a gaiola e ao cicloergômetro há um sistema de monitoramento por sensor (ciclocomputador Cataye, model CC-VL810, Osaka, Japan) (Figura 1D-F). A atividade física das ratas foi avaliada pela movimentação do cicloergômetro, sendo quantificado diariamente através dos sensores que permite o registro das seguintes grandezas físicas: Distância percorrida (km), tempo de atividade (min) e estimativa do gasto calórico (kcal), Tabela 1.

A B C

D E F

Figura 1. Dimensões da gaiola de atividade física voluntária (A); Gaiola de atividade física voluntária com cicloergômetro e comedouro (B), Cicloergômetro (C), Esquema do funcionamento do ciclocomputador com os sensores [Cateye, model CC-VL820, Velo 9, Osaka, Japan] (D); Posicionamento de um sensor na porção externa da GAFV, acoplado ao ciclocomputador (E); Visão interna dos sensores, um aclopado ao cicloergômetro e outro na GAFV (F).

As ratas nulíparas foram colocadas individualmente nas GAFV por um período de 30 dias para a adaptação e receberam durante esse período dieta AIN-93M (REEVES, 1997). Após esse período, as ratas foram classificadas em dois grupos de acordo com o nível de atividade física diário: Inativo (I, n = 11) ou Ativo (A, n=9) de acordo com os parâmetros e valores apresentados na tabela 1. As ratas foram colocados em gaiola padrão de biotério feita de polipropileno (33x40x17cm) para o acasalamento e após a presença de espermatozoide na cavidade vaginal (MARCONDES *et al.*, 2002) (Figura 2), as ratas foram recolocadas individualmente nas GAFV onde uma parte das ratas de cada grupo recebeu dieta a base de caseína de acordo com a AIN-93G (REEVES, 1997), e a outra parte recebeu a mesma dieta, porém com menor quantidade de proteína (8% de proteina) durante toda gestação (Tabela 2 e Figura 3).

Tabela 1: Classificação dos grupos experimentais de acordo com a atividade física diária (distância percorrida, gasto calórico e tempo de atividade) no cicloergômetro.

Grupos experimentais	***n***	**Distância percorrida (km.dia^{-1})**	**Gasto Calórico (kcal.dia^{-1})**	**Tempo de atividade (min.dia^{-1})**
Inativo	*11*	< 1.0	< 10.0	< 20.0
Ativo	*9*	≥1.0≤5.0	≥10.0≤40.0	≥20.0≥ 120

(SANTANA MUNIZ *et al.*, 2014)

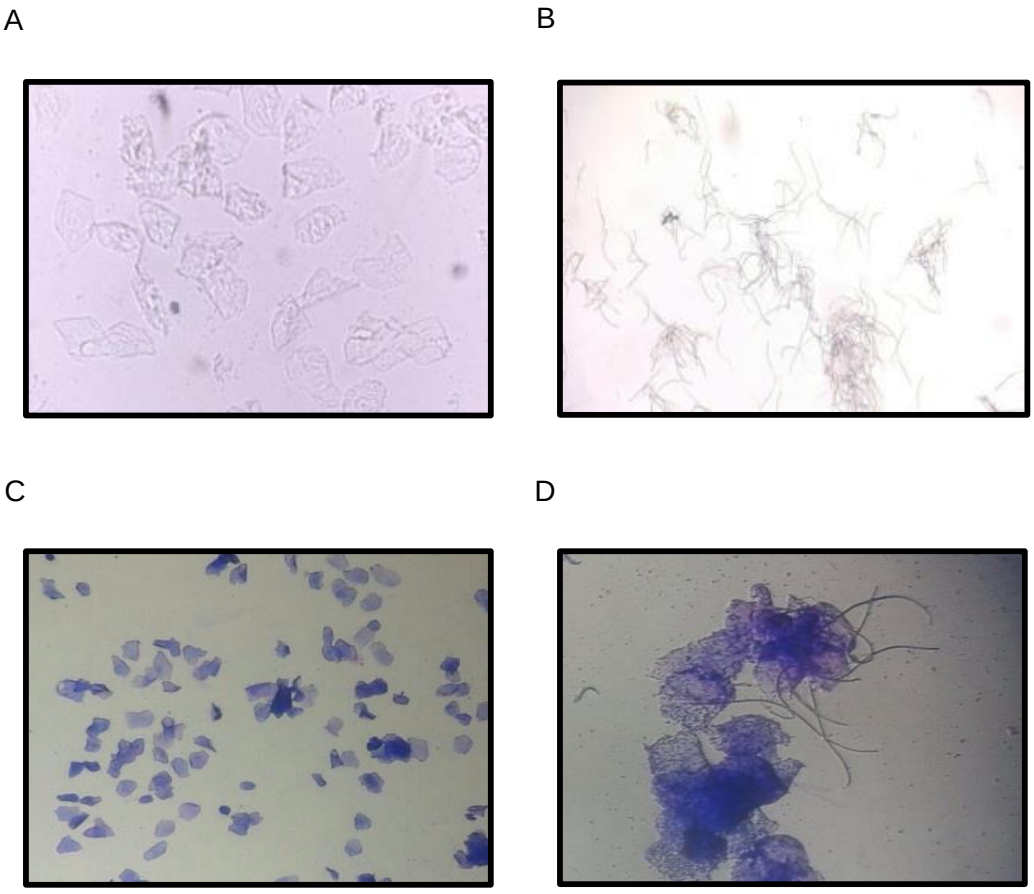


Figura 2. Imagens de lâminas de esfregaço vaginal. Imagens de lâminas de ratas não gestantes: Sem coloração (A) e com coloração (C); Imagens de lâminas de ratas gestantes: Sem coloração (B) e com coloração (D).

Tabela 2. Composição das dietas

Ingredientes	**AIN-93M* g/Kg**	**AIN-93G* g/Kg**	**Hipoproteica g/Kg**
Amido de milho (87% carboidratos), g	465.692	397.486	476.686
Caseína (proteína ≥80%), g	140.0	200.0	94.1
Amido de milho dextrinizado (92% tetrasaccharides), g	155.0	132.0	158.7
Sacarose, g	100.0	100.0	100.0
Óleo de soja, g	40.0	70.0	70.0
Celulose, g	50.0	50.0	50.0
Mix de Mineral (AIN-93M-MX), g	35.0	-	-
Mix de Mineral (AIN-93G-MX), g	-	35.0	35.0
Mix de Vitaminas (AIN-93-VX), g	10.0	10.0	10.0
L-Metionina, g	1.8	3.0	3.0
Bitartarato de Colina (41.1% colina), g	2.5	2.5	2.5
Tert-butylhydroquinone (TBHQ), g	0.008	0.014	0.014

***(REEVES, 1997)

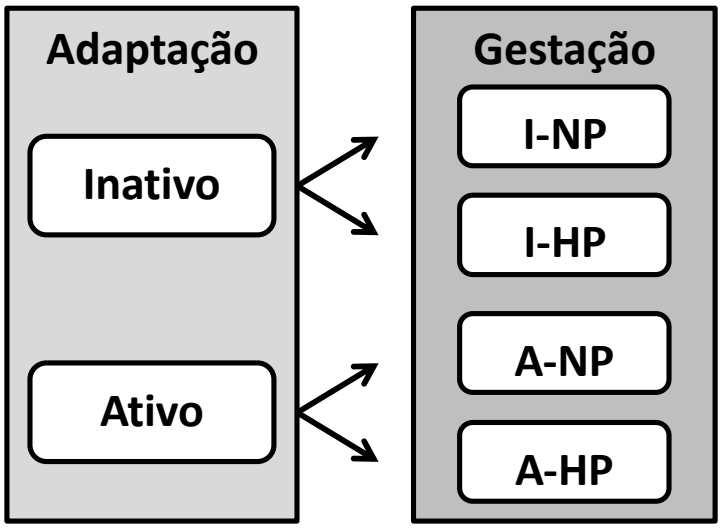


Figura 3. Formação dos grupos experimentais do estudo 2: I-NP: Inativo Normoproteico; I-HP: Inativo Hipoproteico; A-NP: Ativo Normoproteico e A-HP: Ativo Hipoproteico.

5.3 Avaliação do peso corporal e do consumo alimentar das ratas

O peso corporal e o consumo alimentar das ratas foram avaliados a cada três dias durante os períodos de adaptação e gestação. Foi utilizada uma balança eletrônica digital – Marte, modelo S-1000, com capacidade máxima de 1000g e sensibilidade de 0,01g.

5.4 Avaliação da glicemia de jejum das ratas

Ao final do período de adaptação (30º dia) e no 7º, 14º e 20º dia de gestação, foi quantificada a glicemia de jejum. Para quantificação, as ratas foram submetidas a um jejum noturno de 12 horas. Foi utilizado o glicosímetro de marca *Accu-Chek* Performa (Figura 4).

Figura 4. Glicosímetro: Accu-Chek Performa.

5.5 Ensaio molecular

5.5.1 Coleta dos tecidos

Após a eutanásia das ratas (no 20º dia de gestação), as amostras de placenta, cérebro das mães e dos filhotes foram coletadas, congeladas em gelo seco e imediatamente armazenadas em freezer -80°C até a realização das análises pretendidas.

5.5.2 Extração de RNA

O RNA total foi extraído da placenta e do cérebro de filhotes com TRI reagent® (SIGMA- ALDRICH T9424, Spruce Street, St. Louis, MO 63103 USA) de acordo o manual do fornecedor. Foi adicionado 1 ml de TRI reagent® para cada 50-100 miligramas de tecido. A suspensão resultante foi homogeneizada e incubada à temperatura ambiente durante 5 minutos. Foi adicionado 0,2 ml de clorofórmio, as amostras foram homogeneizadas durante 15 segundos, incubadas durante 5 minutos à temperatura ambiente e centrifugadas a 13.500 RPM durante 15 minutos a 4 °C. A fase aquosa, contendo o RNA, foi transferida para um tubo esterilizado e precipitada em isopropanol (0,5 ml). As amostras foram incubadas durante 10 minutos à temperatura ambiente e centrifugadas a 13.500 RPM durante 15 minutos a 4 °C. O sobrenadante foi removido e os sedimentos contendo RNA foram lavados sequencialmente com etanol a 75% e 100%. O sedimento de RNA foi ressuspenso em 100 µl de H_2O (Versol). A concentração e a pureza do RNA (taxa de absorvância de 260/280 nm) foram determinadas num espectrofotômetro (Thermofisher).

5.5.3 Transcrição reversa (RT)

A transcrição reversa foi realizada usando um PrimeScript RT reagent Kit-Perfect Real Time (TAKARA) usando 1 µg de RNA para placenta e cérebro dos filhotes seguindo as instruções do fabricante. Foram adicionados sequencialmente: RNase Free dH2O (3 µl), PrimeScript Buffer 5× (4 µl), Oligo dT - 50 µM (1 µl), Random hexamers - 100 µM (1 µl) e PrimeScript RT Enzyme Mix (1 µl), seguido por 15 minutos de incubação a 37 °C e 15 segundos a 85 °C. Em seguida, o RT foi diluído a 1/10 por adição de 180 µl de água livre de RNase, que resultou o volume final de 200 µl – armazenado a -20 °C. O RT 1/10 foi diluído para RT 1/60 (para as análises de IGF-1, IGF-1r, BDNF e NTRK2) e 1/20 (para análise da NTF4 na placenta) e a técnica de reação em cadeia da polimerase (PCR em tempo real) foi realizada para investigação da expressão gênica.

5.5.4 PCR Quantitativo (qPCR)

A amplificação quantitativa por PCR em tempo real (qPCR) foi realizada utilizando um sistema de PCR em tempo real Rotor-Gene (Labgene Scientific Instruments, Archamps, Fran). As sequências de primers usadas neste estudo estão relatadas na Tabela 3. As reações foram incubadas a 95 °C por 10 min, seguidas por 40 ciclos de desnaturação (95 °C, 10 s), anelamento (58–65 °C dependendo da sequência de primers, 30s) e alongamento (72 °C, 30s). Foram quantificados a expressão de RNAm do Fator de Crescimento Semelhante à Insulina - Tipo 1 (IGF-1), Receptor do Fator de Crescimento Semelhante à Insulina - Tipo 1 (IGF-1R), Fator Neurotrófico Derivado do Cérebro (BDNF), Neurotrofina 4 (NTF4) e Receptor Neurotrófico de Tirosina Quinase Tipo 2 (NTRK2). Os resultados de qPCR de cada gene (incluindo os genes housekeeping) foram expressos como unidades arbitrárias a partir de uma curva de calibração padrão derivada de uma amostra de referência. Amostras de referência para os tecidos foram geradas misturando alíquotas de 5 µl de amostras de cDNA 1/10 (3 do grupo Inativo Normoproteico, 2 do grupo Inativo Hipoproteico, 3 do grupo Ativo Normoproteico e 2 do grupo Ativo Hipoproteico). qPCR para cada amostra foi realizado em duplicata. Os níveis de RNAm dos genes analisados foram normalizados utilizando os níveis de RNAm de dois genes: Proteína Ribossômica L19 (RPL19) e da Beta Actina (Actb).

Tabela 3. Sequência de primers utilizadas para realização do qRT-PCR

	Foward/ Reverse	T	Sequência 5'-3'	Tamanho do Amplicon
IGF-1	F	60°C	GCTCTTCAGTTCGTGTGTGG	108 bp
	R		GCAACACTCATCCACAATGC	
IGF-1r	F	60°C	CTGGTCTCTCATCTTGGATGC	197 bp
	R		GCTTCCCACACACACTTGG	
BDNF	F	60°C	GAGTGAAGATACCATCAGCA	117 bp
	R		ATCTAGGCTACGTGAAGTCT	
NTF4	F	65°C	CTGAGATGTCAGGGAGGAGA	115 bp
	R		ATGGCTTTGCACACCTGTCA	
NTRK2	F	60°C	GTGGTGATTGCCTCTGTGG	149 bp
	R		TTGGAGATGTGGTGGAGAGG	
RPL19	F	58°C	CTGAAGGTCAAAGGGAATGTG	195 bp
	R		GGACAGAGTCTTGATGATCTC	
Actb	F	60°C	AGCCATGTACGTAGCCATCC	231 bp
	R		TCCCTCTCAGCTGTGCTGGTGAA	

5.6 Análise Estatística

Os dados foram analisados estatisticamente através do software GraphPad Prism 5® (GraphPad Software, Inc., La Jolla, CA, USA). Inicialmente foi realizado o teste de normalidade de Kolmogorov-Smirnov. Foi utilizado o teste ANOVA *two-way*, tendo como fatores a atividade física e o tempo ou a atividade física e a dieta. Como pós-teste, foi utilizado o teste de Bonferroni. Um valor de $p<0,05$ foi considerado significante.

6 RESULTADOS

6.1 Artigo 1

Submetido no "*Journal of Developmental Origins of Health and Disease*"

Title: Maternal physical activity-induced adaptive transcriptional response in brain and placenta of mothers and rat offspring

Short-title: Maternal physical activity and neuroplasticity

Authors: Jéssica Fragoso[1], Gabriela Carvalho Jurema Santos[2], Helyson Thomaz da Silva[1], Viviane Oliveira Nogueira[2], Emmanuelle Loizon[3], Hubert Vidal[3], João Henrique Costa-Silva[2], Raquel da Silva Aragão[2], Luciano Pirola[3] and Carol Gois Leandro[2]

Authors Institution:

[1] Department of Nutrition, Federal University of Pernambuco, 50670-901 Recife, PE, Brazil

[2] Department of Physical Education and Sports Science, Federal University of Pernambuco, 55608-680 Vitória de Santo Antão, PE, Brazil

[3] CarMeN (Cardiology, Metabolism and Nutrition) Laboratory; INSERM U1060, Lyon-1 University, South Lyon Medical Faculty, 69921 Oullins, France.

Address of corresponding author:
Carol Góis Leandro
Núcleo de Nutrição, Universidade Federal de Pernambuco. Centro Acadêmico de Vitória – CAV. Phone: (00 55 81) 21268463. Fax: (00 55 81) 21268473. **E-mail:** carolleandro22@gmail.com

List of abbreviations:

I: inactive; A: active; VA: very active; NTFs: Neurotrophic factors; BDNF: brain-derived neurotrophic fator; NTF4/NT-4: neurotrophin-4; NTKR2, or TrkB: neurotrophic tyrosine kinase receptor type 2; IGF-1: insulin-like growth factor 1; IGF-1r: insulin-like growth factor 1 receptor.

Abstract

Maternal physical activity induces brain functional changes and neuroplasticity, leading to an improvement of cognitive functions, such as learning and memory in the offspring. This study investigated the effects of voluntary maternal physical activity on the gene expression of the neurotrophic factors IGF-1, IGF-1r, BDNF and NTRK2 mRNA in the brain of mothers and pups and IGF-1, IGF-1r, BDNF and NTF4 mRNA in the placenta. Female Wistar rats (n=15) were individually housed in voluntary physical activity cages, containing a running wheel, for 4 weeks (period of adaptation) before gestation. Rats were classified as inactive (I); active (A) or very active (VA) according to distance spontaneously travelled daily. During gestation, the dams continued to have access to the running wheel. At the 20th day of gestation, gene expression of neurotrophic factors was analysed in different areas of mother's brain (cerebellum, hypothalamus, hippocampus and cortex), placenta and the offspring's brain. Neurotrophic factors gene expression was evaluated by quantitative PCR. Very active mothers showed upregulation of IGF-1 mRNA in the cerebellum (36.8%) and NTF4 mRNA expression in the placenta (24.3%). In the cortex, there was a tendency of up-regulation of NTRK2 mRNA ($p=0.06$) in the A and VA groups when compared to the I group. There were no noticeable changes in the gene expression of neurotrophic factors in the offspring's brain. Maternal physical activity affects gene expression of some neurotrophic factors in specific areas of the brain and placenta in mothers, but not in pup's brain.

Keywords: Physical exercise; pregnancy; neuroplasticity; rats; neurotrophic factors

Introduction

Human and animal studies have shown that environmental stimuli, such as maternal physical activity, influence the brain development and function of both mother and offspring (ROBINSON e BUCCI, 2014; LABONTE-LEMOYNE *et al.*, 2017; FERRARI *et al.*, 2018). In humans, infants from active mothers during pregnancy (three times per week, at least 20 min/day at 55% of their maximal aerobic capacity) showed a better response to sound discrimination and auditory memory as measured by electroencephalography (LABONTE-LEMOYNE *et al.*, 2017). In rats, young pups born from dams which were active throughout pregnancy showed an increased amount of neuronal and non-neuronal cells in the hippocampus, improved cognitive functions (habituation behaviour and spatial learning) and enhanced memory as tested using a novel object recognition paradigm (ROBINSON e BUCCI, 2014; GOMES DA SILVA *et al.*, 2016). The underlying mechanism of this neuroplasticity can be related to adaptive changes in the expression of neurotrophic and growth factors such as brain-derived neurotrophic factor (BDNF), neurotrophin-4 (NTF4/NT-4), neurotrophic tyrosine kinase receptor type 2 (NTKR2, or TrkB), insulin-like growth factor 1 (IGF-1) and insulin-like growth factor 1 receptor (IGF-1r) (PARNPIANSIL *et al.*, 2003; LEMOYNE *et al.*, 2012; SOLVSTEN *et al.*, 2018).

Neurotrophic factors (NTFs) are a family of peptides involved in the control of growth, survival, and differentiation of neurons. NTFs include neurotrophins, glial cell-line derived neurotrophic factor family ligands and neuropoietic cytokines (SKAPER, 2018). NTFs are expressed in different areas of the brain such as the hippocampus, hypothalamus, cerebellum and cortex (NEEPER *et al.*, 1996; TAPIA-ARANCIBIA *et al.*, 2004; MIKI *et al.*, 2013; SOLVSTEN *et al.*, 2017; SOLVSTEN *et al.*, 2018). In addition, NTFs and growth factors can also regulate the development and efficiency of the placenta (DEY *et al.*, 2004; FOWDEN *et al.*, 2009; KAWAMURA *et al.*, 2009; MAYEUR *et al.*, 2010). A previous study showed that

BDNF is able to increase the growth of blastocysts (embryonic cells), *in vitro*, during the pre-implantation period (KAWAMURA *et al.*, 2009). Similarly, it was demonstrated that IGF-1 stimulates the migration of placental trophoblastic cells, regulating foetus-placental growth (JONES *et al.*, 2013).

The efficiency in the transport of oxygen and nutrients through the placenta is essential for the growth and development of the foetus (CLAPP, 2003; HSIAO e PATTERSON, 2012). Previous studies have shown that regular exercise during pregnancy enhances foetus-placental growth (CLAPP *et al.*, 2000; CLAPP, 2003; AMORIM *et al.*, 2009). Babies from exercising women (intensity between 55% and 60% of the preconception maximum aerobic capacity) showed higher body weight and length at birth than those born from sedentary women (CLAPP *et al.*, 2000). Regular exercise during pregnancy increased intervillous space blood volume, cardiac output and placental function (CLAPP, 2003). In rats, pups from active dams throughout pregnancy showed morphological changes in the placenta at the 19th day of gestation (ROSA *et al.*, 2011). Since the placental development and efficiency are regulated by growth factors (IGF-1/IGF-1r) and neurotrophic factors (BDNF and NTF4), the hypothesis that maternal physical activity can alter gene expression of neurotrophic factors is plausible.

In our previous studies, we demonstrated that maternal voluntary physical activity was able to alter the growth and developmental trajectory of the offspring (SANTANA MUNIZ *et al.*, 2014; FRAGOSO *et al.*, 2017a). Voluntary maternal physical activity increased the indicators of somatic growth (laterolateral axis of skull, longitudinal axis and tail length) of the offspring during lactation (SANTANA MUNIZ *et al.*, 2014). Moreover, there was an earlier occurrence of the day of ear opening, internal auditory conduct opening, eruption of lower incisors and the palmar grasp reflex in pups from the very active dams during lactation (SANTANA MUNIZ *et al.*, 2014). In addition, maternal voluntary physical activity attenuated the effects of maternal low-protein diet (8% protein) on patterns of locomotor activity (distance

travelled, average power, total energy and time of immobility) of the offspring at 60 days old (FRAGOSO *et al.*, 2017a). The underlying mechanisms may be related to the uterine blood flow and enhanced placental transfer of oxygen and diffusible substrates (CLAPP, 2003). Herein, we have used the same experimental model of voluntary maternal physical activity to investigate the physical activity-induced neuroplasticity on transcriptional gene expression of neurotrophic factors.

In the present study, it was tested the hypothesis that voluntary physical activity performed by mothers before and during gestation modulates the expression of some trophic factors in the brain and placenta of dams, while having less readily detectable effects on the expression of neurotrophic factors in the progeny. Thus, the main goal of the present study was to evaluate the effects of voluntary maternal physical activity on gene expression of IGF-1, IGF-1r, BDNF and NTRK2 in the brain of mothers and neonate pups and mRNA expression of IGF-1, IGF-1r, BDNF and NTF4 in the placenta.

Material and methods

The experimental protocol was approved by the Ethical Committee of the Biological Sciences Centre (protocol nº 23076.015984/2015-30), Federal University of Pernambuco, Brazil, and followed the Guidelines for the Care and Use of Laboratory Animals.

Animals

Fifteen virgin female albino Wistar rats (*Rattus norvegicus*) aged 85-95 days were obtained from the Department of Nutrition, Federal University of Pernambuco, Brazil. Animals were maintained at a temperature of 22 ± 1°C with a controlled light–dark cycle (dark 06.00 am – 6.00 pm). Food and water were given *ad libitum* throughout the experiment. The rats were individually housed in voluntary physical activity cages (cages equipped with a running wheel)

for 4 weeks. After this period, females were placed into a standard cage and mated (1 female for 1 male) for a period of 1–5 days. Females had no access to the running wheel during mating. The day on which spermatozoa were present in a vaginal smear was designated as day 0 of gestation. Pregnant dams were then transferred back to their original cages with free access to the running wheel throughout gestation. At day 20 of gestation, dams were anesthetised with xylazine (10 mg/kg, *ip*.) and ketamine (80 mg/kg, *ip*.) prior to decapitation after a 6h fasting period. Experimental analyses were performed in specific brain areas of mothers (cerebellum, hypothalamus, hippocampus and cortex), placenta and the entire brain of male offspring. The tissues collected were stored at −80°C until RNA extraction.

Voluntary physical activity measurements

Female Wistar rats were individually housed in voluntary physical activity cages (with running wheels - 27 cm diameter) for a 4 weeks period of adaptation. A wireless cyclocomputer (Cataye, model CC-VL820, Colorado, USA) was attached in the wheel to calculate and display information related to physical activity, such as distance travelled, duration of activity and estimated calorie burned. These parameters were used to classify the rats according to their level of daily physical activity in: inactive (I), active (A) or very active (VA) according to previous studies (SANTANA MUNIZ *et al.*, 2014; FRAGOSO *et al.*, 2017a). After mating, rats continued to have access to the running wheel during gestation (Figure 1).

Body weight and food intake

Mother's body weight was recorded each three days throughout the experiment. Maternal food consumption was determined by the difference between the amount of food provided at the onset of the dark cycle (06.00 hours) and the amount of food remaining 48 h later. Body weight of the pups and the placental weight were measured at the day of sacrifice

(day 20 of gestation). Body weight was recorded using a Marte Scale (AS-1000) with 0.01 g accuracy.

Blood glucose measurements

Fasting glycaemia levels were evaluated in the last day of adaptation and weekly during gestation using blood samples from the tail vein of the rats, using a glucometer (Accu Check Advantage and Accutrend GCT) and the glucose oxidase method. The animals were fasted six hours prior to glycaemia measurement.

RNA extraction

Total RNA was extracted from brain regions of mothers (cerebellum, hypothalamus, hippocampus and cortex), placenta and brain of offspring with the TRI reagent® (SIGMA-ALDRICH T9424, St. Quentin Fallavier, FR) according to the manufacturer's instructions. Briefly, 1 ml of TRI reagent® was added per 50-100 mg of tissue, the resulting suspension was homogenized and incubated at room temperature for 5 min. Thereon, 0.2 ml of chloroform was added, samples were vortexed for 15 seconds, incubated for 5 minutes at room temperature and centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol were added to precipitate RNA. Samples were incubated for 10 minutes at room temperature and centrifuged at 12,000 g for 15 minutes at 4 °C. The supernatant was removed and RNA-containing pellets were washed sequentially with 75% and 100% ethanol and dissolved in 100 µl RNase free water. RNA concentration and purity (defined by a 260/280 nm absorbance ratio > 1.8) was determined on a Nanodrop 2000 (Thermofisher).

Reverse transcription

Reverse transcription was performed using an PrimeScript RT reagent Kit-Perfect Real Time (TAKARA) using 0.5 μg of RNA for brain of mothers (cerebellum, hypothalamus, hippocampus and cortex) and 1 μg of RNA for placenta and brain of offspring following the manufacturer's instructions. RNase Free H_2O (3 μL), PrimeScript Buffer 5× (4 μL), Oligo dT - 50 μM (1 μl), Random hexamers - 100 μM (1 μl) and of PrimeScript RT Enzyme Mix (1 μl) were sequentially added, followed by a 15 minutes incubation at 37°C and 15 seconds at 85°C. Reverse transcription reactions were brought to 200 μl final volume by adding RNase free water and stored at −20°C.

Quantitative PCR (qPCR)

Real-time quantitative PCR amplification (qPCR) was performed using a Rotor-Gene Real-Time PCR System (Labgene Scientific Instruments, Archamps, France). The sequences of primers used in this study are reported in Table 1. Reactions were incubated at 95°C for 10 min, followed by 40 cycles of denaturation (95°C, 10 s), annealing (58–65°C depending on the primer sets, 30 s) and elongation (72°C, 30 s). It was measured gene expression levels of Insulin-like Growth Factor 1 (IGF-1), Insulin-Like Growth Factor 1 Receptor (IGF-1R), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin 4 (NTF4) and Neurotrophic Tyrosine Kinase Receptor Type 2 (NTRK2, or TrkB). qPCR results from each gene (including the housekeeping genes) were expressed as arbitrary units derived from a standard calibration curve derived from a reference sample. Reference samples for the tissues were generated by mixing 5 μl aliquots from multiple cDNA samples (3 from the Inactive group, 4 from the Active group and 3 from the Very Active group). qPCR for each sample was carried out in duplicate. The mRNA levels of the analysed genes were normalized using the mRNA levels of ribosomal protein L19 (RPL19) and beta actin (Actb).

Statistical analyses

The Kolmogorov–Smirnov test was performed to determine if the data were normally distributed. Measurements of distance travelled, time of activity and estimated calorie burned were analysed by ANOVA two-way (using day and physical activity as factors) followed by the Bonferroni post-hoc test. For body weight, food intake, blood glycaemia, placental weight, number of pups and gene expression, statistical analyses were performed by ANOVA one-way followed by the Tukey's post-hoc test. All data are presented as mean ± S.E.M. Significance was set at $p<0.05$. Data analysis was performed using the statistical program GraphPad Prism 5® (GraphPad Software Inc., La Jolla, CA, USA).

Results

Data on maternal voluntary physical activity parameters during the period of adaptation are schematically presented in Figure 2. After the adaptation period (30 days), rats were classified as inactive, active or very active according to the daily level of physical activity. During adaptation, inactive dams performed less than 1 km/day in the running-wheel. Active dams performed a constant amount of distance travelled, while the very active dams presented a progressive increase of distance travelled. In this period, body weight, food intake and fasting glycaemia did not change among groups (Table 2).

During gestation, dams remained in the special cages with the running wheel. The inactive dams kept the distance travelled less than 1 km/day in the running-wheel. However, active and very active dams reduced the distance travelled between 1 and 3 km/day (Figure 3). Body weight variation (initial and final), fasting glycaemia, number of offspring (males and females) and placental weight were similar when active dams were compared to inactive dams. However, the very active dams presented an increase in body weight gain, food intake and number of pups (Table 2).

The effects of the different behaviours associated to the performance or not of spontaneous maternal physical activity on the gene expression of neurotrophic factors in different brain areas of mothers, placenta and brain of pups were evaluated next. Very active mothers showed increased IGF-1 mRNA in the cerebellum when compared to inactive mothers (Figure 4). In response to physical activity, there were no changes in IGF1, IGF1r, BDNF and NTRK2 mRNA expression in different areas of the brain: hypothalamus, hippocampus and cortex. In the cortex, there was a tendency of up-regulation of NTRK2 mRNA ($p=0.06$) in the active and very active groups when compared to the inactive group (Figure 4).

Voluntary maternal physical activity also induced statistically significant changes in the mRNA expression in the placenta. Very active mothers had increased NTF4 mRNA in relation to inactive mothers (Figure 5). In the brain of pups, voluntary maternal physical activity did not alter mRNA expression of the tested mRNAs (Figure 5).

Discussion

In the present study, a spontaneous active phenotype was observed in a subset of rats. Individual animals presented different levels of physical activity that allowed the categorization of rats as inactive, active and very active groups, according to previous studies (SANTANA MUNIZ *et al.*, 2014; FRAGOSO *et al.*, 2017a). Earlier literature has also shown that such behavioural traits, i.e. the propensity or not for spontaneous physical activity, can be regulated by central (mRNA expression in the central nervous system) and/or peripheral (mRNA expression in skeletal muscle) mechanisms (TSAO *et al.*, 2001; KNAB *et al.*, 2009; KNAB *et al.*, 2012). In order to test the hypothesis that physical activity induces changes in the gene expression of neurotrophic factors in different areas of the brain, we evaluated the expression of IGF-1, IGF-1r, BDNF and NTRK2 mRNA. Previous studies in rats have shown that physical activity on running wheels increased the expression of IGF-1 mRNA, BDNF and TrkB in the

hippocampus, but without alteration of IGF-1r expression (SOLVSTEN *et al.*, 2017; SOLVSTEN *et al.*, 2018). In the present study, the expression of IGF-1 mRNA in the cerebellum was increased in response to physical activity on running wheels. Another study showed that physical activity increased the expression of BDNF in the cerebellum (NEEPER *et al.*, 1996). In the placenta, there was an increase in NTF4, but there were no changes in mRNA of the neurotrophic factors in the offspring's brain.

During gestation, active and very active rats continued to perform physical activity on the running wheel, but with a substantial reduction in the distance travelled in the very active dams (from 12 km/day to 1.9 km/day). This reduction in physical activity levels on the running wheel may be due to a switch in maternal behaviour to favour the disposal of nutrients for the development of the offspring (MOORE, 2012; NEWCOMER *et al.*, 2012). Interestingly, very active dams showed increased number of pups, but there was no difference in the body weight of foetuses. It is probable that the increase in the number of pups in very active dams influenced the increased food intake to ensure energy supply for the developing foetuses. This result is aligned with our previous observations (FRAGOSO *et al.*, 2017a). The number of pups represents one of the variables of the maternal reproductive ability, which is dependent on the quality of the environment (WELLS, 2003). Indeed, mothers can establish different reproductive strategies depending on the environmental context (WELLS, 2003). Thus, the increase in the number of offspring can be considered as a reproductive strategy in response to physiological mechanisms that allow, for example, a high availability of nutrients in the rats that performed physical activity.

In the present study, the expression levels of IGF-1, BDNF, IGF-1r and NTRK2 mRNA did not change in the cortex, hypothalamus and hippocampus from active and very active rats. We used an experimental model of spontaneous physical activity since forced exercise could induce stress and thus influence the expression of neurotrophic factors (KE *et al.*, 2011).

Persistent voluntary physical activity on running wheels induced an upregulation of IGF-1, BDNF and TrkB (NTRK2) mRNA whereas expression levels of IGF-1r mRNA were not altered in the hippocampus (SOLVSTEN *et al.*, 2017; SOLVSTEN *et al.*, 2018). It has been observed that voluntary physical activity on running wheels did not change the expression of IGF-1 and BDNF mRNAs in the prefrontal cortex (SOLVSTEN *et al.*, 2018). On the other side, acute voluntary physical activity on running wheels increased the expression of BDNF in the hippocampus and cortex (NEEPER *et al.*, 1996). BDNF and IGF-1 are survival factors for sympathetic and sensory neurons, mediators for synaptogenesis, neuronal growth and differentiation in the peripheral and central nervous systems. These neurotrophic factors are also important for cognitive functions, such as memory and learning (YAU *et al.*, 2014; ARNARDOTTIR *et al.*, 2016; SKAPER, 2018). Our data showed that 6 weeks of voluntary physical activity on running wheel induced an adaptive mechanism on the expression of neurotrophic factors and growth factors. We speculate that in situations of brain function impairment, such as memory and learning deficits, physical activity can be a good strategy to prevent cognitive decline, for example in neurodegenerative diseases.

Very active dams showed an upregulation of IGF-1 mRNA in the cerebellum. Previous study showed that chronic resistance physical training induced reduction of IGF-1 mRNA in the cerebellum (ANTONIO-SANTOS *et al.*, 2016). Different types of exercise training induce distinct changes on the motor circuits, generating different adaptive responses (BLACK *et al.*, 1990). For example, animals that performed exercise showed an increase of synapses per Purkinje cell as compared to non-exercised control animals (BLACK *et al.*, 1990). The cerebellum plays an important role to optimize motor behaviour, timing procedures, motor control and to prevent body oscillations (LAWRENSON *et al.*, 2018). Moreover, the cerebellum is involved in neurological disorders, extending from motor dysfunction to cognitive and affective impairment (LAWRENSON *et al.*, 2018). It is possible that the increase

of IGF-1 mRNA in response to physical activity on running wheel may act as a neuroprotective factor on the maternal brain (ANDERSON *et al.*, 2002; LLORENS-MARTIN *et al.*, 2008; FERNANDEZ *et al.*, 2010).

It has been demonstrated that the maternal environment determines the structure and function of the placenta, with consequences on the development of the foetus (REBELATO *et al.*, 2013; MANOKHINA *et al.*, 2017). In the present study, we observed an increase in the expression of NTF4 in the placenta of very active mothers as an adaptive response to maternal physical activity. NTF4 is related to placental growth and inhibition of apoptosis promoting the survival of placental cells (KAWAMURA *et al.*, 2009). This function contributes to the maintenance of villi improving placental transfer of substrates (CLAPP, 2003).

Pups from exercised mothers presented improved cognitive functions (habituation behaviour and spatial learning) and memory capability throughout their lifespan (KIM *et al.*, 2007; GOMES DA SILVA *et al.*, 2016). Previous study showed a pattern of expression BDNF mRNA in the hippocampus in pups from exercised mothers (PARNPIANSIL *et al.*, 2003). In contrast, the present study showed that the expression of IGF-1 and BNDF and their receptors (IGF1r and NTRK2) were not altered in the pups' brain in response to maternal physical activity on running wheel. Accordingly, a recent study has demonstrated that exercise during pregnancy did not change BDNF levels in brain of mice offspring at 21 days old (FERRARI *et al.*, 2018). Thus, the underlying mechanism to improve neuroplasticity in pups from exercised mothers possibly is not related to changes in neurotrophic gene expression.

Conclusion

Maternal voluntary physical activity increased expression of IGF-1 mRNA in the cerebellum and NTF4 mRNA in the placenta, but there were no detectable modifications in the brain of pups. Future studies should examine the epigenetic mechanisms underlying these

changes and investigate whether maternal physical activity is able to protect the growth and development of the fetus in adverse situations, such as protein restriction or high fat diet during gestation.

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Legend and Figures

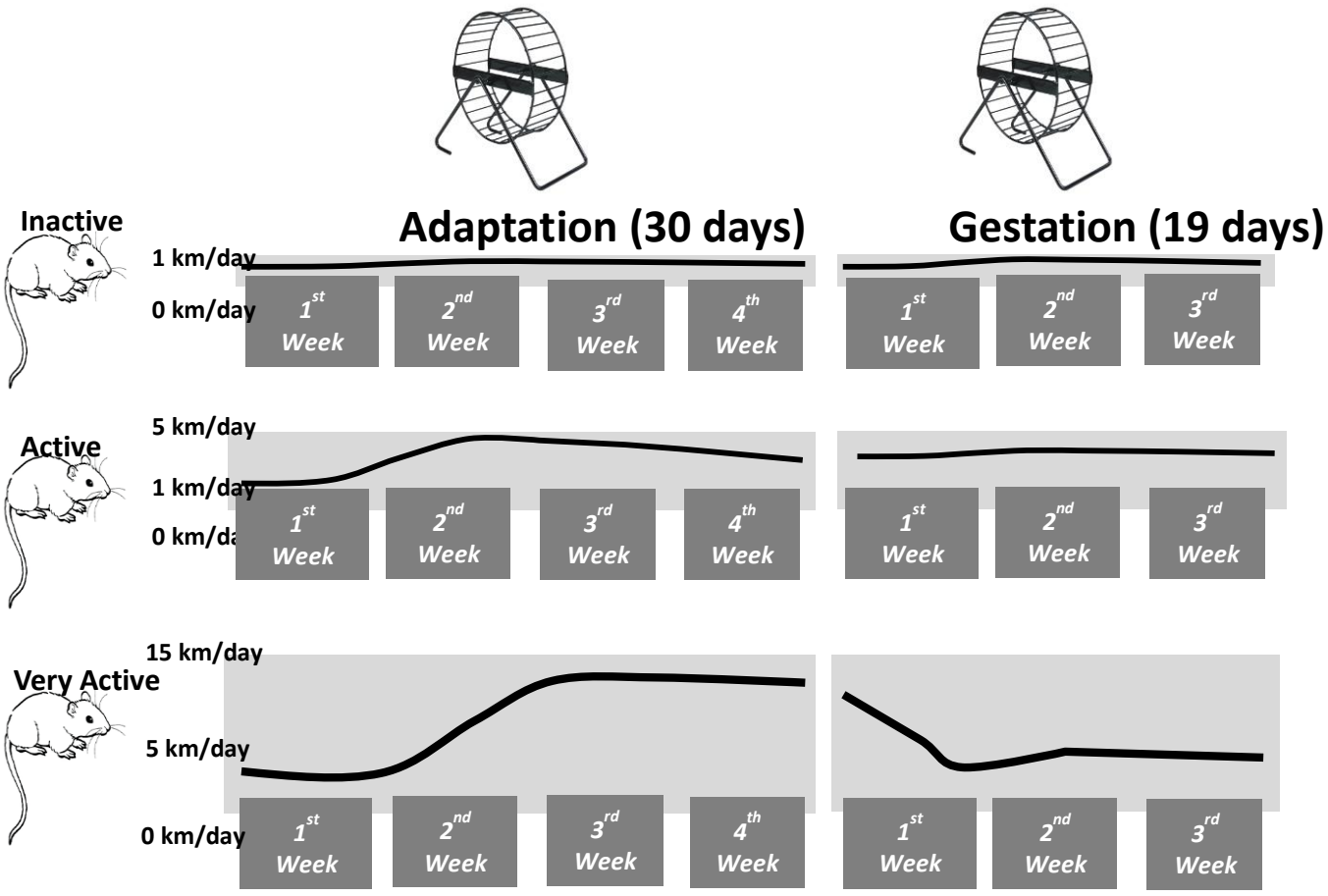
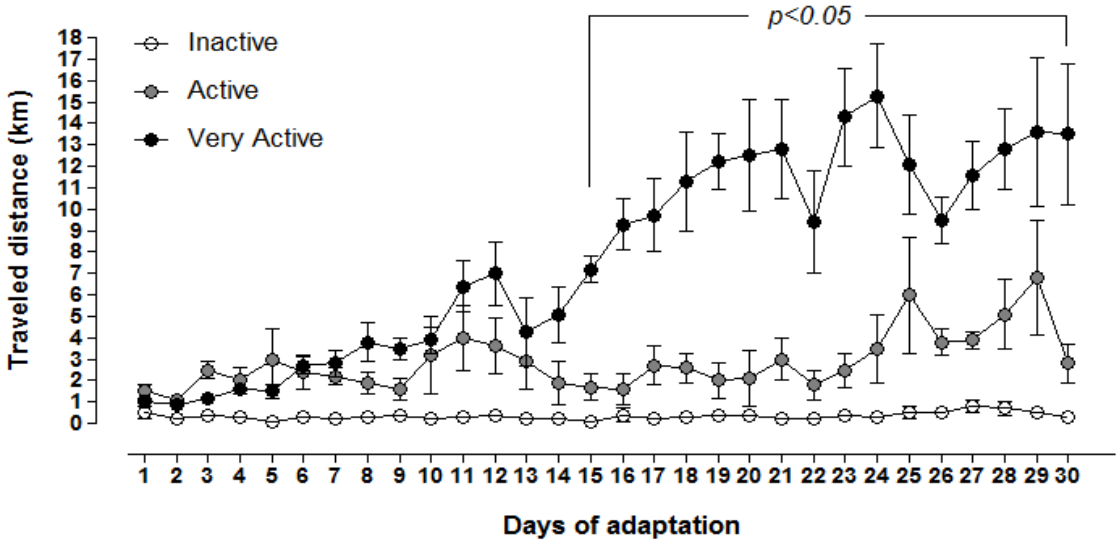
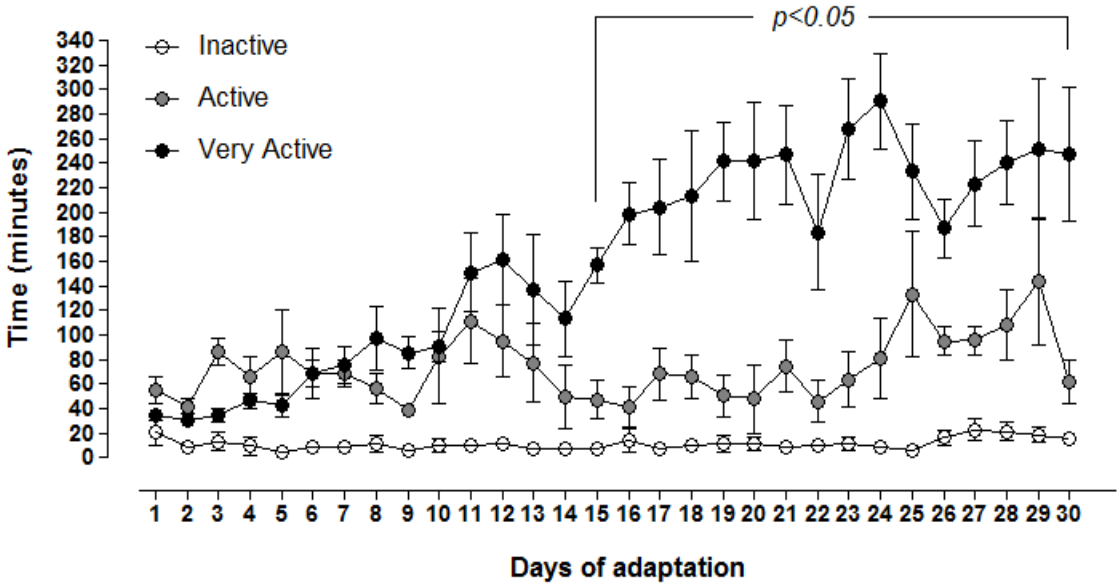


Figure 1. Graphical representation of the maternal voluntary physical activity during the period of adaptation and gestation of inactive, active and very active rats.

A


p<0.05
Inactive
Active
Very Active
Traveled distance (km)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Days of adaptation

B


p<0.05
Inactive
Active
Very Active
Time (minutes)
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Days of adaptation

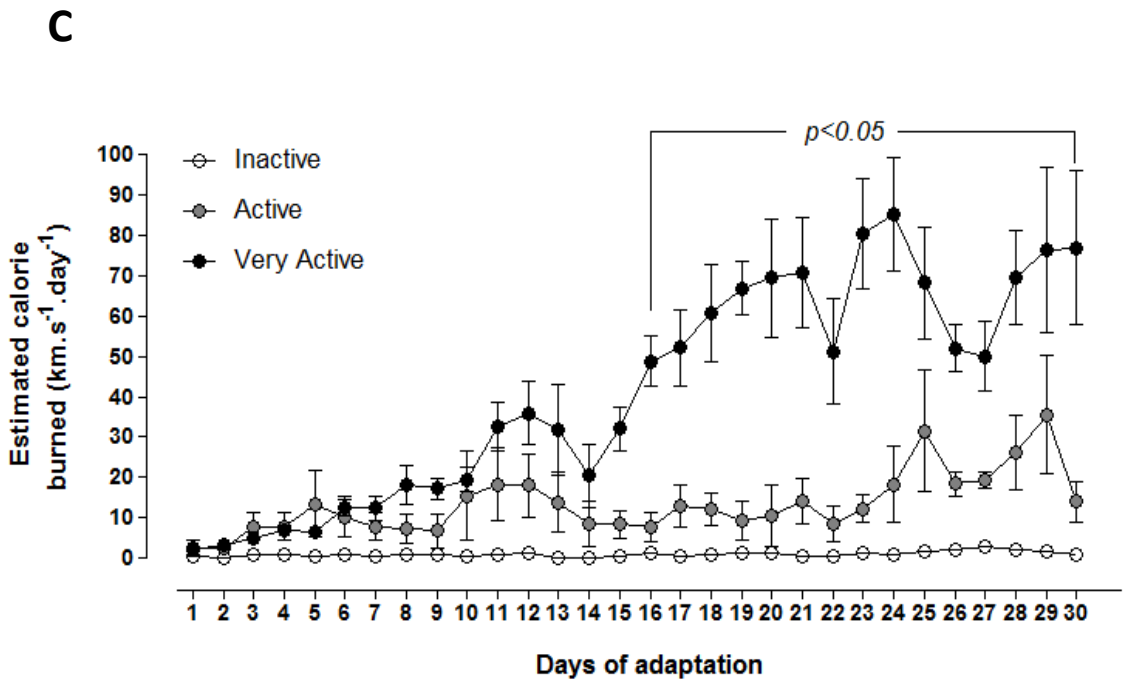
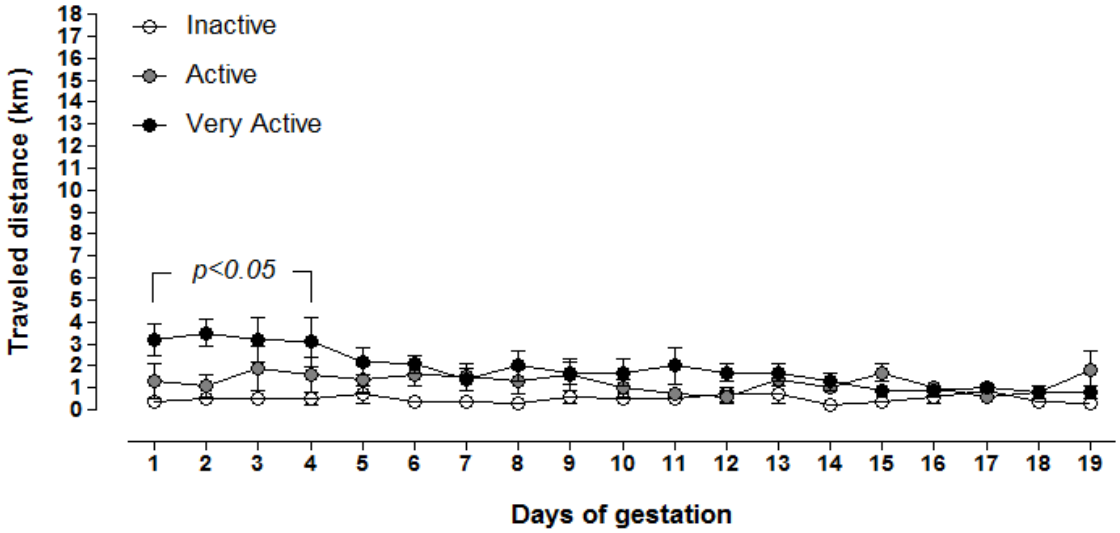
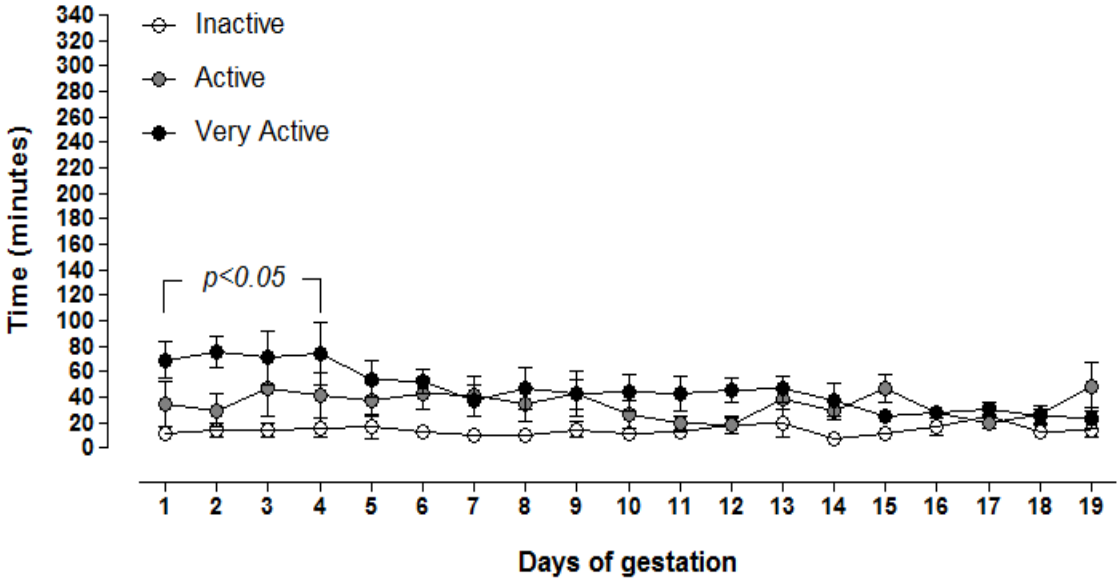


Figure 2. Parameters of voluntary physical activity for Inactive (*n*= 6), Active (*n*= 4) and Very Active dams (*n*= 5). Travelled distance (A), time of activity (B) and estimated calorie burned (C) were recorded during the period of adaptation. Values are presented as mean ± S.E.M. *p<0.05 *vs.* Statistical analysis was performed using two way ANOVA with Bonferroni post hoc test.

A


Inactive
Active
Very Active
Traveled distance (km)
p<0.05
Days of gestation

B


Inactive
Active
Very Active
Time (minutes)
p<0.05
Days of gestation

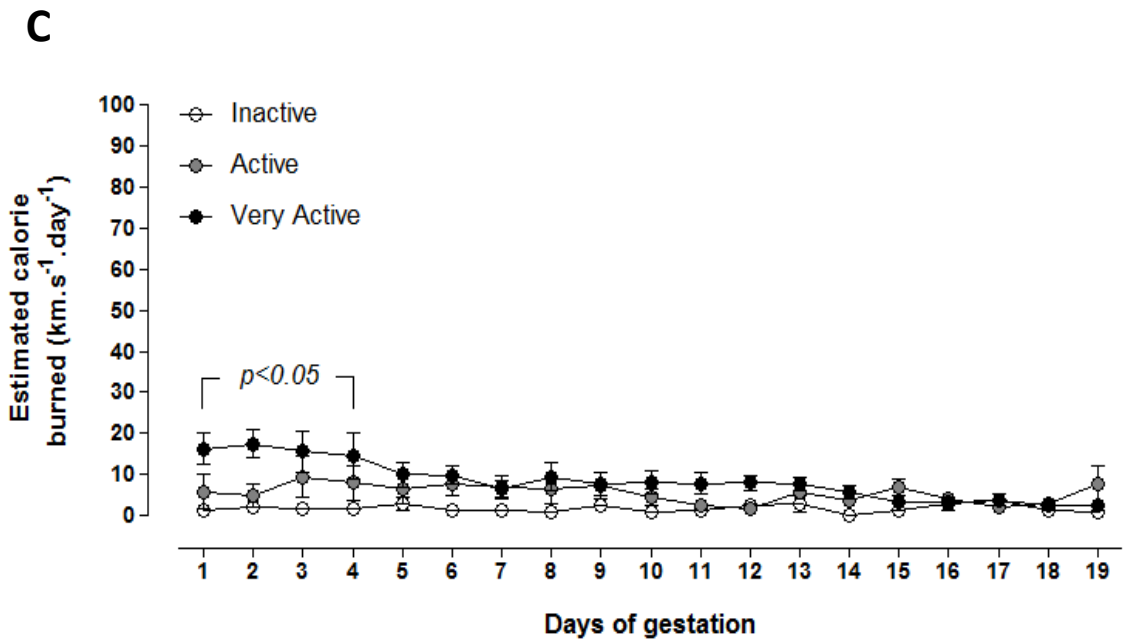
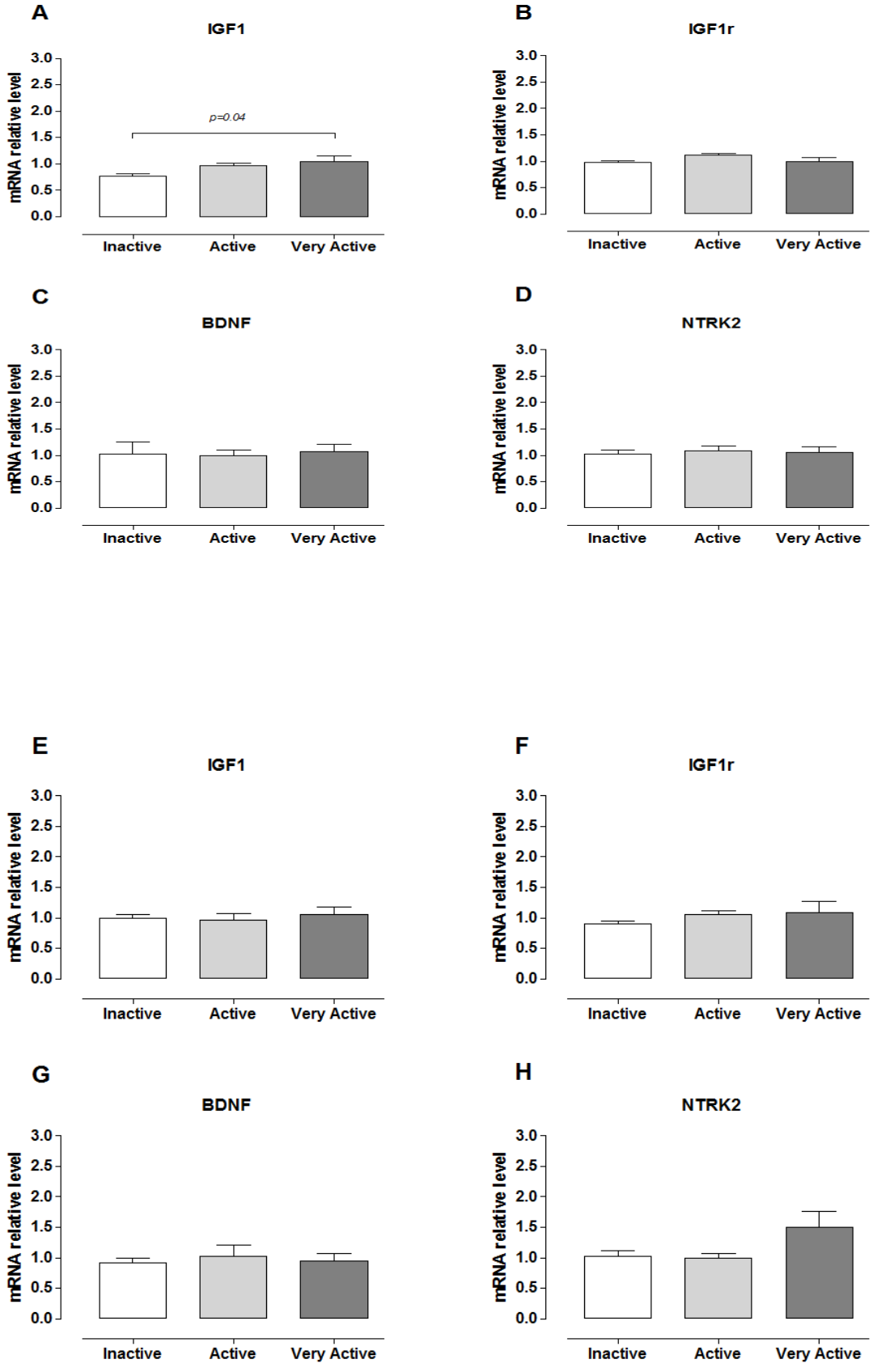
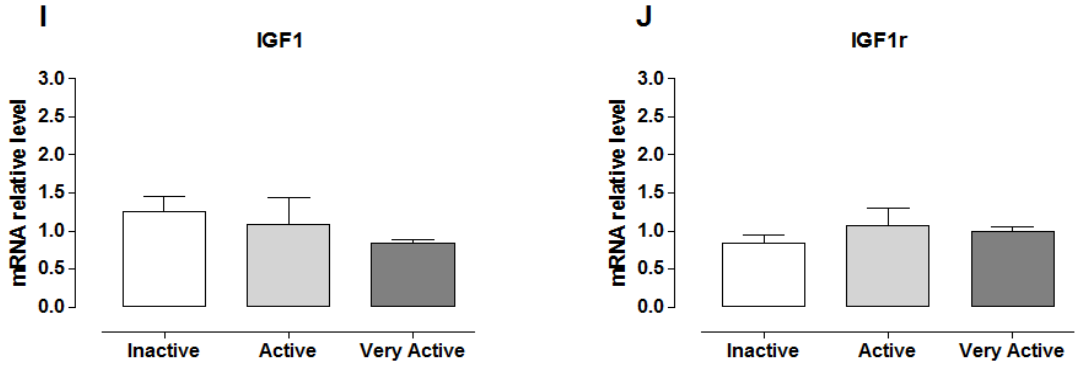
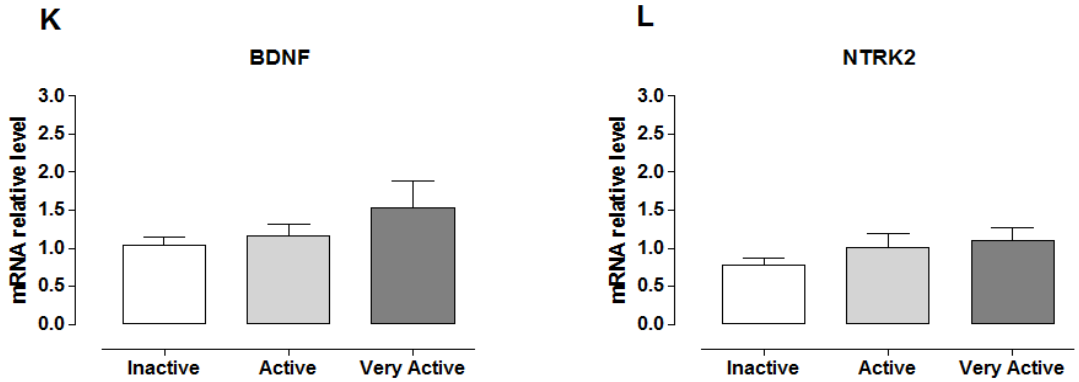
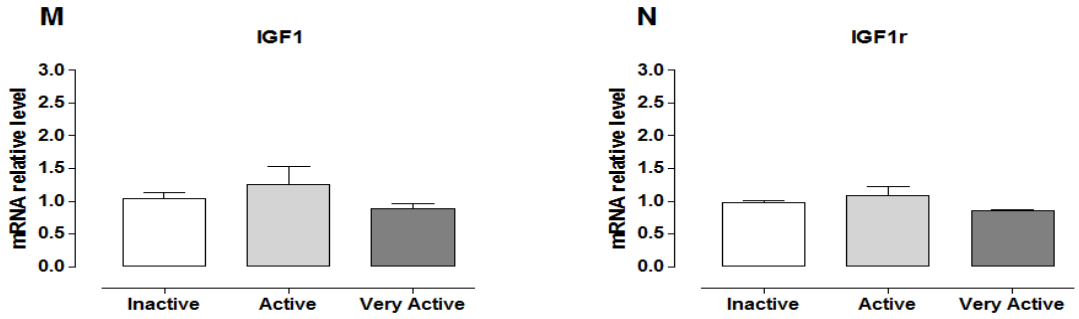


Figure 3. Parameters of voluntary physical activity for Inactive (*n*= 6), Active (*n*= 4) and Very Active dams (*n*= 5). Travelled distance (A), duration of physical activity (B) and estimated calorie burned (C) were recorded during the period of gestation. Values are presented as mean ± S.E.M. *p<0.05 *vs.* Statistical analysis was performed using two way ANOVA with Bonferroni post hoc test.


A
IGF1
p=0.04
B
IGF1r
C
BDNF
D
NTRK2
E
IGF1
F
IGF1r
G
BDNF
H
NTRK2
mRNA relative level
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Inactive
Active
Very Active


I
IGF1
J
IGF1r
mRNA relative level
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Inactive
Active
Very Active


K
BDNF
L
NTRK2
mRNA relative level
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Inactive
Active
Very Active


M
IGF1
N
IGF1r
mRNA relative level
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Inactive
Active
Very Active

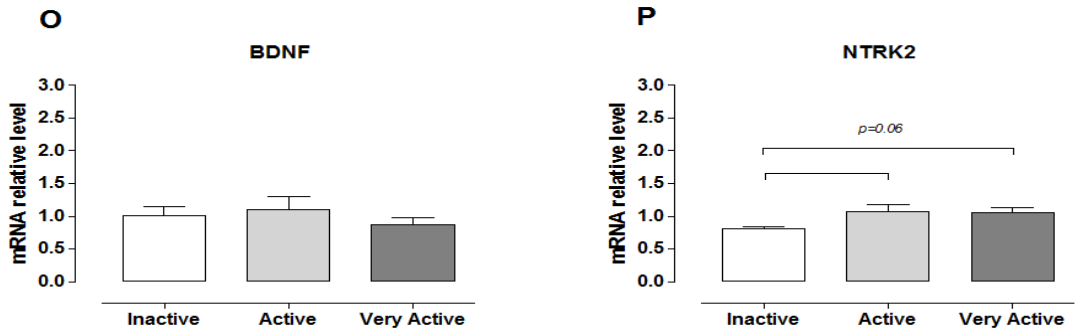
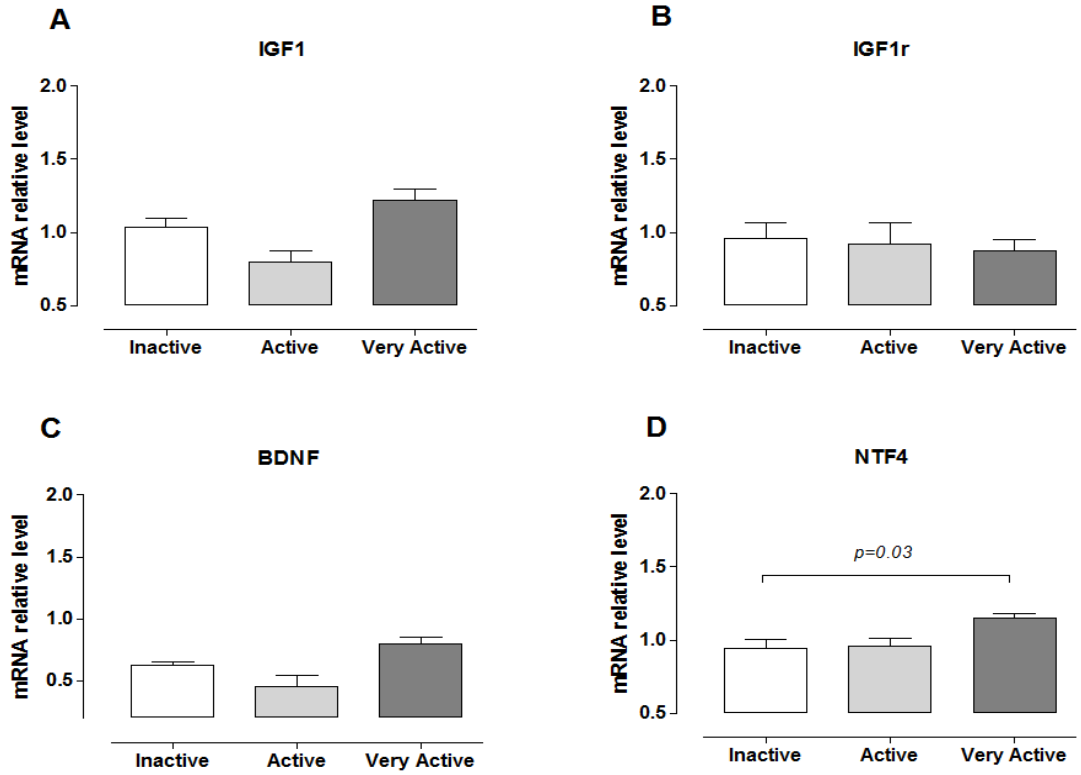

O
BDNF
P
NTRK2
p=0.06
mRNA relative level
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Inactive
Active
Very Active

Figure 4. mRNA expression of IGF1, IGF1r, BDNF and NTRK2 in the cerebellum (A-D), hypothalamus (E-H), hippocampus (I-L) and cortex (M-P). The groups were constituted by: Inactive (*n*=6), Active (*n*=4) and Very Active (*n*=5). Values are presented as mean ± S.E.M. Statistical analysis was performed using one-way ANOVA with Tukey post hoc test.



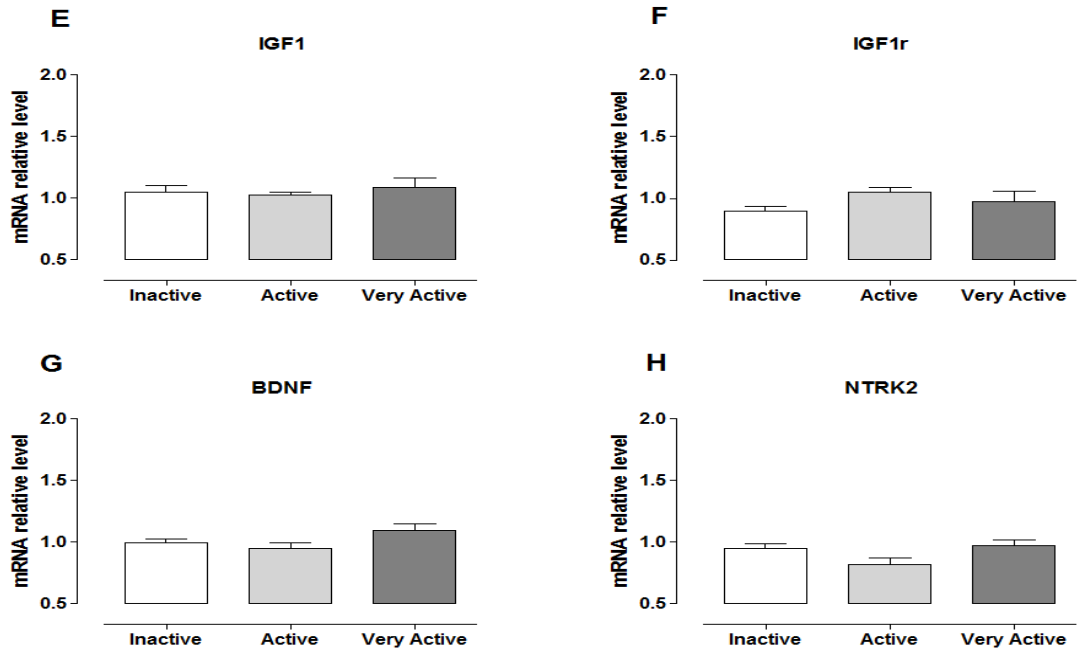


Figure 5. mRNA expression of IGF1, IGF1r, BDNF and NTF4 in the placenta (A-D). The groups were constituted by: Inactive (*n*=6), Active (*n*=4) and Very Active (*n*=5). mRNA expression of IGF1, IGF1r, BDNF and NTRK2 in the brain of offspring (E-H). The groups were constituted by: Inactive (*n*=12), Active (*n*=8) and Very Active (*n*=10). Values are presented as mean ± S.E.M. Statistical analysis was performed using one-way ANOVA with Tukey post hoc test.

Table 1. Primers sequence used to perform qRT-PCR

Gene	Forward/ Reverse	T	Sequence 5'-3'	Amplicon size
Igf1	F	60°C	GCTCTTCAGTTCGTGTGTGG	108 bp
	R		GCAACACTCATCCACAATGC	
Igf1r	F	60°C	CTGGTCTCTCATCTTGGATGC	197 bp
	R		GCTTCCCACACACACTTGG	
Bdnf	F	60°C	GAGTGAAGATACCATCAGCA	117 bp
	R		ATCTAGGCTACGTGAAGTCT	
Ntf4	F	65°C	CTGAGATGTCAGGGAGGAGA	115 bp
	R		ATGGCTTTGCACACCTGTCA	
Ntrk2	F	60°C	GTGGTGATTGCCTCTGTGG	149 bp
	R		TTGGAGATGTGGTGGAGAGG	
RPL19	F	58°C	CTGAAGGTCAAAGGGAATGTG	195 bp
	R		GGACAGAGTCTTGATGATCTC	
Actb	F	60°C	AGCCATGTACGTAGCCATCC	231 bp
	R		TCCCTCTCAGCTGTGCTGGTGAA	

Table 2. Maternal and fetal physiological parameters during adaptation (30 days before pregnancy) and gestation. Values expressed as Mean and S.E.M.

	INACTIVE		**ACTIVE**		**VERY ACTIVE**		**P values**
	Mean	S.E.M	**Mean**	S.E.M	**Mean**	S.E.M	
ADAPTATION							
Initial BW (g)	223.5	4.0	223.0	4.7	221.6	5.0	0.952
Final BW (g)	233.2	5.2	236.5	7.1	231.6	8.2	0.890
Gain of BW (g)	9.7	3.7	13.5	3.6	10.0	4,4	0.784
Food intake (g/day)	13.6	0.6	14.1	1.1	16.0	0.3	0.070
Fasting Glycaemia at day 30 (mg/dL)	103.2	5.9	98.7	3.4	101.2	2.4	0.808
GESTATION							
Initial BW (g)	255.5	8.1	256.3	7.3	245.4	7.8	0.582
Final BW (g)	324.3	13.3	318.5	11.8	350.6	5.5	0.150
Gain of BW (g)	68.8	7.0	62.2	13.8	**105.2**[a,b]	4.9	**0.008**
Food intake (g/day)	17.0	0.7	16.7	0.4	**20.5**[a,b]	0.7	**0.003**
Fasting Glycaemia at day 20 (mg/dL)	63.2	3.5	69.5	4.4	66.8	4.2	0.546
Number of pups	11.0	0.7	11.2	0.6	**13.4**[a]	0.2	**0.022**
Number of female pups	3.3	0.5	3.5	0.9	5.4	0.5	0.055
Number of male pups	7.7	0.7	7.7	0.5	8.0	0.5	0.925
Pups weight (g)	3.6	0.2	3.0	0.3	4.2	0.5	0.092
Placenta weight (g)	0.6	0.08	0.5	0.02	0.5	0.03	0.191

Mothers and placenta: Inactive (n=6), Active (n=4) and Very Active (n=5).
Pups: Inactive (n=12), Active (n=8) and Very Active (n=10). [a]$p<0.05$ *vs* Inactive and [b]$p<0.05$ *vs* Active using one-way ANOVA with Tukey's post-hoc.
BW = Body Weight

6.2 Artigo 2

Title: Effects of maternal undernutrition and physical activity on transcriptional response of neurotrophic factors in the mother brain, placenta and the fetus brain

Short-title: Undernutrition-induced neuroplasticity in active mothers

Authors: Jéssica Fragoso[1,2], Gabriela Carvalho Jurema Santos[2], Helyson Thomaz da Silva[1], Emmanuelle Loizon[3], Viviane Nogueira Oliveira[2], Hubert Vidal[3], Rubem Carlos Araújo Guedes[1], João Henrique Costa-Silva[2], Raquel da Silva Aragão[2], Luciano Pirola[3] and Carol Gois Leandro[1]

Authors Institution:

[1] Department of Nutrition, Federal University of Pernambuco, 50670-901 Recife, PE, Brazil

[2] Department of Physical Education and Sports Science, Federal University of Pernambuco, 55608-680 Vitória de Santo Antão, PE, Brazil

[3] CarMeN (Cardiology, Metabolism and Nutrition) Laboratory; INSERM U1060, Lyon-1 University, South Lyon Medical Faculty, 69921 Oullins, France.

Address of corresponding author:

Carol Góis Leandro

Núcleo de Nutrição, Universidade Federal de Pernambuco. Centro Acadêmico de Vitória – CAV. Phone: (00 55 81) 21268463. Fax: (00 55 81) 21268473. **E-mail:** carolleandro22@gmail.com

Abstract

Maternal protein restriction and physical activity can affect the interaction mother-placenta-fetus. This study quantified the gene expression of BDNF, NTF4, NTRK2, IGF-1 and IGF-1r in the different areas of mother's brain (hypothalamus, hippocampus and cortex), placenta and fetus brain of rats. Female Wistar rats (n=20) were housed in voluntary physical activity cages, containing a running wheel, for 4 weeks before gestation. Rats were classified as inactive or active according to distance spontaneously travelled daily. During gestation, dams remained to have access to the running wheel and half of each group received normoprotein diet (18% protein) and the other half received low-protein diet (8% protein). At the 20th day of gestation, gene expression of neurotrophic factors was analysed by quantitative PCR. Dams submitted to a low-protein diet during gestation showed up-regulation of IGF-1r and BDNF mRNA in the hypothalamus, IGF-1r and NTRK2 in the hippocampus, BDNF, NTRK2, IGF- 1 and IGF-1r in the cortex. In the placenta, there was a down regulation of IGF-1. In fetus brain, there was a down regulation in IGF-1r and NTRK2. Voluntary physical activity attenuated the effects of low-protein diet on IGF-1r in the hypothalamus, IGF-1r and NTRK2 in the hippocampus, IGF-1 in the placenta and NTRK2 in the fetus brain. In conclusion, maternal protein restriction and physical activity can influence the gene expression of BDNF, NTRK2, IGF-1 and IGF-1r in the mother's brain. There is an important interaction between mother and placental development in the control of fetal growth factors gene expression.

Keywords: Physical exercise; low-protein diet, pregnancy; neuroplasticity; rats; neurotrophic factors

Introduction

A balanced maternal diet during gestation and lactation is needed to maintain a full-term pregnancy and cover increased maternal and placental-fetal metabolism and juvenile growth of the offspring. According to the Recommended Dietary Allowances (RDA), in humans, daily caloric intake should increase by approximately 300 kcal during pregnancy and the recommended protein intake should increase to 1.1g of protein/kg/day during pregnancy from 0.8g of protein/kg/day for non-pregnant states (KOMINIAREK e RAJAN, 2016). Critically, maternal malnutrition may impair placentation, with resulting changes in placental size, morphology, and blood flow (BELKACEMI *et al.*, 2010). To the fetus, the subsequently compromised supply of nutrients will affect organogenesis, growth and neural development resulting in intrauterine growth restriction (IUGR) and newborns with low birthweight, with both conditions being associated with short- and long-term effects in development of metabolic diseases and neural and behavioral disturbances (DE BRITO ALVES *et al.*, 2016; FRAGOSO *et al.*, 2017b; DOS SANTOS *et al.*, 2018). Particularly in the central nervous system (CNS), a close relationship between the maternal nutritional status, brain development and cognitive function level has been consistently detected in previous studies according to a recent systematic review (VEENA *et al.*, 2016).

The maternal phenotype, or "maternal capital" is the primary influence on early nutrition and developmental trajectory of offspring (WELLS, 2018). In rodents models of undernourished dams (8% protein), reductions in body weight gain, anemia and microalbunminuria (defined as albumin/creatinine ratio ≥2.5) during gestation have been reported (FIDALGO *et al.*, 2013; DE BRITO ALVES *et al.*, 2014; FRAGOSO *et al.*, 2017b). Undernutrition results in placentas with lower weight and size, reduced blood flow and proliferation of Langhan's cell of the villi, calcification and diminished villous surface (BELKACEMI *et al.*, 2010; TARRADE *et al.*, 2015). In the fetus, maternal protein restriction

will affect the growth and development, proliferation, migration, differentiation, synaptogenesis, myelination, and the expression of multiple growth factors (neurotrophins) (BELKACEMI *et al.*, 2010; AMARAL *et al.*, 2015; GONZALEZ-MACIEL *et al.*, 2015). Most of the evidences at the cellular and molecular levels of these undernutrition-dependent defects is from studies on the placenta, fetuses and the offspring during development. Most notably, significant less attention has been directed towards the neurobiology of undernourished mothers. We hypothesize that maternal neuroplasticity can provide a physiological adaptation to the magnitude and programming of nutritional investment on placental-fetus development. This adaptation would expose the offspring to a nutritional environment that may optimize the offspring's adaptation in response to maternal nutritional constraints.

Brain-derived neurotrophic factor (BDNF) is an important neurotrophin that influence almost all aspects of the CNS development, such as neuronal proliferation, migration and survival, synapse formation, axonal and dendritic plasticity (GARCES *et al.*, 2014). BDNF also has been shown to exert an important role during embryo implantation, placental development, and fetal growth control in mice (MAYEUR *et al.*, 2010). Maternal food restriciton (50% of the food-intake of control mothers) induced IUGR of fetuses at term and decreased the placental BDNF and its functional receptor (NTF4) (MAYEUR *et al.*, 2010). In the brain of growth-restricted rat offspring, BDNF mRNA was up regulated and its tyrosine kinase receptor B (TrkB or NTRK2) gene expression (LEE, DUAN, *et al.*, 2002). Administration of a maternal low-protein diet (7 to 8% casein) to rats results in the development of fewer synapses, synaptic structural changes, decreased dendritic span and arborization (complexity of branching projections) and decreases insulin-like-growth factor (IGF-1) levels and its receptor (IGF-1-r), which influence myelin production (JONES e DYSON, 1981; GONZALEZ-MACIEL *et al.*, 2015; IKEDA *et al.*, 2016). However, less is known about gene expression of neurotrophic factors in different areas of mother's brain and placenta.

Many of the effects of maternal protein restriction are permanent, though some degree of plasticity may be expected by the simultaneous exposure to a stimulating and enriched environment, such as physical activity. Previous studies have shown that maternal physical activity may mitigate or even prevent maternal protein restriction-programmed neuronal growth and development impairments in the offspring (LEANDRO *et al.*, 2012; FIDALGO *et al.*, 2013; FRAGOSO *et al.*, 2017b). Healthy pregnant women without medical contraindications should be encouraged to participate in regular physical activity of moderate aerobic intensity at least 150 min per week (analogically 20-30 min per day on most or all days of the week) (FERRARI e GRAF, 2017). In humans, infants from active mothers during pregnancy (three times per week, at least 20 min/day at 55% of their maximal aerobic capacity) showed a better response to sound discrimination and auditory memory as measured by electroencephalography (LABONTE-LEMOYNE *et al.*, 2017). In rats, young pups born from dams which were active throughout pregnancy showed an increased amount of neuronal and non-neuronal cells in the hippocampus, improved cognitive functions (habituation behaviour and spatial learning) and enhanced memory as tested using a novel object recognition paradigm (ROBINSON e BUCCI, 2014; GOMES DA SILVA *et al.*, 2016). In our previous study, voluntary physical activity on running wheel before and during breeding attenuated the effects of maternal low-protein diet (8% protein) on patterns of locomotor activity of offspring rats at 60 days old (FRAGOSO *et al.*, 2017a). However, less is known about the molecular mechanisms in the brain and placenta that could be related to the gene expression of neurotrophins during gestation in both mother and fetus.

In line with the reported contribution of BDNF, NTF4/NT-4, NTRK2, IGF-1 and IGF-1r in placental development and in the control of fetal growth, the present study tested the hypothesis that maternal neuroplasticity represents the first investment to which offspring are exposed, and hence is the primary influence of early nutrition and physical activity on neural

development. In this study, two maternal environmental stimuli were tested (low-protein diet and physical activity), but the focus is on the proposing that the variability in maternal investment is shaped by trade-offs that emerged during maternal environment. This may help explain placental commitment to maintain intrinsic quality of fetal development. Thus, the main goal of this study was to quantified the gene expression level of BDNF, NTF4/NT-4, NTRK2, IGF-1 and IGF-1r in the different areas of mother's brain (hypothalamus, hippocampus and motor cortex), placenta and foetal's brain of rats.

Material and methods

The experimental protocol was approved by the Ethical Committee of the Biological Sciences Center (protocol nº 23076.015984/2015-30), Federal University of Pernambuco, Recife, PE, Brazil, and we followed the Guidelines for the Care and Use of Laboratory Animals.

Animals and experimental diets

Twenty virgin female albino Wistar rats (*Rattus norvegicus*) aged 85-95 days were obtained from the Department of Nutrition, Federal University of Pernambuco, Brazil. Animals were maintained at a temperature of 22 ± 1°C with a controlled light–dark cycle (dark 06.00 am – 6.00 pm). Food and water were given *ad libitum* throughout the experiment. The rats were individually housed in voluntary physical activity cages (cages equipped with a running wheel) for 4 weeks. After this period, the rats were classified as inactive (I; n=11) and active (A; n=9) and were placed into a standard cage and mated (1 female for 1 male) for a period of 1–5 days. Females had no access to the running wheel during mating. The day on which spermatozoa were present in a vaginal smear was designated as day 0 of gestation. Dams were transferred back to their original cages with free access to the running wheel throughout gestation. Part of the dams received a casein-based diet (AIN-93G diet, containing 18% protein) and the other

part received the same diet, but low amount of protein (8% protein). Thus, groups were formed as follows: inactive normoprotein diet (I-NP, *n*=6); Inactive low-protein diet (I-LP, *n*=5); active normoprotein diet (A-NP, *n*=4) and active low-protein diet (A-LP, *n*=5). At day 20 of gestation, dams were euthanized by decapitation after a 6h fasting period. Experimental analyses were performed in specific brain areas of mothers (hypothalamus, hippocampus and motor cortex), placenta and the brain of offspring males. The tissues collected were stored at −80°C until RNA extraction.

Voluntary physical activity measurements

Female Wistar rats were individually housed in voluntary physical activity cages (with running wheels - 27 cm diameter) for a 4-weeks period of adaptation. A wireless cyclocomputer (Cataye, model CC-VL820, Colorado, USA) was attached in the wheel to calculate and display trip information, such as distance travelled, duration of activity and estimated calorie burned. These parameters were used to classify the rats according to the level of daily physical activity in: inactive (I) or active (A) according to previous studies (SANTANA MUNIZ *et al.*, 2014; FRAGOSO *et al.*, 2017a). After mating, rats continued to have access to the running wheel during gestation.

Body weight and food intake

Dams body weight was recorded each three days throughout the experiment. Maternal food consumption was determined by the difference between the amount of food provided at the onset of the dark cycle (06.00 hours) and the amount of food remaining 48 h later. Body weight of the pups and the placental weight were measured at the day of sacrifice (day 20 of gestation). Body weight was recorded using a Marte Scale (AS-1000) with 0.01 g accuracy.

Blood glucose measurements

Fasting glycaemia levels were evaluated in the last day of adaptation and weekly during gestation using blood samples from the tail vein of the rats, using a glucometer (Accu Check Advantage and Accutrend GCT) and the glucose oxidase method. The animals were fasted six hours prior to glycaemia measurement.

RNA extraction

Total RNA was extracted from brain regions of mothers (hypothalamus, hippocampus and cortex), placenta and the whole brain of offspring with TRI reagent® (SIGMA-ALDRICH T9424, St. Quentin Fallavier, FR) according to the manufacturer's instructions. Briefly, 1 mL of TRI reagent® was added per 50-100 milligram of tissue, the resulting suspension was homogenized and incubated at room temperature for 5 min. Thereon, 0.2 mL of chloroform was added, samples were vortexed for 15 seconds, incubated for 5 minutes at room temperature and centrifuged at 12,000*g* for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube and 0.5 mL of isopropanol were added to precipitate RNA. Samples were incubated for 10 minutes at room temperature and centrifuged at 12,000 *g* for 15 minutes at 4 °C. The supernatant was removed and RNA-containing pellets were washed sequentially with 75% and 100% ethanol and dissolved in 100 µL RNase free water. RNA concentration and purity (defined by a 260/280 nm absorbance ratio > 1.8) was determined on a Nanodrop 2000 (Thermofisher).

Reverse transcription

Reverse transcription was performed using an PrimeScript RT reagent Kit-Perfect Real Time (TAKARA) using 0.5 µg of RNA for brain of mothers (hypothalamus, hippocampus and cortex) and 1 µg of RNA for placenta and brain of offspring following the manufacturer's

instructions. RNase Free H_2O (3 µL), PrimeScript Buffer 5× (4 µL), Oligo dT - 50 µM (1 µL), Random hexamers - 100 µM (1 µL) and of PrimeScript RT Enzyme Mix (1 µL) were sequentially added, followed by a 15 minutes incubation at 37°C and 15 seconds at 85°C. Reverse transcription reactions were brought to 200 µL final volume by adding RNase free water and stored at −20°C.

Quantitative PCR (qPCR)

Real-time quantitative PCR amplification (qPCR) was performed using a Rotor-Gene Real-Time PCR System (Labgene Scientific Instruments, Archamps, France). The sequences of primers used in this study are reported in Table 1. Reactions were incubated at 95°C for 10 min, followed by 40 cycles of denaturation (95°C, 10 s), annealing (58–65°C depending on the primer sets, 30 s) and elongation (72°C, 30 s). mRNA expression levels of insulin-like growth factor 1 (IGF-1), insulin-like growth factor 1 receptor (IGF-1R), brain-derived neurotrophic factor (BDNF), neurotrophin 4 (NTF4) and neurotrophic tyrosine kinase receptor type 2 (NTRK2, or TrkB) were performed. qPCR results from each gene (including the housekeeping genes) were expressed as arbitrary units derived from a standard calibration curve derived from a reference sample. qPCR for each sample was carried out in duplicate. The mRNA levels of the analyzed genes were normalized using the mRNA levels of ribosomal protein L19 (RPL19) and beta actin (Actb).

Statistical analyses

The Kolmogorov–Smirnov test was performed to determine the normal distribution of data. Measurements of distance travelled, estimated calories burned and time of activity were analyzed by two-way ANOVA, followed by Bonferroni's post hoc test. During the adaptation period, statistical analyses of body weight, food intake and fasting glycaemia were performed

by *t* test student (Active *vs* Inactive). During gestation, statistical analyses were performed by using two-way ANOVA, with physical activity and diet as factors, followed by Bonferroni's post hoc tests. All data are presented as means ± S.E.M. Significance was set at $p<0.05$. Data analysis was performed using the statistical program GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Daily physical activity parameters (distance travelled, time spend in the wheel-running and estimated burned calories) of active and inactive dams before gestation are shown in Figure 1 (A-C). Of note, active dams spent around 1 to 2 hours in the wheel-running, performing 4-6 km per day before breeding. Body weight, food intake and fasting glycaemia assessment were not different between active and inactive dams (Table 2). During gestation, maternal exposure to low protein diet did not change the amount of distance travelled and time in the wheel-running (1 – 2 km per day) until the end of gestation (Figure 1A-C). Inactive dams, irrespective to the diet administered, continued performing less than 1 km/day. In addition, I-LP dams showed reduced food intake that was attenuated by voluntary wheel running (Table 3).

We next asked whether changes in the observed maternal active phenotypes were secondary to changes in either physical activity or protein-restriction on the expression of mRNA in different brain areas of mothers, placenta and brain of pups. In the hypothalamus, dams submitted to a low-protein diet during gestation showed up-regulation of IGF-1r and BDNF mRNA (Figure 3A). However, voluntary physical activity was able to attenuate the increase in IGF-1r while inducing further BDNF upregulation in the hypothalamus of active dams (Figure 3A). Likewise, in the hippocampus, maternal low protein diet-induced upregulation of IGF-1r and NTRK2 that were attenuated by voluntary physical activity (Figure 3B). In addition, BDNF was also increased in the hippocampus of active dams subjected to protein restriction.

It was interesting to observe that both maternal stimuli studied here, i.e., protein restriction and physical activity had the same effects on the upregulation of all genes that were evaluated in the motor cortex (Figure 3C). In the placenta, we observed a down regulation of IGF-1 in inactive LP dams (Figure 4), and, interestingly, this effect was attenuated by maternal physical activity.

In offspring brain, there was a down regulation in IGF-1r in response to low-protein diet irrespective of maternal physical activity. BDNF mRNA was up regulated in offspring brain from active dams (Figure 5). We also observed a down regulation of NTRK2 in inactive LP dams (Figure 5). This effect was attenuated by maternal physical activity (Figure 5).

Discussion

The mother brain and placenta interact during pregnancy and mediate maternal adaptations to support eventual environmental disturbances (BEHURA *et al.*, 2018). In the context of developmental neuroplasticity, previous studies have focused on the short- and long-lasting effects of nutrition on offspring, and on how fetuses adapt to diverse nutritional conditions (FERREIRA *et al.*, 2016; QASEM *et al.*, 2016; BERARDINO *et al.*, 2017; THANOS *et al.*, 2018). Conversely, environmental stimuli before and during pregnancy are primarily sensed by mothers, showing that maternal life history is highly relevant to any adaptations of the offspring (WELLS, 2018). In this study, we first used maternal protein restriction during pregnancy to investigate the gene expression of neurotrophic factors. In LP mothers, IGF-1r and BDNF mRNAs were upregulated in the hypothalamus. NTRK2 was upregulated in the hippocampus and all neurotrophic factors were upregulated in the motor cortex of the LP mothers. Changes in the neural structure, function and molecular patterns at the end of gestation period can be expected on certain brain areas that need to be plastic to specific circumstances, and adaptable to environmental cues (KIM *et al.*, 2016). Next, we found that placenta protected the fetal brain except for IGF-1r and NTRK2 mRNA that were

downregulated, probably because of the reduced placental IGF-1 mRNA. The connectivity between mother and placenta (and consequently the fetus) may be adaptable according to the demands of different environmental stimuli. Indeed, maternal physical activity, irrespective to the diet, did not affect the transcriptional response of neurotrophic factors, as supported by previous studies on phenotype flexibility (SANTANA MUNIZ *et al.*, 2014; SENNA *et al.*, 2016; FRAGOSO *et al.*, 2017b). However, some of undernutrition-induced changes on transcriptional response of neurotrophic factors were inadaptable, and active maternal phenotype was not able to revert, for example, the effects on motor cortex in mother's brain. These findings provide new evidence for and insights into how the mother brain, placenta and fetus brain interact during pregnancy in response to different environmental cues.

During gestation, formerly active mothers remain active although with a reduction in the amount of distance travelled (1 – 2 km/day) and time (30 – 40 min/day) in the wheel-running, regardless to the switching to a low protein diet. This result is in accordance with previous studies using the same experimental model (SANTANA MUNIZ *et al.*, 2014; FRAGOSO *et al.*, 2017b). In addition, food intake, weight of placenta and the body weight of the fetus were similar among groups. Paradoxically, in energetic terms, the maternal dietary intake during pregnancy has relatively modest effects on fetal energy requirements even after addressing placental costs (BUTTE e KING, 2005; TARRADE *et al.*, 2015; WELLS, 2018). It is interesting to observe that active mothers exercised approximately 1 to 2 hours on the wheel-running (4 – 6 km/day) before breeding. Active mothers probably allocated relatively more energy to gestation by increasing basal oxygen consumption and fat free-mass (FIDALGO *et al.*, 2013), and dams with high levels of daily physical activity may increase the protein anabolism and promote nutritional investment to their offspring during pregnancy even with low-protein diet (LEANDRO *et al.*, 2012; FIDALGO *et al.*, 2013). Thus, it is plausible to

consider maternal voluntary physical activity as a proactive prediction on the part of the mother in order to buffer an eventual injured growth trajectory on the part of the offspring.

IGF-1r mRNA was up regulated in hippocampus, hypothalamus and motor cortex of dams brain during gestation. In this respect, our data converge with previous studies (PATZ e WAHLE, 2004; COUPE *et al.*, 2009; KIM *et al.*, 2016). IGF-1r has a potent effect on cellular neuroplasticity in neuroepithelial cell types, and shows a relatively stable pattern of expression from early development to maturity (DYER *et al.*, 2016). The increased IGF-1r expression highlights the association between regions of increased neurogenesis and the active transport mechanism that allows peripheral circulating IGF-1 to cross the blood brain barrier and allows mother brain to be responsive to undernutrition. Physical activity mitigated the up regulation of IGF-1r in all areas studied except for motor cortex. Peripheral IGF-1 levels are quickly increased in humans in response to physical exercise and an adaptive response of its receptors was seen in the brain (DYER *et al.*, 2016; WRIGLEY *et al.*, 2017). In turn, normalization of brain IGF-1r mRNA may be caused not only by increased circulating levels of IGF-1 but also by the increased brain blood flow induced by exercise or IGF-1 itself (WRIGLEY *et al.*, 2017).

The placenta plays a foremost role in the development of the fetal brain and also influences maternal brain function, by a mechanism that may include neurotrophic and growth factors (DYER *et al.*, 2016; BEHURA *et al.*, 2018). Our data showed that IGF-1 mRNA was down regulated in the placenta of LP mothers, but maternal physical activity was able to attenuate this reduction. A previous study showed that maternal exercise (treadmill, 20 m/min for 20 min/day, over 19 days) significantly increases plasma IGF-1 concentration in the late period of pregnancy (TURGUT *et al.*, 2006). Brains from fetuses showed reduced IGF-1r gene expression in response to maternal low-protein diet and physical activity. The pattern of changes in placental and fetal IGF-1 mRNA mirrors weight gain and neural development (KIM *et al.*, 2016). Indeed, our recent data from malnutrition-induced delayed neurodevelopment in

rats offspring showed that physical features, reflex ontogeny and somatic growth were affected in pups from protein-restricted mothers and physical activity acted as a buffer for these effects (FRAGOSO *et al.*, 2017b). The present study confirms the importance of both IGF-1 and IGF-1r as the molecular mechanism related to adaptive response to malnutrition and physical activity during embryonic development and placental growth.

In mother brain, BDNF mRNA was up regulated in response to protein restriction and physical activity in both hypothalamus and motor cortex. In adult rats, a close relationship between the nutritional status and BDNF level in different areas of the brain has been observed and may modulate neuronal proliferation, neuritogenesis, axonal and dendritic plasticity, synapse formation, stimulation of neurotransmitter or neuropeptide synthesis and release (COUPE *et al.*, 2009). In addition, one of the most well established brain metabolic changes evoked by exercise is indeed the increase of BDNF and NTRK2 mRNA (DE ASSIS *et al.*, 2018). These observations indicate that maternal physical activity, initiated prior to pregnancy induces maternal-foetal adaptations and can be considered as a maternal investment countering the effects of maternal undernutrition. The underlying mechanism can be related to epigenetic modulation induced by physical activity that regulates gene expression (GOMEZ-PINILLA *et al.*, 2011). In line with this hypothesis, we found that maternal physical exercise induces up regulation of BDNF and its receptor NTRK2. In the case of these changes occurring during the critical period of foetal development, nutrition and physical activity assume important roles in the control of gene transcription in the context of the long-term effects of developmental plasticity.

In accordance with previous observations (LEE, DUAN, *et al.*, 2002; LEE, SEROOGY, *et al.*, 2002; COUPE *et al.*, 2009), our findings showed that, in developing rats, BDNF mRNA levels are significantly augmented in fetal brains during sensitive developmental windows. Both maternal undernutrition and physical activity enhanced BDNF mRNA in fetal brains. The

environmental stimuli acting in the critical period of CNS development may elicit molecular signals to protect brain development. Thus, BDNF could be implicated in the metabolic adjustment during food-restricted conditions and energetic expenditure that may also participate in the protection of the CNS against excitotoxic damage under these conditions (COUPE *et al.*, 2009). BDNF modulates survival, differentiation, and activity of neurons by binding to its high affinity receptor, NTRK2, which is responsible for the initiation of intracellular signaling cascades and the regulation of the local availability and responsiveness to BDNF (DE ASSIS *et al.*, 2018). We showed that the gene-expression of NTRK2 is down regulated in fetus brain. Maternal LP diet may especially have affected the neurotrophic BDNF/NTRK2 pathway during the perinatal life. However, pups from active LP mother did not change mRNA NTRK2. Thus, nutrition and physical activity can be considered benefic investments during pregnancy, and fetal life is a critical period not only for structural and functional development of brain, but also for epigenetic influences. Collectively, these traits underpin the maternal and placental capacity for safeguarding the magnitude of prenatal growth as a valuable marker of the intrinsic quality of the neuroplasticity.

Conclusion

In conclusion, maternal protein restriction and physical activity can influence the gene expression of BDNF, NTRK2, IGF-1 and IGF-1r in different areas of mother brain. There is an important interaction between mother and placental development in the control of fetal growth factors gene expression. In the present study we demonstrated maternal neuroplasticity representing the first investment for offspring and the primary influence of early nutrition and physical activity on neural development. Our observation also suggest an important role of placenta in the maintenance of fetal neurodevelopment.

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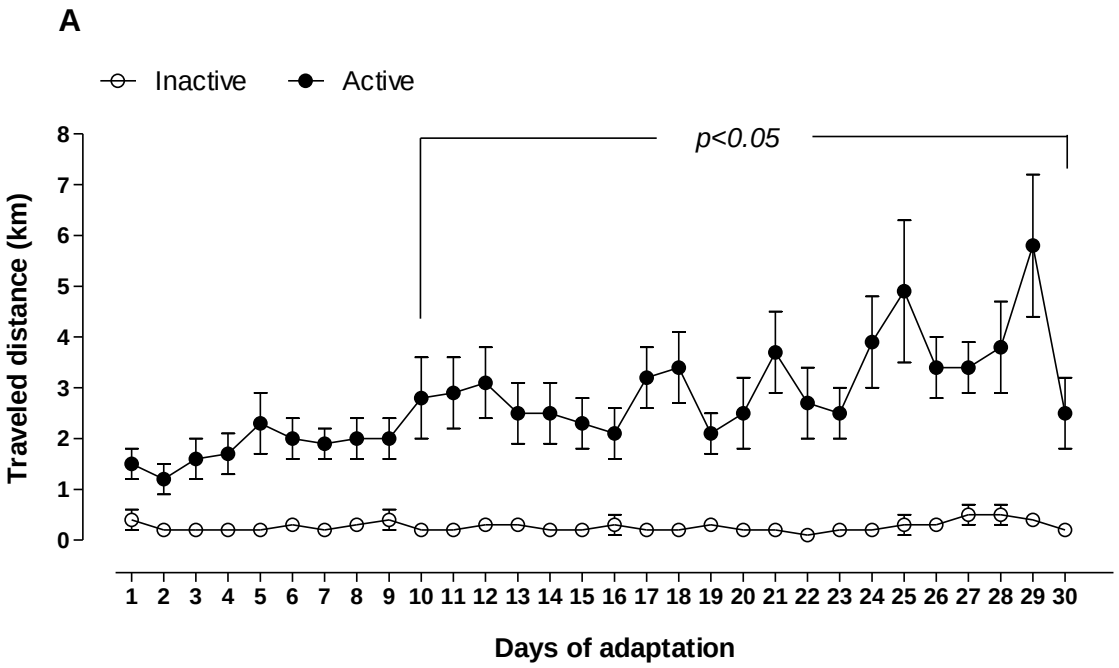
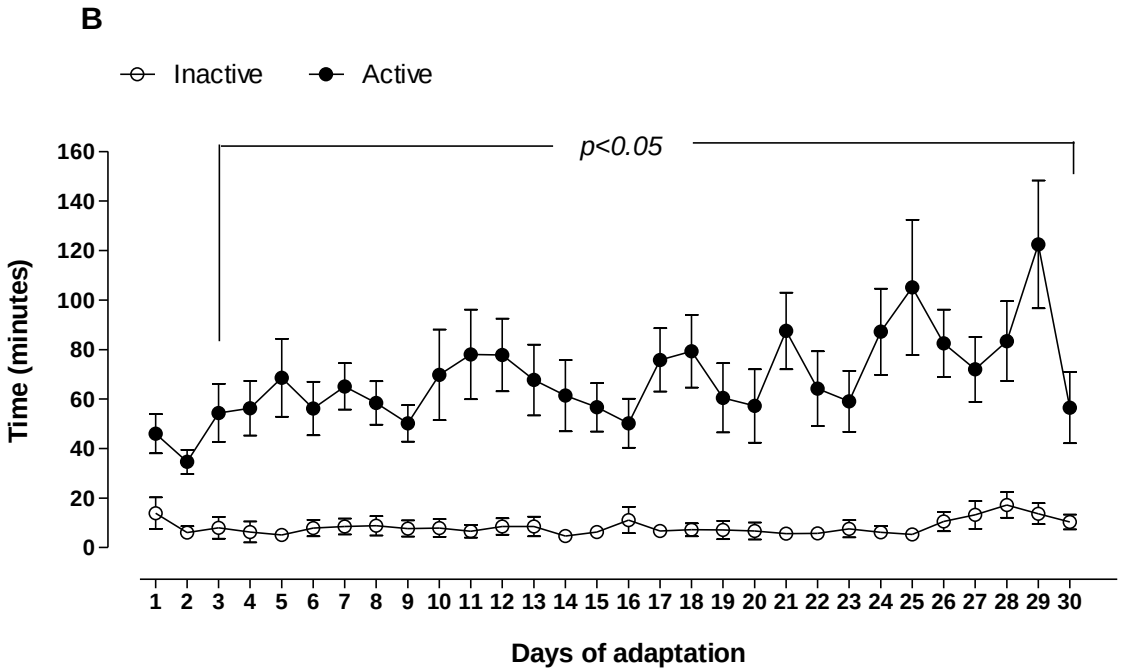
Legend and Figures





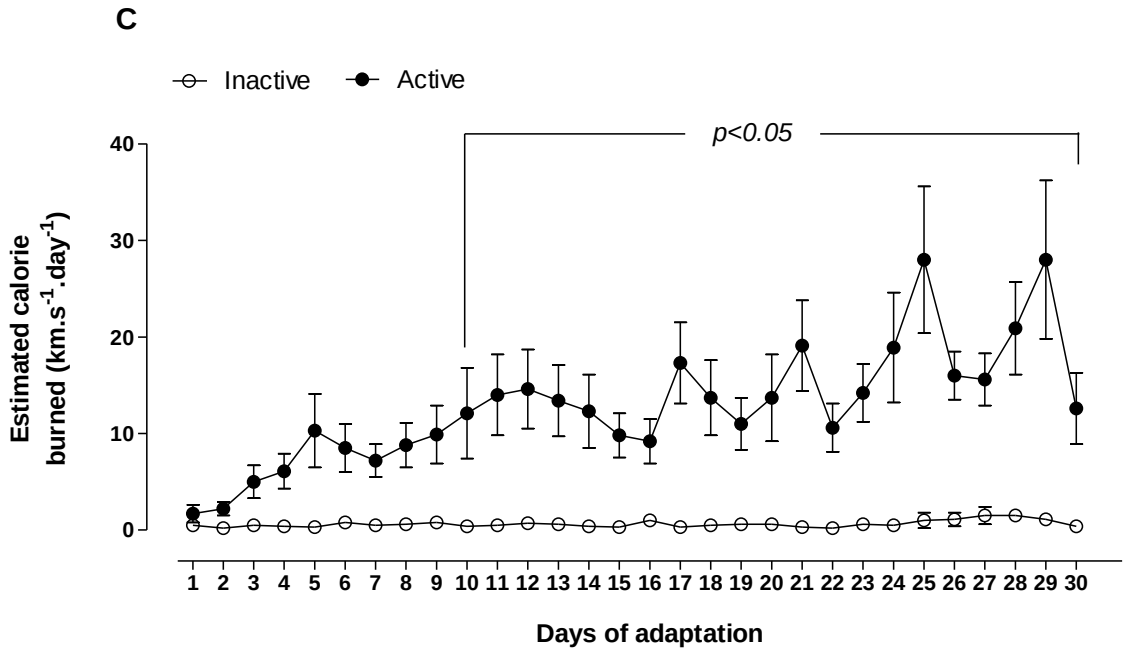
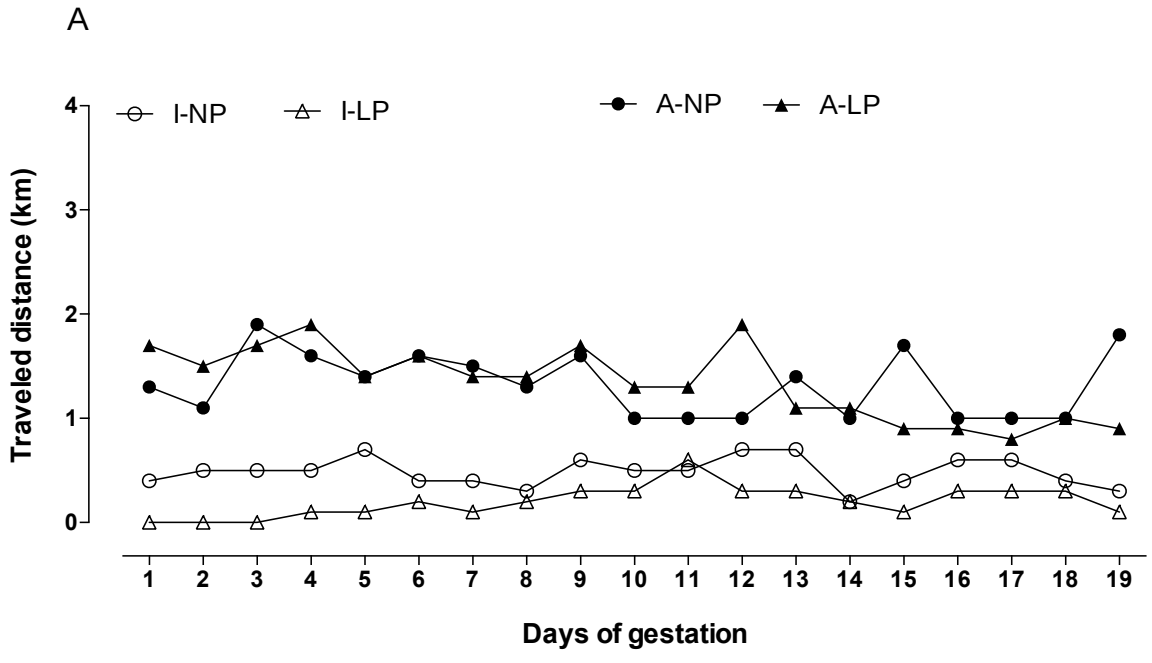
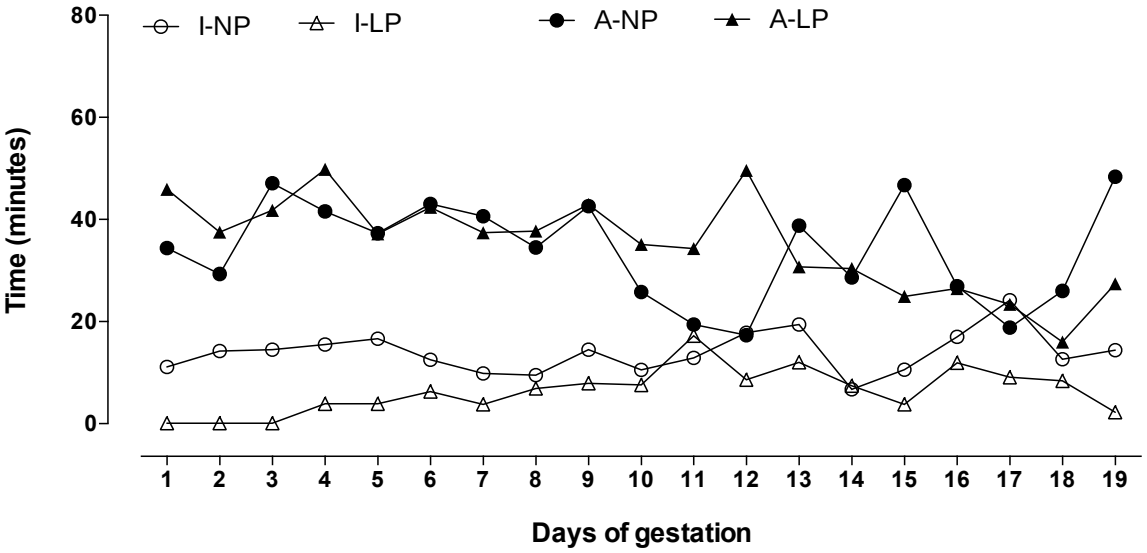


Figure 1. Parameters of voluntary physical activity for Inactive (*n*= 11) and Active (*n*= 9) dams. Travelled distance (A), time of activity (B) and estimated calorie burned (C) were recorded during the period of adaptation. Values are presented as mean ± S.E.M. *p<0.05 *vs*. Inactive. Statistical analysis was performed using two way ANOVA with Bonferroni post hoc test.



B


I-NP
I-LP
A-NP
A-LP
Time (minutes)
0
20
40
60
80
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Days of gestation

C

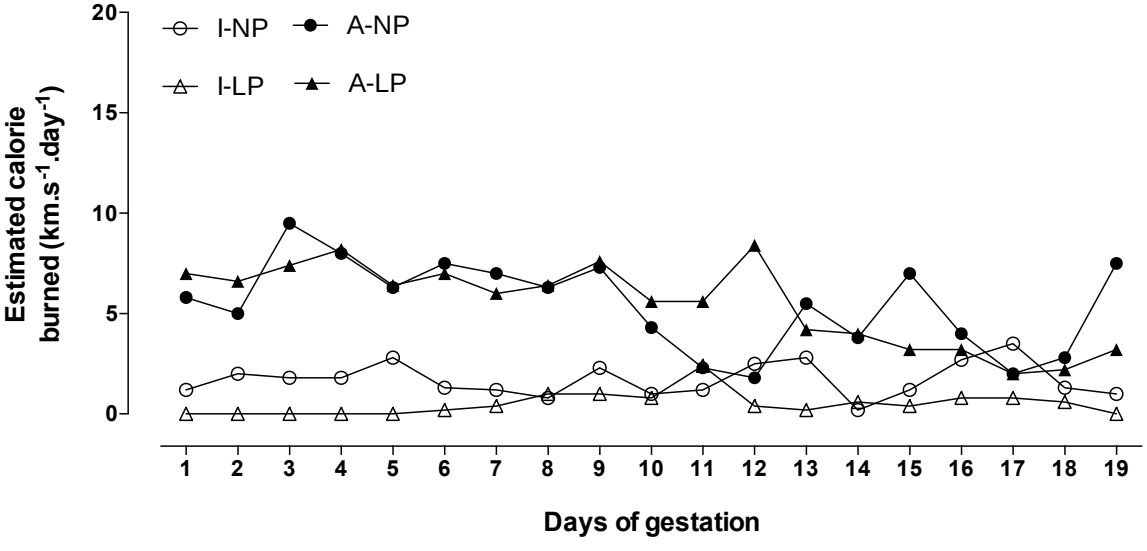
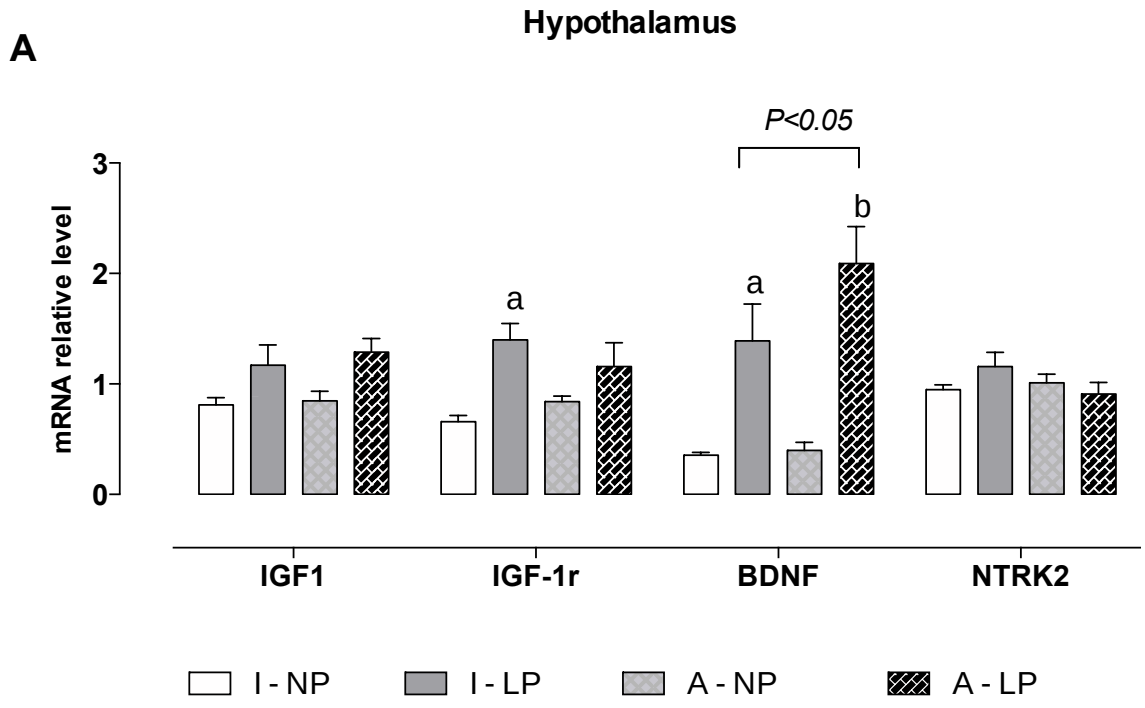
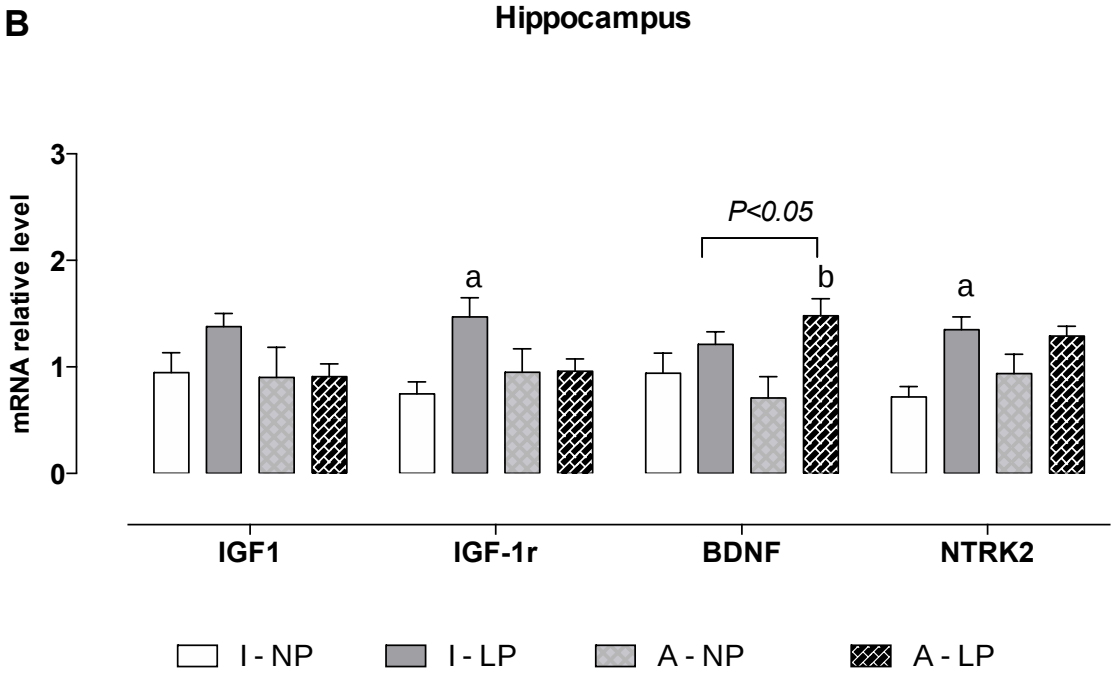

I-NP
A-NP
I-LP
A-LP
Estimated calorie burned (km.s-1.day-1)
0
5
10
15
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Days of gestation

Figure 2. Parameters of voluntary physical activity for Inactive Normoprotein (I-NP, n=6), Inactive Lowprotein (I-LP, n=5), Active Normoprotein (A-NP, n=4) and Active Lowprotein (A-LP, n=5) dams. Travelled distance (A), time of activity (B) and estimated calorie burned (C) were recorded during the period of gestation. Values are presented as mean ± S.E.M. Statistical analysis was performed using two way ANOVA with Bonferroni post hoc test.




B
Hippocampus
mRNA relative level
3
2
1
0
P<0.05
a
b
a
IGF1
IGF-1r
BDNF
NTRK2
I - NP
I - LP
A - NP
A - LP

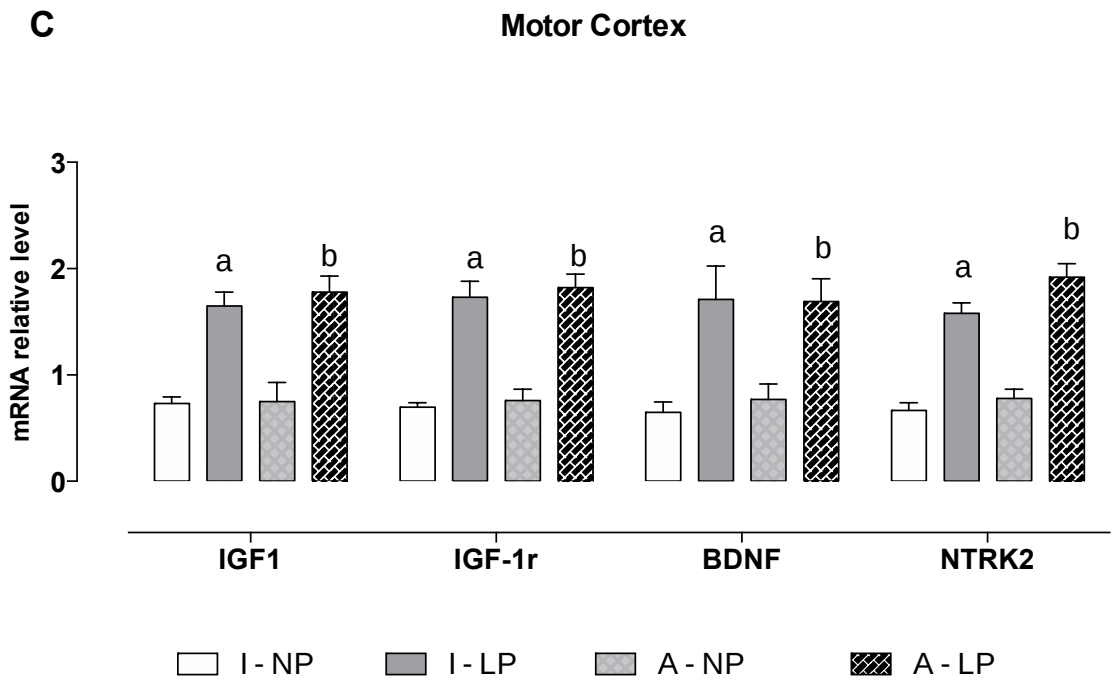


Figure 3. mRNA expression of IGF-1, IGF-1r, BDNF and NTRK2 (TrkB) in the hypothalamus (A), hippocampus (B) and cortex (C). The groups were constituted by: Inactive Normoprotein (I-NP, n=6), Inactive Lowprotein (I-LP, n=5), Active Normoprotein (A-NP, n=4) and Active Lowprotein (A-LP, n=5). Values are presented as mean ± S.E.M. [a] $p<0.05$ *vs.* I-NP and [b] *vs.* A-NP. Statistical analysis was performed using two way ANOVA with Bonferroni post hoc test.

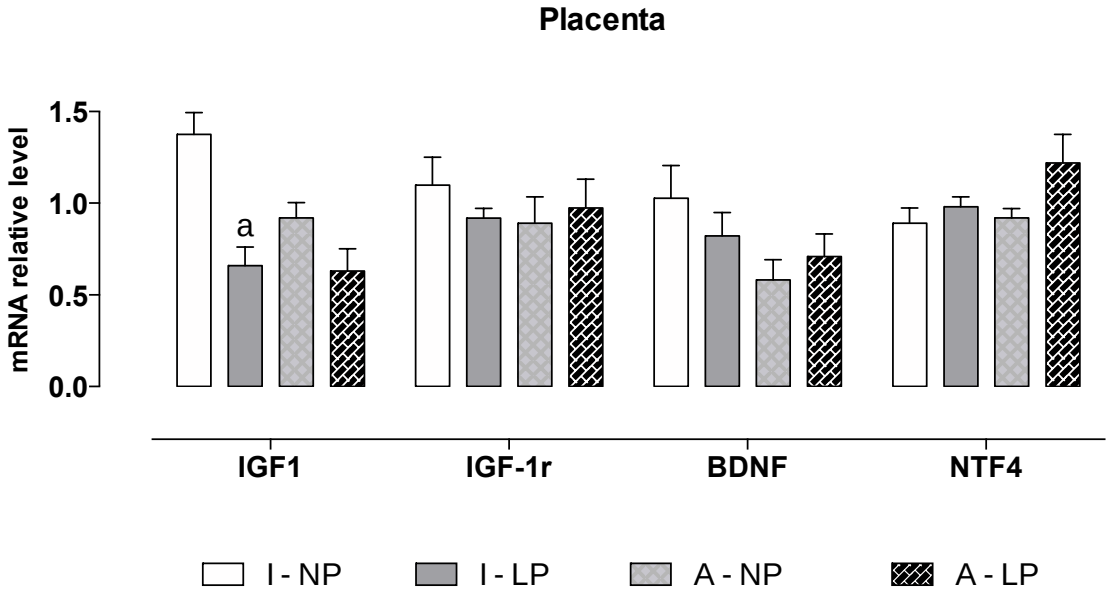


Figure 4. mRNA expression of IGF-1, IGF-1r, BDNF and NTF-4 (NT-4) in the placenta. The groups were constituted by: Inactive Normoprotein (I-NP, n=6), Inactive Lowprotein (I-LP, n=5), Active Normoprotein (A-NP, n=4) and Active Lowprotein (A-LP, n=5). Values are presented as mean ± S.E.M. [a] $p<0.05$ *vs.* I-NP. Statistical analysis was performed using two way ANOVA with Bonferroni post hoc test.

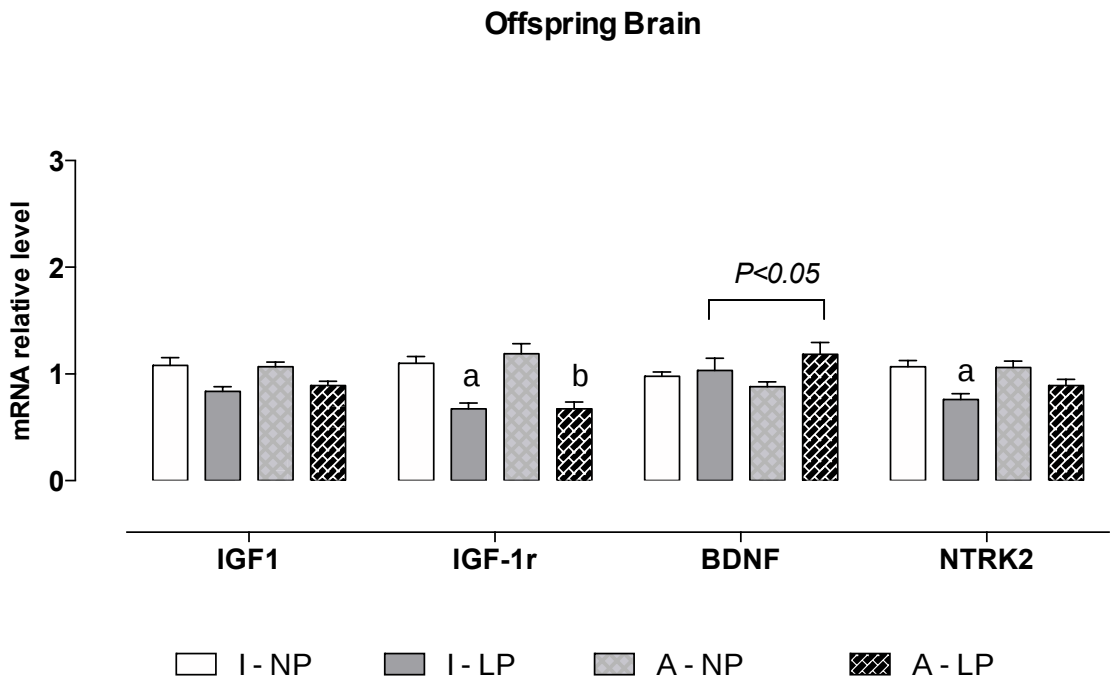


Figure 5. mRNA expression of IGF1, IGF1r, BDNF and NTRK2 (TrkB) in the offspring brain. The groups were constituted by: Inactive Normoprotein (I-NP, n=12), Inactive Lowprotein (I-LP, n=10), Active Normoprotein (A-NP, n=8) and Active Lowprotein (A-LP, n=10). Values are presented as mean ± S.E.M. [a] $p<0.05$ *vs.* I-NP and [b] *vs.* A-NP. Statistical analysis was performed using two way ANOVA with Bonferroni post hoc test.

Table 1. Primers sequence used to perform qRT-PCR

Gene	Foward/ Reverse	T	Sequence 5'-3'	Amplicon size
Igf1	F	60°C	GCTCTTCAGTTCGTGTGTGG	108 bp
	R		GCAACACTCATCCACAATGC	
Igf1r	F	60°C	CTGGTCTCTCATCTTGGATGC	197 bp
	R		GCTTCCCACACACACTTGG	
Bdnf	F	60°C	GAGTGAAGATACCATCAGCA	117 bp
	R		ATCTAGGCTACGTGAAGTCT	
Ntf4	F	65°C	CTGAGATGTCAGGGAGGAGA	115 bp
	R		ATGGCTTTGCACACCTGTCA	
Ntrk2	F	60°C	GTGGTGATTGCCTCTGTGG	149 bp
	R		TTGGAGATGTGGTGGAGAGG	
RPL19	F	58°C	CTGAAGGTCAAAGGGAATGTG	195 bp
	R		GGACAGAGTCTTGATGATCTC	
Actb	F	60°C	AGCCATGTACGTAGCCATCC	231 bp
	R		TCCCTCTCAGCTGTGCTGGTGAA	

Table 2. Maternal physiological parameters during adaptation (30 days before pregnancy). Groups: Inactive (n=11) and Active (n=9). Values expressed as Mean ± S.E.M. using *t* test student.

	INACTIVE		ACTIVE		P values
	Mean	S.E.M	**Mean**	S.E.M	
ADAPTATION					
Initial Body Weight (g)	228.4	4.5	226.7	4.3	0.791
Final Body Weight (g)	237.5	6.4	239.6	4.7	0.802
Gain of Body Weight (g)	9.1	3.2	12.9	3.6	0.436
Food intake (g/day)	14.4	0.7	14.1	0.5	0.800
Fasting Glycaemia at day 30 (mg/dL)	104.9	3.5	101.1	3.0	0.432

Table 3. Maternal physiological parameters during gestation.

	I-NP		I-LP		A-NP		A-LP	
	Mean	S.E.M	**Mean**	S.E.M	**Mean**	S.E.M	**Mean**	S.E.M
GESTATION								
Initial Body Weight (g)	255.5	8.1	256.4	12.4	256.2	7.3	261.8	8.4
Final Body Weight (g)	324.3	13.3	308.0	4.5	318.5	11.8	337.0	12.1
Gain of Body Weight (g)	68.8	7.0	51.6	9.2	62.2	13.8	75.2	6.2
Food intake (g/day)	17.0	0.7	**16.2**	0.7	16.7	0.4	**18.9***	0.8
Fasting Glycaemia at day 20 (mg/dL)	63.2	3.5	76.2	3.5	69.5	4.4	76.6	5.1
Number of fetus	11.0	0.7	8.0	2.0	11.2	0.6	10.2	1.8
Number of female fetus	3.3	0.5	2.6	1.2	3.5	0.9	3.4	0.9
Number of male fetus	7.7	0.7	5.4	2.0	7.7	0.5	6.8	1.0
Fetus body weight (g)	3.6	0.2	4.0	0.5	3.0	0.3	3.7	0.4
Placenta weight (g)	0.6	0.08	0.6	0.06	0.5	0.03	0.6	0.04

Mothers and placenta: Inactive Normoprotein (I-NP, n=6), Inactive Lowprotein (I-LP, n=5), Active Normoprotein (A-NP, n=4) and Active Lowprotein (A-LP, n=5). **Pups:** I-NP (n=12), I-LP (n=10), A-NP (n=8) and A-LP (n=10). Data are shown in mean±S.E.M. and analyzed by two-way ANOVA, Bonferroni's post hoc test was used. *$p<0.05$: A-LP *vs* I-LP.

7 CONSIDERAÇÕES FINAIS

No presente estudo, vimos que redução no aporte de proteína durante a gestação alterou a expressão de fatores neurotróficos no córtex (aumento de IGF-1, IGF-1r, BDNF e NTRK2), hipotálamo (aumento de IGF-1r e BDNF) e hipocampo (aumento de IGF-1r e NTRK2) da mãe, na placenta (diminuição de IGF-1) e no cérebro do feto (diminuição de IGF-1r e NTRK2). Porém, a prática de atividade física antes e durante a gestação foi capaz de atenuar alguns efeitos da desnutrição proteica sobre o hipotálamo (IGF-1r) e hipocampo (IGF-1r e NTRK2) da mãe, a placenta (IGF-1) e o cérebro do feto (NTRK2). Esses resultados contribuem para o entendimento da neuroplasticidade frente à estímulos ambientais como a desnutrição proteica e a atividade física.

A neuroplasticidade pode ser entendida como a capacidade do sistema nervoso em se adaptar (através de mudanças estruturais, funcionais e/ou moleculares) em resposta à diferentes demandas ambientais. Os nossos resultados demonstram que as diferentes regiões do cérebro materno analisadas foram responsivas a desnutrição proteica, alterando assim a expressão de fatores neurotróficos. Interessantemente apesar da redução na expressão de IGF-1 na placenta, não houve alteração do peso do feto e da expressão de IGF-1 e BDNF no cérebro dos filhotes. Visto que esses fatores neurotróficos estão envolvidos no controle do crescimento de neurônios e regulam o desenvolvimento sináptico, o binômio mãe-feto (via placenta) foi eficiente para tamponar alguns efeitos deletérios da desnutrição proteica sobre o crescimento e desenvolvimento fetal.

A prática de atividade física é um dos estímulos ambientais que tem sido estudado para compreender com o organismo materno e fetal se adaptam a tal condição. Nossos resultados demonstraram que a prática de atividade física de forma isolada não foi capaz de alterar a expressão dos genes analisados no cérebro da mãe, placenta e no cérebro dos filhotes. Porém, os efeitos protetores da atividade física são demonstrados quando o organismo é desafiado por outros insultos ambientais, como a desnutrição. No presente estudo, vimos que a atividade física atenuou os efeitos da desnutrição proteica sobre o hipotálamo (IGF-1r) e hipocampo (IGF-1r e NTRK2) da mãe, a placenta (IGF-1) e o cérebro do feto (NTRK2). Essas observações indicam que a atividade física materna, iniciada antes da gestação, induz adaptações materno-

fetais e pode ser considerada como um investimento materno que contraria os efeitos da desnutrição materna.

Apesar da proteção na expressão de fatores neurotróficos no cérebro dos filhotes no início da vida em resposta a desnutrição, futuros estudos podem ser conduzidos para acompanhar o desenvolvimento da prole durante a infância e adolescência. É interessante também avaliar o nível de proteína dos genes quantificados nesse estudo e os possíveis mecanismos epigenéticos envolvidos nas alterações encontradas em resposta a desnutrição proteica e atividade física materna. É relevante a realização de testes que avaliem as funções cerebrais, como habilidade cognitiva, memória e aprendizagem, para verificar como as modulações na expressão gênica no início da vida em resposta a desnutrição proteica e a atividade física podem influenciar a funcionalidade do cérebro da prole ao longo da vida.

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ANEXO A – PARECER DA COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL

Universidade Federal de Pernambuco
Centro de Ciências Biológicas

Av. Prof. Nelson Chaves, s/n
50670-420 / Recife – PE – Brasil
fones: (55 81) 2126 8840 | 2126 8351
fax: (55 81) 2126 8350
www.ccb.ufpe.br

Recife, 26 de junho 2015.

Ofício nº 63/15

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: Profª. Carol Virginia Góis Leandro
Centro Acadêmico de Vitoria de Santo Antão (CAV)
Universidade Federal de Pernambuco
Processo nº 23076.015984/2015-30

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado "**Atividade Física Voluntária materna e dieta hipoprotéica: efeito sobre a morfologia da placenta e a expressão de genes de enzimas-chave do ciclo glicose-ácido graxo e de marcadores epigenéticos de filhotes recém-nascidos".**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Biotério do Departamento de Nutrição/ UFPE; Animais: rato heterogênico; Linhagem: Wistar; Idade: 85-95 dias; Peso: 220-260g; Sexo: macho (14) e fêmea (56); Nº total de Animais: 70.

Atenciosamente,

Prof. Dr. Pedro V. Carelli
Presidente da CEUA / CCB - UFPE
UFPE SIAPE 1801584

ANEXO B – COMPROVANTE DE SUBMISSÃO DO ARTIGO 1

03-Jan-2019

Dear Dr. Leandro:

Your manuscript entitled "Maternal physical activity-induced adaptive transcriptional response in brain and placenta of mothers and rat offspring" has been successfully submitted online and is presently being given full consideration for publication in the Journal of Developmental Origins of Health and Disease.

Your manuscript ID is DOHaD-01-19-OA-1084.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to Manuscript Central at https://mc.manuscriptcentral.com/dohad and edit your user information as appropriate.

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Sincerely,
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