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GELSON MARCOS RODRIGUES JUNIOR

EFEITOS DO ESTRESSE AGUDO SOBRE A PROGRAMAÇÃO EPIGENÉTICA DO CÉREBRO DE RATOS PRATICANTES E NÃO PRATICANTES DE EXERCÍCIO FÍSICO

Londrina
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Reabilitação (Programa Associado UEL/UNOPAR), como requisito parcial à obtenção do título de Mestre em Ciências da Reabilitação.

Orientador: Prof. Dr. Marcus Vinícius de Matos Gomes

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Londrina, 27 de fevereiro de 2014.

DEDICATÓRIA

À pessoa que sempre acreditou no meu potencial e nunca me deixou desistir dos meus sonhos: minha mãe (*in memorian*).

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“Quem não se movimenta, não sente as correntes que o prendem”.

Rosa Luxemburgo

RODRIGUES JUNIOR, Gelson Marcos. **Efeitos do estresse agudo sobre a programação epigenética do cérebro de ratos praticantes e não praticantes de exercício físico**. 2014. 69 fls. Dissertação (Mestrado em Ciências da Reabilitação) – Universidade Norte do Paraná, Londrina, 2014.

RESUMO

Um número crescente de evidências tem indicado o envolvimento de mecanismos epigenéticos com as respostas biológicas adaptativas ao estresse. Neste contexto, este estudo objetiva avaliar o efeito do estresse sobre a metilação global do DNA *em* células encefálicas de ratos e sua relação com a expressão dos genes *Dnmt1* e *Bdnf.* Adicionalmente, avaliamos o potencial da prática de exercício física (natação) em modular os efeitos moleculares relacionados ao estresse. Para isto foram utilizados ratos Wistar, estratificados em três grupos: grupo estresse (ST), de animais submetidos ao protocolo validado de estresse por restrição aos 75 dias pós-natal (DPN); grupo exercício físico e estresse (EX-ST), de animais praticantes de natação durante os dias 35 a 74 DPN e ao protocolo de estresse aos 75 DPN; e o grupo controle (CTL), sem intervenção. Subsequentemente os animais foram sacrificados para a obtenção de amostras do hipocampo, córtex, hipotálamo e substância cinzenta periaquedutal (PAG). O perfil de metilação global do DNA foi quantitativamente avaliado pelo método de ELISA de auto rendimento especifica para metilação. A expressão dos genes *Dnmt1* e *Bdnf* foi avaliada por PCR em tempo real. O estresse demonstrou induzir uma hipometilação global do DNA no hipocampo, córtex e PAG dos animais sedentários. No entanto, essa alteração não foi observada nos animais praticantes de atividade física. Além disso, enquanto o estresse foi associado à diminuição da expressão do gene *Dnmt1* no córtex dos animais sedentários, foi associado a um aumento da expressão do *Dnmt1* na PAG nos animais praticantes de exercicio físico. Em relação ao gene *Bdnf*, o estresse foi associado a um aumento significativo na expressão na PAG dos animais sedentários, enquanto que nos animais praticantes de atividade física foi associado à uma diminuição da expressão no córtex e um aumento no hipotálamo. Em suma, nossos dados evidenciam que o estresse comportamental induz hipometilação global do DNA no hipocampo, córtex e PAG, além de sugerirem que no córtex esta alteração pode estar associada à diminuição da expressão do gene *Dnmt1*, além de na PAG estar associada ao aumento da expressão do *Bdnf*. Adicionalmente, nossos dados revelam o potencial da prática de exercício físico em diminuir a intensidade das alterações na metilação global do DNA induzidas pelo estresse no hipocampo, córtex e PAG além de modular os efeitos sobre a expressão dos genes *Dnmt1* no córtex e PAG, e do *Bdnf* no córtex e no hipotálamo do cérebro de ratos.

Descritores: Estresse; Epigenética; Metilação do DNA; *Dnmt1*; *Bdnf*; Exercício Físico.

RODRIGUES JUNIOR, Gelson Marcos. **Effects of acute stress on epigenetic programming of rat brains in practitioners and non-practitioners of physical exercise**. 2014. 69 fls. Dissertação (Mestrado em Ciências da Reabilitação) – Universidade Norte do Paraná, Londrina, 2014.

ABSTRACT

A large amount of evidence has indicated the involvement of epigenetic mechanisms on the biological adaptive responses to behavioral stress. In this context, the present study aimed to assess the effect of stress on global DNA methylation in brain cells of rats and its relation to the expression of *Dnmt1* and *Bdnf* genes. Further we evaluated the potential of physical exercise (swimming) in modulating stress-related molecular effects. Male Wistar rats were stratified into three groups: stress group (ST), of animals submitted to a validated stress protocol at 75 postnatal day (PND); physical exercise and stress group (EX-ST), of animals submitted to swimming at 35-74 PND and the stress protocol at 75 PND; and control group (CTL) with no intervention. Subsequently the animals were sacrificed to obtain samples from hippocampus, cortex, hypothalamus, and periaqueductal gray (PAG). The global DNA methylation profile was quantified using a high-throughput ELISA-based method. The expressions of *Dnmt1* and *Bdnf* genes were evaluated by real time PCR. Stress induced a decrease of the global DNA methylation in the hippocampus, cortex and PAG of sedentary animals. However, this alteration was not observed in the exercised animals. Furthermore, while stress was associated with decreased expression of *Dnmt1* gene in the cortex of sedentary animals, in the exercised animals it was associated to an increased expression of *Dnmt1* in the PAG. In relation to *Bdnf* stress was associated with a significant increase in the expression of PAG in sedentary animals, whereas in the exercised animals it was associated with a decrease in the expression in the cortex and an increase in the hypothalamus. In summary, our data show that behavioral stress induces global DNA hypomethylation in the hippocampus, cortex and PAG. Furthermore, our evidences indicate a relationship between this epigenetic alteration with a decreased expression of the *Dnmt1* gene in the cortex and also with the increased expression of the *Bdnf* gene in PAG. Additionally, our data reveal the potential of physical exercise in attenuating the intensity of the changes induced by stress in the global DNA methylation of hippocampus, cortex and PAG, and in modulating the effects on the expression of *Dnmt1* in the cortex and PAG, and *Bdnf* in the cortex and hypothalamus of the rat brain.

Key words: Stress; Epigenetics. DNA methylation; *Dnmt1*; *Bdnf*; Physical Exercise.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

ACTH	Adenocorticotropina
ANOVA	Analysis of variance
Bdnf	Brain derived neurotrophic factor
CRH	Hormônio liberador da corticotrofina
CTL	Controle
DNA	Desoxiribonucleic acid
DNMT	DNA-metiltransferase
DPN	Dias pós-natal
EX-ST	Exercício-estresse
GABA	Ácido gama-amino-butírico
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
HPA	Eixo hipotálamo-hipófise-adrenal
IAM	Infarto Agudo do Miocárdio
PAG	Periaqueductal gray
PCR	Polymerase Chain Reaction
SE	Sistema Endócrino
SN	Sistema Nervoso
SNA	Sistema Nervoso Autônomo
ST	Estresse
UEL	Universidade Estadual de Londrina
UNOPAR	Universidade Norte do Paraná

SUMÁRIO

1 INTRODUÇÃO

A sobrevivência do ser humano é dependente de um equilíbrio dinâmico, desempenhado por todos os órgãos e tecidos do corpo, que através de suas funções específicas ajudam a manter a homeostasia[1,2]. Nesse sentido o estresse tem se tornado nos últimos anos um importante fator de desequilíbrio da homeostasia, e consequentemente das relações pessoais e de saúde da humanidade[3].

Frente a uma sociedade moderna que exige grande capacidade de adaptação física, mental e social os indivíduos estão frequentemente expostos a situações de conflitos, ansiedades, angústias e desestabilizações emocionais. Neste contexto, o estresse emerge como uma consequência direta aos persistentes esforços adaptativos da pessoa à sua situação existencial[4].

No Brasil estima-se uma incidência populacional de estresse em torno de 32%, no entanto a intensidade de sua manifestação parece estar associada à atividade profissional, podendo atingir até 70% nestas situações[5].

Em relação aos aspectos neurofisiológicos do estresse, sabe-se que as principais áreas cerebrais ativadas no mecanismo adaptativo do estresse são o eixo hipotálamo-hipófise, sendo esse eixo responsável pela liberação de fatores e hormônios que desencadeiam a ativação do córtex da glândula adrenal[1]. Juntos eles compoem o chamado eixo HPA (eixo hipotálamo-hipófise-adrenal).

Além disso, um importante papel nas respostas adaptativas ao estresse tem sido atribuído a outras áreas cerebrais, tais como o hipocampo[6], o córtex e a substância cinzenta periaquedutal (PAG)[7].

Em estudo recente utilizando células do sangue periférico humano, foram evidenciadas alterações loci específicas de metilação do DNA associadas ao estresse[8]. Porém outros tecidos não puderam ser analisados neste estudo em virtude da inacessibilidade para a obtenção de células encefálicas e de tecidos hígidos dos indivíduos.

Além disso, dados prévios da literatura indicaram a vulnerabilidade da expressão de alguns genes frente a situações de estresse, incluindo entre estes os genes das DNA-metiltransferases (*Dnmt*)[9–16] e o fator neurotrófico derivado do cérebro (*Bdnf*)[17–22].

A prática regular de atividade física tem sido evidenciada como

intervenção profilática e terapêutica para diversas disfunções, dentre elas o estresse. O aumento das respostas adaptativas do eixo HPA provocada pelo exercício físico realizado voluntariamente por animais melhora a resposta adaptativa ao estresse e diminui a ansiedade relacionada a esse evento[6].

Acredita-se que a identificação de variações do padrão epigenético do DNA e da expressão gênica de ratos expostos ao estresse possam gerar conhecimentos neurofisiológicos adicionais e os estudos realizados em modelos experimentais de ratos praticantes e não-praticantes de atividades físicas possam revelar dados adicionais e extrapoláveis à população humana sobre a importância da prática de atividade física na prevenção de doenças.

Assim, este estudo teve por objetivo identificar o efeito do estresse agudo sobre o padrão epigenético de células encefálicas de ratos, além de identificar as variações na expressão dos genes *Dnmt1* e *Bdnf* consequentes a esse efeito. Paralelo a isso, também foi objetivo observar a participação da prática de exercício físico em modular os efeitos do estresse agudo sobre o perfil global de metilação do DNA e as variações na expressão dos genes *Dnmt1* e *Bdnf* em células encefálicas de ratos.

2 REVISÃO DE LITERATURA – CONTEXTUALIZAÇÃO

2.1 CONSIDERAÇÕES INICIAIS SOBRE O ESTRESSE

As primeiras definições fisiológicas de estresse datam de 1965, quando Selye o descreveu como uma reação inespecífica do organismo frente a qualquer exigência[23]. O estresse é um desequilíbrio substancial entre a capacidade de demanda (física ou psicológica) e a capacidade de resposta, que repercute em consequências importantes[24].

O estresse pode ser classificado como físico, psíquico, por sobrecarga, por monotonia, agudo (durando momentos, horas ou dias, se dissipando) e crônico (persistindo por mais tempo). O estresse prepara o organismo para a luta ou fuga, por conta da ativação do sistema endócrino[25].

Além disso, o estresse pode ser resposta a um evento positivo ou negativo. Se a pessoa reage bem a uma determinada situação, o estresse pode ser subclassificado como eustresse, onde as reações fisiológicas desencadeadas são decorrentes de situações agradáveis e prazerosas. Em resposta a um evento negativo, o estresse é denominado distresse, onde as reações fisiológicas desencadeadas incluem distúrbios de sono, dificuldade para relaxar, irritação, ansiedade, cansaço excessivo, angústia, dentre outros[26].

Sendo assim, o estresse nem sempre é um fator de desgaste emocional e físico, e sim um mecanismo natural de defesa do organismo. Recebem-se estímulos internos e externos e dependendo da forma com que esses estímulos são enfrentados, poderão provocar alterações psicológicas e biológicas negativas[3].

De forma geral, quando o termo estresse é empregado, refere-se às características geradas pelos seus efeitos negativos no organismo, logo, ao distresse.

Basicamente, o estresse pode ser definido como uma reação complexa e global do organismo, envolvendo componentes físicos, psicológicos, mentais e hormonais frente a situações que representem um desafio maior e que ultrapassem sua capacidade de enfrentamento, visando adaptar o indivíduo à nova situação[27].

2.2 Aspectos Fisiológicos e Neurobiológicos do Estresse

A sobrevivência de um indivíduo é dependente da manutenção de condições constantes no meio interno, desempenhadas, de acordo com suas funções específicas, por todos os órgãos e tecidos do organismo. Esse equilíbrio das condições constantes do organismo é designado por homeostasia[1].

O organismo está constantemente alternando seu estado de homeostasia com estado de estresse. Quando uma situação estressante é detectada pelo Sistema Nervoso (SN) provocando o desequilíbrio da homeostasia, o organismo passa a desenvolver uma série de reações, captadas por receptores do sentido e por mecanismos de *feedback*, que são encaminhados aos efetores da periferia do corpo, visando o reequilíbrio da homeostasia[2,25,28]. A resposta ao estresse se dá de forma temporal, iniciando-se em milissegundos após o estímulo estressante e durando até dias[29].

A manutenção da homeostasia é realizada principalmente pelo Sistema Nervoso Autônomo (SNA), tanto no repouso quanto em situação de estresse. Este sistema coordena respostas reflexas em sistemas específicos como o cardíaco e o gastrointestinal, correlacionando reações globais com comportamentos voluntários[28].

Além do SNA, o mecanismo fisiológico do estresse também tem influência do Sistema Endócrino (SE) pelo eixo hipotálamo-hipófise, ou eixo Hipotálamo-Pituitária-Adrenal (HPA)[3]. Além disso, um importante papel nas respostas adaptativas ao estresse tem sido atribuído a outras áreas cerebrais como o hipocampo[6].

Com a ativação do eixo HPA, os neurônios do núcleo paraventricular do hipotálamo são estimulados a secretar o hormônio de liberação de corticotrofina (CRH), que é transportado para a adeno-hipófise, onde induz a liberação do hormônio adrenocorticotropina (ACTH), que por sua vez estimula a síntese e secreção de hormônios glicocorticoides pelo córtex da adrenal, elevando a concentração destes na corrente sanguínea, principalmente do cortisol em humanos, e corticosterona em roedores[30].

Os efeitos da corticosterona se desenvolvem através de receptores de mineralocorticoides e de glicocorticoides. Os receptores de mineralocorticoides possuem grande afinidade pela corticosterona, sendo esta transportada para o

interior do núcleo celular, atuando então na transcrição gênica e influenciando na síntese de proteínas específicas[29,31].

Os glicocorticoides apresentam a função de preparar o organismo para as respostas fisiológicas ao estresse. Porém, em situação de persistência ou intensidade exagerada do estímulo estressante, essa substância pode fazer com que o eixo HPA torne-se hiperreativo, acarretando em prejuízos ao organismo[27].

A corticosterona mobiliza a energia armazenada nas células e dessa forma potencializa os efeitos mediados pelo SNA Simpático. Também atua no controle da atividade do eixo HPA e na finalização da resposta ao estresse, utilizando-se de uma realimentação inibitória em áreas cerebrais extra-hipotalâmicas, hipotálamo e hipófise[31].

Outras substâncias têm sido estudadas visando a compreender a neurofisiologia do estresse, dentre elas estão a noradrenalina, a dopamina, a serotonina, o ácido gama-aminobutírico (GABA), a glicina e o glutamato[32].

Subsequentemente à cascata de eventos cerebrais e endócrinos envolvidos com a resposta adaptativa ao estresse, respostas cardiovasculares importantes, resultantes da mediação do SNA Simpático, também são evidenciadas, como o aumento da pressão arterial e da frequência cardíaca, vasodilatação a nível musculoesquelético e vasoconstrição a nível cutâneo, que acarretam em queda na temperatura cutânea e aumento na temperatura corporal[33].

Apesar de controvérsias, o estresse também pode ser considerado um fator de risco modificável para o desenvolvimento de doenças cardiovasculares. O que se sabe é que o estresse mental pode ser o "gatilho" para um evento cardíaco agudo como a isquemia miocárdica, infarto agudo do miocárdico (IAM) e outros eventos coronários[34].

O estresse pode acarretar em efeitos deletérios para áreas cerebrais como o hipocampo, principalmente em situações onde há concentrações elevadas de corticosterona, que induzem o remodelamento neural e diminuem a neurogênese no hipocampo[27].

O estresse também parece ser um dos principais fatores ambientais predisponentes à depressão. Em grande parte dos casos, os episódios depressivos são precedidos pela ocorrência de fatores estressantes, principalmente no que diz respeito a questões psicossociais. Na depressão pode ser observado níveis basais elevados de corticosterona, que prejudicm o controle inibitório do eixo HPA[27].

2.3 Fundamentos Epigenéticos Aplicados ao Estresse

Um número crescente de estudos tem investigado o envolvimento de mecanismos epigenéticos em áreas específicas do cérebro em concomitância a respostas comportamentais adaptativas[6].

Definem-se como epigenéticos os mecanismos capazes de controlar a atividade e a expressão gênica das células, sem que a sequência do DNA seja modificada. Entre as principais modificações epigenéticas conhecidas incluem-se a metilação do DNA e as modificações pós-traducionais de histonas[35,36].

A metilação do DNA consiste na adição de um radical metil (CH_3) no carbono 5 de citosinas geralmente seguidas de guaninas (dímeros CpG) por ação de enzimas DNA metiltransferases (DNMTs)[35].

Estima-se que aproximadamente 80% de todos os dímeros CpGs do genoma humano sejam metilados, porém os mecanismos onde a metilação do DNA controlam a expressão gênica ainda não são completamente elucidados. Geralmente, a metilação de citosinas presentes em aglomerados de CpGs (ilhas CpGs) localizadas na região promotora de genes está associada com a estrutura compactada da cromatina e silenciamento gênico[37].

Muitos estudos têm focado a elucidação dos processos que envolvem a metilação do DNA devido ao seu importante papel no controle da expressão gênica nas fases iniciais do desenvolvimento embrionário e na diferenciação celular (fenômeno conhecido como reprogramação epigenética) e seu papel na etiologia de doenças. Uma das principais características da metilação do DNA é a manutenção do padrão preestabelecido durante a diferenciação celular nas células-filhas (pós-divisão celular). No entanto, estudos recentes têm revelado uma vulnerabilidade dos padrões de metilação em regiões cromossômicas específicas quando expostos a fatores ambientais[38–40].

As histonas, estruturas proteicas associadas ao DNA que organizam estruturalmente a cromatina, também podem sofrer alterações epigenéticas. A dupla hélice do DNA se conforma estruturalmente dando duas voltas nas histonas, formando a estrutura básica de condensação do DNA, denominada nucleossomo. Este é composto por dois complexos idênticos, cada um constituído de 4 histonas, formando um octâmero. As proteínas histonas presentes em número de dois em cada nucleossomo são: H2A, H2B, H3 e H4, além de uma molécula da proteína H1,

que também contribui para a condensação do DNA[41,42]. Dentre as principais modificações das histonas, podem ser destacadas a metilação, a acetilação, ubiquitinilação, fosforilação, sumoilação, ADP-ribosilação, deaminação e isomerização da prolina[41,43].

Em relação à metilação do DNA, muitas patologias podem ser causadas por alterações desse mecanismo. Dentre elas, o câncer é a doença em que o envolvimento de alterações epigenéticas está mais bem caracterizado[44]. Com base nas características de instabilidade e manutenção (transmissão célula-célula) do padrão epigenético, acredita-se que alterações do controle epigenético possam ser induzidas por fatores ambientais e dessa forma estarem associadas à origem de algumas doenças humanas. Assim, estudos nessa área podem revelar, a nível molecular, a associação entre fatores externos e a origem de doenças no homem[45].

Pouco se sabe ainda sobre o papel exercido pelos mecanismos epigenéticos no controle neurocomportamental e psiquiátrico, porém alterações do padrão de metilação do DNA têm sido evidenciadas na etiologia de distúrbios como a depressão[19,46–49], esquizofrenia[50,51], distúrbio bipolar[50,52,53], distúrbios pós-traumáticos do estresse[54,55], autismo[56,57] e dependências químicas[58]. Adicionalmente, evidências sugerem uma relação entre alterações epigenéticas em genes específicos do córtex cerebral e a etiologia da doença de Alzheimer[59].

Recentemente, muito tem sido discutido na literatura sobre a relação entre alterações de metilação causadas pela exposição a experiências desfavoráveis nas fases iniciais da vida e a incidência de distúrbios de comportamento que se manifestam tardiamente[11,22,60–65].

Particularmente em relação ao estresse, foi documentada a ocorrência de alterações de histonas em células do hipocampo após o evento agudo de forma mais notável do que em evento de estresse crônico[66]. Entretanto, ainda são escassos os dados sobre o envolvimento da metilação do DNA frente a situações de estresse.

Além disso, estudos recentes em modelos experimentais têm demonstrado a vulnerabilidade do controle da expressão gênica em áreas cerebrais relacionadas ao estresse, como o hipocampo, quando submetidas a outros tipos de variações ambientais. Por exemplo, mudanças na expressão de genes como o fator neurotrófico derivado do cérebro (*Bdnf*) foram evidenciadas em resposta ao exercício físico voluntário[67], pressupondo uma interação entre a prática de exercícios físicos e

os efeitos do silenciamento de genes desencadeadores da resposta ao estresse.

2.4 EXERCÍCIO FÍSICO

O exercício físico é compreendido como qualquer atividade associada à movimento corporal produzido por músculos que resulta em maior dispêndio de energia, desde que seja estruturado, repetitivo e aplicado de forma proposital[68].

As alterações fisiológicas que o exercício físico promove no organismo são bem documentadas e o número de estudos relacionados ao exercício físico aumentou progressivamente de 1966 a 2005[69].

As alterações biológicas associadas ao exercício físico podem ser classificadas em agudas imediatas, agudas tardias (ou subagudas) e crônicas. Os efeitos agudos imediatos ocorrem durante e imediatamente após o exercício, como aumento da frequência cardíaca, da ventilação pulmonar e sudorese. Os efeitos agudos tardios ocorrem de 24 a 48 horas após o exercício físico e compreendem discreta redução dos níveis pressóricos, melhora na função endotelial e na potencialização da ação e aumento da sensibilidade insulínica na musculatura esquelética[70,71]. Já os efeitos crônicos compreendem adaptações que diferem um indivíduo treinado de outro sedentário, e podem ser citadas a bradicardia de repouso, hipertrofia muscular e hipertrofia do ventrículo esquerdo, alterações autonômicas e hemodinâmicas, incremento de demandas metabólicas, diminuição da pressão arterial sistólica, aumento do débito cardíaco, diminuição da resistência vascular periférica e aumento do consumo de oxigênio[72].

É evidente o interesse do conhecimento de aspectos moleculares do efeito do exercício físico e/ou atividade física[69], no entanto pouco se sabe ainda a respeito de alterações epigenéticas consequentes ao exercício físico.

Dados recentes demonstram, a nível molecular, que indivíduos praticantes de atividade física programada de 26 a 30 minutos por dia apresentam um aumento significante do perfil de metilação global do DNA no sangue periférico quando comparados a praticantes de apenas 10 minutos diários ou menos de atividade física regular[73]. Além disso, relatos prévios da literatura evidenciaram a relação entre modificações de histonas e variações na expressão gênica em

músculos esqueléticos reforçando a participação de mecanismos epigenéticos no controle da plasticidade musculoesquelética em situações de saúde ou em comorbidades[74].

Além disso, os efeitos do exercício físico a nível molecular também compreendem o aumento da expressão do gene *Bdnf* em áreas cerebrais. Este gene é um importante mediador molecular da plasticidade funcional e estrutural no cérebro e atua em várias funções cerebrais como neuroplasticidade, neurogênese, reparo e diferenciação celulares[75]. Por outro lado, níveis alterados da expressão deste gene têm sido descritos em várias doenças neurológicas e psiquiátricas[18]. Sendo assim, diversos estudos têm sido conduzidos para elucidar a conexão entre a prática regular de exercício físico e o aumento da concentração de *Bdnf*[76], fazendo-se necessária a elucidação do papel deste gene na inibição de efeitos deletérios conferidos a situações de estresse.

3 ARTIGO

ACUTE STRESS AFFECTS THE GLOBAL DNA METHYLATION PROFILE OF RAT BRAIN: ATTENUATION BY PHYSICAL EXERCISE PRACTICING

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Abstract

Evidences have indicated the involvement of epigenetic mechanisms on the adaptive responses to stress. Thus, the present study aimed to assess the effect of stress on global DNA methylation in rat brains and its relation to the expression of *Dnmt1* and *Bdnf* genes. Further we evaluated the potential of physical exercise (swimming) in modulating stress-related molecular effects. Male Wistar rats were submitted to acute restraint stress at the 75 postnatal day (PND) and as the physically active group rats submitted to swimming at 35-74 PND and to the stress protocol at 75 PND were considered. Samples from hippocampus, cortex, hypothalamus, and periaqueductal gray (PAG) were obtained. The global DNA methylation profile was quantified using a high-throughput ELISA-based method. The expressions of *Dnmt1* and *Bdnf* were evaluated by real time PCR. Stress induced a decrease on global DNA

methylation in the hippocampus, cortex and PAG of sedentary animals. However, this alteration was not observed at the trained animals. Furthermore, stress was associated with decreased expression of *Dnmt1* in the cortex of exercised animals, and associated to increased expression of *Dnmt1* in the PAG. In relation to *Bdnf* stress was associated with a significant increase in the expression of PAG in sedentary animals, whereas it was associated with a decrease in the expression in the cortex and an increase in the hypothalamus of the trained animals. In summary, our data show that stress induces global DNA hypomethylation in the hippocampus, cortex and PAG. Moreover, our evidences indicate a relationship between this epigenetic alteration with decreased expression of *Dnmt1* in the cortex and also with increased expression of *Bdnf* in PAG. Additionally, our data reveal the potential of physical exercise in attenuating the changes induced by stress in the global DNA methylation of hippocampus, cortex and PAG, and in modulating the effects on the expression of *Dnmt1* in the cortex and PAG, and *Bdnf* genes in the cortex and hypothalamus of rat brain.

Keywords: Stress. Epigenetics. DNA Methylation. Bdnf. Dnmt1.Physical Exercise.

Introduction

Stress has become an important imbalance factor of personal relationships and health of mankind (Araldi-Favassa et al., 2005). The main adaptive mechanism of stress involves the hypothalamic-pituitary axis. Moreover, an important rule on the adaptive responses to stress has been attributed to other brain areas such as the hippocampus (Collins et al., 2009), periaqueductal gray matter (PAG), and cortex (Brandão et al., 2003).

From a molecular point of view, recent data have indicated the involvement of several genes in the adaptive response to stress, such as *Bdnf* (brain-derived neurotrophic factor), which is involved in the improvement of neuronal plasticity (Fuchikami et al., 2010) and *Dnmt1* (DNA

methyltransferase), which plays an important role in maintaining DNA methylation after mitosis, but its function in the central nervous system remains unclear (Feng et al., 2011).

The participation of epigenetic changes in the molecular adaptive responses to behavioral stress has been questioned for some time in the scientific community. However, the idea of DNA methylation as an important epigenetic mechanism involved in stress is a relative new concept (Trollope et al., 2012; Stankiewicz et al., 2013).

In a recent study performed by Unternaehrer et al., loci specific changes in DNA methylation of peripheral blood cells were associated to behavioral stress. However, the implications to brain cells have not been evaluated in this study due to obvious inaccessibility in healthy individuals.

In this context, studies involving experimental models are necessary to reveal the participation of epigenetic mechanisms such as DNA methylation in the modulation of neurophysiological responses induced by stress.

Thus, the aim of the present study was to identify the effect of acute stress on the global DNA methylation profile of brain cells of rats, and on the expression of the *Dnmt1* and *Bdnf* genes.

In addition, considering previous report of the potential of physical activity in changing brain-specific epigenetic patterns (Cotman et al., 2007), we evaluated the potential of physical activity (swimming) in modulating the impacts of stress on DNA methylation profile and on *Dnmt1* and *Bdnf* gene expressions.

Material and Methods

Animals and Experimental Design. Experimental procedures were carried out following protocols approved by the ethical review committee of the University of Londrina, Londrina, Parana, Brazil. Non related male rats were kept under standard conditions (temperature 25 ± 1°C, photoperiod 12h light/12h dark) and with water and food *ad libitum*, in the Central Animal House of the University of Londrina. The animals were classified into three groups, containing 4-6 animals each: 1) stress group (ST): animals that were submitted to the acute restraint stress, as previously demonstrated (Reis et al., 2011), at the 75 postnatal day (PND); 2) physical activity group (EX-ST): animals that were submitted to physical exercise (swimming for 60 minutes/day) from the 35PND to the 74PND followed by the acute restraint stress at 75 PND; and 3) control group (CTL): animals that were not submitted to interventions.

Acute Restraint Stress. In the morning period (07:00 a.m - 12:00 p.m), the animals were transported to the experimental room in their home cages and allowed to adapt to this environment for at least 30 minutes. After this period, the animals were submitted to the protocol of acute restraint stress (Reis et al., 2011) being placed in a metal cylinder of 6.5 cm of diameter and 15 cm of length where they remained closed for 60 minutes.

Physical Exercise. The animals of the EX-ST group were submitted to physical training (swimming) without stimuli or charges, according to Martins-Pinge et al. The swimming sessions were performed in the morning (11:00 a.m. - 1:00 p.m.) in a glass tank filled with lukewarm water (31±1 °C) with 4000 cm^2 surface area and 60 cm deep. The training consisted of 4 weeks (20 sessions) of swimming being held 5 days a week and 60 minutes per day. During the first week, the training was graded, beginning with 15 minutes on the first day, 30

minutes on the second day, and 45 minutes on the third day, for adaptation to the training process. From the fourth day on, each session consisted of 60 minutes of swimming until 74PND. After each swimming session, the animals were dried with towel and held for a period of readjustment in collective box, and then returned to their individual cages.

Sample Collection. The animals were killed by decapitation and the brain was removed from the skull for a period of 6-8 minutes for each animal. Samples from hypothalamus, frontal region of cortex, PAG, hippocampus and blood were immediately obtained. Brain areas were sectioned according to the anatomical atlas of Paxinos and Watson, and the tissues were stored at -80°C.

Global DNA Methylation Profile. Genomic DNA was obtained by standard salting out protocol and the global DNA methylation profile was evaluated by dosage (percentage) of the methyl groups (CH_3) using the Imprint Methylated DNA Quantification Kit (Sigma-Aldrich®), as previously described (Gomes et al., 2012). Briefly, the methylation status of each sample was calculated by the amount of methylated cytosines in the sample (5mC) relative to global cytidine (5mC + dC) in a positive control (100% methylated) that had been previously methylated and a no template control sample (0% methylated) using absorbance readings at 450nm and following the formula: A450sample-A450NTC)/(A450met-A450NTC)x100. All samples were analyzed in triplicate.

Quantitative analysis of Gene Expression. RNA samples were obtained using Trizol (Invitrogen ®) and the reverse transcription was performed using the High Capacity Kit (Applied Biosystems ®), according to manufacturers' recommendations. Expression levels of *Dnmt1* and *Bdnf* genes were evaluated by Real-Time PCR (Applied Biosystems ®) using the

Taqman detection system (Applied Biosystems ®). For the normalization of difference in the amount of cDNA we used the *Gapdh* gene as endogenous control. The experiments were performed in triplicate.

Corticosterone levels. Blood samples were centrifuged at 2300 rpm for 20 minutes and the plasma frozen for subsequent analysis by radioimmunoassay as previously described (Saia et al., 2011).

Statistical Analysis. Analyzes of data normality were performed using the Kolmogorov-Smirnov test. The Mann-Whitney test was used to compare the dosage of serum corticosterone from the groups ST and CTL. Analysis of variance with multiple comparisons was carried out using the ANOVA test and Dunn's post-test to compare the data of global DNA methylation profile among the groups ST, CTL and EX-ST, and also the relative levels of quantitative expression of *Bdnf* and *Dnmt1* genes. The GraphPrism 6.0 software was used for statistical analysis, considering $P<0.05$.

Results

Corticosterone levels. Increased levels of serum corticosterone were observed in the animals from the ST group in comparison to the CTL group ($P=0.0043$) confirming the efficacy of the acute restraint stress protocol in inducing stress related-physiological changes (Figure 1).

Effect of the stress on global DNA methylation. Comparative analyzes of the mean percentages of the global DNA methylation profile revealed statistically significant decrease in the hippocampus, cortex and PAG of animals that were submitted to the acute restraint stress (ST group) when compared to the CTL group ($P<0.05$).

In contrast, no significant change in DNA methylation was observed in animals from the EX-ST group in comparison to the animals from the ST group and CTL group, revealing the potential of the physical exercise in modulating the effects of stress on the global methylation profile (Figure 2).

Quantitative Expression of Dnmt1. No significant alteration in the expression of the *Dnmt1* gene was associated to stress when compared the ST and CTL groups. However, statistically significant decrease in the expression of *Dnmt1* was found in the cortex of animals from the EX-ST group while an increased expression was evidenced in the PAG (Figure 3).

Quantitative Expression of Bdnf. Statistically significant increase in the expression of the *Bdnf* gene was observed in the PAG of animals from ST group in comparison to CTL group ($P<0.05$). Nevertheless, a statistically significant decrease in the expression of *Bdnf* was associated to the stress in the EX-ST group in the cortex ($P<0.05$). Specifically in the hypothalamus a significant increase in the expression of *Bdnf* was observed in the EX-ST group ($P<0.05$) (Figure 4).

Discussion

In the present study we observed, in experimental model, the potential of behavioral stress to induce global DNA hypomethylation in the hippocampus, cortex and PAG. Additionally, we speculate based on our data that stress-induced demethylation in the cortex might be associated with decreased expression of *Dnmt1* gene in this area, although it does not occur in the hippocampus and PAG.

Although a significant amount of evidence has linked stress with changes in methylation at loci specific, little is known about the relation between this event and behavioral changes involving the DNA molecule as a whole.

In a study proposed by Guo et al., external stimuli in vivo demonstrated that modifications of the DNA methylation profile in mature neurons, making evident the vulnerability of DNA methylation in brain areas after environmental stimuli.

Contextualizing to the literature, our data of association of DNA hypomethylation in the hippocampus with stress corroborate the evidence obtained by Chertkow-Deutsher et al., in which DNA hypomethylation in the hippocampus was correlated to disorders of posttraumatic stress.

The idea of DNA methylation as an epigenetic mechanism for controlling gene expression in neurons and for neurobiological control is relatively recent, although it is already known the role of the enzyme *Dnmt1* in the acquisition of learning and memory in mature neurons, by maintaining DNA methylation and post-mitotic stability of marks, as well as the physiological implications of the decreased expression of *Dnmt1* to the neuronal morphology, synaptic plasticity, memory and learning (Feng et al., 2010).

The hippocampus plays essential role in cognitive and memory function, as well as it is heavily involved in the adaptive response to stress. Essentially, the activation of the adrenal gland by hypothalamic-pituitary axis leads to release of glucocorticoids, activating the hippocampus which consequently acts inhibiting the initial process of physiological stress (Joca et al., 2003).

Based on our findings, we speculate that the alteration of DNA methylation in the hippocampus might be related to the inhibitory effects of the hypothalamic-pituitary axis, in the adaptive responses of acute stress. However, future studies are needed to confirm this hypothesis.

In regard of the correlation between changes in DNA methylation in the cortex and PAG with behavioral stress, as far as we conceived, no corresponding evidence was reported until now in the literature.

The prefrontal cortex is related to the hypothalamic-pituitary axis in an inhibitory form, because it is also a sensitive area to glucocorticoids (Ruiz et al., 2007). Moreover, the prefrontal cortex plays a role in executive functions, decision-making, and it is also responsible for feelings of pleasure (Blaze and Roth, 2013).

The PAG is involved in behavioral and cardiovascular autonomic functions, particularly in the adaptive responses of fear and pain (Pelosi et al., 2007), in addition to being associated to escaping behavior in animals (Brandão et al., 2003).

Considering our evidence of decreased DNA methylation in the cortex and PAG after stress, we hypothesized the involvement of this epigenetic alteration in the molecular mechanisms related to the possible pathophysiological changes involving the cortex and the PAG, such as changing decision-making or changes in the escape mechanism and cardiovascular control, respectively, consequent to stress. Future studies are needed to confirm this hypothesis.

Interestingly, the analysis involving the findings of DNA methylation changes in the hippocampus, cortex and PAG in comparison to gene expression of *Dnmt1* showed a correlation only in the cortex, where the DNA demethylation seems to be associated with decreased expression of *Dnmt1*. However, in the hippocampus and PAG, the demethylation of DNA induced by stress demonstrated to be independent of changes in the expression of *Dnmt1*, indicating in these cases the participation of other factors such as *Dnmt3* enzymes or even other non-identified active demethylation mechanism.

The *Bdnf* gene plays a recognized role in mediating synaptic plasticity and behavioral responses to aversive social experiences (Fuchikami et al., 2010). Moreover, *Bdnf* is important in the control of biological activities in several brain areas (Karpova, 2013), so that disorders in *Bdnf* expression are related to the course and development of several neurological and psychiatric diseases (Boulle et al., 2012). Additionally, increased expression of the *Bdnf* gene in the hypothalamus is considered an important mark for the involvement of this gene in the synaptic plasticity in response to stress (Rage et al., 2002).

In the present study we observed a significant increase in the expression of *Bdnf* exclusively in the PAG of animals submitted to behavioral stress, indicating the participation of *Bdnf* in the modulation of PAG activity after stress. Somehow, this evidence supports previous data (Siuciak et al., 1995, 1998; Brandão et al., 2003) and suggests a relation between the increased expression of *Bdnf* in the PAG and changes in the neurobiology of fear, anxiety, cardiovascular control and analgesia. Moreover, when comparing our data concerning to the expression of *Bdnf* and the effects on DNA methylation, we can hypothesize a relation between the loss of control in the expression of *Bdnf* and significant decrease in DNA global methylation in the PAG. However, further studies are needed to elucidate this association.

When considering the potential of physical activity in modulating stress-induced molecular changes observed in the present study, the animals submitted to swimming present a diverse profile of DNA methylation in the hippocampus and PAG significantly different from sedentary animals.

Similarly, a significantly different pattern of response to stress was observed when considered the *Dnmt1* gene expression in the cortex and PAG of the exercised and sedentary animals. In relation to the *Bdnf* gene, distinct pattern of this gene expression was also observed when compared to the cortex, hypothalamus and PAG of both exercised and sedentary animals.

Considering the evidences related to global DNA methylation, the expression of *Dnmt1* and *Bdnf* genes, our data demonstrate the potential of physical activity in modulating the molecular effects induced by stress in the hippocampus, cortex, hypothalamus and PAG.

Consequently these data lead us to hypothesize that such molecular modulation induced by physical exercise has significant repercussions in the control of neurobiological activities related to each of these brain areas, as previously demonstrated considering the hippocampus and the involvement in the cognitive function (Cotman and Berchtold, 2002; Berchtold et al., 2005; van Praag et al., 2005; Cotman et al., 2007; O'Callaghan et al., 2007; Gomes-Pinilla et al., 2011), as well as in the cortical activity (Marmigere et al., 1998; van Praag et al., 2005; Cotman et al., 2007; McEwen and Gianaros, 2011).

In summary, our data reveal that behavioral stress induces DNA hypomethylation in the hippocampus, cortex and PAG, and affects control of the expression of *Dnmt1* in the cortex and PAG, and of *Bdnf* in the PAG. Additionally, our data show the potential of physical

activity in attenuating the intensity of the stress-induced global DNA methylation in the hippocampus, cortex and PAG, besides modulating the effects on the expression of *Dnmt1* and *Bdnf* genes.

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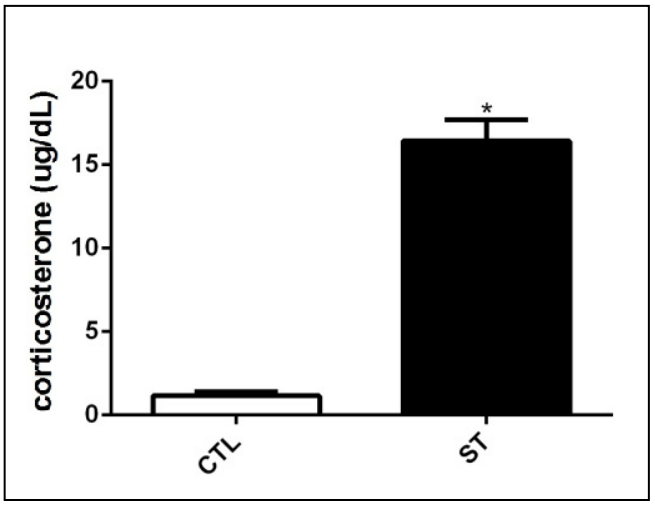

Figure 1. Serum corticosterone levels between CTL and ST groups. CTL: control group; ST: stress group. *P=0.0043. Mann-Whitney test.

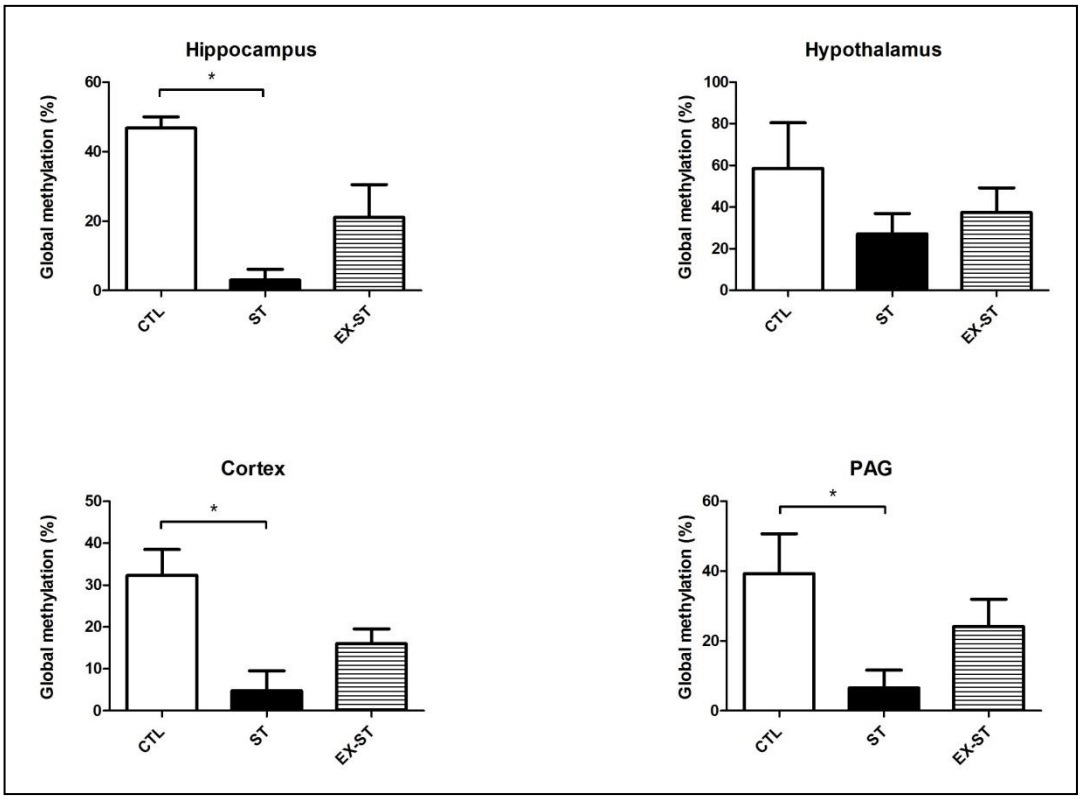

Figure 2. Global DNA methylation profile in the hippocampus, hypothalamus, cortex and PAG in the CTL, ST and EX-ST groups. CTL: control group; ST: stress group; EX-ST: exercise + stress group. *P<0.05. ANOVA test, Dunn's post-test.

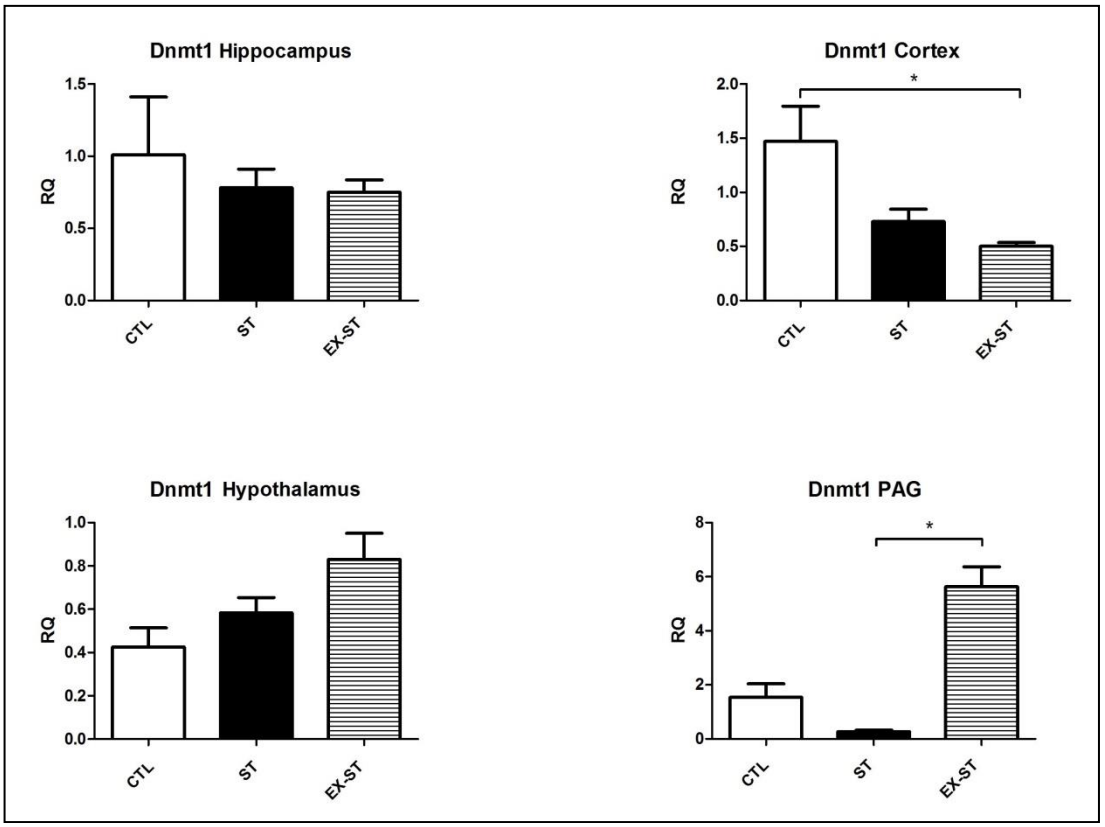

Figure 3. Quantitative expression of *Dnmt1* among the CTL, ST and EX-ST groups. CTL: control group; ST: stress group; EX-ST: exercise + stress group. **P*<0.05. ANOVA test, Dunn's post-test.

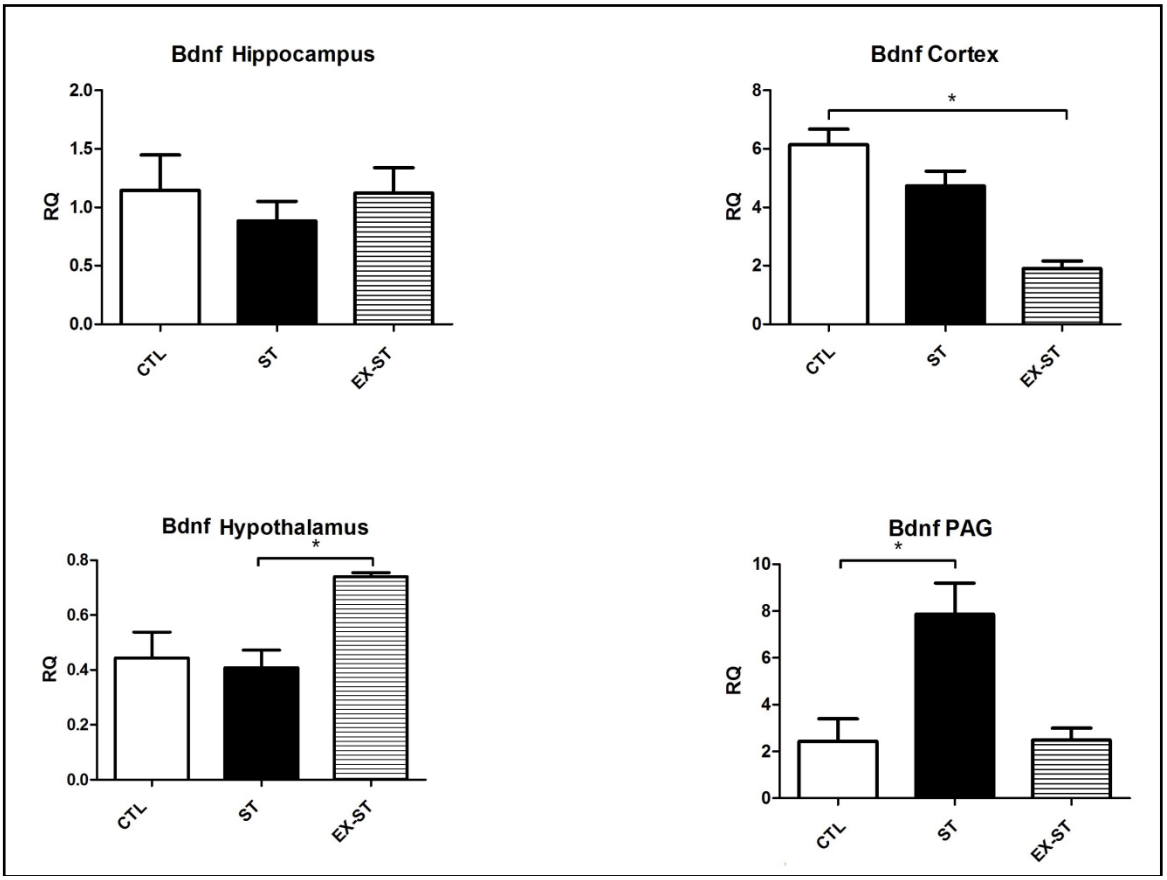

Figure 4. Quantitative expression of *Bdnf* among the CTL, ST and EX-ST groups. CTL: control group; ST: stress group; EX-ST: exercise + stress group. **P*<0.05. ANOVA test, Dunn's post-test.

CONCLUSÃO GERAL

Com base nas evidências do presente estudo podemos concluir que o estresse comportamental induz hipometilação global do DNA no hipocampo, córtex e PAG, sugerindo a participação da metilação do DNA na neurobiologia molecular do estresse.

Além disso, nossos dados sugerem que a demetilação induzida pelo estresse no córtex e na PAG possa estar associada à diminuição da expressão do gene *Dnmt1* nessas regiões. Similarmente, a demetilação da PAG pode estar associada ao aumento da expressão do *Bdnf* nesta região.

No tocante ao efeito da prática de atividade física sobre os efeitos moleculares induzidos pelo estresse, nossos dados demostram que animais praticantes de natação apresentam alterações da metilação global do DNA no hipocampo, córtex e PAG em intensidades menores do que as observadas em animais sedentários, indicando assim um efeito atenuador da prática de atividade física sobre os efeitos moleculares induzidos pelo estresse.

Adicionalmente, a prática de atividade física demonstrou potencial em modular os efeitos do estresse sobre a expressão dos genes *Dnmt1* na PAG e no córtex, assim como os efeitos do estresse sobre a expressão do gene *Bdnf* no córtex, hipotálamo e PAG.

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ANEXOS

ANEXO A – Normas de formatação do periódico *Neuroscience*

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Neuroscience publishes papers describing the results of original research on any aspect of the scientific study of the nervous system. Any paper, however short, will be considered for publication provided that it reports significant, new and carefully confirmed findings with full experimental details.

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INTRODUCTION

Neuroscience publishes the results of original research on any aspect of the scientific study of the nervous system. Papers most suitable for publication are those that report new observations that directly contribute to our understanding of how the nervous system works. Any paper, however short, will be considered for publication provided that it reports significant, new and carefully confirmed findings with full experimental details.

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A Special Issue is not a loose collection of topically related articles but a concerted attempt to provide an overview of the status of an emerging field. Cross references between the articles are strongly encouraged.

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