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ESCOLA DE CIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO
BIOLOGIA CELULAR E MOLECULAR

JULIANO VIANA BORGES

EFEITOS DO ISOLAMENTO E SUPORTE SOCIAL SOBRE INDICADORES EPIGENÉTICOS E NÍVEIS DE BDNF EM HIPOCAMPO DE RATOS CRONICAMENTE ESTRESSADOS

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PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

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Orientadora: Elke Bromberg

Tese apresentada como requisito para a obtenção do grau de Doutor pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

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Aprovado em 28 de Março de 2019

BANCA EXAMINADORA:

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PORTO ALEGRE

2019

À minha família, por todo apoio e incentivo.

Sem vocês esse sonho não se tornaria realidade.

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RESUMO

O estresse é caracterizado como um conjunto de ações tomadas em reposta á situações que alterem a fisiologia normal do organismo. Quando crônico, leva á alterações da homeostasia corporal somado á prejuízos físicos e mentais. O isolamento social já foi apontado como uma causa de estresse para espécies de animais com características de convívio social. Essa privação social pode induzir danos ao organismo, com modificações no Sistema Nervoso Central (SNC), que muitas vezes culminam em quadros de ansiedade e depressão, assim como déficits de memória. As alterações do SNC podem ser associadas a variações moleculares, como do fator neurotrófico derivado do cérebro (BDNF), que é muito importante para a fisiologia e plasticidade neuronal. O suporte provido pela convivência social poderia atenuar os efeitos do estresse crônico e isolamento social, melhorando aspectos moleculares, físicos e cognitivos. O isolamento social de animais adultos ainda é pouco explorado na literatura científica, portanto o objetivo deste trabalho foi investigar os efeitos do isolamento e suporte social sobre: alterações moleculares dos níveis e expressão de BDNF no hipocampo; mecanismos epigenéticos de acetilação das histonas e metilação do DNA no hipocampo; sobre parâmetros comportamentais de memória e ansiedade em animais adultos cronicamente estressados, com idade de três e dezessetes meses. A exposição de animais de três meses ao isolamento social e estresse crônico mostrou que a privação social é prejudicial para a memória, entretanto, o aumento da ansiedade só pode ser observado em animais isolados e submetidos ao protocolo de estresse crônico imprevisível (CUS). O ganho de peso durante o experimento foi menor em animais que eram estressados. O BDNF é diminuído em animais isolados em comparação

aos acompanhados e a expressão da enzima HDAC5 foi aumentada apenas em animais que eram isolados e estressados. A acetilação de H4K12 foi maior em hipocampos de animais acompanhados, e H3K9 foi diminuída em animais que eram isolados e estressados. Os animais de dezessete meses também tiveram a memória prejudicada pelo isolamento em relação àqueles que eram acompanhados, já a ansiedade foi menor apenas nos animais que eram acompanhados e sem estresse. O ganho de peso durante o experimento foi menor em animais que eram estressados pelo CUS. A expressão de BDNF foi maior em hipocampos de animais acompanhados. As expressões de HDAC5 e DNMT1 foram maiores em animais que eram isolados e estressados, entretanto, a expressão de DNMT3a foi indiferente aos protocolos de isolamento e estresse. Em conclusão, o estudo mostrou que o isolamento social de animais adultos pode induzir alterações epigenéticas e exacerbar o efeito do estresse crônico em parâmetros comportamentais e moleculares, indicando que o suporte social pode ser eficaz na atenuação de alguns efeitos prejudiciais causados pelo estresse.

Palavras chaves: suporte social, isolamento social, estresse, acetilação, metilação, memória, BDNF.

ABSTRACT

Stress is characterized by a set of actions taken in response to situations that alter the normal physiology of the organism. When chronic, the stress leads to alteration of body homeostasis, physical and mental impairment. Social isolation has already been pointed out as a cause of stress for animal species with social conviviality characteristics. The social deprivation can induce damages to the organism, leading to changes in the Central Nervous System (CNS), which can culminate in anxiety and depression, as well as memory deficits. These CNS changes may be associated with molecular variations, such as brain-derived neurotrophic factor (BDNF), which is very important for neuronal physiology and plasticity. The support provided by social coexistence can attenuate the effects of chronic stress and social isolation, improving physical and cognitive aspects. The social isolation of adult animals still is little explored in the scientific literature, so the objective of this work was to investigate the effects of isolation and social support on: level and expression of BDNF in the hippocampus; molecular mechanisms of histones acetylation and methylation of DNA in the hippocampus; on behavioral parameters of memory and anxiety in chronically stressed adult animals, aged three and seventeen months. The exposure of three-month old animals to social isolation and chronic stress showed that social isolation was detrimental to memory, but the increase in anxiety can only be observed in animals that were isolated and submitted to the unpredictable chronic stress protocol (CUS). The weight gain during the experiment was lower in animals that were stressed by CUS. BDNF was decreased in isolated animals in comparison to accompanied groups, and HDAC5 expression was increased only in animals that were isolated and stressed. Acetylation of H4K12 was higher in the hippocampus of

accompanied animals, and H3K9 was decreased in animals that were isolated and stressed. Seventeen-month old animals also had memory impaired by isolation compared to accompanied, and anxiety was lower only in animals that were accompanied and non-stressed. The weight gain during the experiment was lower in animals that were stressed by CUS. The expression of BDNF was higher in the hippocampus of accompanied animals. Expression of HDAC5 and DNMT1 were higher in animals that were isolated and stressed, whereas DNMT3a expression was indifferent to the isolation and stress protocols. In conclusion, the study showed that the social isolation of adult animals can induce epigenetic alterations and to exacerbate the effect of chronic stress in cognitive and molecular parameters, indicating that social support can be effective in attenuating some harmful effects caused by stress.

Keywords: social support, social isolation, stress, acetylation, methylation, memory, BDNF.

LISTA DE ABREVIATURAS

ACTH - Hormônio adrenocorticotrófico

BDNF - Fator Neurotrófico Derivado do Cérebro

C - Citosina

CA - Corno de Amon

CH3 - Metil

CRH - Hormônio Liberador da Corticotrofina

CUS - *Chronic Unpredictable Stress*

DNA - Ácido Desoxirribonucleico

DNMT - Dna Metiltransferase

G - Guanina

H - Histona

HAT - Histona Acetiltransferase

HDAC - Histona Deacetilase

HPA - Hipotálo-Pituitária-Adrenal

IBGE - Instituto Brasileiro de Geografia e Estatística

LTM - *Long-term Memory*

LTP – Long-term Potentiation

K - Lisina

NAD - Dinucleótido de nicotinamida e adenina

PB - Pares de Base

R - Arginina

RNA - Ácido Ribonucleico

SAM - S-adenosilmetionina

SNC - Sistema Nervoso Central

STM - *Short-term Memory*

Trkb - Receptor Cinase B da Tropomiosina

tRNA - Ácido Ribonucleioco Transportador

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1. INTRODUÇÃO

1.1 EPIGENÉTICA

O termo epigenética foi apresentado primeiramente na década de 1940 por Conrad Waddington e se refere ás inúmeras modificações moleculares necessárias para realizar o acesso ao DNA (RICHARDS, 2006). Atualmente esse termo já recebeu diversas descrições funcionais, relacionadas principalmente aos processos da regulação da expressão gênica. Os diferentes tipos de células do organismo apresentam padrões próprios de expressão gênica, condizente com a necessidade e demanda de cada tecido corporal. Essa característica epigenética que diferencia a funcionalidade das mais variadas células presentes no organismo (JIRTLE; SKINNER; CAROLINA, 2007).

Os mecanismos epigenéticos agem no sistema nervoso central (SNC) que é uma estrutura de grande plasticidade e necessita de uma constante adaptação ao meio. As características do SNC são viáveis graças aos processos epigenéticos, que desempenham papel primordial no desenvolvimento, sobrevivência e diferenciação celular, assim como os processos de formação, consolidação e armazenamento de memórias (LANDGRAVE-GÓMEZ; MERCADO-GÓMEZ; GUEVARA-GUZMÁN, 2015).

Os diferentes eventos epigenéticos têm a capacidade de alterar os padrões de expressão gênica, podendo ativar ou reprimir a maquinaria de transcrição, a partir da alteração da conexão do DNA junto as proteínas de enovelamento genômico

(HE; LEHMING, 2003). O conjunto dessas proteínas, também chamadas de histonas (H2A, H2B, H3 e H4) formam um complexo octâmero que constitui o principal componente da cromatina. Cada classe de histonas possui dois exemplares na constituição da cromatina, e por sua vez, essas proteínas são envoltas de sequências de DNA contendo cerca de 148 pares de base (pb). Adicionalmente á esse complexo se liga a histona H1, cuja função seria de estabilização de todo conjunto. O complexo de cromatina com o seu DNA enovelado recebe o nome de nucleossoma (ONUFRIEV; SCHIESSEL, 2019).

A cromatina possui dois estados básicos de estrutura no genoma: heterocromatina e eucromatina. Essas estruturas são influenciadas pelo ambiente e são modificadas através de enzimas epigenéticas (Figura 1). A heterocromatina está associada á condensação do nucleossoma e consequentemente a diminuição da expressão gênica. Já a eucromatina está associada á menor condensação do nucleossoma, o que torna o DNA mais exposto, facilitando processos como replicação, reparo do DNA e transcrição gênica (TAMARU, 2010).

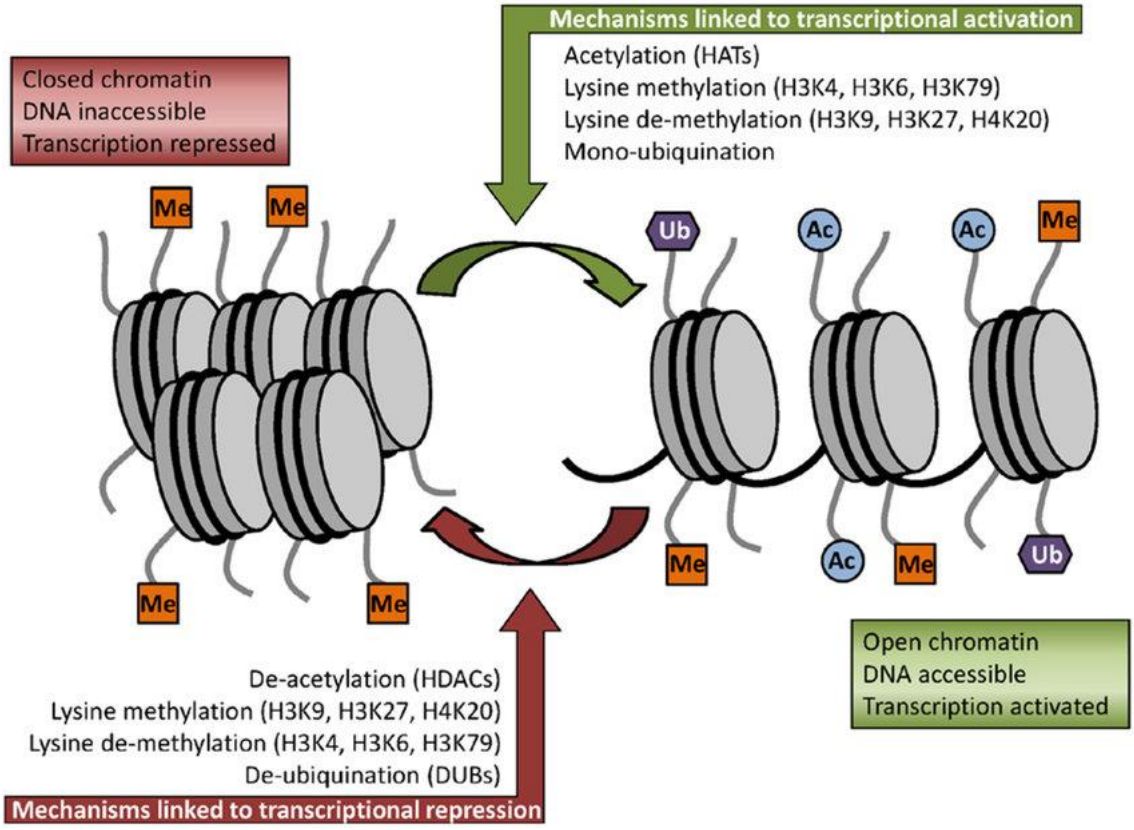

Figura 1- Mecanismos epigenéticos que induzem a modificação química da cromatina em eucromatina e heterocromatina (ULLAH, 2015).

As histonas presentes na cromatina podem ser alvos de modificações pós-traducionais que alteram a sua conformação química, induzindo alterações na acessibilidade da maquinaria transcricional junto ao material genético. Dentre essas modificações podemos citar a acetilação e a metilação, que tem ações distintas no complexo DNA-cromatina; enquanto a acetilação está associada á aumento da expressão gênica, a metilação pode induzir tanto a inibição, quanto a ativação da expressão, e isso é dependente dos locais de ligação e enzimas ativas na reação. Portanto essas alterações epigenéticas desempenham um papel muito importante no controle da expressão gênica ao longo do genoma (KOUZARIDES, 2007).

1.1.1 ACETILAÇÃO

A acetilação é um processo de adição de um grupamento acetil na ramificação N-terminal das histonas, isso faz com que a interação de cargas entre DNA e cromatina fique enfraquecida, devido á alteração de polaridade no complexo. Após a acetilação e consequente diminuição de interação entre as estruturas, o DNA fica mais exposto para que ocorra a transcrição gênica. Em eucariotos a acetilação induz a neutralização das cargas positivas da lisina presente na cauda N-terminal das histonas, enfraquecendo assim a ligação entre o DNA (que possui carga negativa) e as histonas. Esse processo está associado à transcrição e também pode regular os mecanismos de replicação e reparo ao DNA (ZUPKOVITZ et al., 2006).

Existem alguns tipos de enzimas fundamentais para que o processo possa acontecer e estas são conhecidas como histonas acetiltransferases (HATs). Estas enzimas são as responsáveis pela adição de grupamento acetil aos resíduos de lisina das histonas, e dessa maneira induzem a acetilação. Pode-se dizer, assim, que as HATs são facilitadoras do processo de transcrição. Em contrapartida, existem as histonas deacetilases (HDACs), que fazem a retirada do grupamento acetil, restauram a carga positiva das histonas e aumentam a interação entre DNA e histonas. Esse processo torna a cromatina mais condensada e reprime a transcrição gênica (TESSARZ; KOUZARIDES, 2014).

A acetilação pode ocorrer em diversos pontos da cauda N-terminal das histonas: os pontos de ligação mais descritos até o momento são da histona H3, nas lisinas 9 e 14; e lisinas 5 e 12 da H4 (KOUZARIDES, 2007). As HATs são divididas em dois grupos, A e B. O grupo A é composto de enzimas que se localizam

preferencialmente no núcleo, que desempenham variadas funções e são classificadas de acordo com sua conformação estrutural e função (HODAWADEKAR, MARMORSTEIN, 2007). Por outro lado as HATs do tipo B se localizam no citoplasma das células, acetilando histonas recentemente sintetizadas (BENSON et al., 2007).

As HDACs por sua vez, estão divididas em quatro classes, da seguinte maneira: classe I - HDACs 1, 2, 3 e 8; classe II - HDACs 4, 5, 6, 7, 9 e 10; classe III - que são as sirtuínas, numeradas de 1 á 7; e a classe IV - HDAC11. As classes I, II e IV possuem similaridade na sua estrutura e utilizam Zn^{2+} como cofator da sua reação enzimática, entretanto as sirtuínas, que representam a classe III, fazem uso do NAD^{+} (Dinucleótido de nicotinamida e adenina) para realizar sua ação (RUIJTER et al., 2003).

Dentre as enzimas HDACs, a HDAC5 integrante da classe II, vem ganhando atenção devido a sua associação com distúrbios neurológicos, como a depressão, ansiedade e Alzheimer. Ela está presente em células do cérebro, sendo encontrada em diversas estruturas relacionas ao sistema límbico, como o hipocampo, córtex pré-frontal, amígdala e núcleo accumbens (RENTHAL et al., 2007; AGIS-BALBOA et al., 2013). Prévios estudos já demonstraram sua associação com alterações comportamentais e cognitivas. A ação epigenética da HDAC5 é associada ao controle do estímulo emocional crônico, levando á adaptações emocionais no núcleo accumbens (RENTHAL et al., 2007).

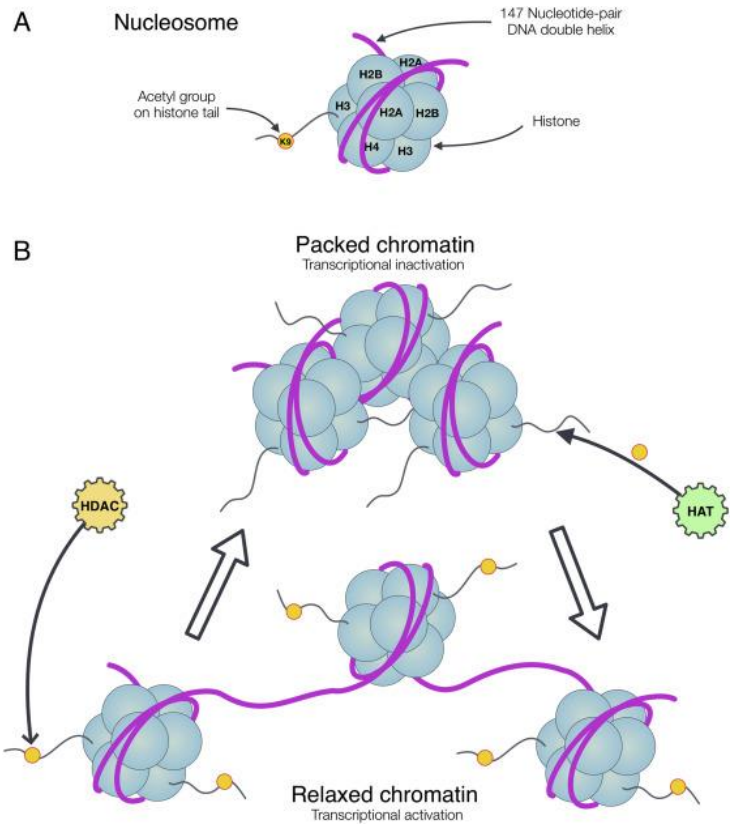

Figura 2. Processo de acetilação e deacetilação que induzem a alteração da conformação do nucleossoma (HUYNH; EVERTS; AMPORNARAMVETH, 2017).

No hipocampo, por exemplo, o aumento da expressão HDAC5 foi associado com diminuição na capacidade de resiliência em animais expostos ao estresse crônico social. Evidências também sugerem a associação do aumento da expressão da HDAC5 com a diminuição da efetividade de medicamentos antidepressivos. Devido a esse fato, as enzimas de controle epigenético, de acetilação e metilação, têm se tornado alvo de pesquisas para desenvolvimento de novos fármacos voltados ao tratamento de déficits cognitivos e transtornos neuropsiquiátricos (SANANBENESI; MUNGENAST; TSAI, 2010; AKBARIAN; , 2011).

A inibição das HDACs pelo Butirato de Sódio já se mostrou eficiente na neuroproteção de modelos animais, pois sua utilização é associada com a melhora

da memória de animais submetidos a estresse por separação materna e estresse crônico leve (VALVASSORI et al., 2015). Essa melhora da memória parece estar relacionada com a elevação dos níveis do fator neurotrófico derivado do cérebro (BDNF), uma neurotrofina de extrema importância na melhora das funções cerebrais, pois é importante para os processos de crescimento, diferenciação e sobrevivência neuronal, sendo (ORTIZ et al., 2014).

O estresse crônico e o isolamento social têm influência sobre os processos epigenéticos, como a acetilação de histonas. Já foi demonstrada a associação dessas duas situações com a diminuição da acetilação em algumas estruturas do SNC, dentre elas podemos citar o hipocampo. Além da diminuição na acetilação das histonas, também já se observou que ocorre um aumento da ativação e expressão das enzimas HDACs (JIANG, HUILI, 2018). O aumento dessas enzimas e consequente diminuição da acetilação estão associados á diminuição da expressão gênica no SNC, o que culmina com a diminuição dos níveis de algumas proteínas importantes para o funcionamento do cérebro, como o BDNF (LI et al., 2016).

1.1.2 METILAÇÃO

A metilação é um dos principais mecanismos epigenéticos a modificar os níveis de expressão gênica. Esta reação contribui para uma série de processos biológicos de grande importância no desenvolvimento e manutenção do organismo. Um exemplo disso é o *imprinting* genômico, um mecanismo de regulação da

expressão gênica, cuja função é inibir a transcrição gênica oriunda de um dos alelos parentais, tal como ocorre na inativação do cromossomo X (WANG; WANG, 2013).

A metilação é o processo de adição de um grupamento metil (CH3) aos sítios de metilação, presentes nas histonas e no DNA. Nas histonas, a metilação pode acontecer em resíduos de lisina (K), mas também em alguns de arginina (R), sendo apontada como um dos principais eventos de modificações pós-traducionais das histonas H3 e H4. A metilação de lisina é um mecanismo específico que tem ação distinta dependente do sítio de ligação ao qual ocorre. Quando esse processo acontece nos sítios H3K4, H3K36 e H3K79 é associado com ativação na transcrição a partir da RNA polimerase II. Por outro lado, quando ocorre nos sítios H3K9, H3K27 e H4K20 promove repressão da transcrição (BANNISTER; KOUZARIDES, 2005). A reversão da metilação pode acontecer á partir de enzimas conhecidas como desmetilases, que exercem efeitos distintos confirme o sítio de ligação onde realizam sua ação, podendo inibir ou ativar a transcrição (SHI et al., 2004; SCHNEIDER et al., 2005).

No DNA, a metilação acontece por uma ligação covalente entre o grupamento CH3 e o carbono localizado na posição 5’ do nucleotídeo citosina (C). Em mamíferos, isso acontece constantemente em sítios específicos denominados de ilhas CpG, que são sequências curtas contendo cerca de 200pb com alta frequência de dinucleotídeos C e G (guanina). Essas sequências estão presentes ao longo do genoma e geralmente são locais ricos em regiões de promotores gênicos (WANG; LEUNG, 2004). Evidências sugerem que a hipermetilação e a hipometilação das ilhas CpG exercem importante função na expressão gênica, estando associadas à diminuição e ao aumento da expressão de genes, respectivamente (BIRD, 2008). Entretanto, o local onde a metilação acontece determinará a resposta perante essa

reação, por exemplo, quando realizada nos promotores está associada ao silenciamento gênico, já quando ocorre na região codificadora induz a transcrição gênica (BOYES; BIRD, 1992; YANG et al, 2014).

Para que a metilação no DNA possa acontecer são necessárias ações enzimáticas que catalisem a transferência do grupamento metil ao complexo. As enzimas que desempenham esse papel pertencem a família das DNA metiltransferases (DNMTs), incluindo as DNMT1, DNMT2, DNMT3a e DNMT3b (Figura 3). Essas enzimas conseguem ativar a metilação a partir da transferência do CH3 oriundo do substrato S-adenosilmetionina (SAM) para os sítios de ligação (KIM; SAMARANAYAKE; PRADHAN, 2009).

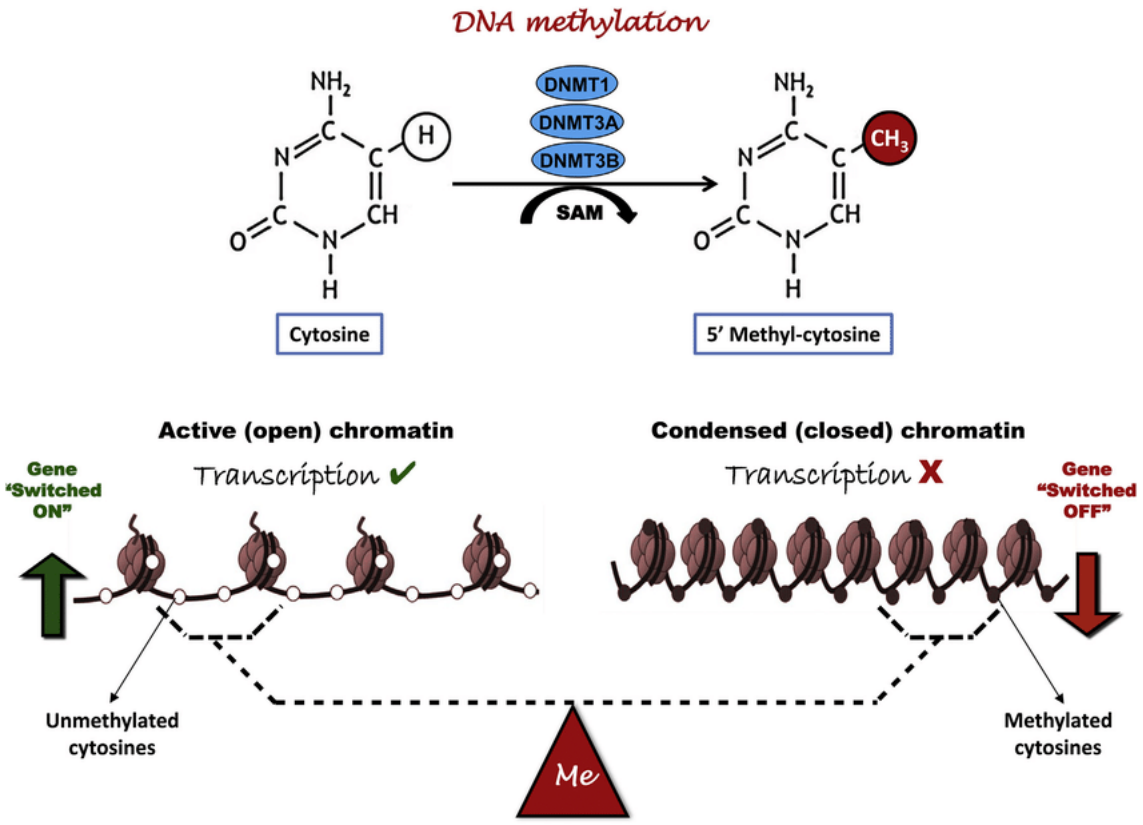

Figura 3. Processo de metilação do DNA realizado pelas enzimas DNMT (AGRAWAL et al., 2018).

As DNMTs apresentam papéis distintos: a DNMT1 mantém o padrão de metilação durante a replicação celular; DNMT2 apesar da similaridade estrutural tem papel na metilação de RNA transportador (tRNA) e não no DNA; as DNMT3a e DNMT3b por sua vez, estão associadas com à catalisação da via *de novo* de metilação, nas moléculas de DNA dupla fita previamente metilado durante o desenvolvimento embrionário (NEWELL-PRICE; CLARK; KING, 2000; JURKOWSKI et al., 2008; OKANO et al., 1999).

A DNMT1 é a isoforma mais frequente em células somáticas e classificada como uma enzima de manutenção, pois reestabelece a metilação no DNA de novas células, após a replicação do DNA (FOULKS et al., 2012). Portanto, ela é muito importante nos processos de proliferação e sobrevivência celular, além de garantir o *imprinting genômico* (SINGH; SHARMA; CAPALASH, 2013). Por outro lado, a DNMT3 é muito importante nos períodos de desenvolvimento embrionário, assim como na gametogênese (FOULKS et al., 2012).

Nos últimos anos, estudos têm sugerido que a metilação está associada à diversas patologias, incluindo o câncer e doenças do SNC. No câncer, a gênese tumoral estaria associada a alterações nos padrões de metilação do DNA, nas ilhas CpG. Estas alterações levariam à ativação de diversos oncogenes e ao silenciamento de genes supressores tumorais, favorecendo, assim, a carcinogênese. (FREW et al., 2008; SARKAR; ROSENTHAL, 2013). Em relação às patologias do SNC, as alterações na metilação foram associadas com a diminuição de neurotrofinas, levando ao envelhecimento neuronal, distúrbios de memória, plasticidade sináptica, resultando em distúrbios neurológicos e processos neurodegenerativos (GAPP et al., 2014).

O BDNF é umas das principais neurotrofinas conhecidas pela comunidade científica e sua conexão com o receptor TrkB está associada com uma diversidade de eventos bioquímicos que abrangem a sobrevivência e diferenciação neuronal e plasticidade sináptica (Figura 4). A sua diminuição parece estar atrelada ao aparecimento de alguns distúrbios do SNC, como o Alzheimer, Parkinson e a depressão induzida pelo de estresse (NAGAHARA; TUSZYNSKI, 2011; STEIN; DANIELS, 2008).

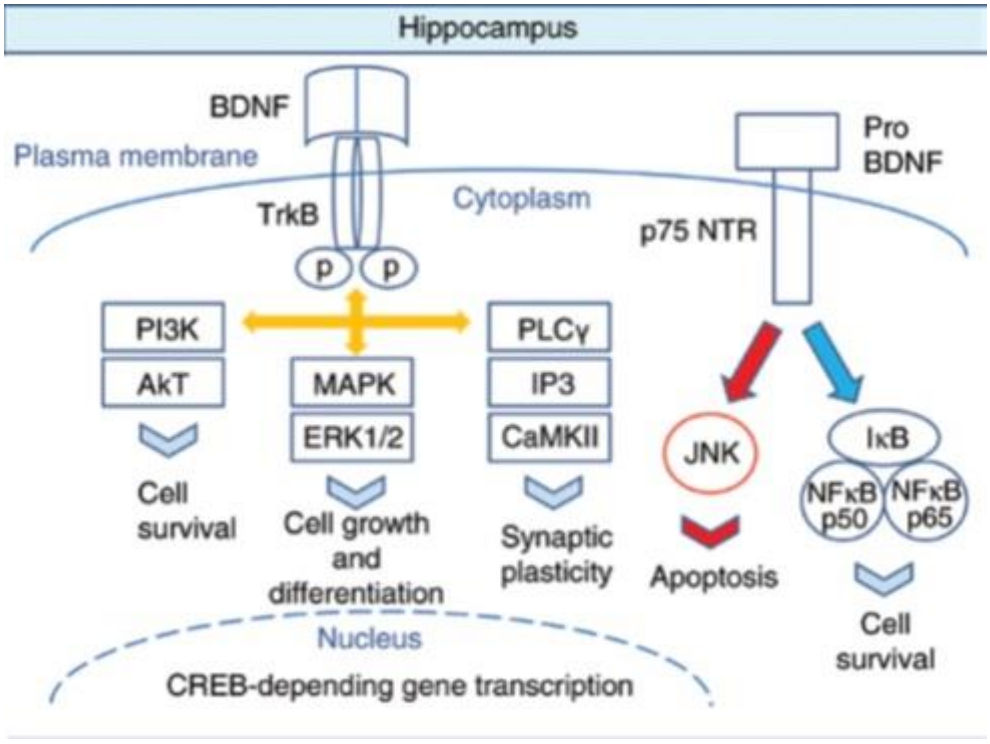

Figura 4. Cascatas bioquímicas relacionadas à ligação do BDNF com seu receptor TrkB (ZALETEL, 2017a).

Em relação à depressão, pesquisas vêm demonstrando cada vez mais, a associação desta patologia com aumento da metilação nos promotores do BDNF, levando a diminuição da expressão desse gene nos neurônios (MARTINOWICH et

al., 2003; FUCHIKAMI et al., 2011). Suportando esses estudos, o uso de antidepressivos se relacionou ao aumento dos níveis de BDNF, e a outros eventos epigenéticos. Logo, a diminuição nas concentrações da neurotrofina parece ser uma peça chave no aparecimento da depressão (JUSE, 2009; BOULLE et al., 2011).

O estresse crônico representa um dos fatores de risco para o desenvolvimento da depressão, sendo também uma condição que pode induzir modificações epigenéticas em vários tecidos. No SNC, o estresse é associado a alterações na conformação das histonas e na metilação do DNA (TSANKOVA et al., 2006; SAUNDERSON et al, 2013). O estresse crônico pode ter uma origem multifatorial, sendo classificado conforme os tipos de estressores, que abrangem os físicos, psicológicos ou sociais. O estresse social pode ser gerado por separação materna, traumas na infância e adolescência, e isolamento social (ROMANO-L; GARC, 2015; LEWIS et al., 2017).

A literatura possui alguns trabalhos associando o efeito da separação materna com os processos epigenéticos. Esses estudos indicam que a separação materna induz alterações nos processos de metilação e acetilação, culminando em aumento de inflamação, déficits cognitivos, diminuição de BDNF e aumento de glicocorticoides (MPOFANA; DANIELS; MABANDLA, 2016). Por outro lado, estudos sobre o isolamento social de animais adultos e sua associação aos mecanismos epigenéticos ainda são pouco abordados na comunidade científica, que costuma focar preferencialmente no efeito a curto e longo prazo do estresse, separação materna ou isolamento social nas fases iniciais da vida.

1.2 ESTRESSE, ISOLAMENTO E ACOMPANHAMENTO SOCIAL

O termo “estresse” foi introduzido para a comunidade científica por Hans Selye em 1936, para descrever o processo de adaptação realizado a partir de uma série de modificações fisiológicas que acontecem no organismo perante situações que alteram a homeostase. Os estressores podem englobar situações que se mostrem desafiadoras e que levam á necessidade de uma adaptação homeostática do organismo (MCEWEN, 2000). Ao longo da vida estamos expostos a uma diversidade de situações que afetam a homeostasia corporal, e forçam nosso organismo a adaptar-se mediante diferentes mecanismos fisiológicos (CUESTA; SINGER, 2012).

Os principais mecanismos de adaptação fisiológica ao estresse são o sistema nervoso autônomo e o eixo hipotálamo-pituitária-adrenal (HPA), que são acionados em reposta ao estresse induzindo uma resposta de “luta ou fuga” pelo aumento da liberação de adrenalina e aumento dos hormônios glicocorticoides circulantes (cortisol em humanos e corticosterona em roedores), respectivamente. A ativação do eixo HPA é gerida por uma cascata de reações hormonais: primeiramente o núcleo paraventricular do hipotálamo libera o hormônio liberador de corticotrofina (CRH); o CRH por sua vez chega á glândula pituitária e induz a secreção do hormônio adenocorticotrófico (ACTH); quando o ACTH é aumentado na circulação ele chega até a glândula adrenal (localizada sobre os rins), e finalmente ocorre a liberação dos glicocorticoides (Figura 5) (ULRICH-LAI; HERMAN, 2014).

A elevação dos níveis de glicocorticoides desencadeia diversos processos regulatórios fisiológicos sobre: o metabolismo, o sistema imune, o SNC, sobre a

reatividade vascular às catecolaminas, entre outras respostas associadas à regulação perante as condições de estresse. Além disso, esses hormônios estimulam diversas outras respostas no organismo, como inibição da ativação demasiada do próprio eixo HPA, pelo sistema de *feedback* negativo; coíbe respostas excessivas ao estresse; e ainda atua na consolidação dos diversos tipos de memória (AMSTERDAM et al., 2015; MYERS; MCKLVEEN; HERMAN, 2014; ROY et al., 1999).

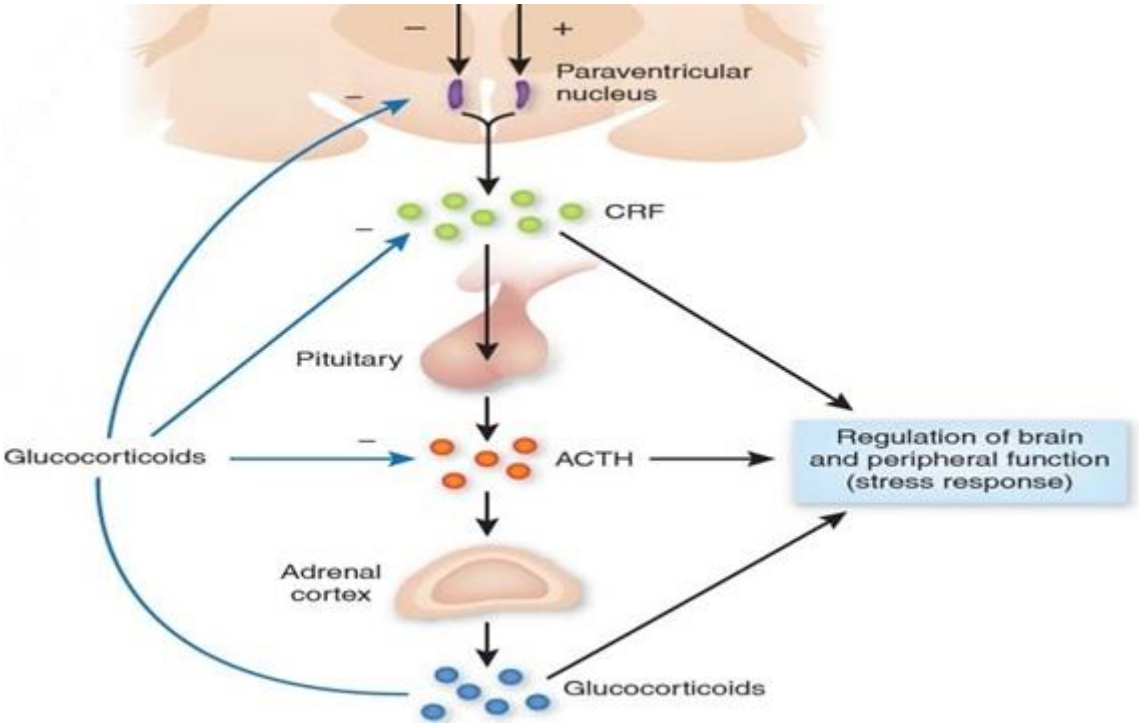

Figura 5. Cascata de reações do eixo HPA (RAABE; SPENGLER, 2013).

Entretanto, algumas situações levam à exacerbação dos limites de adaptação homeostática, com desregulação do eixo HPA e níveis hormonais, devido ao aumento do tempo e/ou intensidade do estressor ao qual o organismo é exposto. Esse extravasamento do limiar fisiológico acarreta em prejuízos ao organismo,

levando a perda da homeostasia, que muitas culminam em disfunções dos parâmetros cognitivos (MCEWEN, 2000; HERMAN; CULLINAN; HERMAN, 1997).

Em relação à duração do estresse, podemos classificá-lo em agudo, aquelas situações em que o indivíduo é exposto de maneira única ao estressor, ou crônico, caracterizado pela exposição contínua ou em períodos prolongados aos agentes estressores (PACÁK; PALKOVITS, 2001). O estresse agudo de baixa intensidade está relacionado com uma a melhor adaptação fisiológica, entretanto, o crônico está mais associado com quadros patológicos, visto que este exige uma maior demanda homeostática devido a constante exposição do organismo aos estressores (MCEWEN; MAGARINOS, 1968).

Em modelos animais, o estresse crônico pode ser simulado de diferentes maneiras e assim realizar a avaliação das respostas do organismo perante o estresse e a partir disso é possível fazer a translação para o que acontece nos seres humanos (MCCORMICK; GREEN, 2013). Nesses animais, já foram evidenciadas diversas alterações comportamentais e cognitivas, como ansiedade, depressão e problemas de memória. As variações cognitivas costumam aparecer devido à susceptibilidade das estruturas presentes no SNC, como o hipocampo, ás alterações fisiológicas oriundas do estresse (IDE et al., 2015)..

O hipocampo é constituído por duas porções principais: o giro denteado e o Corno de Amon que é subdividido nas regiões CA1, CA2, CA3 e CA4. Essa estrutura é de extrema importância nos processos de aprendizado e formação de memórias, atuando também na inibição da ativação demasiada do eixo HPA (DEKEYZER et al., 2017; TANTI et al., 2012; KIM; YOON, 1998). O estresse crônico e a elevação excessiva dos níveis de glicocorticoides estão associados com

prejuízos nas atividades do hipocampo. Essa ação hormonal acontece devido a essa estrutura ser rica em receptores corticoides, que o torna propenso á alterações em quadros de estresse. Portanto, as situações estressantes podem provocar importantes alterações hipocampais, incluindo a diminuição da neurogênse e arborização dendrítica, assim como ansiedade e prejuízo da memória (Figura 6) (MCEWEN et al, 2013; MCEWEN; GRAY; NASCA, 2015)

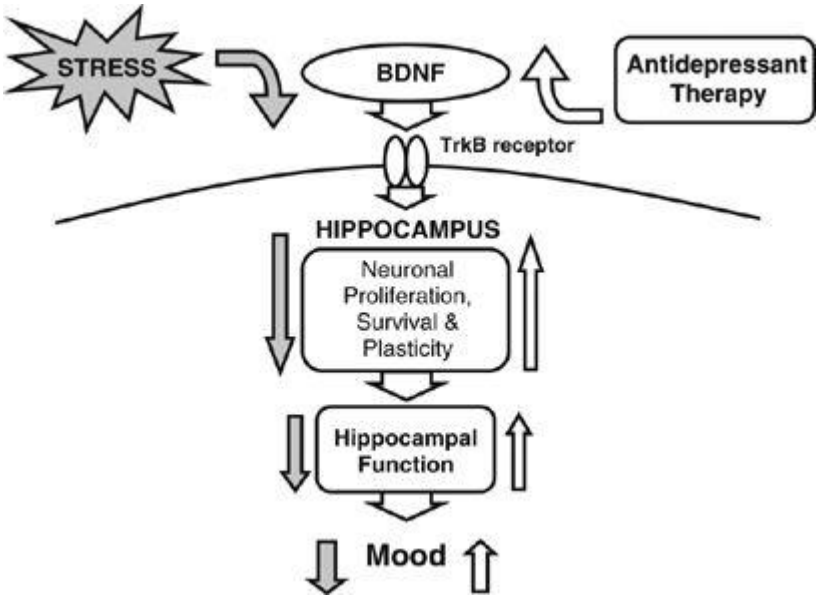

Figura 6. Respostas do hipocampo perante quadros de estresse (GROVES, 2007).

Além das alterações cognitivas, a literatura sugere que o estresse induz alterações epigenéticas no SNC, como acetilação e metilação. No hipocampo estresse já foi associado com alteração na metilação em animais submetidos á protocolos de estresse crônico, levando a diminuição da expressão de BDNF. Por sua vez, a acetilação é diminuída no hipocampo de animais expostos a situações estressoras (MCEWEN et al, 2013; TSANKOVA et al., 2006; RENTHAL et al., 2007).

O protocolo de estresse crônico imprevisível (do inglês “*chronic unpredictable stress”* – CUS) vem sendo muito utilizado atualmente para indução de estresse e depressão em animais. O CUS utiliza variados estressores, que visam simular o alguns tipos de estresse: como privação de água, alimento e sono, troca dos ciclos de luz claro-escuro, entre outros (GRØNLI et al., 2005). Um ponto imprescindível desse protocolo é a alternância de estressores e horários ao longo dos dias em que são empregados. Esse cronograma variado com diferentes tipos de estressores, intensidades e horários acontece para que não haja uma adaptação à rotina, assim o estresse se torna uma novidade para o animal, influenciando a atividade do eixo HPA e suas respostas (GAMARO et al., 2003). Por outro lado, a exposição repetida aos mesmos estressores pode atenuar a resposta do eixo HPA, gerando uma habituação aos estímulos, fato que evidencia a importância da randomização do protocolo (WEINBERG et al., 2008).

Estudos já demonstraram que o CUS assim como outros tipos de estresse crônico, pode induzir uma série de sintomas cognitivos e comportamentais, incluindo prejuízo de memória, ansiedade e depressão (WILLNER; MUSCAT; PAPPT, 1992). Animais expostos ao protocolo CUS também já apresentaram modificações epigenéticas em comparação com aqueles animais não estressados e essas modificações foram mostradas tanto para acetilação de histonas, quanto para a metilação das ilhas CpG no DNA (LI et al., 2017; PATCHEV et al., 2015).

A acetilação das histonas H3 e H4 parece sofrer efeito dos protocolos de estresse crônico, pois estas apresentam diminuição no nível de acetilação, resultando em decréscimo da expressão gênica (HAN et al., 2014; LIU et al, 2014). Mais precisamente, os estudos apontam que o CUS está associado á diminuição da acetilação dos resíduos de lisina presentes nas histonas, como por exemplo, em

H3K9 e H4K12, em células do hipocampo (FERLAND; SCHRADER, 2011). Complementar ao processo de redução na acetilação de histonas é possível observar o aumento da atividade da HDAC5. Em contrapartida, o aumento da atividade dessa enzima pode ser revertido pela ação de antidepressivos, indicando a associação da HDAC5 com os mecanismos moleculares da depressão (KARNIB et al., 2019).

A literatura se mostra restrita em termos de resultados que comparem os níveis de metilação do DNA em animais expostos ao CUS. Entretanto, trabalhos que fazem uso de outros protocolos mostram que a metilação é aumentada em animais expostos ao estresse crônico. Pesquisas mostram o aumento da expressão de DNMT1 em regiões hipocampais de animais submetidos á estresse pré-natal ou por separação materna (MONK; CHAMPAGNE; PEN, 2012; PARK et al., 2018). Interessantemente, as modificações dos níveis de metilação em animais expostos à separação materna parecem perdurar por longos períodos, sendo observadas até mesmo em animais adultos (ANIER et al., 2014). Estudos anteriores também demonstraram que o CUS age no hipocampo, prejudicando o processo de formação de novas memórias e isso ainda é associado com diminuição na concentração e expressão de BDNF, neurotrofina primordial para o processamento das memórias (LIU et al., 2014).

Outra maneira de induzir o estresse crônico em roedores é a partir do isolamento social. Pelo fato desses animais (assim como humanos) possuírem característica a vida social, a privação desse tipo de interação acaba gerando um ambiente estressor (CRUCES et al., 2014). O isolamento social é um protocolo de estresse crônico, amplamente difundido na literatura científica, utilizado para indução de depressão e ansiedade (KOKARE et al., 2010). A privação social induz diversas

respostas em roedores, como aumento da agressividade, déficits de memória, ansiedade e depressão. Estudos ainda sugerem importantes alterações no hipocampo, que apresenta diminuição da arborização dendrítica, neurogênese e plasticidade sináptica (VARTY; MARSDEN; HIGGINS, 1999; LU, 2003; FLORES; SILVA-GOMEZ, 2003)**.**

A maioria dos estudos que buscam uma associação entre privação social e mecanismos epigenéticos prioriza o isolamento nas fases iniciais da vida, portanto, a separação materna surge como principal objeto de estudo para essa avaliação. A privação de contato materno está associada a uma diversidade de fatores ligados ao metabolismo do eixo HPA, induzindo aumento de corticosterona, alteração no número de receptores de glicocorticoides no hipocampo e aumento de CRH em regiões do sistema límbico (PLOTSKY et al., 2005). Esse tipo de estresse social demonstrou uma possível ação sobre os mecanismos epigenéticos, com diferentes resultados nos processos de acetilação de histonas, metilação do DNA e alteração nos níveis de BDNF (CARVALHO et al., 2017; PARK et al., 2018).

A literatura apresenta diferentes resultados associando a separação materna e acetilação de histonas. Alguns grupos de pesquisa mostraram que acetilação de H3 diminui em hipocampos de animais submetidos à separação materna quando comparados ao grupo controle acompanhado, entretanto, outros estudos indicam não haver diferença entre os grupos. O cronograma do protocolo parece influenciar os padrões de acetilação, pois trabalhos que utilizaram períodos maiores de separação materna encontraram as maiores diferenças (WOO et al., 2017; SURI; BHATTACHARYA; VAIDYA, 2014; BURENKOVA; ALEKSANDROVA; ZARAYSKAYA, 2019). A separação materna também parece estar associada ao aumento da atividade das enzimas HDACs no hipocampo desses animais (IGNÁCIO

et al., 2017). Por outro lado, a metilação parece ser mais sensível à separação materna, visto que se apresenta alterada em grande parte dos trabalhos que utilizam o protocolo, resultando em aumento da metilação das ilhas CpG e da atividade das DNMTs no hipocampo desses animais (WEI; HAO; KAFFMAN, 2014; IGNÁCIO et al., 2017).

O isolamento social na fase adulta (assim como nos primeiros períodos da vida) pode ser prejudicial, gerando comportamentos depressivos e agressivos, ansiedade e prejuízos cognitivos quando se compara á animais mantidos em ambientes sociais. A falta de interação também pode interferir na gênese de subpopulações neuronais e neurotransmissores em diferentes regiões do hipocampo (PERIĆ et al., 2019; MA et al., 2016; CHAN et al., 2017). A privação social nos adultos induz diminuição na concentração de BDNF no hipocampo, o que está intimamente associado ao prejuízo dos diferentes tipos de memória (aversiva, espacial e de trabalho) apresentados por esses animais (MA et al., 2016; GONG et al., 2017). O BDNF do hipocampo parece ser sensível ao isolamento social, visto que a sua expressão é diminuída a partir de variados períodos de isolamento social. Alguns trabalhos já evidenciaram que a neurotrofina é influenciada por períodos que compreendem desde trinta dias até oito semanas de isolamento (GIUSEPPINA et al., 2011; SCACCIANOCE et al., 2006).

Embora o isolamento social de animais adultos possua inúmeros resultados bem definidos e descritos na literatura científica, a sua associação aos mecanismos epigenéticos ainda é pouco explorada, tornando-se um excelente tópico para novas abordagens e estudos.

Diferente do padrão imposto pelo isolamento social surge o ambiente de interação social, que é de extrema importância para a grande maioria das espécies

de mamíferos. Animais com esse tipo de característica social, quando vivem junto aos seus semelhantes têm maior resiliência frente às experiências adversas (Figura 7). Os termos utilizados atualmente para nomear esse benefício provido por essa interação são o suporte ou tamponamento social (do inglês "*social buffering*") (DAVITZ; MASON, 1954). Esse efeito amenizador de situações adversas já foi evidenciado em diversas espécies de mamíferos, abrangendo ratos, porcos e humanos, mostrando um efeito benéfico de amplo espectro (DAVITZ; MASON, 1954; HENNESSY; MAKEN; GRAVES, 2000; THORSTEINSSON; JAMES; GREGG, 1998).

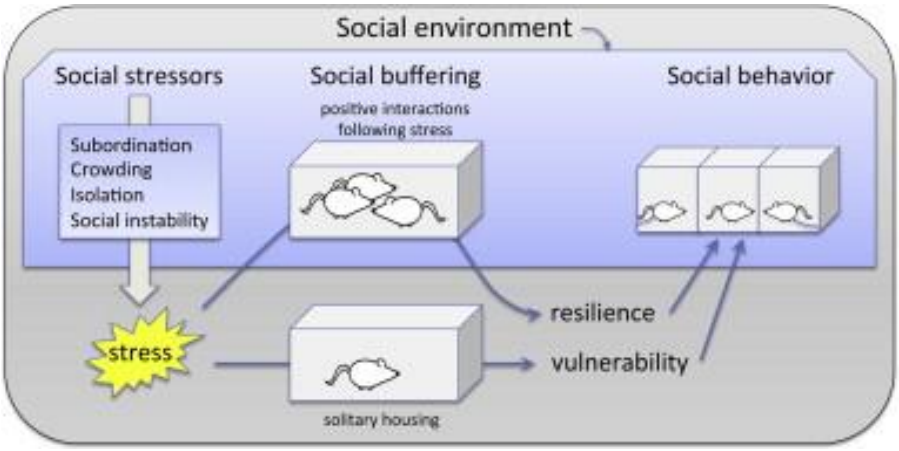

Figura 7. Efeitos benéficos do suporte social em relação ao isolamento (BEERY; KAUFER, 2015).

O suporte social parece ter ação sobre aspectos fisiológicos, contribuindo para diminuição da ativação do eixo HPA, reduzindo os níveis de glicocorticoides plasmáticos e as respostas ao estresse. A amenização das respostas ao estresse ainda está associada com melhora na condição cognitiva e mental (LEVINE et al., 2000). Diversos estudos sugerem que o tamponamento social tem efeitos positivos em animais previamente estressados e os autores indicam que a busca pela

interação social após períodos de estresse pode ativar circuitos neurais de recompensa, aliviando as emoções negativas e diminuindo as respostas estressoras (ARON et al., 2019; DAVITZ; MASON, 1954). Em roedores, a exposição a novos ambientes induz ativação do eixo HPA, porém animais acompanhados demonstram atenuação dessa resposta fisiológica em relação aos animais isolados, que apresentam maiores níveis de glicocorticoides (WILSON, 2000).

Biologicamente o suporte social está associado aos neuropeptídios ocitocina e vasopressina que exercem papel importante na regulação do comportamento social (CARTER, 2003; DONALDSON, 2008). Essas moléculas desempenham funções periféricas quando são secretadas na circulação sanguínea, entretanto, no SNC agem como neuromoduladores em estruturas do sistema límbico (hipotálamo, amígdala e hipocampo) (LANDGRAF; NEUMANN, 2004). A ocitocina também parece ser importante na amenização de respostas ao estresse, atenuando a ativação do eixo HPA, indicando um possível mecanismo dos efeitos benéficos associados ao suporte social (WANG et al., 2018).

O suporte social materno ou realizado por outros acompanhantes aparentam ser importantes para os processos de aprendizado e memória. Esse efeito pode ser elucidado pela utilização do teste de reconhecimento de objeto, onde os animais que recebem o suporte social tendem a gastar maior tempo explorando novo objeto (indicativo de boa memória), ao serem comparados aos animais isolados. A memória aversiva, medida pelo teste de esquiva inibitória também parece estar aprimorada em animais acompanhados, indicando que o suporte social é benéfico para diversos tipos de memória (KOSTEN et al., 2007). A melhora observada na memória desses animais parece estar associada ao aumento de BDNF (LI et al., 2016). Além de induzir aumento do BDNF e seus receptores, o acompanhamento aparenta diminuir

a ansiedade de animais expostos ao ambiente social (RAVENELLE et al., 2014). Sendo assim, o suporte social tem influência sobre mecanismos que levam ao aumento do BDNF e culminam na melhora da atividade neuronal (Figura 8).

Estudos que associem o acompanhamento social e mecanismos epigenéticos em animais adultos ainda são escassos na literatura, mas é sugerido que esse ambiente de integração social possa ter efeito sobre esses mecanismos. Por exemplo, a metilação em hipocampos de ratos mantidos em ambiente de suporte social induziu a diminuição da metilação no hipocampo de ratos em comparação á animais isolados submetidos a um protocolo de estresse crônico. (THEILMANN et al., 2016). No entanto, ainda não possuímos a descrição sobre os padrões de acetilação de animais expostos ao isolamento e suporte social na vida adulta, assim como sua associação ao metabolismo do BDNF e status cognitivo.

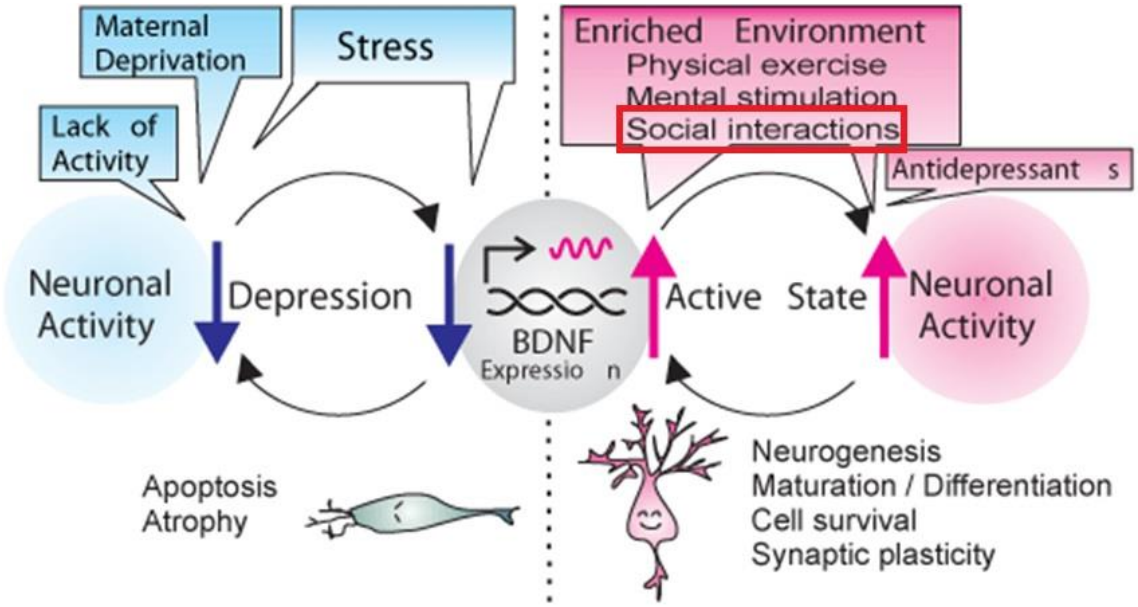

Figura 8. Efeitos do enriquecimento ambiental e suporte social na atividade neuronal (SAKATA, 2014).

O suporte social é uma área que necessita de maior atenção, visto que sua associação aos mecanismos epigenéticos pode fornecer ferramentas importantes no entendimento e amenização dos transtornos neurológicos. Sendo assim, são necessários mais trabalhos que busquem uma associação dos mecanismos epigenéticos de acetilação e metilação, junto ao suporte social e sua conexão com a atividade do BDNF em áreas neuronais relacionadas com os processos cognitivos, como memória e ansiedade.

1.3 MEMÓRIA

A memória e aprendizado são processos que trabalham associados para o armazenamento de novas informações no cérebro. Esse mecanismo possui três etapas distintas: 1) Aquisição: situação de primeiro contato dos nossos sistemas sensoriais a um novo estímulo ou informação; 2) Consolidação: é uma série de processos moleculares que ocorre em áreas do SNC (com destaque para o hipocampo), que induzem a expressão de proteínas promotoras da plasticidade sináptica e que facilitam a formação das memórias; 3) Evocação: é o processo de recordação daquelas memórias que foram produzidas a partir da modificação de redes sinápticas no período da consolidação (ALBERINI, 2005; NADER, 2003).

As memórias são elementos lábeis e podem ser classificadas de acordo com sua essência e também quanto a sua duração. Portanto, elas podem ser classificadas em declarativa (ou explícita) ou memória não declarativa (ou de procedimentos) (WILLINGHAM; WILLINGHAM, 2010). As declarativas são aquelas relacionadas aos fatos que ocorrem no nosso dia-a-dia (por exemplo: qual foi o

nosso café da manhã; ou onde fomos ao final de semana) e estão relacionadas á redes neurais presentes no hipocampo, que é um dos principais responsáveis pela consolidação de memórias (UNSEY, 1996; EICHENBAUM, 1999). Por sua vez, as memórias não declarativas ou de procedimentos são aquelas que adquirimos de maneira gradual (como por exemplo, aprender a tocar um instrumento musical), estas estão associadas a redes motoras e sensoriais de áreas encefálicas referentes àquelas habilidades (LIBBEN; TITONE, 2008). Em relação ao tempo de armazenamento, as memórias podem receber duas classificações: memórias de curta duração (do inglês "*short-term memory"* ou STM), que podem ficar retidas por um período reduzido de minutos á algumas horas após o estímulo; e também possuímos a memória de longa duração (*"long-term memory"* ou LTM), que podem perdurar de dias, até mesmo por toda a vida (FUSTER, 1998).

Os eventos epigenéticos aparentam estar ativos na formação de novas memórias, visto que a transcrição gênica e tradução de proteínas são necessárias para indução da plasticidade sináptica e consolidação de memórias (SWANK; SWEATT, 2001). A acetilação de histonas se mostrou envolvida ao processo de memória, pois a diminuição da atividade das enzimas HATs está associada à diminuição da formação de LTM, e em contrapartida a utilização de inibidores de HDAC melhora a LTM (VECSEY et al., 2010). A acetilação também se mostrou envolvida com o processo de potenciação de longa duração (LTP), que é de suma importância para a plasticidade sináptica e consequentemente para a aprendizagem e memória. Além disso, a metilação também parece ser importante nos processos de armazenamento de memórias (GUPTA et al., 2010). Alguns estudos sugerem que a modificação no padrão de expressão das DNMTs pode influenciar a aprendizagem e memória, pela diminuição da expressão gênica. Essas alterações

também ocorrem com a metilação de histonas, que apresentam diferentes tipos de resposta, dependendo dos resíduos de histonas que sofrem o processo (GUPTA-AGARWAL et al., 2012; FENG et al., 2010).

As modificações epigenéticas no cérebro podem resultar em diferenças nas concentrações e expressão de algumas neurotrofinas, afetando as vias de sinalização destas moléculas, e assim alterando os processos de aprendizagem e memória. O BDNF é um exemplo dessas moléculas, pois ativa diversas cascatas bioquímicas intracelulares, desempenhando um papel muito importante nos processos de sobrevivência, diferenciação e gênese neuronal (CHEN; CHEN; GENE, 2017). A principal conexão celular do BDNF é via receptor TrkB, e é sugerido que essa ligação esteja associada aos processos de aprendizagem e memória, assim como regulação da plasticidade e formação de memórias (BLANK et al., 2016). Em contrapartida a falta da neurotrofina, está associada com a diminuição da arborização dendrítica e morte celular, gerando prejuízos cognitivos (NEIDL et al., 2016).

A comunidade científica possui conhecimentos bem estabelecidos sobre os mecanismos de aprendizagem, formação e armazenamento de memórias, entretanto, esses parâmetros cognitivos ainda são pouco associados aos mecanismos epigenéticos, que podem ocorrer em resposta perante situações de isolamento e suporte social de animais adultos. Ainda é uma incógnita se os animais isolados são mais propensos aos efeitos negativos do estresse, ou até mesmo se o suporte social poderia amenizar as respostas físicas e cognitivas oriundas do estresse, portanto neste trabalho tentamos elucidar essas questões.

2. OBJETIVOS

2.1 GERAL

Verificar o efeito do suporte social sobre os níveis de BDNF, acetilação de histonas e metilação do DNA em ratos submetidos á protocolos de isolamento e estresse crônico imprevisível.

2.2 ESPECÍFICOS

- Verificar o efeito do suporte social, isolamento, estresse crônico e envelhecimento no hipocampo de ratos adultos jovens e de media idade sobre:
  - A acetilação das histonas H3 e H4;
  - Os níveis de expressão de HDAC5;
  - Expressão das enzimas DNMT1 e DNMT3a
  - Os níveis e expressão de BDNF**;**
- Observar o efeito do suporte social e estresse crônico sobre a memória e ansiedade.

3. RESULTADOS

3.1 ARTIGO CIENTÍFICO 1

Social isolation and social support at adulthood affect epigenetic mechanisms, brain-derived neurotrophic factor levels and behavior of chronically stressed rats.

3.2 ARTIGO CIENTÍFICO 2

Effects of social isolation and social buffering at middle age adulthood on expression of DNA methyltransferases, histone deacetylase and brain-derived neurotrophic factor and behavioral status of chronically stressed rats.

ARTIGO CIENTÍCO 1

Social isolation and social support at adulthood affect epigenetic mechanisms, brain-derived neurotrophic factor levels and behavior of chronically stressed rats.

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Original paper

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Highlights

- Social isolation increased HDAC5 expression and decreased ackH3K9 and ackH4k12 levels.
- Isolation-induced epigenetic changes were associated to BDNF and memory impairments.
- Unpredictable stress affected ackH3K9, HDAC5 and induced anxiety-like behaviors.
- Effects of stress on HDAC5 expression were limited to isolated animals.
- Accompaniment promoted social buffering of the stress effects on HDAC5.

Abstract

Epigenetic modulation of brain-derived neurotrophic factor (BDNF) provides one possible explanation for the dysfunctions induced by stress, such as psychiatric disorders and cognitive decline. Interestingly, social support can be protective against some of these effects, but the mechanisms of social buffering are poorly understood. Conversely, early isolation exacerbates the responses to stressors, although its effects in adulthood remain unclear. This study investigated the effects of social isolation and social buffering on hippocampal epigenetic mechanisms, BDNF levels and behavioral responses of chronically stressed young adult rats. Male Wistar rats (3 months) were assigned to accompanied (paired) or isolated housing. After one-month half of each group was submitted to a chronic unpredictable stress (CUS) protocol for 18 days. Among accompanied animals, only one was exposed to stress. Behavioral analysis encompassed the Open field, plus maze and inhibitory avoidance tasks. Hippocampal H3K9 and H4K12 acetylation, HDAC5 expression and BDNF levels were evaluated. Isolated housing increased HDAC5 expression, decreased H3K9 and H4K12 acetylation, reduced BDNF levels, and impaired long-term memory. Stress affected weight gain, induced anxiety-like behavior and decreased ackH3K9 levels. Interactions between housing conditions and social stress were seen only for HDAC5 expression, which showed a further increase in the isolated+CUS group but remained constant in accompanied animals. In conclusion, social isolation at adulthood induced epigenetic alterations and exacerbated the effects of chronic stress on HDAC5. Notwithstanding, social support counteracted the adverse effects of stress on HDAC5 expression.

Keywords: Social buffering, Acetylation, Histone, Histone Deacetylase, Memory, Chronic Unpredictable Stress

1. Introduction

The reactions of the organism to stressful situations are usually analyzed based on the classic concept of the fight or flight response. In this concept, the brain perceives and determines what is threatening, and activates the sympathetic nervous system and the hypothalamic-pituitary-adrenocortical (HPA) axis, leading to the recruitment of different organs and systems for a concerted effort to combat or escape from threat. Although providing a good characterization of responses to stress, the fight or flight concept is incomplete from the standpoint of human beings [1]. A remarkable response of humans to stress is the tendency to affiliate, that is, to come together in groups to provide and receive joint protection in threatening times [2,3]. Social support seems to have a protecting effect against the negative outcomes of stress exposure [4,5,6]. Conversely, social isolation and feelings of loneliness are important stressors by itself, being associated with alterations of the neuroendocrine response to stress and predisposition to different mental health dysfunctions, such as anxiety, depression and cognitive decline [7,8].

The effects of social isolation have been investigated in many animal models. Studies with mice and rats have shown that social isolation is associated with important alterations in brain neurochemistry, structure and function, inducing behavioral changes manifested as depressive and anxiety-like symptoms [9,10,11]. It is also known that isolated animals usually show exacerbated behavioral and neuroendocrine responses to chronic stressors [12]. However, these studies were mostly conducted to investigate the effects of maternal separation or early isolation on behavioral and endocrine responses to stress at adulthood [13,14]. Thus, the

effect of adult isolation on the behavioral and physiological responses to chronic stress remain unclear.

The social contact seeking that humans show after stress exposure can also be observed in other mammals with distinct levels of social bonding, including rats [15]. Additionally, social support, or group housing, can decrease plasma glucocorticoid levels and reduce the reactions to stress in different animal models [9,16,17,18,19,20]. Although the behavioral effects of social support are well documented, studies concerning the mechanism implicated that social buffering of stress is mostly restricted to the role of the HPA axis, oxytocin and vasopressin [9]. However, in the last decade it became increasingly evident that epigenetic mechanisms provide one possible explanation for the lasting impact that a history of stress exposure can have on future stress reactivity and maladaptation [21]. Thus, it would be interesting to investigate if social buffering could also be acting through modulation of epigenetic mechanisms. If this is the case, it could be potentially effective to prevent exacerbated reactions or dysfunctional adaptations in response to stress.

One of the most susceptible brain regions to the effects of chronic stress is the hippocampus, a component of the limbic system that regulates motivation, emotion, and processing of declarative memories [22,23]. Chronic stress impairs neurogenesis, plasticity and neuronal survival in the hippocampus [24,25]. These changes have been associated with psychiatric and cognitive dysfunctions and, more recently, have been investigated from the epigenetic point of view [26].

Among the epigenetic mechanisms activated in the hippocampus by chronic stress is the modulation of histone acetylation [27]. Histone acetylation promotes gene transcription by reducing the interaction of histones with DNA (allowing the

coupling of transcriptional machinery to DNA) or serving as a recognition site for gene transcription promoters [28]. Histone acetylation is modulated by the activity of acetyltransferases (HATs), responsible for the increase in acetylation, and deacetylases (HDACs), responsible for the decrease in acetylation [29].

Different stress protocols, including acute and chronic restraint, social defeat and chronic unpredictable stress (CUS), are able to simultaneously increase the activity of HDACs and decrease histone acetylation. In this context, the activity of HDAC5 and the acetylation of H3 (K9) and H4 (K12) has drawn attention because of their role in the regulation of brain-derived neurotrophic factor (BDNF) expression [27,30,31,32,33,34,35]. BDNF is an important modulator of neurotransmission [36,37,38], neuroplasticity [39,40] and neuronal survival [41]. Chronic stress was already shown to decrease BDNF levels in humans [42,43] and animals [44]. Moreover, lower levels of BDNF in serum were associated to neurodegenerative diseases [45,46] and psychiatric disorders [47,48,49]. Thus, there are suggestions that the maladaptive effects of chronic stress on mental health are, at least partially, associated to the epigenetic modulation of BDNF levels [12,50].

The current study was designed to explore the effects of social isolation and social buffering on epigenetic and behavioral responses to chronic stress. More specifically, we investigated hippocampal H3K9 and H4K12 acetylation, HDAC5 expression and BDNF levels, as well as behavioral responses, in young adult rats maintained in different housing conditions (isolation or accompanied housing) and exposed to chronic stress (CUS protocol). Our main hypotheses were that: (1) isolation and chronic stress would lead to negative outcomes on the investigated variables; (2) isolation would magnify the effects of chronic stress; (3) paired housing

would be protective against epigenetic, BDNF and behavioral alterations induced by chronic stress.

2. Material and methods

2.1 Animals

Adult male Wistar rats (three-month-old, 465-573 g, n=46) were obtained from the university breeding facility (Centro de Modelos Biológicos Experimentais/ Pontifícia Universidade Católica do Rio Grande do Sul, CeMBE/PUCRS). Animals were maintained in standard cages with sawdust bedding, room temperature of 21±1^{o}C, a 12-h light/dark schedule and *ad libitum* access to standardized pellet food and water. The experiments were carried out in conformity with the Guide for the Care and Use of Laboratory Animals and performed according to the recommendations of the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI). Experimental protocols were approved by the Ethics Committee for the Use of Animals of the Pontifical Catholic University (CEUA, registration No. 7142). All efforts were made to reduce sample size and minimize animal suffering.

2.2 Experimental design

All animals were weighted and randomly divided in two experimental groups: Accompanied (two animals/ home cage) and Isolated (one animal/home cage). After one month, half of the animals of each group were submitted to a 20 days' stress

protocol (see below), whereas the other half remained in the standard housing conditions during this period. Thus, four experimental subgroups emerged: Accompanied (two animals/home cage); Accompanied + CUS (two animals/home cage and one of them daily submitted to the CUS protocol); Isolated (one animal/ home cage); Isolated + CUS (one animal/home cage daily submitted to the CUS protocol).

2.3 Chronic unpredictable stress (CUS) protocol

The CUS protocol, also known as chronic unpredictable mild stress protocol, is widely used [51]. Composed by diverse micro-stressors, presented in a random and unpredictable fashion, the CUS protocol was designed according to the literature to induce a mild to moderate intensity stress [52,53]. The stress protocol (Table 1) lasted 14 days, was interrupted during four days for behavioral tasks, and resumed for another 4 days. Two days after the end of the CUS protocol animals were weighted and euthanized by decapitation.

Table 1. Schedule of stressors used during the chronic unpredictable stress (CUS) treatment.

Stress	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Water deprivation	10h->	10h					
Wet bedding		13-17h					
Light 24h			8h->	8h			
Imobilization				16-16:45h			
Food deprivation					11h->	11h	
Strobe light						14-16h	
Cage tilt							7-11h

2.4 Behavioral tasks

The behavioral tasks were run on four consecutive days in the following sequence: Open field, plus maze, inhibitory avoidance training and testing sessions.

2.4.1 Open field

Open field testing was performed as previously described [54]. In short, animals were placed in a 40 × 45 × 50 cm high open field cage divided into 12 equal-sized sections under red lighting for 5 min. Between each session, feces and urine were removed from the apparatus. Animals were videotaped and locomotor and exploratory responses (latency to start locomotion, section crossings and rearings) were scored offline by blind experimenters with high inter-rater reliability (Pearson's r>0.9).

2.4.2 Elevated plus maze

The elevated plus maze test is a standard method to assess the anxiety-like behaviors in rodents [55]. The apparatus consisted of two open and two closed arms with the same size (50 × 10 cm) elevated 50 cm above the floor. The closed arms were surrounded by 40 cm high walls. Animals were placed in the central square of the plus maze apparatus (10 × 10 cm), facing the open arm, and allowed to explore the maze during five minutes. Between animals, feces and urine were removed from the apparatus. All animals were videotaped and the number of entries and time spent

in open versus closed arms were scored offline by blind experimenters with high inter-rater reliability (Pearson's r>0.9).

2.4.3 Inhibitory avoidance

The inhibitory avoidance task was performed to evaluate long term aversive memory and followed the procedures previously described [56]. The apparatus was an acrylic box (50 × 25 × 25 cm) whose floor consisted of parallel-caliber stainless-steel bars (1 mm diameter) spaced 1 cm apart, and a platform that was 7 cm wide and 2.5 cm high. During the training session animals were placed on the platform and their latency to step down on the grid with all four paws was measured. Animals received a 0.4-mA, 3.0 seconds foot shock after stepping down on the grid and were immediately removed from the apparatus. The test session was carried out 24 hours after training, no foot shock was given and the step-down latency (maximum of 180 seconds) was used as a measure of memory retention.

2.5 Biochemical analysis

Animals were euthanized by decapitation 48 hours after the last session of the CUS protocol. Brains were immediately removed and the hippocampus rapidly dissected and snap-frozen in nitrogen. All samples were stored at -80^{o}C until further analysis, as explained bellow.

2.5.1 Analysis of histone acetylation by Western Blot

The dissected and nitrogen frozen hippocampi samples were homogenized, placed in EDTA-free (Sigma-Aldrich, St. Louis, MO, USA) solution 1x containing a protease inhibitor cocktail tablet, and stored at −80°C for subsequent analysis. For histone extraction, PBS buffer (Phosphate-Buffered-Saline) containing 250 uL Triton and 10 mg NaN3 was added to the homogenate samples to a 50 mL final volume. After 10 minutes on ice, samples were centrifuged at 6500g for 10 minutes at 4°C. The supernatant was collected and acid extraction (0.2-N HCl) of histones was carried out overnight at 4°C. Samples were centrifuged once again (6500 g for 10 minutes at 4°C), supernatants saved, and the protein content was determined using the Coomassie Blue method, with bovine serum albumin as a standard [57]. Western blot analysis of acetylated H3K9 (ackH3K9) and H4K12 (ackH12) was done as follows. Twenty-five µg total protein was separated on a 10% SDS polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween 20 and were incubated overnight with the following antibodies: anti-histone H3 (ab1791, Abcam) at 1:3000, anti-acetyl histone H3 (Lys-9, ab10812, Abcam) at 1:500, anti-histone H4 (ab10158, Abcam) at 1:200 and anti-acetyl histone H4 (Lys-12, K12, ab61238, Abcam) at 1:700. Goat anti-rabbit (ab97051, HRP) radish-conjugated secondary antibodies were used and detected using ECL Western Blotting Substrate Kit (Abcam, Cambridge, UK). Pre-stained molecular weight protein markers (SuperSignal Molecular Weight Protein Ladder, Thermo Scientific, Rockford, USA) were used to determine the detected bands molecular weight and confirm target specificity of antibodies. Analysis of band intensities were performed in a Carestream

Gel Logic 2200 PRO Imaging System and the associated Image Analysis Software. Data for acetylated histones were corrected for the amount of total histone protein.

2.5.2 Analysis of HDAC5 gene expression by real-time PCR

Total cellular RNA of hippocampus was extracted with SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's protocol. RNA was re-suspended in nuclease-free water and was quantitated by spectrophotometry. The total RNA was used for reverse transcription (RT) reactions. RT reactions were performed using Invitrogen Superscript IV One-Step RT-PCR System (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and this was followed by real-time PCR of the target gene. TaqMan probes and the One-Step RT-PCR System (Applied Biosystems, Foster City, CA) were used in our experiments. PCR reactions were performed using 20x Assays-On-Demand Gene Expression Assay Mix (containing unlabeled PCR primers and Taq-Man probe) and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. PCR conditions were 95°C for 10 minutes, followed by 95°C for 15 seconds and 60°C for 1 minute repeated for 40 cycles. Experiments were performed in duplicate for each data point. Beta-actin was evaluated as an internal RNA control. Quantitative values were obtained from the cycle number (CT value) at which the increment in fluorescent signal associated with an exponential growth of PCR products started to be detected. The amount of target gene mRNA expression was normalized to the endogenous level of Beta-actin. Analysis was performed by obtaining the relative threshold cycle (ΔCT), in relation to the CT of the control gene in order to measure the relative expression level ($2^{-\Delta\Delta CT}$) of the target gene [58].

Primer sequences for HDAC5 were: 5'CAGCCAGAAGATGTACGCCA3' (*forward*) and 5'GCTGTGATGGCTACGGAGTT3' (*reverse*). For Beta-actin they were 5'ACCGAGCATGGCTACAGCGTCACC3'(*forward*), 5'GTGGCCATCTCTTGCTCGGAGTCT3'(*reverse*).

2.5.3 Analysis of BDNF by ELISA

Hippocampus samples were homogenized by gently grinding in 0.1M phosphate buffer solution with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The homogenates were immediately centrifuged at 2000 g for 5 minutes and the supernatant was collected and frozen at −80°C until further analysis. BDNF levels were evaluated with a commercial sandwich-ELISA kit (Milipore, USA) following the manufacturer's instructions. In short, samples were added in duplicate to the microtiter plates (96 well flat-bottom), incubated for 24 hours at 4^{o}C and rinsed four times with wash buffer. After that, biotinylated mouse anti-human BNDF monoclonal antibody (diluted 1:1000 in sample diluent) was added to each well and incubated for 3 hours at room temperature. Wells were once again washed and then incubated with streptavidin–horseradish peroxidase conjugate solution (diluted 1:1000) for 1 hour at room temperature. After the addition of substrate and stop solution, the amount of BDNF was determined (absorbance set at 450 nm). The standard curve ranged from 15.63 to 500 pg/ml of BNDF and showed a direct relationship between optical density and BDNF concentration. Total protein was measured by Bradford's method [57] using bovine serum albumin as standard.

2.6 Statistical analysis

Parametric data are expressed as mean ± standard deviation and were analyzed with two-way ANOVAs, with housing conditions (accompanied or isolated) and stress (submitted or not submitted to the CUS protocol) as the between-group variables. The non-parametric data of the inhibitory avoidance test are expressed as median (interquartile ranges) and were analyzed with Kruskal-Wallis tests, followed by Wilcoxon (for dependent variables) and Mann-Whitney (for independent variables) tests whenever appropriate. $P<0.05$ was considered to indicate statistical significance.

3 Results

3.1 Weight gain during the experimental period

The two-way ANOVA indicated a significant main effect of stress [$F(1,40) = 23.076$, $p<0.001$] on weight gain. As can be seen in Figure 1, animals submitted to the CUS protocol showed significantly less weight gain than animals that were not submitted to the stress protocol. However, there was no effect of housing condition or any interaction between housing condition and stress on weight gain (all $p>0.05$).

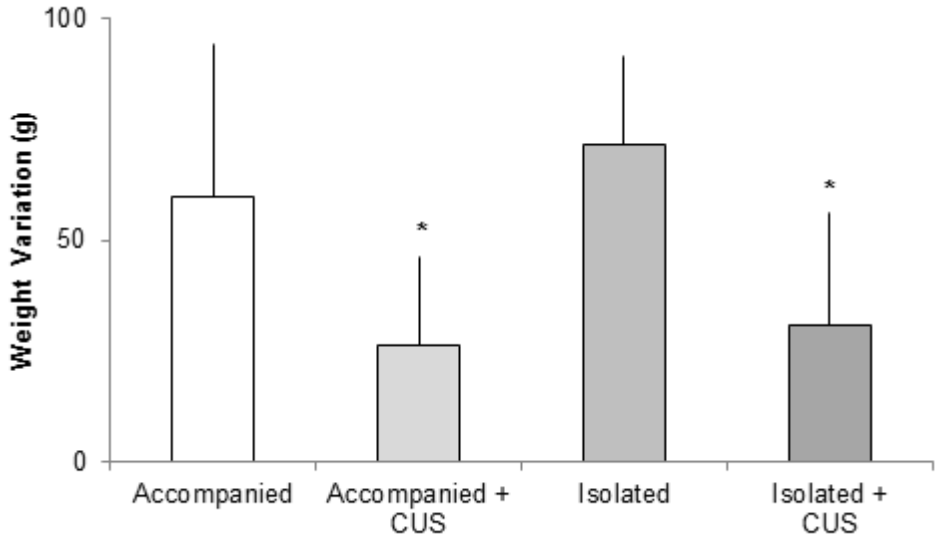

Figure 1. Weight gain of rats during the experimental procedures, calculated as the difference between weight at the start (when animals were assigned to the different housing conditions) and at the end (immediately before animals were euthanized) of the experiment. Statistical analysis was performed using two-way analysis of variance with housing conditions (accompanied or isolated) and stress (submitted or not submitted to the CUS protocol) as fixed factors. Data are expressed as mean ± standard deviation. n=10-12 *per* group. *p<0.001 in comparison to the accompanied-only and isolated-only subgroups, indicating a significant effect of stress.

3.2 Open field

The results obtained in the open field task can be seen in Table 2. The two-way ANOVAs identified neither significant effects of housing conditions and stress, nor significant interactions between housing conditions and stress, on latencies to start locomotion, crossings and rearings (all p>0.05).

3.3 Elevated Plus Maze

The results of the Plus maze task can also be seen in Table 2. The two-way ANOVA indicated significant effects for stress only on the time spent in open [F(1,39)= 6.436, p=0.017] and closed [F(1,39)= 5.786, p=0.023] arms. Animals submitted to the CUS protocol spent significantly less time in the open arms, and consequently more time in the closed arms, than animals that were not submitted to the stress protocol. No significant effects of housing condition, or interactions between housing condition and stress, were seen on any of the Plus maze variables (all p>0.05).

Table 2. Effects of housing conditions and stress on Open Field and Plus maze parameters.

		Groups			
		Accompanied	Accompanied +CUS	Isolated	Isolated +CUS
Open Field	Latency(s)	3.5±1.67	2.71±1.05	3.20±1.5	3.82±2.03
	Crossing(n)	71.9±34.39	90.61±13.45	82.3±18.12	91.54±28.9
	Rearing(n)	29.9±9.64	34.33±7.4	29.25±5.95	30.95±7.05
Plus Maze	Open Arm Time (%)	14.23±10.07	12.79±6.27*	13.53±3.84	4.56±1.43*
	Closed Arm Time (%)	75.48±13.96	76.46±11.93**	78.33±5.85	91.46±6.69**
	Open Arm Entries (n)	1.50±1.3	1.00±1.41	2.00±1.56	0.69±0.85
	Closed Arm Entries (n)	4.12±2.1	3.25±2.52	3.20±1.98	3.15±2.3

Statistical analysis was performed using two-way analysis of variance, with housing conditions (accompanied or isolated) and stress (submitted or not submitted to the CUS protocol) as fixed factors. Data are expressed as mean ± standard deviation. n = 10-12 per group; *p<0.05 for the time spent in open arms in comparison to the accompanied-only and isolated-only subgroups, indicating significant effects of the CUS protocol; **p<0.05 for the time spent in closed arms in comparison to the

accompanied-only and isolated-only subgroups, indicating significant effects of the CUS protocol.

3.4 Inhibitory avoidance

As indicated by the Kruskal-Wallis test, latency to step down the platform in the training session was not significantly different (p=0.441) between the accompanied [180 (21,75/180)], accompanied + CUS [52,2 (11,77/125,86)], isolated [9,56 (6,03/47,61)] and isolated + CUS [10,21 (5,87/37,35)] groups. Although the latency to step down increased significantly from the training to the test session in all experimental groups, as indicated by the Wilcoxon test (all p<0.05), further analysis with the Mann-Whitney post hoc test indicated that the isolated and isolated + CUS group had a worse performance on the memory retention test than the accompanied and accompanied + CUS groups (all p<0.05) (Figure 2). On the other hand, no significant differences were identified between the accompanied and accompanied + CUS group (p=0.236) and between the isolated and isolated + CUS groups (p=0.744).

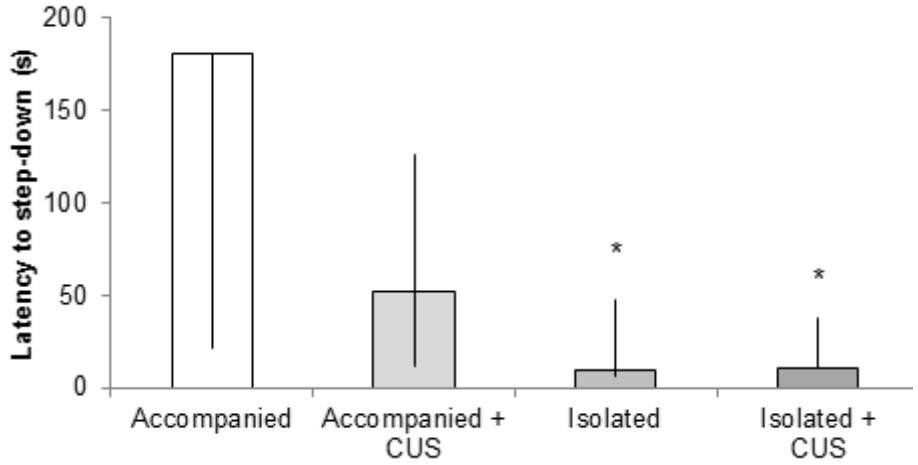

Figure 2. Long-term retention of inhibitory avoidance memory in animals submitted to different housing and stress conditions. The retention test was run 24 h after the training session. Statistical analysis was performed using Kruskal-Wallis test and Mann-Whitney's post hoc test. Data are expressed as median and interquartile range. n=10-12 *per* group. *p<0.05 in comparison to the accompanied subgroups, indicating a significant housing effect.

3.5 Histone acetylation

Significant main effects of housing condition were found for ackH3K9 [$F(1,14) = 26.473$, $p < 0.001$] and ackH4K12 [$F(1,18) = 11.733$, $p=0.003$]. As can be seen in Figure 3, isolated animals had lower levels of acetylated histones than accompanied animals. A main effect of stress was seen only on H3K9 acetylation [$F (1,14) = 6.752$, $p = 0.021$], which decreased in animals submitted to the CUS protocol. No significant interactions between housing conditions and stress were seen on H3K9 and H4K12 acetylation (all $p>0.05$).

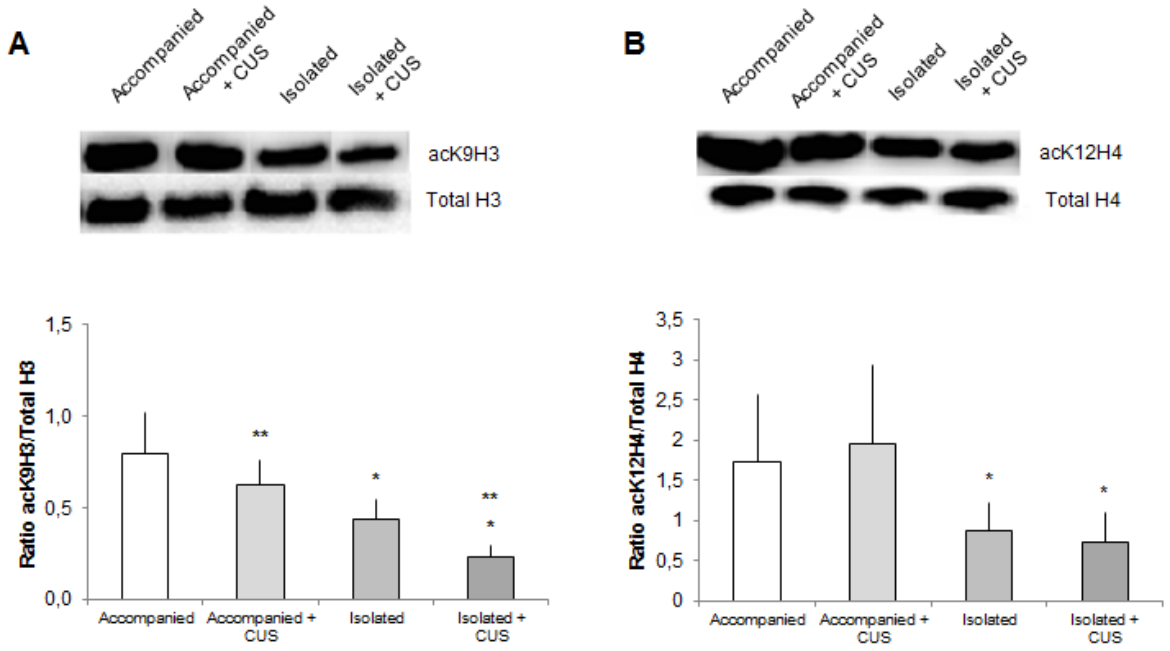

Figure 3. Quantification and representative western blots of (A) acetylated histone 3 lysine 9 [acK9(H3)] and (B) histone 4 lysine 12 [ackH4(K12)] in the hippocampus of rats exposed to different housing conditions and stress. Statistical analysis was performed using two-way analysis of variance with housing conditions (accompanied or isolated) and stress (submitted or not submitted to the CUS protocol) as fixed factors. n= 4-6 *per* group. Data are expressed as mean ± standard deviation. *p<0.01 in comparison to the accompanied subgroups, indicating a significant housing effect; **p<0.05 in comparison to the accompanied-only and isolated-only subgroups, indicating a significant effect of stress.

3.6 HDAC5 gene expression

The two-way ANOVA indicated significant effects of housing condition [F (1,9)= 327.95, p<0.001] and stress [F(1, 9) = 154,31, p<0.001] on the HDAC5 gene expression, as well as a significant interaction between housing condition and stress [F(1,9)= 144.78, p<0.001]. As can be seen in Figure 4, the expression of the HDAC5

gene was higher in isolated than in accompanied animals. Animals submitted to the CUS protocol also showed higher levels of HDAC5 expression than animals that were not submitted to this stress protocol. The interaction between housing condition and stress can also be seen in Figure 4, which shows a greater effect of the CUS protocol on isolated animals in comparison to accompanied animals. This pattern of results suggests that accompaniment can mitigate the effects of CUS on HDAC5 expression.

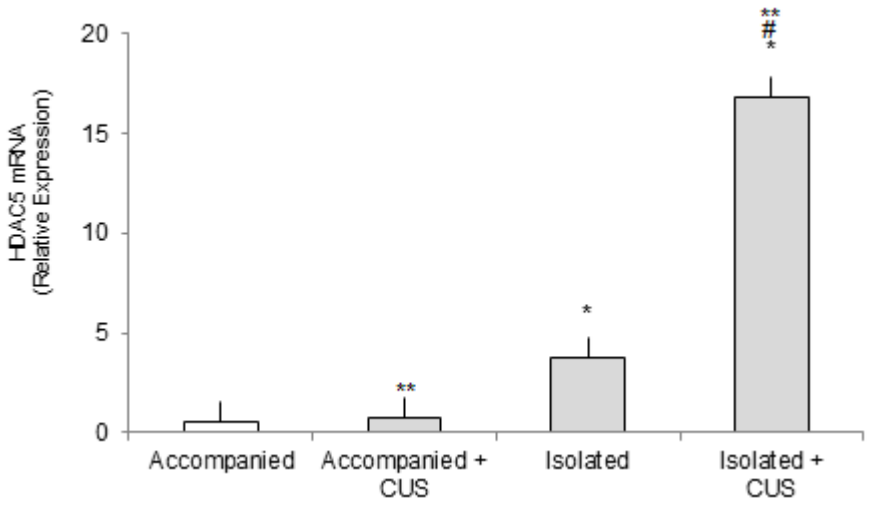

Figure 4. Hippocampal alterations in the expression of the HDAC5 gene in response to different housing conditions and stress. Samples were normalized to Beta-actin expression and run in duplicate. Statistical analysis was performed using two-way analysis of variance with housing conditions (accompanied or isolated) and stress (submitted or not submitted to the CUS protocol) as fixed factors. Data are expressed as mean ± SD. *p<0.001 indicating the housing effect; ** p<0.001 indicating the stress effect; #p<0.001 indicating the interaction between housing condition and stress.

3.7 BDNF levels

The results obtained for BDNF levels can be seen in Figure 5. The two-way Anova indicated significant housing effects on hippocampal BDNF [$F(1,18) = 22.469$, $p < 0.001$], with higher levels of this neurotrophin in accompanied than in isolated animals. However, no significant effects of stress, or interactions between stress and housing conditions, were seen on the BDNF levels (all $p > 0.05$).

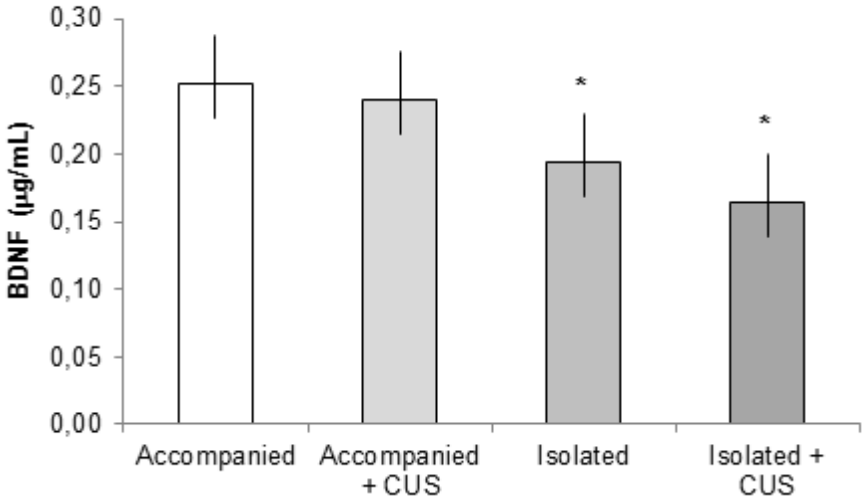

Figure 5. Alterations of BNDF levels in the hippocampus of rats submitted to different housing conditions and stress. Statistical analysis was performed using two-way analysis of variance with housing conditions (accompanied or isolated) and stress (submitted or not submitted to the CUS protocol) as fixed factors. Data are expressed as mean ± standard deviation. n=5-6 *per* group. *p<0.001 in comparison to the accompanied subgroups, indicating significant housing effects.

4. Discussion

One of the main hypotheses of this study was that social isolation and chronic unpredictable stress of young adult rats would lead to negative outcomes on epigenetic mechanisms known to modulate hippocampal BDNF levels and affect behavior. Accordingly, our results indicated increased HDAC5 expression, decreased

histone acetylation (acKH3K9 and acKH4K12), lower BDNF levels and impaired long-term memory in isolated animals. Moreover, the stress protocol used in this study was capable of affecting weight gain, inducing anxiety-like behavior and decreasing ackH3K9 levels and increasing HDAC5 expression. We also hypothesized that social isolation would magnify the effects of chronic stress on the investigated variables. However, further worsening of the effects of the CUS protocol by isolation were limited to the HDAC5 expression, as indicated by the significantly higher expression in the isolated + CUS animals in comparison to the isolated-only animals. Our third assumption was that paired housing would be protective against the alterations induced by chronic stress. Support for this supposition was also limited to the HDAC5 expression, as suggested by the lack of significant effects of the CUS protocol on the HDAC5 expression of accompanied animals, in opposition to its effect on socially isolated animals.

Locomotor and exploratory activities in the open field were unchanged by chronic stress and housing conditions. Although different studies indicated decreased or increased open field activity after induction of social isolation [59,60,61] or CUS [51,62,63,64,65], our results are consistent with the findings of other research groups, which reported no alterations in locomotor and exploratory behavior of animals exposed to these treatments [66,67,68,69]. As pointed out by Hu and collaborators (2010), it is possible that these inconsistent findings among research groups are the result of confounding factors such as the modification of the stress protocol and stimuli intensity, behavioral measure methodology, the variety in animal species and the method of interpretation of results [66].

Analysis of weight gain and anxiety-like behaviors, evaluated with the plus maze task, also indicated no significant effects of isolation. In fact, this is not a

surprising result. A recent review concluded that social isolation has only a small effect on rodent defense behavior [70], and the lack of significant effects on plus maze results is not uncommon [68,71,72]. However, animals submitted to CUS had a reduction in weight gain, indicating that this mild stress protocol had a negative impact on them. Moreover, animals exposed to the CUS protocol spent significantly less time in the open arms of the plus maze in comparison to animals that were not submitted to this stress protocol. Most studies that use CUS protocols report weight decreases [51,73,74,75] and increased anxiety-related defense behaviors [74,75].

Although locomotor and exploratory activities, weight gain and anxiety-like behaviors were not affected by social isolation, the negative effects of this experimental protocol became evident by the decreased hippocampal acetylation of H3K9 and H4K12, the increased HDAC5 expression and the decreased BDNF levels in comparison to accompanied animals. Interestingly, this pattern of results appears to be conserved from early development into adulthood. Maternal separation is associated with reduced levels of total, exon I and exon IV BDNF mRNA, lower BDNF protein levels, decreased acetylation of histone H3 and H4 at the BDNF promoter IV and increased HDAC5 mRNA [76, 77]. Li and collaborators (2016) reported a decrease in histone acetylation and BDNF protein expression after social isolation in early adolescent animals and our results clearly suggest that the isolation of young adult rats has a negative impact on H3K9 and H4K12 acetylation, which is associated to an increase in HDAC5 expression and BDNF decrease [78]. In fact, decreases in hippocampal BDNF levels are a common finding in studies of social isolation and have been associated to impaired synaptic plasticity, neurogenesis and neuronal survival, besides behavioral dysfunctions such as depression, anxiety and memory impairments (for a review see reference [12]). In line with these evidences,

our results indicated that animals of the isolated subgroups had impaired long-term memory in the inhibitory avoidance task when compared to the accompanied subgroups.

Consolidation of inhibitory avoidance memory is known to be dependent on the extracellular release of BDNF and its interaction with tropomyosin-related kinase B (TrkB) [79,80,81]. There are also evidences that histone acetylation (including H3K9 and H4K12) begins a gene expression program that leads to hippocampal memory consolidation [82]. Accordingly, factors that decrease histone acetylation (such as aging) are associated to impairment of aversive memories [83], whereas factors that are able to increase acetylation (such as physical exercise or HDAC inhibitors) are associated with inhibitory avoidance improvement [82,84,85]. Moreover, inhibition of HDACs facilitates long-term potentiation in the CA1 area of the dorsal hippocampus, a cellular plasticity mechanism involved in the establishment of inhibitory avoidance memory [86,87,88]. Thus, our results clearly show an association of social isolation with epigenetic mechanisms potentially involved in the decrease of BDNF levels and memory impairment. However, our study design does not allow the establishment of causal relationships between these variables. Thus, the clinical relevance of our findings should be further investigated in studies planned to evaluate the causal relations between epigenetic modifications, alterations in BDNF levels and behavioral outcomes. Therefore, it would be interesting to evaluate the effect of experimental procedures known to depress the expression of HDAC5 or increase the expression of BDNF, such as pharmacological interventions, viral-mediated BDNF overexpression or HDAC5 knockdown models [30,84,85,89,90,91], and verify if they are able to revert the effects of social isolation.

Histone modification of the BDNF gene in the hippocampus is likely to play a critical role in the response to stressful environments. Different stress protocols (including acute and chronic restrain, social defeat and CUS) are able to induce epigenetic effects through decreases in histone acetylation, increases in HDAC expression and/or reduction of BDNF expression [27,31,32,34]. In this study, stress effects decreased H3K9 acetylation and increased HDAC5 expression. However, the worsening of the epigenetic effects of the CUS protocol by social isolation were limited to the expression of HDAC5, which showed higher levels in the isolated + CUS subgroup in comparison to the isolated-only subgroup. Seo and collaborators (2016) combined maternal separation and chronic restrain stress and also observed that maternal separation exacerbated the effects of the stress protocol on HDAC5 expression. However, the authors also found a further reduction of histone H3 and H4 acetylation at BDNF promoter IV and a further decrease in BDNF mRNA (both total and at exon IV) in animals that were submitted to the restrain stress in addition to the maternal separation. [76]. The critical elements responsible for the extent of the effects of isolation on the responses of animals to other chronic stressors have not yet been identified, but it is likely that the developmental stage of the animals, the type and duration of the isolation and stress protocols play a significant role on the outcomes seen in different studies [51,92]. Moreover, the methods used to investigate the epigenetic (total histone acetylation vs chromatin immunoprecipitation assays directed to specific BDNF promoters) and BDNF alterations (protein levels vs mRNA expression, total hippocampus vs hippocampal subregions) could also contribute to some of the discrepancies seen between the studies [30,31,32]. Notwithstanding, this is the first study to explore the interactions of social isolation and chronic stress on adult animals. The results obtained for the HDAC expression in

isolated +CUS animals warrant further investigations on the effects of isolation on epigenetic mechanisms of chronically stressed animals. Thus, future studies should evaluate the effects of more intense stress protocols (the CUS protocol of this study can be classified as mild to moderate) and broaden the epigenetic variables to be investigated (e.g. evaluating histone methylation and demethylases).

Besides evaluating the effects of housing conditions and stress on animals, our study was also designed to evaluate the possible effects of social buffering on additional epigenetic and behavioral effects induced by the CUS protocol on isolated animals. However, the analysis of social buffering was limited by the fact that, out of all variables investigated (anxiety-like behaviors, memory, BDNF levels, epigenetic variables), only HDAC5 expression showed significant effects of isolation on the stressed animals. So, if social buffering effects were to occur, only HDAC5 expression would be capable to indicate them. Howsoever, no effects of the CUS protocol were seen on the HDAC5 expression of accompanied animals. This result suggests that social buffering could be potentially involved in the modulation of HDAC5 expression and warrants further investigation on this issue. It is important to note that the role of histone remodeling in the pathophysiology and treatment of psychiatric disorders has been underscored by studies showing that drugs, experimental (such as sodium butyrate) or therapeutic (such as antidepressant and anxiolytics), capable to inhibit HDAC5 effects can revert disturbances of the epigenetic control of BDNF levels [30,32,76,93]. Thus, our results suggest that social buffering could act on some of the mechanisms targeted by these pharmacological interventions, i.e. modulation of the effects of HDAC5.

In conclusion, the results of this study indicate that social isolation and mild CUS protocols are able to induce epigenetic alterations in the hippocampus of adult

animals. However, social isolation effects were more extensive and the only ones that lead to decreased BDNF levels and memory impairment. They also worsened the effects of the CUS protocol on HDAC5 expression. Moreover, the lack of effects of the CUS protocol on HDAC5 expression suggest that social buffering can act through epigenetic mechanisms to counteract the harmful effects of stress. Thus, this study adds to the knowledge of the epigenetic effects of social isolation in adulthood, a developmental time window in which epigenetic mechanisms have been scarcely explored. Moreover, the possibility of social buffering effects on HDAC5 expression seen in this study warrant further investigations. There are surprisingly few studies on the mechanisms through which social support operates. Affiliative behavior, group cohesion and liking are natural responses seen in humans exposed or anticipating stressful events [1,15,94]. Moreover, the effects of social buffering in humans are far reaching, being able to aid in the health outcomes of diseases that affect different organs and systems and even increase longevity [9]. Thus, social support is a field that deserves much more attention than it has received until now.

Author contributions

E. Bromberg conceived, designed and supervised the experiments. J.V. Borges, B.S. de Freitas, V. Antoniazzi, C.S. dos Santos, K. Vedovelli, V.N. Pires and L. Paludo performed the experiments. E. Bromberg and J.V. Borges analyzed the data. M.N.M. de Lima contributed reagents and aided in manuscript writing. E. Bromberg and J.V. Borges wrote the manuscript. All authors provided final approval for the submission of the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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ARTIGO CIENTÍFICO 2

Effects of social isolation and social buffering at middle age adulthood on expression of DNA methyltransferases, histone deacetylase and brain-derived neurotrophic factor and behavioral status of chronically stressed rats.

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Artigo em preparação para submissão em periódico

Original Paper

Effects of social isolation and social buffering at middle age adulthood on expression of DNA methyltransferases, histone deacetylase and brain-derived neurotrophic factor and behavioral status of chronically stressed rats.

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Highlights

- The isolation increased HDAC5 and DNMT1 expression.
- Isolated animals presented lower BDNF expression associated with memory impairments.
- Chronic unpredictable stress was associated with increased HDAC5 and DNMT1 expression;
- The stress protocol affected HDAC5 and DNMT1 expression only in isolated animals.
- Social buffering promoted by accompaniment ease the stress effects on HDAC5, and DNMT1 expression.

Abstract

Early social isolation and chronic stress have negative effects on mental health, increasing the risk for conditions such as depression, anxiety and cognitive decline. Animal models suggest that these dysfunctions are associated to epigenetic alterations capable to decrease BDNF expression and protein levels. Previous results showed by our group indicate the effects of social isolation on BDNF modulation, epigenetics modifications and behavior of young adult. Moreover, the potential of social buffering to prevent, or limit, the negative effects of stress on epigenetic mechanism needs more exploration. This study investigated the effects of social isolation and social buffering on the hippocampal expression of epigenetic mediators (HDAC5, DNMT1, DNMT3a) of BDNF expression and the behavioral outcomes of chronically stressed middle-aged rats. Male Wistar rats (17 months) were assigned to accompanied (paired) or isolated housing. Each group was submitted to the CUS protocol for 30 days. Among accompanied animals, only one was exposed to stress. Behavioral analysis encompassed the Open Field and Inhibitory Avoidance tasks. Hippocampal HDAC5, DNMT1, DNMT3a and BDNF expression were evaluated by RT-PCR. Isolated housing increased HDAC5 and DNMT1 expression, reduced BDNF expression, induced anxiety-like behavior and impaired long-term memory. Stress induced weight loss, anxiety-like behavior and increased DNMT1 expression. Interactions between housing conditions and social stress were seen for HDAC5 and DNMT1 expression. Accordingly, the highest levels of HDAC5 and DNMT1 expression were seen in the isolated+CUS group. Accompained animals showed no significant effects of stress on the expression of HDAC5 and DNMT1. In conclusion, negative outcomes of social isolation and protecting effects of accompaniment involve epigenetic mediators potentially involved in BDNF expression and behavior modulation.

Keywords: Social buffering, Acetylation, Histone, Histone Deacetylase, Memory, Chronic Unpredictable Stress

1. INTRODUCTION

The stressful events can affect the normal homeostasis of organs and systems, as central nervous system (CNS), shaping brain structures and synaptic plasticity [1]. When brain recognizes alterations it could be threatening, and activates the cascade of hypothalamic-pituitary-adrenal (HPA) axis, which turns the body ready to fight of flight [2]. During lifetime individuals are exposed to different stressful situations, it can promote chronic stress and activate some neuronal areas, including HPA axis and limbic system which is composed of structures related to memory and learning, as the prefrontal cortex and hippocampus. The social isolation is a harmful condition linked with stress, which increase the glucocorticoids levels and is associated with impairment of cognitive parameters [3,4]. Social isolation in rodents has been associated with changes in synaptic plasticity, in concentrations of neurotrophins and neurotransmitters, and behavioral changes such as anxiety and depression [5,6]. However, most studies presented until now, had explore the outcomes of maternal separation or early-life isolation on physiology and mental aspects and its neuroendocrine responses at adulthood [7,8]. The response to middle-age adulthood social isolation remains unclear, therefore, is a field to be filled in literature.

Unlike social isolation, the accompaniment social or social buffering is a good alternative to attenuate the damages generated by stressful events. The social buffering is important to mammals' health, contributing to improve physiological aspects, including decreased activation of HPA axis, and psychological parameters, improving memory and learning [9,10]. Social bonding can decrease the release of corticosterone and then to ease body reactions to stress [11,12].

Most of studies which try to explain the stress mechanisms usually are interested about physiologic and behavioral conditions of animal models [13]. However, in the last years, the scientific community turned the attention to epigenetics mechanisms, and this new trend focuses to elucidate how these epigenetics factors could affect reactions related to stressful events.

The chronic stress affects many different brain areas, as the hippocampus. This structure is part of limbic system, and is related with cognitive, behavioral and emotional responses [14,15]. The social stress and isolation leads to deficient process of learning and memory formation, interfering on long-term potentiation (LTP), and alteration of molecules concentration as brain-derived neurotrophic factor (BDNF) [16,17]. The BDNF plays a key role on modulation of neurotransmission, neuroplasticity, and neuronal survive [18,19,20,21]. This molecule had been associated with some psychiatric disorders as schizophrenia, anxiety and depression [22,23,24]. The reduction of BDNF expression was already identified in the hippocampus of patients with schizophrenia and depression [25,26]. In this way, there are suggestions of the influence of epigenetic mechanisms associated to mental disorders predisposed to appear due chronic stress and social isolation.

Among these epigenetics factors, there are the acetylation of histones and methylation of DNA that can induce or repress gene expression. The acetylation of histones is induced by histone acetyl-transferases (HATs) and inhibited by histone deacetylases (HDACs). These enzymes alter the interaction between chromatin e and DNA, then inducing (allowing the coupling of transcriptional machinery to DNA) or inhibiting the gene expression [27]. Thus, the hyperacetylation increases transcriptional activity, whereas hypoacetylation decreases the gene expression [28]. The HDAC5 is present in the limbic areas as hippocampus and was already

associated with stressful events indicating a possible association with social isolation and chronic stress [29,30].

The reaction of DNA methylation is catalyzed by DNA methyltransferases (DNMTs) family, which is divided in subtypes [31]. The DNMT1 and DMNT3a are the most subtypes predominant in the brain [32]. Evidence suggests that epigenetic changes on BDNF gene expression are associated with depression and stress models, and with the action of antidepressant drug [33,34]. The alterations on BDNF gene expression were already associated with the actions of HDAC5 and DNA methyltransferases [35,36]. However, the association of these epigenetic enzymes, BDNF and adult social isolation remains unexplored in the literature.

The aim of this study was the investigation of social isolation and social buffering effects on epigenetic and behavioral responses to chronic stress. More specifically, we investigated the expression of BDNF and genes related with epigenetic mechanisms, as HDAC5, DNMT1 and DNMT3a in the hippocampus, as well as behavioral responses, in middle age adult rats maintained in different housing conditions (isolation or pair housing) and exposed to chronic stress (CUS protocol). Our main hypotheses were that: (1) isolation would lead to negative outcomes on the investigated variables; (2) chronic stress would magnify the effects of social isolation; (3) paired housing would be protective against epigenetic, BDNF and behavioral alterations induced by chronic stress.

2. METHODS

2.1 ANIMALS

Middle-aged Adult male Wistar rats (seventeen-month-old, n=46) were obtained from the university breeding facility (Centro de Modelos Biológicos Experimentais/ Pontifícia Universidade Católica do Rio Grande do Sul, CeMBE/PUCRS). Animals were maintained in standard cages with sawdust bedding, room temperature of 21±1oC, a 12-h light/dark schedule and ad libitum access to standardized pellet food and water. The experiments were carried out in conformity with the Guide for the Care and Use of Laboratory Animals and performed according to the recommendations of the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI). Experimental protocols were approved by the Ethics Committee for the Use of Animals of the Pontifical Catholic University (CEUA, registration No. 7142). All efforts were made to reduce sample size and minimize animal suffering.

2.2 EXPERIMENTAL DESIGN

At the start of the experiment all animals were weighted and randomly divided in four experimental groups: Accompanied (two animals/home cage); Accompanied + CUS (two animals/home cage and one of them daily submitted to the CUS protocol); Isolated (one animal/ home cage); Isolated + CUS (one animal/home cage daily submitted to the CUS protocol).

2.3 CHRONIC UNPREDICTABLE STRESS (CUS) PROTOCOL

The CUS protocol used is also known as chronic unpredictable mild stress protocol [37]. Composed by a diversity of micro-stressors, presented in a random and unpredictable fashion, the CUS protocol was designed according to the literature to induce a moderate intensity stress [38,39]. The stress protocol (Table 1) lasted four weeks, and was interrupted for three days at last week for behavioral tasks, and resumed for another two days. At the 31th day after the beginning of the CUS protocol animals were weighted and euthanized by decapitation.

Table 1. Schedule of stressors used during the chronic unpredictable stress (CUS) treatment.

Stress	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Water deprivation	10h->	10h					
Wet bedding		13-17h					
Light 24h			8h->	8h			
Imobilization				16-16:45h			
Food deprivation					11h->	11h	
Strobe light						14-16h	
Cage tilt							7-11h

2.4 BEHAVIORAL TASKS

The behavioral tasks were run on three consecutive days in the following sequence: on the first day was performed the Open Field, on the second and third day was performed the training and testing sessions of Inhibitory Avoidance, respectively.

2.4.1 OPEN FIELD

Open-field testing was performed as previously described [40]. In short, animals were placed in a 40 × 45 × 50 cm high open-field cage divided into 12 equal-sized sections under red lighting for 5 min. Between each session, feces and urine were removed from the apparatus. Animals were videotaped and locomotor, exploratory and anxiety responses were scored offline by blind experimenters with high inter-rater reliability (Pearson's r>0.9). The number of squares the animal crossed (number of crossings), the number of rearings and the proportion of time spent in the inner zone were determined as measures of locomotion, exploratory behavior and anxiety, respectively.

2.4.2 INHIBITORY AVOINDANCE

The Inhibitory Avoidance task was performed to evaluate long term aversive memory and followed the procedures previously described [41]. The apparatus was an acrylic box (50 × 25 × 25 cm) whose floor consisted of parallel-caliber stainless-steel bars (1 mm diameter) spaced 1 cm apart, and a platform that was 7 cm wide and 2.5 cm high. During the training session animals were placed on the platform and their latency to step down on the grid with all four paws was measured. Animals received a 0.4-mA, 3.0-s foot shock after stepping down on the grid and were immediately removed from the apparatus. The test session was carried out 24 hours after training, no foot shock was given and the step-down latency (maximum of 180 seconds) was used as a measure of memory retention.

2.5 HIPPOCAMPUS SAMPLES FOR BIOCHEMICAL ANALYSIS

Animals were euthanized by decapitation 48 hours after the last session of the CUS protocol. Brains were immediately removed and the hippocampus rapidly dissected and snap-frozen in nitrogen. All samples were stored at -80oC until further analysis, as explained bellow.

2.6 ANALYSIS OF HDAC5, DNMT1 AND DNMT3a GENE EXPRESSION BY REAL-TIME PCR

Total cellular RNA of hippocampus was extracted with SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's protocol. RNA was re-suspended in nuclease-free water and was quantitated by spectrophotometry. The total RNA was used for reverse transcription (RT) reactions. RT reactions were performed using Invitrogen Superscript IV One-Step Rt-PCR System (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and this was followed by real-time PCR of the target gene. TaqMan probes and the One-Step RT-PCR System (Applied Biosystems, Foster City, CA) were used in our experiments. PCR reactions were performed using 20× Assays-On-Demand Gene Expression Assay Mix (containing unlabeled PCR primers and Taq-Man probe) and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. PCR conditions were 95°C for 10 min, followed by 95°C for 15 s and 60°C for 1 min repeated for 40 cycles. Experiments were performed in duplicate for each data point. Beta-actin was evaluated as an internal RNA control. Quantitative values

were obtained from the cycle number (CT value) at which the increment in fluorescent signal associated with an exponential growth of PCR products started to be detected. The amount of target gene mRNA expression was normalized to the endogenous level of Beta-actin. Analysis was performed by obtaining the relative threshold cycle (ΔCT), in relation to the CT of the control gene in order to measure the relative expression level (2–ΔΔCT) of the target gene [42].

Table 2. Primers sequences for RT-PCR

Primers	Foward Sequence (5'-3')	Reverse Sequence (5'-3')
BDNF	AAGCTCAACCGAAGAGCTAAAT	CTGAGGGAACCCGGTCTCA
HDAC5	CAGCCAGAAGATGTACGCCA	GCTGTGATGGCTACGGAGTT
DNMT1	CCTAGTTCCGTGGCTACGAGGAGAA	TCTCTCTCCTCTGCAGCCGACTCA
DNMT3a	GCCGAATTGTGTCTTGGTGGATGACA	CCTGGTGGAATGCACTGCAGAAGGA
B-ACTN	ACCGAGCATGGCTACAGCGTCACC	GTGGCCATCTCTTGCTCGGAGTCT

2.7 STATISTICAL ANALYSIS

Parametric data are expressed as mean ± standard deviation and were analyzed by two-way ANOVAs, with housing condition (accompanied or isolated) and stress (exposed or not exposed to the CUS protocol) as between-group variables. The two-way ANOVAs were followed by one-way ANOVAs and Tukey post hoc test whenever appropriate. Non-parametric data are expressed as median (interquartile ranges) and were analyzed with Kruskal-Wallis tests, followed by Mann-Whitney test. $P<0.05$ was considered to indicate statistical significance.

3. RESULTS

3.1 WEIGHT GAIN DURING THE EXPERIMENTAL PERIOD

The two-way ANOVA revealed a significant main effect of stress [$F(1,43) = 122.828$, $p<0.001$] on weight alterantion during the experimental period. No significant main effect of housing condition, or interaction between housing condition and stress, was identified by the statistical analysis (all $p>0.05$). As can be seen in Figure 1, experimental groups submitted to the CUS protocol showed a much greater weight loss than animals that were not submitted to stress.

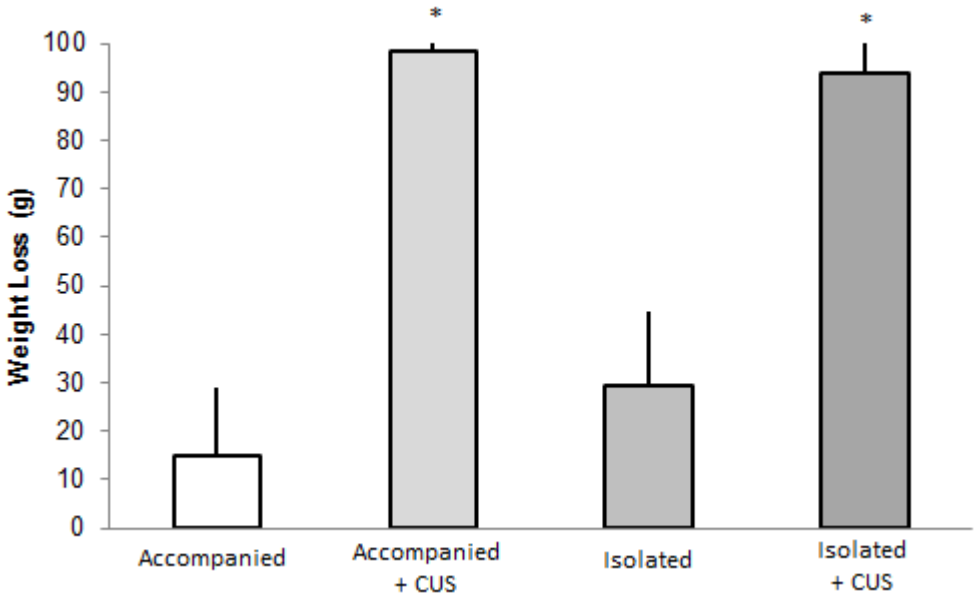

Figure 1. Weight gain of rats during the experimental procedures, calculated as the difference between weight at the start (when animals were assigned to the different housing conditions) and at the end (immediately before animals were euthanized) of the experiment. Statistical analysis was performed using two-way analysis of variance (2-way ANOVA) with housing conditions (accompanied or isolated) and

stress (submitted or not submitted to the CUS protocol) as fixed factors. Data are expressed as mean ± standard deviation. n=10-12 *per* group. *p<0.001 in comparison to the accompanied subgroups, indicating a significant effect of stress.

3.2 OPEN FIELD

The two-way ANOVA showed a significant effect of housing condition [$F(1,33) = 18.287$, $p<0.001$] on the latency to start locomotion and on the time spent in the center of the apparatus [$F(1,33) = 4.301$, $p=0.046$]. As can be seen in Table 3, isolated animals took longer to start locomotion and spent less time in the center zone, suggesting that they are more anxious than the accompanied animals. The statistical analysis of the time spent in the inner zone also indicated a significant main effect of stress [$F(1,33) = 9.461$, $p=0.004$], which could also be seen on the number of crossings [$F(1,39) = 5.735$, $p=0.022$]. These results indicate that animals submitted to the CUS protocol spent less time in the center zone and made more crossings, implying greater anxiety and hyperactivity in comparison to animals not submitted to stress. No interactions were found between housing condition and stress on the variables described above. On the other hand, a significant interaction between housing condition and stress [$F(1,40) = 4.686$, $p=0.037$] was found for rearings, despite the lack of main effects of housing condition [$F(1,40) = 0.933$, $p=0.341$] and stress [$F(1,40) = 0.010$, $p=0.921$] on this variable. Table 3 indicates that animals of the isolated + CUS group made more rearings than animals of the other experimental groups.

Table 3. Open Field behavior

	Adult rats				
Group	Latency to Start (s)	Number of crossings	Number of rearings	Duration in centre (s)	n
A	0,89 ± 0,38*	36,4 ± 8,31	19,7 ± 5,39	29,16 ± 9,24*	9-10
A+C	1,11± 0,74**	43,2 ± 10,61*	16,6 + 2,22	18,55 ± 10,17	9-10
I	2,81 ± 1,48	39,11 ± 7,96	17,9 ± 5,58	21,65 ± 11,29	9-10
I+C	3,23 ± 2,02	45,7 ± 7,52*	21,3 ± 4,98*	13,26 ± 6,44	9-10

Open-field behavior was analyzed by two-way ANOVA. Data are expressed as mean ± SEM ($p<0,05$). The * at start latency indicate the difference between the Accompanied to isolated groups, and the ** represent the difference of Accompanied + CUS to Isolated + CUS; The * at crossing results indicates the differences between stressed and non-stressed groups; The rearing number was affected by isolation plus CUS; The * for time spent in centre of Opend Field indicate the difference between Accompanied from Isolated + CUS group.

Legend Groups: A = Accompanied; A+C = Accompanied + CUS; I = Isolated; I+C = Isolated Group.

3.3 INHIBITORY AVOIDANCE

As indicated by the Kruskal-Wallis test, latency to step down the platform in the training session was not significantly different ($p=0.837$), between the accompanied [180 (152,99/180], accompanied + CUS [71,97 (40,25/180)], isolated [16,21 (12,28/27,03)] and isolated + CUS [10,51 (7,85/16,32)] groups. Although the latency to step down increased significantly from the training to the test session in all experimental groups, as indicated by the Wilcoxon test (all $p<0.05$), further analysis with the Mann-Whitney post hoc test indicated that the isolated and isolated + CUS group had a worse performance on the memory retention test than the accompanied

and accompanied + CUS groups (all $p<0.05$) (Figure 2). On the other hand, no significant differences were identified between the accompanied and accompanied + CUS group ($p=0.085$) and between the isolated and isolated + CUS groups ($p=0.124$).

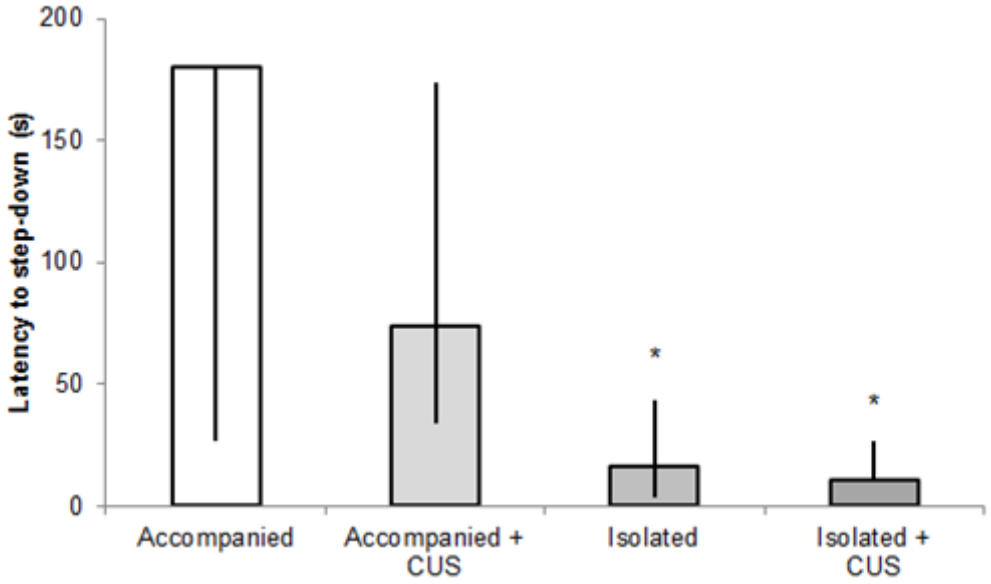

Figure 2. Long-term retention of Inhibitory Avoidance memory in animals submitted to different housing and stress conditions. The retention test was run 24 h after the training session. Statistical analysis was performed using Kruskal-Wallis test and Mann-Whitney's post hoc test. Data are expressed as median and interquartile range. n=10-12 *per* group. *$p<0.001$ in comparison to the accompanied subgroups, indicating a significant housing effect.

3.4 GENE EXPRESSION

BDNF

A significant main effect of housing condition [F(1,18) = 29.879, p<0.001] was indicated by the two-way ANOVA on the BDNF gene expression. As can be seen in Figure 3, isolated animals had lower levels of BDNF expression than accompanied animals. Neither main effects of stress nor interactions between housing condition and stress were seen on BDNF expression (all p >0.05).

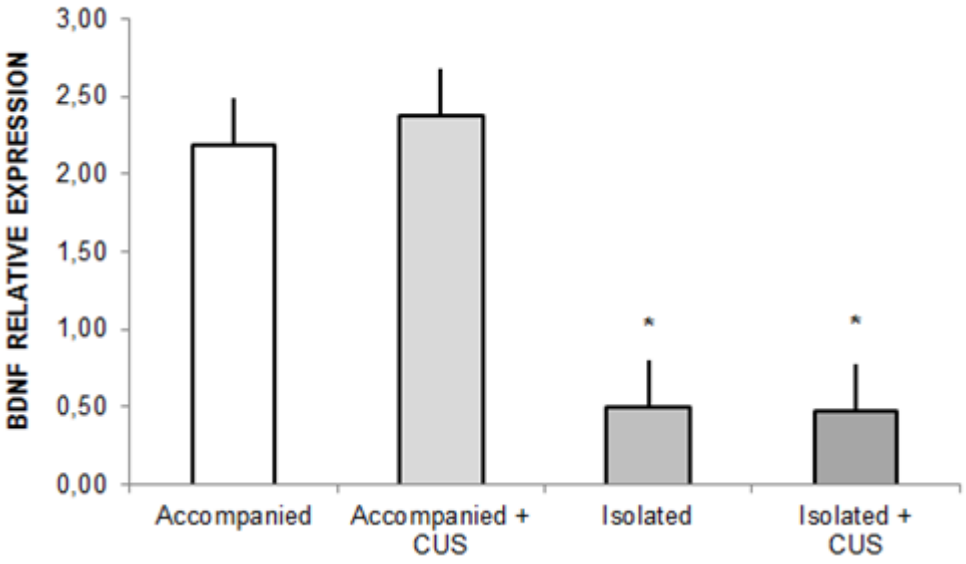

Figure 3. Alterations of BNDF expression in the hippocampus of rats submitted to different housing conditions and stress. Samples were normalized to Beta-actin gene expression and run in duplicate. Statistical analysis was performed using two-way ANOVA with housing conditions (accompanied or isolated) and stress (submitted or not submitted to the CUS protocol) as fixed factors. Data are expressed as mean ± standard deviation. n=4 *per* group. *p<0.001 in comparison to the accompanied subgroups, indicating significant effect of housing condition.

HDAC5

The statistical analysis indicated significant main effects of the housing condition [F(1,15) = 18.045, p<0.001], but not of stress [F(1,15) = 2.910, p=0.116], on

the HDAC5 expression. However, an interaction between housing condition and stress on HDAC5 expression was found [F(1,15) = 10.178, p=0.009]. As can be seen in Figure 4, the HDAC5 expression was significantly higher in isolated animals. Moreover, only isolated animals were sensible to the CUS protocol, as indicated by their levels of HDAC5 expression, which were the highest among all experimental groups. This pattern of results suggests that accompaniment can mitigate the effects of CUS on HDAC5 expression.

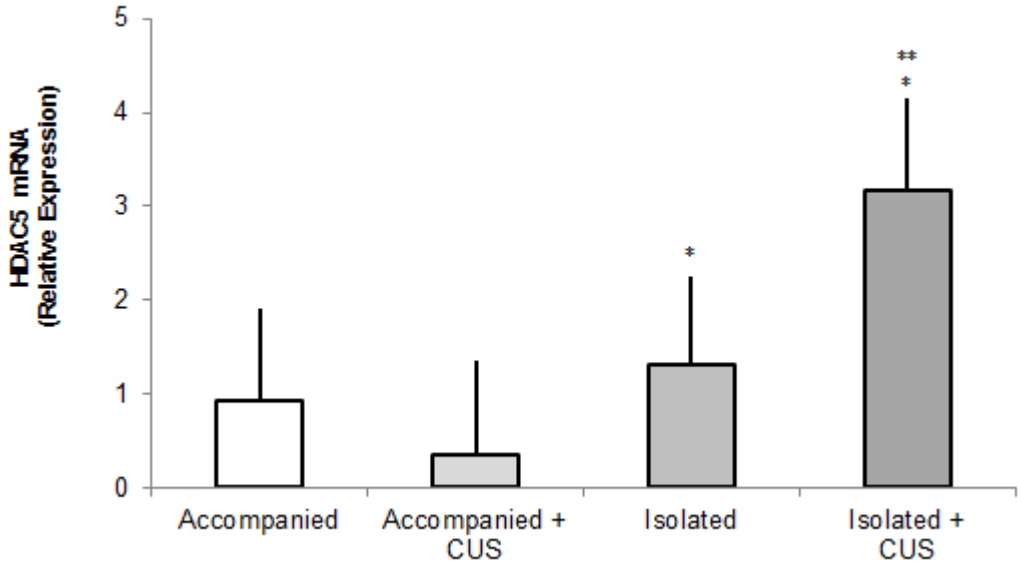

Figure 4. Hippocampal alterations in the expression of the HDAC5 gene in response to different housing conditions and stress. Samples were normalized to Beta-actin gene expression and run in duplicate. Statistical analysis was performed using two-way analysis of variance (2-way ANOVA) with housing conditions (accompanied or isolated) and stress (submitted or not submitted to the CUS protocol) as fixed factors. n= 4 *per* group. Data are expressed as mean ± SEM. *p<0.05 indicating the housing condition effect; **p<0.05 indicating the interaction between housing condition and stress.

DNMT1

Main effects of housing condition [F(1,16) = 22.017, p=0.001] and stress [F(1,16) = 15.133, p=0.002] on the expression of DNMT1 were identified by the two-way ANOVA. Moreover, a significant interaction between housing condition and stress was found [F(1,16) = 14.294, p=0.003]. As can be seen in Figure 5a, animals submitted to social isolation had higher levels of DNMT1 expression than accompanied animals, and experimental groups submitted to the CUS protocol had higher levels of DNMT1 expression than groups that were not submitted to stress. Importantly, stress effects were more pronounced on isolated than accompanied animals, suggesting that social support can mitigate the effects of CUS on DNMT1 expression.

DNMT3a

No main effects of housing condition [F(1,17) = 0.157, p=0.699] and stress [F(1,17) = 2.536, p=0.135] on DNMT3a expression were identified with the two-way ANOVA. Interactions between housing conditions and stress were also absent [F(1,17) = 0.085, p=0.775]. Thus, no significant group differences were found for the DNMT3a expression (Figure 5b).

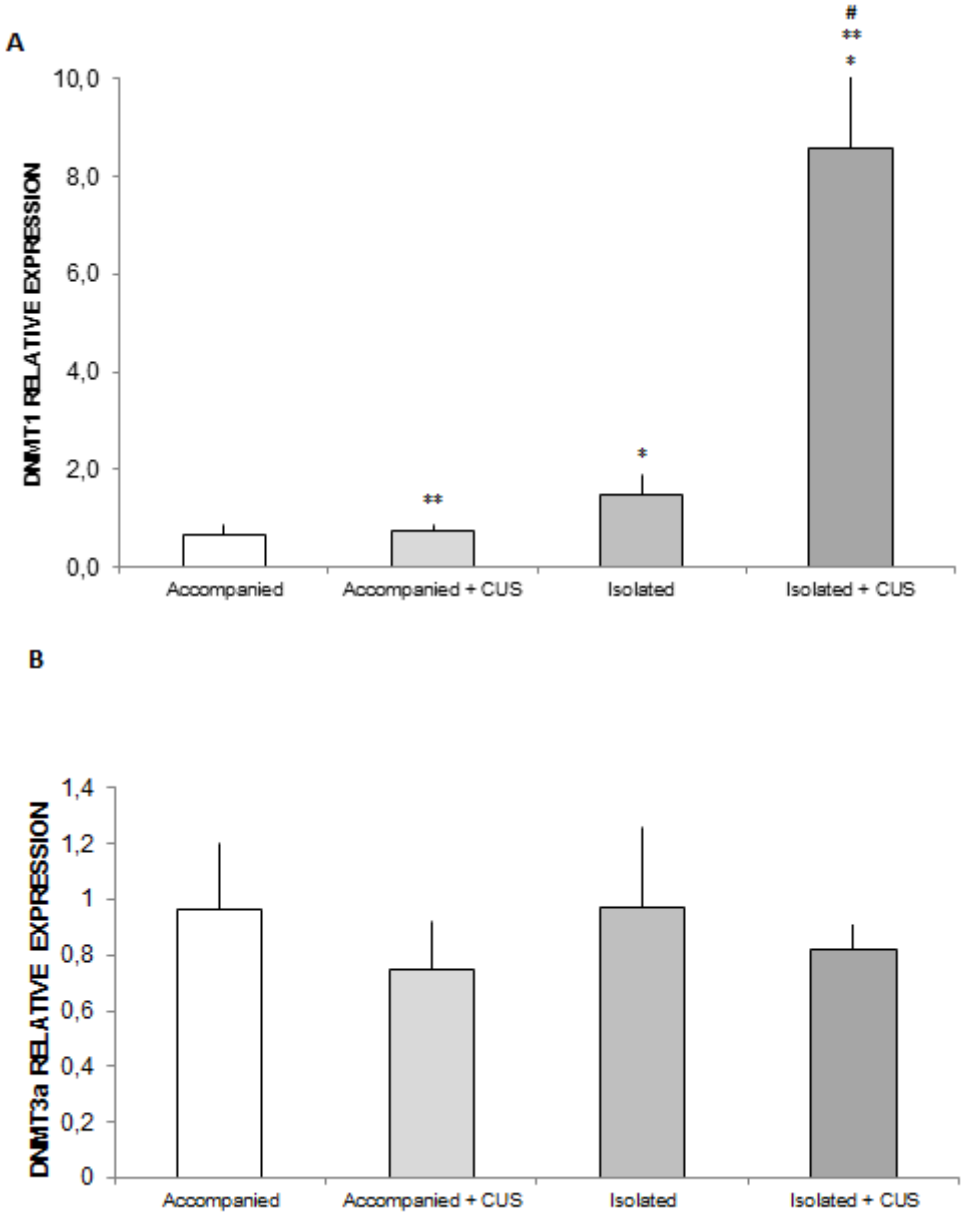

Figure 5. A. Alterations of DNMT1 expression in the hippocampus of rats submitted to different housing conditions and stress. Samples were normalized to Beta-actin gene expression and run in duplicate. Statistical analysis was performed using two-way ANOVA with housing conditions (accompanied or isolated) and stress (submitted or not submitted to the CUS protocol) as fixed factors. n= 4 *per* group. Data are expressed as mean ± standard deviation. *p<0.001 indicating the housing effect; ** p<0.001 indicating the stress effect; $^{\#}$p<0.001 indicating the interaction

between housing condition and stress. **B.** The DNMT3a expression in the hippocampus of rats submitted to different housing conditions and stress. Samples were normalized to Beta-actin gene expression and run in duplicate. Statistical analysis was performed using two-way ANOVA with housing conditions (accompanied or isolated) and stress (submitted or not submitted to the CUS protocol) as fixed factors. Data are expressed as mean ± standard deviation. n=4 *per* group (p>0,05).

4. DISCUSSION

The main findings of this study indicate that social isolation in middle-aged rats has epigenetic effects on hippocampus, increasing HDAC5 and DNMT1 expression. These alterations were associated to lower BDNF levels in the hippocampus, anxiety-like behaviors and impaired long-term memory. The CUS protocol induced important behavioral and physiological alterations, as can be seen by the anxiety-like behaviors and an important weight loss of the stressed animals. These effects of stress were associated whit a significant increase in DNMT1 expression. Interestingly, interactions between housing condition and stress were found for the HDAC5 and DNMT1 expression, indicating that the CUS protocol worsened the effect of social isolation on these variables. Importantly, our results also suggest that accompaniment can mitigate the effects of chronic stress on DNMT1 and HDAC5 expression, indicating that social buffering mechanisms involve epigenetic modulation of stress effects.

Isolated and stressed animals displayed behavioral alterations compatible with increased anxiety, as can be seen by the associations of decreased time spent in the

inner zone of the open field arena and increased latency to start locomotion or hyperlocomotion. These results are in agreement with a large body of evidences describing the association of social isolation and CUS protocols on anxiety-like behaviors [43,44]. On the other hand, social isolation and stress had no significant effects by itself on exploratory activity. These results are in accordance with previous studies of our and other research groups [45, 46,16]. Even so, the combination of these conditions affected exploratory activity, as can be seen by the increased number of rearings in the isolated + CUS groups, suggesting that social isolation and chronic stress have synergic effects on the investigative behavior of a novel environment [47,48,49,50].

The anxiety-like behavior of isolated animals was associated with decreased BDNF expression and increased HDAC5 and DNMT1 expression. Reduced BDNF protein and mRNA expression levels are a common finding in studies of social isolation, suggesting that BDNF has a main role in mediating behavioral alterations associated whit isolation, such as anxiety and memory impairment [51,52,53,54,55]. Epigenetic changes to early-life social isolation are in accordance with the concept that pre- and postnatal periods are sensitive phases of developmental plasticity, in which increases in the expression of HDAC5 and DNMT1 levels were shown to be associated with decreased BDNF expression [56]. In contrast, whether social isolation and loneliness in adults also induces epigenetic changes is less clear. Besides the present study, there is only one other investigation on this issue. Borges and colleagues evaluated the effects of social isolation at young adulthood and showed that decreased BDNF levels were associated to increased HDAC5 expression, decreased histone acetylation (H3K9 and H4K12) and impaired long-term memory on the inhibitory avoidance task, clearly implicating epigenetic

alterations in the responses to social deprivation [45]. The present study corroborates these findings by reproducing, in middle-aged animals, the increased HDAC5 activity and the impaired long-term memory on the inhibitory avoidance task seen in isolated young adult animals [57]. Moreover, we expand former findings by showing that, besides increasing the expression of deacetylases, social isolation at adulthood can also increase the expression of DNA methyltransferases. Former studies already showed the association of decreased histone acetylation, increased DNA methylation and modifications in BDNF expression mechanisms involved in memory consolidation [56,58].

Among the epigenetic mediators evaluated in this study, only DNMT1 expression showed significant alterations in animals submitted to the CUS protocol. The increase in DNMT1 expression seen in stressed animals was not followed by alterations in BDNF expression. A former study of our research group with the CUS protocol also showed epigenetic alterations in the absence of BDNF alterations [45] However, studies with other stress protocols, such as chronic restrain, found increased expression of HDAC5 and DNMT1 in association with decreases BDNF levels [59]. It is possible that these conflicting findings are the result of confounding factors such as the intensity of the different stress protocols, the strain and developmental stage of animals [46] .

The DNMT3a gene expression was not different among the groups, as showed by Mallei and coworkers (2018), who didn’t observe a significant difference on DNMT3a gene expression in the hippocampus, after the social defeat protocol (60). This unaltered gene expression could be explained by the intensity of protocol used in our work, since others studies which evidenced such epigenetic modification applied more severe protocols as the restraint and footshock (59,61). From the

studies described above, it seems that more intense stress protocols and younger ages lead to broader effects on the epigenetic mediators of BDNF expression, as suggested by the study of Seo and colleagues, which used adolescent animals and chronic restrains, in comparison to the study of Borges and colleagues and the present study, which evaluated the effects of a mild stress protocol on young and middle-aged animals [17,59,45]. However, studies specifically designed to confirm this hypothesis are needed.

In conclusion, social isolation at middle age showed broader effects on behavioral and epigenetic alterations associated with BDNF expression than the CUS protocol. Moreover, some of the negative outcomes induced by social isolation were exacerbated by stress, namely HDAC5 and DNMT1 expression, which showed the highest levels in the isolated + CUS groups. Accompaniment animals, on the other hand, promoted social buffering of the stress effects on HDAC5 and DNMT1, warranting further studies of the epigenetic mechanisms involved in social buffering.

Author contributions

E.Bromberg conceived, designed and supervised the experiments. J.V. Borges, B.S. de Freitas, V. Antoniazzi, C. S. dos Santos and V.N.Pires performed the experiments. E. Bromberg and J.V. Borges analyzed the data. M.N.M. de Lima contributed reagents and aided in manuscript writing. E. Bromberg and J.V.Borges wrote the manuscript. All authors provided final approval for the submission of the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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4. CONSIDERAÇÕES FINAIS

O isolamento social e estresse crônico podem causar algumas modificações importantes no SNC, levando a prejuízos moleculares, físicos e mentais. Neste trabalho observou-se que o estresse crônico e isolamento social induzem algumas modificações nos mecanismos epigenéticos no hipocampo de ratos adultos jovens e de meia idade, somando-se a prejuízos em parâmetros comportamentais e cognitivos, como aumento da ansiedade e déficit de memória (MCEWEN; GRAY; NASCA, 2015; TSANKOVA et al., 2006; WILLNER, 2017).

A acetilação de histonas foi um mecanismo que apresentou efeito oriundo do isolamento e estresse crônico. Em animais adultos jovens a acetilação de H4K12 foi observada em concentrações diferentes quando comparamos os animais isolados aos acompanhados. Os animais que foram mantidos em ambiente de suporte social possuíam maiores índices de acetilação nesse resíduo de histona (LI et al., 2017). Por sua vez, a acetilação de H3K9 também apresentou diferença nos resultados comparativos entre os grupos experimentais, indicando que essa histona é susceptível ao estresse oriundo do isolamento e do CUS. Na análise de acetilação dessa histona, percebemos que o grupo acompanhado sem qualquer tipo de estresse apresentou maior acetilação em relação aos animais que pertenciam aos grupos de isolamento social (CAROLINA et al., 2016). Entretanto, a acetilação da histona H3K9 no grupo de animais acompanhados e submetidos ao CUS foi diferente apenas do grupo que permaneceu isolado e estressado pelo protocolo, indicando um possível efeito de exacerbação do CUS junto ao isolamento social na acetilação dessa histona (LI et al., 2017).

A enzima HDAC5 parece estar envolvida nas respostas ao estresse que acontecem no hipocampo, pois sua expressão se mostrou influenciada pelo isolamento social e estresse crônico. Os grupos experimentais de animais adultos jovens e de meia idade apresentaram resultados similares referentes a expressão gênica dessa enzima no hipocampo, indicando que ela é influenciada de maneira semelhante independentemente da idade dos animais. Portanto, os animais nas duas idades apresentaram aumento significativo desta enzima quando eram empregados o isolamento social e protocolo CUS (SEO et al., 2016). Sendo assim, o grupo isolado + CUS apresentou a maior expressão relativa de HDAC5 em comparação aos outros três grupos experimentais.

A metilação é outro evento epigenético que parece estar associado aos mecanismos do estresse, que podem ser originados a partir do ambiente de isolamento social, assim como dos protocolos de estresse crônico (SEO et al., 2018). No presente estudo foi avaliada a expressão (apenas em animais de meia idade) de duas enzimas que induzem a metilação ao DNA, que são a DNMT1 e DNMT3a. A expressão dessas enzimas apresentou padrões diferentes de expressão, visto que a DNMT1 se mostrou diferente entre os grupos experimentais. Em contrapartida, a expressão de DNMT3a foi indiferente aos variados estímulos impostos aos grupos experimentais (MALLEI; IERACI; POPOLI, 2018). Sendo assim, observou-se um aumento da expressão de DNMT1 nos animais do grupo isolado com adição do protocolo CUS, em comparação aos outros grupos experimentais (PARK et al., 2018). Semelhante aos resultados observados para a expressão de HDAC5, a enzima DNMT1 parece necessitar de um estímulo de estresse mais intenso para que ocorra o aumento da sua expressão.

O BDNF que é uma neurotrofina que se mostra lábil a diferentes estímulos físicos e psicológicos, apresentou resultados similares entre os animais jovens e de meia idade (SEO et al., 2016). A expressão de BDNF também já foi associada com os eventos epigenéticos, que induzem diferentes padrões de expressão no hipocampo. As enzimas HDAC5 e DNMT1 parecem influenciar a expressão gênica do BDNF no hipocampo (PARK et al., 2018). Nos animais de três meses foi analisada a concentração do BDNF presente no hipocampo, e evidenciou-se que os animais que permaneceram acompanhados, independente da utilização do protocolo de estresse, apresentaram maiores níveis da neurotrofina, o que representa um fator positivo, visto que o BDNF é de extrema importância para os mais variados processos neurológicos. Nos animais de meia idade o BDNF foi avaliado perante a sua expressão gênica, e foi constatado maiores níveis naqueles animais que permaneceram acompanhados durante o experimento. Portanto, o suporte social parece estar associado tanto ao aumento da expressão gênica, quanto da concentração da neurotrofina no hipocampo, o que pode resultar no favorecimento e melhora de parâmetros comportamentais e cognitivos cognitivos (ZALETEL, 2017b).

Os aspectos físicos também são influenciados pelo estresse crônico, e no presente trabalho isso foi avaliado pela variação de peso dos animais durante o período experimental (WILLNER, 2017). Os animais mais jovens ainda estavam no período de desenvolvimento corporal, portanto a tendência era de que ganhassem peso durante o experimento. Dito isso, os resultados de variação de peso nessa idade se apresentaram da seguinte maneira: os grupos que passaram pelo protocolo de estresse crônico imprevisível (acompanhado estressado e isolado estressado) obtiveram menor ganho de massa corporal em relação aos animais que não foram submetidos ao CUS. Entretanto, nos animais de meia idade se observou uma perda

de peso corporal para todos os grupos experimentais, mas novamente os animais estressados foram diferentes daqueles que não foram expostos aos CUS. Sendo assim, os animais estressados apresentaram uma perda de peso maior do que os animais não estressados (ARAKAWA, 2003). O Protocolo CUS é amplamente utilizado na indução de depressão nos animais, o que poderia indicar uma possível associação do estresse crônico e depressão com a variação de peso corporal, independentemente da idade em que essas situações se apresentem (JAIME et al., 2016).

Foi demonstrado neste trabalho que o isolamento social e estresse crônico podem ser prejudiciais para alguns parâmetros comportamentais e cognitivos, como ansiedade e memória. No artigo 1, onde foram analisados animais adultos jovens, a ansiedade foi vista de maneira aumentada apenas nos animais que eram isolados e estressados pelo CUS (HU et al., 2017). Por outro lado, para os ratos de meia idade analisados no artigo 2, foi evidenciada uma diferença entre os animais acompanhados sem estresse quando comparados aos outros três grupos, indicando uma possível atenuação da ansiedade provida pelo suporte social (MATTHEWS; FORBES; REID, 1995; NAKAYASU; KATO, 2011).

Nos artigos 1 e 2 foi demonstrado que animais isolados apresentam índices de memória inferiores àqueles que foram mantidos em ambientes de suporte social. Esse tipo de suporte social parece ser eficaz independentemente da idade dos animais, visto que tanto os adultos jovens, quanto os de meia idade apresentaram melhora da memória aversiva, em relação aos animais que permaneceram isolados (ZHANG et al., 2016). Esse aumento da capacidade da memória parece estar associado ao aumento do BDNF, visto que a concentração e expressão dessa

neurotrofina também era maior nos grupos acompanhados (PANJA; BRAMHAM, 2014). Evidências também sugerem que os mecanismos epigenéticos possam estar associados á formação de memórias, pela acetilação de histonas H3 e H4 que ativam a expressão gênica do hipocampo, facilitando a consolidação da memória (CAROLINA et al., 2016). Estudos ainda apontam a associação das enzimas HDACs com memória, visto que a inibição destas induz melhora nos resultados do teste da esquiva inibitória (BARICHELLO et al., 2015).

Os resultados apresentados no presente estudo indicam que o suporte social pode ser uma alternativa interessante para atenuação dos efeitos causados pelo estresse crônico e isolamento social. Esse tipo de interação social parece ser importante em aspectos comportamentais e cognitivos, como na ansiedade e memória que são elementos importantes para a saúde mental e que proporcionam uma melhor qualidade de vida ao indivíduo. Associado a isso, o suporte social apresentou efeitos nos mecanismos epigenéticos de acetilação, metilação e níveis de BDNF. Portanto, esses resultados mostram que o suporte social apresenta um promissor efeito na amenização das respostas adversas do organismo.

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