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ELLEN SCOTTON

O PAPEL DO BDNF E DA PRDX-1 NA PROTEÇÃO CONTRA O DANO OXIDATIVO CENTRAL EM RATOS ANEDÔNICOS SUBMETIDOS A UM PROTOCOLO DE ESTRESSE CRÔNICO MODERADO

Porto Alegre, 2019

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Dissertação apresentada como requisito parcial para obtenção de título de Mestre em Psiquiatria e Ciências do Comportamento à Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Psiquiatria e Ciências do Comportamento.

Orientador: Prof. Dr. Maurício Kunz

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SUMÁRIO

LISTA DE ABREVIATURAS

ACTH	Corticotrofina (do inglês *adrenocorticotropic hormone*)
BDNF	Fator neurotrófico derivado do cérebro (do inglês *brain-derived neurotrophic factor*)
BH4	Tetrahidrobiopterina
CAT	Catalase
CPF	Córtex pré-frontal
CRH	Hormônio liberador de corticotrofina (do inglês *corticotropin-releasing hormone*)
CUMS	Estresse crônico moderado e imprevisível (do inglês *chronic unpredictable mild stress*)
DM	Depressão Maior
DRT	Depressão resistente ao tratamento
DSM	Manual Diagnóstico e Estatístico de Transtornos Mentais (do inglês *Diagnostic and Statistical Manual of Mental Disorders*)
EROs	Espécies reativas de oxigênio
GCs	Glicocorticóides
GPx	Glutationa peroxidase
GR	Glutationa redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
H_2O_2	Peróxido de hidrogênio
HPA	Hipotálamo-pituitária-adrenal
IDO	Indoleamina 2,3-dioxigenase

IFN	Interferon
IL	Interleucina
iNOS	Óxido nítrico sintase induzível (do inglês *inducible nitric oxide synthase*)
IRNDs	Inibidores da recaptação de noradrenalina e dopamina
IRSNs	Inibidores da recaptação de serotonina e noradrenalina
ISRSs	Inibidores seletivos da recaptação de serotonina
LPS	Lipopolissacarídeo
NF-κB	Fator nuclear Kappa B (do inglês *nuclear factor kappa B*)
NMDA	N-metil-d-aspartato
Nrf2	Fator nuclear eritróide 2 relacionado ao fator 2 (do inglês *nuclear factor erythroid 2-related factor 2*)
MAPK	Proteínas quinases ativadas por mitógeno (do inglês *mitogen-activated protein kinases*)
MDA	Malondialdeído
OMS	Organização Mundial da Saúde
PCR	Proteína C reativa
PRDX	Peroxirredoxina
RGs	Receptores de glicocorticoides
SNC	Sistema nervoso central
SOD	Superóxido dismutase
TAC	Capacidade antioxidante total (do inglês *total antioxidant capacity*)
TNF	Fator de necrose tumoral (do inglês *tumor necrosis factor*)

TOS	Estado oxidante total (do inglês *total oxidant status*)
TrkB	Receptor tropomiosina quinase B (do inglês *tropomyosin receptor kinase B*)

APRESENTAÇÃO

Este trabalho consiste na dissertação de mestrado intitulada "O papel do BDNF e da PRDX-1 na proteção contra o dano oxidativo central em ratos anedônicos submetidos a um protocolo de estresse crônico moderado", apresentada ao Programa de Pós-Graduação em Psiquiatria e Ciências do Comportamento da Universidade Federal do Rio Grande do Sul, em 29 de março de 2019. O trabalho é apresentado em três partes, conforme segue:

- **Parte I:** Resumo, *Abstract*, Introdução, Justificativa e Objetivos.
- **Parte II:** Metodologia, Resultados e Discussão apresentados no formato do artigo científico intitulado: "*The protective role of BDNF and PRDX-1 against central oxidative damage in anhedonic rats submitted to a chronic unpredictable mild stress protocol*". Os resultados discutidos são provenientes de duas frentes de investigação:

a) Estudo experimental: avaliamos o comportamento, peso corporal, peso relativo das glândulas adrenais, bem como medidas centrais do fator neurotrófico derivado do cérebro (BDNF) e parâmetros oxidativos em animais submetidos a um protocolo de estresse crônico moderado e imprevisível.

b) Bioinformática: realizamos uma análise de bancos de dados de proteômica de três estudos que realizaram protocolos experimentais semelhantes ao do presente trabalho, a fim de identificar proteínas diferencialmente expressas em processos biológicos envolvendo estresse oxidativo e inflamação em animais anedônicos.

- **Parte III:** Considerações Finais e Perspectivas.

Além disso, na sequência constam as Referências e na seção de Anexos encontram-se a carta de aprovação do projeto pela Comissão de Ética no Uso de Animais (CEUA/HCPA), e as instruções para a submissão de manuscritos na revista *Brain, Behavior and Immunity*.

PARTE I

RESUMO

A ativação crônica do eixo hipotálamo-pituitária-adrenal (HPA) e o aumento sustentado de glicocorticoides têm sido associados à depressão maior (DM), e também a alterações envolvendo neurotrofinas e marcadores de estresse oxidativo em resposta à inflamação. O presente estudo teve como objetivo avaliar medidas centrais do fator neurotrófico derivado do cérebro (BDNF), de dano oxidativo, e da capacidade antioxidante total de ratos submetidos ao estresse crônico moderado e imprevisível (CUMS), além de investigar a relação entre os níveis de BDNF e proteínas diferencialmente expressas envolvidas em processos biológicos de estresse oxidativo e inflamação. Ratos Wistar machos foram submetidos ao CUMS por seis semanas. Conforme as taxas de preferência por sacarose, os animais foram classificados como anedônicos ou não-anedônicos. Após a coleta dos tecidos cerebrais, foram avaliados o dano oxidativo, a capacidade antioxidante total e os níveis de BDNF. A fim de estender a discussão sobre possíveis mecanismos envolvendo os achados experimentais, adicionalmente, foi realizada uma análise de bioinformática reunindo resultados de proteômica de outros estudos com protocolo experimental semelhante, visando identificar proteínas envolvidas com o estresse oxidativo e vias inflamatórias diferencialmente expressas em animais anedônicos. O CUMS esteve associado ao aumento das concentrações de BDNF e à diminuição da capacidade antioxidante total, além de não ter sido identificado dano oxidativo aos lipídios e proteínas nos animais estressados. Além disso, a abordagem proteômica de bioinformática indicou que animais anedônicos apresentam um aumento na expressão de peroxirredoxina-1 (PRDX-1) e uma diminuição na expressão de proteínas envolvidas com sinalização apoptótica e inflamatória (RELA, ASK-1 e TAK-1) no hipocampo. Essas evidências sugerem que o BDNF e a PRDX-1 podem representar uma resposta inicial contra o estresse, com papel compensatório, prevenindo o dano oxidativo a lipídios e proteínas através da modulação da defesa antioxidante, principalmente em animais anedônicos.

Palavras-chave: Depressão maior; comportamento anedônico; estresse crônico moderado e imprevisível; preferência por sacarose; BDNF; PRDX-1; estresse oxidativo; espécies reativas de oxigênio; capacidade antioxidante total; proteômica.

ABSTRACT

Chronic activation of the HPA axis and sustained increase of glucocorticoids have been associated in major depression (MD), and also related to changes involving neurotrophins and oxidative stress markers in response to inflammation. This study aimed to evaluate central measures of brain-derived neurotrophic factor (BDNF), oxidative damage and total antioxidant capacity of rats submitted to chronic unpredictable mild stress (CUMS) and investigate the relationship between BDNF levels and differentially expressed proteins involved in oxidative stress and inflammatory biological processes. Wistar male rats were subjected to CUMS for six weeks. Based on the sucrose preference test, animals were divided into anhedonic and non-anhedonic clusters. After brain tissue collection, oxidative damage, total antioxidant capacity, and BDNF levels were evaluated. In order to extend discussion of possible mechanisms involving neurobiological findings, a bioinformatics approach was performed to identify proteins involved with oxidative stress and inflammation pathways that were differentially expressed in anhedonic animals from other studies with similar experimental protocol. CUMS was associated with an increase in BDNF concentrations accompanied by a decrease in total antioxidant capacity, besides the absence of oxidative damage to lipids and proteins in stressed animals. Withal, bioinformatics proteomic approach indicated that anhedonic animals showed a peroxiredoxin-1 (PRDX-1) up-regulation and a down-regulation of proteins involved with apoptotic and inflammation signaling (RELA, ASK-1 and TAK-1) in the hippocampus. These evidences suggest that BDNF and PRDX-1 may represent an initial response against stress, with a compensatory role, preventing oxidative damage to lipids and proteins through the modulation of antioxidant defense, mainly in anhedonic animals.

Keywords: Major depressive disorder; anhedonic behavior; chronic unpredictable mild stress; sucrose preference; BDNF; PRDX-1; oxidative stress; reactive oxygen species; total antioxidant capacity; proteomics

1 INTRODUÇÃO

1.1 Depressão Maior

A Depressão Maior (DM) é um transtorno psiquiátrico grave, crônico, altamente incapacitante e frequentemente associado a outras comorbidades, além de apresentar risco de suicídio substancialmente elevado (1–3). As manifestações da doença acarretam no prejuízo do funcionamento e na diminuição da qualidade de vida do indivíduo acometido, bem como elevam de forma importante os gastos no âmbito da saúde pública (4,5).

O diagnóstivo da DM é realizado de acordo com critérios descritos no Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-V), e a sintomatologia varia de acordo com o endofenótipo apresentado. De forma geral, as manifestações incluem humor deprimido ou irritação, alterações do sono e do apetite, agitação ou retardo psicomotor, fadiga, sentimento de inutilidade ou culpa excessiva, falta de concentração, pensamento recorrente de morte e/ou ideação suicida (6–8). A anedonia, por sua vez, é um sintoma caracterizado pela ausência quase completa de sensação de prazer, motivação e interesse, sendo uma manifestação central no endofenótipo melancólico (9).

A prevalência da DM é estimada em 4,4%, sendo uma doença que se manifesta usualmente no início da idade adulta (10–13). Segundo dados recentes da Organização Mundial da Saúde (OMS), a DM é considerada a principal causa de incapacidade na população em geral, assumindo o quarto lugar entre as doenças com maior número de pessoas acometidas no mundo. Até o ano de 2020, a estimativa é de que a DM esteja em segundo lugar dentre as doenças com maior prevalência mundial (13).

Considerando as terapias farmacológicas disponíveis atualmente, apenas metade dos pacientes atinge a remissão completa dos sintomas, mesmo após várias tentativas de tratamento. Além disso, a remissão está vinculada a altos índices de recaída, principalmente durante o primeiro ano (14). Apesar dos antidepressivos serem responsáveis por resultados

importantes em parte dos indivíduos com DM, o tratamento convencional apresenta muitos efeitos adversos indesejáveis, como boca seca, tontura, ganho de peso e disfunção sexual, o que resulta na baixa adesão dos pacientes (2). Ainda, cerca de 20% dos indivíduos acometidos seguem sintomáticos após dois anos de tratamento (15).

Estima-se que 44% dos pacientes não respondem a duas tentativas consecutivas de tratamento com antidepressivos, apresentando depressão resistente ao tratamento (DRT). A elevada prevalência, somada à diminuição ainda mais acentuada da qualidade de vida, à baixa produtividade, ao maior número de hospitalizações, bem como seu alto impacto econômico, fazem da DRT um dos maiores desafios da psiquiatria moderna (16–19). Recentemente, Bergfeld e colaboradores reportaram que indivíduos com DRT apresentaram índices de tentativa e execução de suicídio, respectivamente, duas e dez vezes maiores, do que indivíduos depressivos não resistentes (20,21).

1.1.1 Neurobiologia

Alterações estruturais e funcionais em diversas regiões corticais envolvidas com o processamento de emoções, controle cognitivo, aprendizagem, formação de memórias e funções executivas estão presentes na DM (22,23). Estudos *post-mortem* com indivíduos depressivos reportam alterações em estruturas associadas fortemente com sinais clássicos da DM, como o córtex pré-frontal (CPF) e hipocampo (24–26). Além disso, estudos de imagem demonstram uma diminuição no volume dessas estruturas em pacientes depressivos, o que reforça a ideia de uma diminuição no número de neurônios e de células gliais em regiões córtico-límbicas de indivíduos com DM (27–31).

Desde os anos 50, a primeira linha de tratamento antidepressivo é composta por medicamentos que atuam sobre a recaptação de monoaminas, como os inibidores seletivos da recaptação de serotonina (ISRSs), inibidores da recaptação de serotonina e noradrenalina (IRSNs) e inibidores da recaptação de noraepinefrina e dopamina (IRNDs) (32). A utilização

desses fármacos é baseada na teoria monoaminérgica da DM, a qual propõe que as manifestações clínicas da doença são provenientes de uma redução na disponibilidade desses neurotransmissores ou de alterações funcionais da transmissão monoaminérgica de regiões cerebrais específicas (33,34).

A alteração no sistema serotoninérgico, por sua vez, pode ser considerada um mediador central na manifestação dos sintomas depressivos, uma vez que esse sistema tem origem nos núcleos dorsal e medial da rafe, e se projeta para diversas áreas corticais e subcorticais, tais como CPF, hipocampo, amígdala e estriado. Desse modo, a neurotransmissão serotoninérgica influencia os sistemas dopaminérgico, noradrenérgico, glutamatérgico, colinérgico e GABAérgico, tendo assim a capacidade de regular o humor, o sono e o apetite, funções que se encontram alteradas na DM (35).

Nesse contexto, alterações na biossíntese da serotonina também parecem estar envolvidas na fisiopatologia da DM, como postulado por Lapin e Oxenkrug em 1969, com a criação da "hipótese serotoninérgica". Essa teoria propõe que alterações no metabolismo do triptofano, precursor da serotonina, resultem na diminuição da síntese desse mediador químico, levando à ocorrência de sintomas depressivos (36). Sugere-se ainda que o prejuízo na síntese de serotonina seja mediado por fatores ambientais, como a exposição crônica ao estresse, e pela modulação da resposta inflamatória via ativação do eixo HPA.

1.1.2 Hipótese inflamatória

Como descrito previamente, um alto índice de pacientes deprimidos tratados com os antidepressivos convencionais não apresenta remissão completa dos sintomas ou demonstra ser refratário ao tratamento (37,38). Evidências crescentes também indicam que a DM está associada a comorbidades de cunho inflamatório, como *diabetes mellitus* (39), obesidade (40) e doenças reumáticas (41), além de estar relacionada a outros fatores que aumentam a produção de mediadores inflamatórios, como o estresse psicossocial, diminuição no sono,

abuso sexual na infância e isolamento social (42,43). Nesse sentido, a DM parece apresentar um importante componente inflamatório em suas bases etiológicas (44,45).

Diversos estudos demonstram que os níveis periféricos de proteínas envolvidas com a resposta inflamatória encontram-se alterados em pacientes com DM. Uma meta-análise de Dowlati e colaboradores indicou aumento significativo nos níveis circulantes do fator de necrose tumoral alfa (TNF-α) e de interleucina (IL) 1 beta (IL-1β) (46), enquanto outra meta-análise demonstrou aumento plasmático de proteína C reativa (PCR), IL-1β e IL-6, ambos em pacientes depressivos (47). Ainda, um estudo epidemiológico demonstrou que níveis elevados de PCR e IL-6 detectados no início do estudo foram associados à presença de sintomas da DM após doze anos de seguimento (48).

Além das citocinas estarem aumentadas perifericamente, esses marcadores inflamatórios podem atingir o sistema nervoso central (SNC) atravessando a barreira hematoencefálica através dos órgãos circunventriculares ou pela via aferente do nervo vago (49–51). No SNC as citocinas podem ser produzidas pela microglia, astrócitos e neurônios e atuam como transmissores gliais, podendo modificar funções neuronais através de sua ligação com um amplo número de receptores (44).

As células da microglia são macrófagos residentes no SNC, os quais atuam na regulação da resposta neuroimune central. Em condições fisiológicas, a microglia se apresenta como uma rede amplamente distribuída que estabelece contato com dendritos e sinapses próximas, podendo remodelar essas conexões e secretar neurotrofinas como o BDNF, o qual exerce papel neuroprotetor, preservando as funções desse microambiente (52). Frente a um sinal gerado por dano celular, a microglia altera sua morfologia passando a secretar citocinas e quimiocinas que recrutam outras células do sistema imune, a fim de promover o reparo e remodelamento tecidual (53).

Tipicamente, a ativação da microglia com fenótipo tipo “M2” está associada a um perfil anti-inflamatório, representado pelo aumento de BDNF, da IL-4, e da atividade fagocítica. Em contraste, a ativação do fenótipo "M1" está associada à maior expressão da enzima óxido nítrico sintase induzível (iNOS), produção de espécies reativas de oxigênio (EROs) e de IL-1β, além da diminuição da secreção de fatores neurotróficos, configurando um perfil pró-inflamatório. Embora a ativação da microglia não possa ser dicotomizada entre ativação tipo “M1” e ativação tipo “M2”, sugere-se que a intensidade dessas respostas antagônicas possa determinar se a atividade microglial resultará na depuração de detritos teciduais e na resolução da resposta inflamatória, ou se será estabelecido um processo de neuroinflamação crônica, levando a disfunção e morte celular (52).

As citocinas pró-inflamatórias tais como interferon (IFN), IL-1β e TNF-α podem reduzir a disponibilidade de monoaminas como serotonina, dopamina e noradrenalina através do aumento da expressão e função dos seus transportadores pré-sinápticos. Ainda, o aumento de citocinas pode resultar na geração de EROs, provocando a oxidação de um cofator enzimático essencial para a síntese de dopamina, a tetrahidrobiopterina (BH4) (54,55). Esses processos podem provocar um prejuízo na via de recompensa, estando associados com a manifestação da anedonia (56).

O aumento da resposta inflamatória também pode diminuir a disponibilidade de monoaminas, desviando o triptofano, precursor da serotonina, para a via das quinureninas, através da ativação da enzima indolamina 2,3-dioxigenase (IDO). A microglia ativada pode converter quinurenina em ácido quinolínico, um agonista do receptor N-metil-d-aspartato (NMDA), o que juntamente com a redução na recaptação e maior liberação astrocitária de glutamato pode resultar na amplificação da neurotransmissão glutamatérgica, além de diminuir a concentração de BDNF e interferir na sinalização de seu receptor tropomiosina quinase B (TrkB), afetando os processos de neurogênese e plasticidade neuronal. Esses

desfechos possivelmente estão relacionados com a piora cognitiva dos pacientes com DM (54,55,57,58).

Quando a via das quinureninas é estimulada ocorre a elevação da condutância da membrana ao cálcio, o aumento da lipoperoxidação e da produção de mediadores inflamatórios, o estímulo da iNOS e da apoptose neuronal, além do surgimento de sinais clínicos da DM (57,58). Nesse sentido, além de diminuir a disponibilidade de triptofano, o aumento da atividade da IDO em resposta à inflamação resulta na amplificação da neurotransmissão glutamatégica, no aumento de estresse oxidativo e intensifica o processo de neurodegeneração (59). Em modelos pré-clínicos o bloqueio dos receptores NMDA através da utilização de quetamina, ou a utilização de inibidores da IDO, diminuiu o comportamento tipo-depressivo em camundongos (60,61).

Em suma, uma vez no cérebro as citocinas podem influenciar os sistemas de neurotransmissão, induzir apoptose, diminuir a neurogênese e prejudicar os mecanismos de neuroplasticidade, podendo afetar circuitos que regulam o comportamento e estão relacionados com a manifestação de sintomas clássicos da DM, como a anedonia e a ansiedade (54,55,62).

1.1.2.1 Inflamação e dessensibilização do eixo HPA

O estresse é considerado um estado de desequilíbrio homeostático frente à exposição a estressores psicológicos, ambientais ou fisiológicos. Esses eventos geram múltiplas alterações neuroquímicas, imunes e hormonais, levando a ativação de uma resposta mediada principalmente pelo eixo HPA (31). A partir de um estímulo estressor, ocorre a liberação do hormônio liberador de corticotrofina (CRH) pelo hipotálamo com a consecutiva síntese e liberação da corticotrofina (ACTH) pela adenohipófise, o qual atua sobre a glândula adrenal, estimulando a síntese de glicocorticóides (GCs), principalmente o cortisol, a espécie humana (63).

Em uma resposta aguda ao estresse os GCs apresentam propriedades imunossupressoras e anti-inflamatórias, inibem a proliferação de linfócitos, reduzem a expressão de citocinas pró-inflamatórias como TNF-α e IL-6 e estimulam o aumento da expressão de citocinas anti-inflamatórias, como a IL-10 (64). Além disso, os receptores de GCs (RGs) – principalmente os receptores de GCs α (RGα) – respondem promovendo um *feedback* negativo sobre a produção de CRH, a fim de preservar a homeostase do organismo. O estresse crônico, por sua vez, aumenta de forma significativa os níveis periféricos de IL-6, o que acaba por intensificar a liberação de cortisol e diminuir a sensibilidade do eixo HPA (42,65).

Em geral, respostas sustentadas ao estresse envolvem concentrações persistentemente altas de GCs, além de níveis aumentados de adrenalina e noradrenalina, o que acaba por prejudicar o funcionamento do eixo HPA, estimulando a inflamação (66). A ativação prolongada do eixo HPA e aumento crônico de GCs têm sido evidenciados na DM, bem como a diminuição da sensibilidade do eixo HPA desses indivíduos frente ao tratamento com dexametasona, um corticoide com potente ação anti-inflamatória (67,68). Essa dessensibilização do eixo HPA parece ser intermediada pela resistência aos GCs, uma vez que na DM e em situações de estresse crônico ocorre a diminuição da expressão dos RGs no hipotálamo, prejudicando o *feedback* negativo necessário para desativar o eixo HPA (65,69). Ainda, o prejuízo no *feedback* negativo potencializa a sinalização imune, através do aumento dos níveis de citocinas e de células pró-inflamatórias (70,71).

Da mesma forma que a resistência aos GCs potencializa a inflamação, as citocinas pró-inflamatórias atuam sobre os RGs, diminuindo sua expressão e função, agravando ainda mais o cenário inflamatório (72). Achados de Carvalho e colaboradores evidenciam o aumento de cortisol e de IL-6 em sangue periférico de pacientes com DRT (73). Os mesmos indivíduos também apresentaram indícios de resistência aos GCs em um ensaio *in vitro*

envolvendo a exposição de suas células linfomononucleares a um insulto e posterior tratamento com cortisol e dexametasona (74). Em conjunto, os achados envolvendo a resistência aos GCs, o aumento na produção de cortisol, bem como a resposta inflamatória, demonstram que esses mecanismos são coexistentes em muitos pacientes depressivos, evidenciando a relação entre a inflamação e a resposta ao estresse na fisiopatologia da DM.

1.1.3 Estresse oxidativo

Durante o metabolismo celular energético aproximadamente 5% do oxigênio não é reduzido diretamente a água, gerando moléculas altamente reativas conhecidas como EROs. As principais EROs incluem o ânion superóxido, o radical hidroxila e o peróxido de hidrogênio (H_2O_2) (75,76). Em baixas concentrações as EROs desempenham funções importantes na regulação de processos como a fagocitose, apoptose, ativação de fatores de transcrição e sinalização celular, estando em equilíbrio com os sistemas antioxidantes (77). Condições adversas podem gerar um desequilíbrio no estado redox celular, tanto pela produção aumentada de EROs, como pela diminuição na capacidade de defesa antioxidante, levando ao estresse oxidativo (76). Essa situação pode provocar dano em biomoléculas como lipídios, proteínas e DNA. O cérebro, por sua vez, é um órgão altamente suscetível aos efeitos prejudiciais das EROs, devido à sua alta demanda metabólica e aos menores níveis de antioxidantes (78).

A carbonilação de proteínas é um marcador muito associado a dano irreversível promovido pelo estresse oxidativo. Esse processo pode resultar no prejuízo das atividades transportadoras e receptoras das proteínas, levando à perda de sua funcionalidade e também à oxidação de lipídios (79,80). Estruturalmente os ácidos graxos são um dos componentes principais das membranas celulares, sendo importantes na manutenção de sua integridade e fluidez. A peroxidação lipídica é um processo que age sobre a composição e organização celular, promovendo alterações covalentes de biomoléculas e prejudicando a homeostasia do

organismo. Adicionalmente, a peroxidação lipídica tem um envolvimento bem documentado nos processos de morte celular e em várias doenças (81). Diversos estudos já identificaram níveis séricos aumentados de malondialdeído (MDA), um subproduto reativo da lipoperoxidação, em pacientes com DM (82–84). Uma meta-análise recente mostrou que compostos associados com a peroxidação lipídica e com a oxidação do DNA, 8-hidroxi-2-deoxiguanosina e F2-isoprostanos, respectivamente, são os marcadores de estresse oxidativo mais consistentemente associados com a DM (85).

O sistema de defesa enzimático é composto por muitas enzimas. Dentre elas, destacam-se a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx). As suas atividades representam a primeira linha de proteção antioxidante, tendo um papel fundamental nos mecanismos e estratégias de defesa do organismo (86). Em um cenário de estresse oxidativo, a SOD é a primeira enzima antioxidante responsável por proteger as células dos danos ocasionados pelas EROs, seguida da ação da CAT (87,88). Alterações na atividade da SOD são usualmente encontradas em pacientes deprimidos, mas os achados são inconsistentes quanto à direção dessas alterações (89). Algumas evidências apontam para uma diminuição da atividade da SOD (90,91), bem como da CAT (92) na DM. Em contrapartida, o aumento das atividades de ambas as enzimas também tem sido demonstrado em outros estudos (93,94). Recentemente, Tsai e Huang encontraram níveis séricos de SOD e CAT aumentados durante a fase aguda da DM (95).

As peroxirredoxinas (PRDX), por sua vez, são proteínas antioxidantes que agem em conjunto com outros sistemas de defesa diminuindo os substratos utilizados na formação do H_2O_2 e modulando a sua sinalização (96–98). Kim e colaboradores demonstraram que a PRDX-1 estava *up-regulated* na microglia, após exposição ao lipopolissacarídeo (LPS). A reversão desse aumento esteve associada à morte celular mediada por H_2O_2, sugerindo um papel protetor da PRDX-1 frente a um contexto pró-inflamatório (99). Ainda, outro estudo

identificou que níveis diminuídos de PRDX-6 estavam associados a alterações da morfologia celular e morte neuronal no córtex de roedores expostos ao tratamento com corticosterona (100).

Além das defesas antioxidantes enzimáticas, alguns compostos não enzimáticos importantes são provenientes da dieta, como as vitaminas E (tocoferol) e C (ácido ascórbico), o ácido úrico e o zinco (101). A glutationa, por sua vez, é o composto não enzimático antioxidante mais abundante no organismo, e desempenha um papel importante na proteção celular e de biomoléculas contra as EROs, além de ser um marcador endógeno sensível de estresse oxidativo. Ela pode ser encontrada na forma reduzida (GSH) ou oxidada (GSSG), por ação das enzimas glutationa redutase (GR) e GPx, respectivamente, sendo a razão GSH/GSSG amplamente utilizada para estimar o estado redox dos sistemas biológicos (102,103). Poucos estudos investigaram anormalidades relacionadas à GSH na DM, mas um estudo *post-mortem* detectou níveis diminuídos no CPF de indivíduos deprimidos (104).

A capacidade antioxidante total (TAC), por sua vez, pode fornecer informações sobre o estado antioxidante total de um indivíduo, incluindo antioxidantes ainda não reconhecidos ou não facilmente detectados (105). Contudo, esse ensaio avalia a capacidade antioxidante proveniente de compostos não enzimáticos, através de reações de oxidação e redução, geralmente não avaliando as atividades enzimáticas (106). Poucos estudos avaliaram a TAC em pacientes depressivos, todavia, alguns achados indicam que indivíduos com DM apresentam TAC diminuída, em detrimento do estado oxidante total (TOS) aumentado (107). Ainda, uma meta-análise conduzida por Liu e colaboradores corrobora esses dados, uma vez que também identificou níveis diminuídos de TAC e níveis aumentados de radicais livres, bem como de produtos provenientes de dano oxidativo em pacientes depressivos quando comparados a controles (108).

Níveis elevados de estresse oxidativo estão usualmente associados com prejuízo cognitivo e são considerados um dos potenciais mecanismos envolvidos com a neuroprogressão e o envelhecimento precoce nos transtornos de humor (88). Um grande número de evidências indica que a produção exacerbada de EROs, levando ao aumento do estresse oxidativo, pode ser responsável por alterações neuronais que induzem a morte celular, promovendo, consequentemente, a atrofia de regiões específicas (109). Um menor volume hipocampal tem sido associado à resposta antidepressiva mais lenta e ao aumento de estresse oxidativo periférico na depressão tardia (110–112).

Em conjunto, essas evidências dão suporte ao envolvimento do estresse oxidativo na fisiopatologia da DM. Estudos clínicos e pré-clínicos que acessaram os efeitos de antidepressivos indicam que eles podem agir sobre as EROs sequestrando os radicais livres e suprimindo a via de estresse oxidativo. A ação dos antidepressivos contra os danos induzidos pelo desequilíbrio do estado redox parece mediar a remissão dos sintomas depressivos, bem como a recuperação dos pacientes (113). Crescentes evidências demonstram um potencial antioxidante dos antidepressivos, indicando que eles são capazes de restaurar e normalizar a atividade de enzimas como SOD, CAT e GPx (114,115), além de aumentar as concentrações de GSH e TAC (116), bem como diminuir os níveis de oxidação do DNA, de lipídios e de proteínas, atenuando também a morte celular induzida pela sinalização do H_2O_2 (117).

1.1.3.1 Estresse oxidativo, inflamação e BDNF

Evidências indicam que as bases biológicas da DM envolvem a interação entre fatores biológicos e ambientais, além de má adaptação na resposta ao estresse. Contudo, a forma como esses fatores são orquestrados e interagem entre si ainda não está bem elucidada (9). O aumento de marcadores de estresse oxidativo e de inflamação tem sido associado com a DM (89,118). Nesse contexto, as EROs, além de provocar dano às biomoléculas, atuam como mediadores na transdução de sinal de vias inflamatórias envolvendo o fator de transcrição

fator nuclear Kappa B (NF-κB) e as proteínas quinases ativadas por mitógeno (MAPK). Sugere-se ainda que a não adaptação das células frente às alterações do estado redox, a subsequente morte celular, e os danos provocados pelos mediadores inflamatórios estejam amplamente associados com a neuroprogressão da DM. Dessa forma, a ativação do sistema imune e o aumento de estresse oxidativo induzem uma cascata de eventos orquestrada por fatores de transcrição como o fator nuclear eritróide 2 relacionado ao fator 2 (Nrf2) e o NF-kB, apresentando efeitos sinérgicos sobre a patogênese da DM (119).

O fator de transcrição Nrf2 exerce um papel fundamental na homeostase redox. Em baixas concentrações de EROs, ele é ativado e estimula a transcrição de genes com papel antioxidante, levando a efeitos protetores (119). Mellon e colaboradores reportaram que genes regulados pelo Nrf2 estavam aumentados em pacientes com DM, sugerindo que uma resposta antioxidante estava sendo requerida, e tiveram sua expressão diminuída após o tratamento antidepressivo (120). Além disso, um recente estudo pré-clínico mostrou que a suscetibilidade à DM era resultado de um estado persistente de estresse oxidativo, mediado por uma disfunção do Nrf2, o que foi revertido após tratamento com antioxidantes (121).

Em contrapartida, o aumento de EROs promove a ativação e translocação do NF-kB para o núcleo, podendo ativar cascatas inflamatórias, pró-oxidantes ou antioxidantes, dependendo do contexto celular (119). A ativação inicial do eixo HPA e a elevação de GCs são tipicamente associadas a respostas anti-inflamatórias, incluindo o bloqueio da sinalização do NF-kB (122,123). No entanto, o estresse pode aumentar os efeitos do NF-κB, intensificando a inflamação (124–126). Koo e colaboradores concluíram que a sinalização IL-1β/NF-κB é ativada pelo estresse crônico, e que essa via foi necessária para o desenvolvimento de comportamento anedônico, bem como para os efeitos anti-neurogênicos observados. Ainda, sugere-se que o bloqueio do NF-κB possa inibir a ação de outras citocinas pró-inflamatórias implicadas na resposta ao estresse e na DM (127).

Strawbridge e colaboradores reportaram que concentrações aumentadas de biomarcadores inflamatórios estavam relacionadas à resposta parcial ao tratamento com antidepressivos, enquanto a inflamação persistentemente aumentada foi capaz de predizer os indivíduos não respondedores (128). Nesse contexto, outro estudo já havia demonstrado que os pacientes depressivos com concentrações mais elevadas de IL-6 eram menos propensos a responder ao tratamento antidepressivo (129).

Inúmeras evidências clínicas e pré-clínicas indicam que a diminuição dos níveis de BDNF, uma neurotrofina amplamente distribuída no cérebro e na periferia, pode ser revertida com tratamento antidepressivo, havendo, em partes, a melhora de sintomas da DM. Desse modo, fica claro que o mecanismo de resposta farmacológica dos antidepressivos está diretamente relacionado à ação do BDNF (130). Somado ao desequilíbrio do eixo HPA e à diminuição dos níveis de BDNF, Kunugi e colaboradores mostraram que os receptores RG interagem diretamente com os receptores TrkB, e que a exposição persistente aos GCs reduz essa interação, diminuindo a sinalização BDNF/TrkB e, consequentemente, colaborando com prejuízo dos efeitos neurotróficos (131).

O BDNF participa diretamente em processos como o desenvolvimento neuronal, neurogênese, plasticidade sináptica e arborização dendrítica, além de participar da consolidação da memória (132). De forma interessante, além de suas funções associadas à resposta imune, o fator de transcrição NF-kB também pode atuar na sobrevivência celular e na plasticidade sináptica. Uma revisão recente reuniu diversas evidências, sugerindo que o NF-kB regula a expressão do BDNF, e que o BDNF pode induzir a ativação do NF-kB. É proposto que esse *feedback* positivo possa estar envolvido com as ações antidepressivas que levam ao aumento da neurogênese e da plasticidade neuronal, com a restauração da transmissão sináptica e com alterações comportamentais positivas, embora esse mecanismo não esteja completamente elucidado (133).

A ativação prolongada do eixo HPA com aumento persistente de GCs, bem como alterações envolvendo o estado redox e a inflamação em resposta ao estresse, usualmente levam ao prejuízo da neurogênese, o que parece estar associado à neuroprogressão na DM (89,134). Em conjunto, esses resultados sugerem que o prejuízo nos níveis de BDNF e na sua via de sinalização, bem como o perfil pró-inflamatório e pró-oxidativo podem estar relacionados com a diminuição da resposta ao tratamento antidepressivo.

1.2 Resiliência e suscetibilidade ao estresse

Perante a exposição ao estresse, a maior parte dos indivíduos exibe uma resposta adaptativa efetiva que viabiliza a superação da adversidade, caracterizando-os como resilientes. Em contrapartida, uma parcela menor da população demonstra uma habilidade limitada de lidar com esses eventos, apresentando um comprometimento das respostas adaptativas e consequentemente sendo mais suscetível ao desenvolvimento de doenças associadas ao estresse, como a DM (135,136).

Em um recente estudo clínico, García-Leon e colaboradores reportaram que a resiliência está relacionada com diferentes manifestações subjetivas de estresse em adultos saudáveis. Seus resultados sugerem que a resiliência atua como um amortecedor da percepção ao estresse, e que a diminuição da auto percepção permite que os eventos estressores sejam enfrentados com mais sucesso (137). Ainda, estudos comparando técnicas comportamentais, moleculares e eletrofisiológicas indicam que a resposta não adaptativa ao estresse envolve alterações em circuitos neurais específicos que regulam a recompensa, a resposta emocional, o medo e o comportamento social, prejudicando o enfrentamento do evento estressor (138). É importante destacar que o fenótipo resiliente parece estar relacionado a um processo neurobiológico distinto, não representando apenas a ausência de vulnerabilidade (139).

Dois modelos animais têm sido muito utilizados para estudar o paradigma de suscetibilidade e resiliência ao estresse. No modelo de derrota social os animais demonstram

diferenças individuais mais pronunciadas, apresentando uma desvantagem em comparação ao modelo de estresse crônico moderado e imprevisível (CUMS) (140). Contudo, a exposição a esse modelo geralmente identifica um maior número de animais resilientes ao estresse, o que se assemelha ao contexto clínico da DM, onde a vulnerabilidade individual interage com os eventos adversos que o indivíduo experimenta ao longo da vida, sendo um bom modelo para estudar os mecanismos envolvidos com a resiliência ao estresse (141). No modelo de CUMS, por sua vez, tem sido demonstrado, que cerca da metade dos animais expostos ao protocolo de estresse desenvolvem comportamento anedônico, sugerindo que ele seja uma ferramenta robusta para o estudo da suscetibilidade ao estresse (142–145). Alguns resultados baseados no protocolo de CUMS indicam que os animais suscetíveis apresentam diferenças, por exemplo, na neurogênese hipocampal (146) na performance cognitiva (147) na resposta inflamatória (143) e nos padrões de estresse oxidativo (148) em relação aos animais resilientes.

1.3 CUMS

Os modelos animais neuropsiquiátricos representam uma ferramenta pré-clínica extremamente desafiadora, dada a natureza subjetiva de muitos sintomas, a ausência de biomarcadores específicos, e as dificuldades de transpor os achados experimentais para o contexto clínico dos transtornos psiquiátricos. No entanto, o progresso na compreensão da fisiopatologia, bem como o avanço acerca dos possíveis tratamentos e a melhora da resposta terapêutica podem ser beneficiados por essa abordagem (149).

Idealmente, a fim de que um modelo animal seja adequado para o estudo de alguma doença, ele deve obedecer a três validades: de face, de construto e preditiva. A validade de face se refere à semelhança entre a sintomatologia clássica da doença e o comportamento desenvolvido pelos animais frente ao protocolo experimental. A validade de construto consiste em reproduzir características das bases biológicas da doença e a validade preditiva,

por sua vez, avalia o quanto as alterações comportamentais podem ser prevenidas ou revertidas através de fármacos classicamente utilizados no tratamento clínico (150).

Dentre os modelos animais utilizados no estudo da DM, destaca-se o modelo de CUMS, que surgiu em meados dos anos 80, baseado na perda de resposta à recompensa em animais submetidos a um cronograma variável de estressores e na reversão desse comportamento após tratamento antidepressivo (151,152). No CUMS, os animais são expostos diariamente a diferentes estressores, a fim de estimular a resposta ao estresse prevenindo uma possível adaptação. Dentre os estudos descritos na literatura, encontram-se variações nos protocolos experimentais. Contudo, uma recente revisão de Willner indicou que os estressores mais utilizados consistem na exposição à maravalha molhada, inclinação da caixa moradia, inversão do ciclo claro-escuro, privação de água e de comida e a superpopulação de roedores em uma mesma caixa (153).

Após verificar-se alta reprodutibilidade, ficou estabelecido que a exposição ao CUMS é capaz de induzir a diminuição do consumo ou da preferência por sacarose em animais suscetíveis, evidenciando a manifestação do comportamento anedônico. Esses achados promoveram de forma crescente o interesse da comunidade científica em utilizar esse modelo experimental para o estudo da DM. Transpondo para o contexto clínico, essa característica faz referência a um sintoma central da DM, a anedonia, e, portanto, fornece validade de face ao modelo experimental (154,155).

O modelo de CUMS também é amplamente utilizado como um indutor do desequilíbrio do eixo HPA e de inflamação. Um estudo conduzido por Liu e colaboradores identificou um aumento na concentração plasmática de IFN, TNF-α, e na atividade da IDO, além da diminuição nas concentrações de serotonina no CPF de ratos expostos ao CUMS (156). Ainda, um estudo de Rosseti e colaboradores demonstrou que o comportamento anedônico estava associado à presença de neuroinflamação, caracterizada pelo aumento de IL-

1, IL-6 e do marcador de ativação microglial CD11b. Essas alterações foram detectadas nos animais que demonstraram uma diminuição no consumo da sacarose, mas não nos animais resilientes (143). Recentemente, Xie e colaboradores reportaram que animais estressados com altos níveis de IL-1β e IL-6 apresentaram menores concentrações de BDNF no CPF (157). Além disso, já havia sido demonstrado que a IL-1β se comportou como um mediador do efeito antineurogênico, e que estava associada ao comportamento anedônico em ratos submetidos ao estresse agudo ou crônico (158).

Diferenças no padrão de estresse oxidativo de animais submetidos ao CUMS também tem sido descritas (159–161). Entretanto, um número limitado de estudos avaliando os parâmetros oxidativos e o estado redox em estruturas do SNC de animais sucetíveis e resilientes ao estresse foi realizado. Wang e colaboradores reportaram o aumento da oxidação proteica e da atividade da CAT, e diminuição da atividade da SOD em hipocampo e córtex de animais com comportamento anedônico em comparação a animais resilientes e controles. Ao mesmo tempo, esse estudo não identificou alterações referentes à ocorrência de peroxidação lipídica nessas estruturas (148).

Considerando os dados apresentados, conclui-se que o modelo de CUMS apresenta as três validades – de face, de construto e preditiva – requeridas, sendo representativo para o estudo da DM. Ainda, considerando a forte relação existente entre a resposta ao estresse e o desenvolvimento da DM, sugere-se que o CUMS seja uma ferramenta robusta para estudar as características e os mecanismos envolvidos na resiliência e suscetibilidade envolvendo o estresse oxidativo, a inflamação e a neurogênese.

1.4 Proteômica e biologia de sistemas na DM

Abordagens independentes de hipótese, como a proteômica e a genômica, são consideradas ferramentas robustas para identificar mecanismos envolvidos com a fisiopatologia de diversas doenças, incluindo transtornos psiquiátricos. Considerando as

modificações pós-traducionais e a provável inconsistência na regulação do processo de tradução, a proteômica parece apresentar algumas vantagens como instrumento de investigação. Devido à dificuldade de acessar amostras de líquor e de tecido cerebral em humanos, e considerando evidências que apontam para uma disfunção na permeabilidade da barreira hematoencefálica na DM – viabilizando o trânsito de proteínas entre o cérebro e a periferia – alguns estudos têm investido na avaliação proteômica a partir de sangue periférico de pacientes depressivos. Xu e colaboradores, por exemplo, identificaram alterações na expressão de proteínas envolvendo o metabolismo lipídico e imunoregulação em pacientes depressivos, reforçando o envolvimento desses mecanismos na DM (162).

Além disso, o modelo animal de CUMS também têm sido utilizado como um instrumento de estudo para avaliações proteômicas, evidenciando proteínas diferencialmente expressas em estruturas relevantes para a patogênese da DM, a partir de análises via bioinformática. Um estudo de Yang e colaboradores identificou alterações em processos biológicos envolvendo o metabolismo da glutationa e o metabolismo energético no CPF de animais expostos ao CUMS (163). Achados recentes também sugerem um prejuízo no metabolismo de aminoácidos, a desregulação do metabolismo do glutamato, alterações no metabolismo de ácidos graxos bem como a expressão anormal de proteínas relacionadas à sinapse no hipocampo de animais suscetíveis ao estresse (164), além de alterações envolvendo proteínas importantes para a neurogênese (165). Ainda, Zhou e colaboradores reportaram a alteração de proteínas relacionadas à neurotransmissão glutamatérgica e ao processo de sinapse na amigdala de ratos expostos ao estresse crônico, sugerindo que mudanças na regulação e na estrutura dessas proteínas possam estar envolvidas com o prejuízo dos mecanismos de neuroplasticidade em animais suscetíveis ao estresse (166).

2 JUSTIFICATIVA

A DM é um transtorno psiquiátrico grave e altamente incapacitante que apresenta um elevado risco de suicídio (1–3). Atualmente, estima-se que apenas metade dos pacientes atinja a remissão completa dos sintomas, havendo altos índices de recaída, principalmente durante o primeiro ano (14). Evidências indicam que a DM é uma doença heterogênea e complexa, que resulta da interação entre diversos fatores biológicos e ambientais, o que dificulta o completo entendimento de sua neurobiologia. Um aspecto consistentemente associado à depressão é a hiperativação do eixo HPA, acompanhada do aumento persistente de GCs e da alteração da resposta adaptativa ao estresse (9). Cerca de 85% dos estudos envolvendo a DM reportam resistência aos GCs e exacerbação da sinalização inflamatória nos pacientes (167).

Ainda, condições adversas podem gerar um desequilíbrio no estado redox celular. Desse modo, sugere-se que uma não adaptação das células frente a essas alterações, com subsequente morte celular e danos provocados pelos mediadores inflamatórios, estejam relacionados com a neuroprogressão da DM (88,119). A alteração de marcadores de estresse oxidativo e de inflamação parece afetar parâmetros neurotróficos como o BDNF, o que pode estar relacionado à redução de neuroplasticidade associada à DM (89,118,131). No entanto, ainda não está claro como a susceptibilidade ao estresse crônico e a anedonia estão associadas a essas alterações.

O CUMS é um modelo amplamente utilizado para promover o desequilíbrio do eixo HPA e a inflamação, além de ser capaz de reproduzir o paradigma de resiliência e vulnerabilidade ao estresse, induzindo a manifestação do comportamento anedônico em animais suscetíveis (142–145). Nesse sentido, visando compreender melhor a relação entre os componentes já descritos na patogênese da DM e a resposta ao estresse, se faz necessário estudar os efeitos do estresse crônico sobre o comportamento, parâmetros oxidativos e antioxidantes, bem como sobre as concentrações de BDNF em animais submetidos ao CUMS.

Ainda, considerando a complexidade dos mecanismos envolvidos no comportamento anedônico, torna-se válido executar através da bioinformática, a avaliação de bancos de dados de proteômica provenientes de estudos com protocolos semelhantes ao desse experimento. Nesse sentido, informações referentes ao enriquecimento de processos biológicos envolvendo estresse oxidativo e inflamação, além da identificação de proteínas diferencialmente expressas em animais anedônicos, podem ser úteis para corroborar os resultados experimentais do presente estudo e sugerir mecanismos envolvidos com a resposta ao estresse.

3 OBJETIVOS

3.1 Objetivo geral

Investigar os níveis de BDNF, a ocorrência de dano oxidativo e a capacidade antioxidante total em animais submetidos ao protocolo de CUMS.

3.2 Objetivos específicos

• Avaliar o comportamento tipo-depressivo utilizando os testes de preferência por sacarose, nado forçado e campo aberto;

• Classificar os animais em anedônicos ou não-anedônicos de acordo com alterações na preferência por sacarose;

• Verificar a relação entre o peso da adrenal e a massa corporal nos diferentes grupos experimentais;

• Avaliar a peroxidação lipídica, a carbonilação de proteínas e a capacidade antioxidante total no CPF e hipocampo dos diferentes grupos experimentais;

• Mensurar a concentração de BDNF hipocampal nos diferentes grupos experimentais;

• Analisar bancos de dados de proteômica de estudos com protocolo experimental semelhante, e identificar proteínas envolvidas com o estresse oxidativo e com a sinalização da resposta inflamatória diferencialmente expressas em animais anedônicos;

• Relacionar a resposta ao estresse com os desfechos avaliados.

PARTE II

4 ARTIGO

4.1 Carta de submissão

14/03/2019 Email – Ellen Scotton – Outlook

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Submission no: BBI_2019_225
Submission title: The protective role of BDNF and PRDX-1 against central oxidative damage in anhedonic rats submitted to a chronic unpredictable mild stress protocol
Corresponding author: Dr Mauricio Kunz
Listed co-author(s): Professor Rafael Colombo, Professor Adriane Rosa, Miss Fernanda E. Valiati, Dr Tiago F. Lopez, Miss Giovana Bristot, Miss Alessandra E. Guerra, Mr Gabriel H. Hizo, Miss Gabriela M.P. Possebon, Miss Ellen Scotton, Miss Tuani M. Silva, Dr Mirian Salvador, Miss Jéssica C. Reis, Miss Luiza P.Géa

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4.2 Manuscrito

Title page

Title: The protective role of BDNF and PRDX-1 against central oxidative damage in anhedonic rats submitted to a chronic unpredictable mild stress protocol.

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Abstract

Chronic activation of the HPA axis and sustained increase of glucocorticoids have been associated in major depression, and also related to changes involving neurotrophins and oxidative stress markers in response to inflammation. This study aimed to evaluate central measures of brain-derived neurotrophic factor (BDNF), oxidative damage and total antioxidant capacity of rats submitted to chronic unpredictable mild stress (CUMS) and investigate the relationship between BDNF levels and differentially expressed proteins involved in oxidative stress and inflammatory biological processes. Wistar male rats were subjected to CUMS for 6 weeks. Based on the sucrose preference test, animals were divided into anhedonic and non-anhedonic clusters. After brain tissue collection, oxidative damage, total antioxidant capacity, and BDNF levels were evaluated. In order to extend discussion of possible mechanisms involving neurobiological findings, a bioinformatics approach was performed to identify proteins involved with oxidative stress and inflammation pathways that were differentially expressed in anhedonic animals from other studies with similar experimental protocol. CUMS was associated with an increase in BDNF concentrations accompanied by a decrease in total antioxidant capacity, besides the absence of oxidative damage to lipids and proteins. Withal, bioinformatics proteomic approach indicated that anhedonic animals showed a peroxiredoxin-1 (PRDX-1) up-regulation and a down-regulation of proteins involved with apoptotic and inflammation signaling (RELA, ASK-1 and TAK-1) in the hippocampus. These evidences suggest that BDNF and PRDX-1 may represent an initial response against stress, with a compensatory role, preventing oxidative damage to lipids and proteins through the modulation of antioxidant defense, mainly in anhedonic animals.

Keywords

Major depression; anhedonic behavior; CUMS; sucrose preference; BDNF; PRDX-1; oxidative stress; proteomics

Highlights

- Increased BDNF levels, but no oxidative damage in the hippocampus from animal exposed to the CUMS.
- Anhedonic rats show PRDX-1 up-regulation and RELA, ASK-1 and TAK-1 down-regulation.
- BDNF and PRDX-1 may have a compensatory role and attenuate oxidative damage.

1. Introduction

Major depressive disorder (MDD) is a severe, chronic, highly disabling psychiatric disorder often associated with comorbidities, besides presenting a high risk for suicide (Buckner et al., 2019; Cameron et al., 2014; Lépine and Briley, 2011). Anhedonia is a central hallmark of MDD, which is characterized by lack of pleasure and loss of reactivity to positive stimuli (Nasca et al., 2015; Otte et al., 2016). MDD symptoms lead to impairment in functioning and decrease in quality of life, representing an important outgoing in public health costs (Ferrari et al., 2013; Luppa et al., 2007).

According to the World Health Organization (WHO), MDD is considered the primary cause of disability in the population. By the year 2020, MDD is estimated to rank second place among the diseases with the highest global prevalence ("WHO | Depression and Other Common Mental Disorders," n.d.). Evidence indicates that the biological bases of MDD include interactions between genetic, epigenetic, biochemical and environmental factors, as well as hormonal changes related to stress response. Both oxidative stress and enhancement of the inflammatory signal play a role in the susceptibility to stress. However, the way these factors are orchestrated and interact is not well elucidated yet (Otte et al., 2016).

Most individuals exhibit an effective adaptive response to stress, which characterizes them as resilient. In contrast, others demonstrate a limited ability to coping with stress, presenting maladaptive responses that lead to stress-related diseases, such as MDD (Hjemdal et al., 2011; Wang et al., 2014). Under an acute stress response, glucocorticoids (GCs) exhibit immunosuppressive and anti-inflammatory properties (Liu et al., 2017). In turn, chronic stress significantly increases the peripheral levels of pro-inflammatory cytokines, which intensifies cortisol release, promotes desensitization of the hypothalamic-pituitary-adrenal (HPA) axis and enhance oxidative stress (Pariante and Miller, 2001; Raedler, 2011).

Persistent activation of the HPA axis and a sustained increase of GCs have been described in MDD (Hasler et al., 2004; Ising et al., 2007). Recently, Horowitz and colleagues have shown that about 85% of MDD studies report resistance to GCs and exacerbation of inflammatory signaling in depressive patients (Horowitz and Zunszain, 2015). Stress and augmented GCs concentration are associated with increased production of reactive oxygen species (ROS) and oxidative stress (Bjelaković et al., 2007; Flaherty et al., 2017; You et al., 2009). Several studies have already identified elevation in serum levels of malondialdehyde (MDA) in patients with MDD (Islam et al., 2018; Khajehnasiri et al., 2013; Mazereeuw et al., 2015). Also, a recent meta-analysis showed that increased lipid peroxidation and DNA oxidation are the most consistently stress markers elevated in MDD, usually with small to moderate effect sizes (Black et al., 2015). Moreover, antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) present alterations in their activity, and increased activities of both enzymes might be indicators of acute depressive episodes in MDD patients (Tsai and Huang, 2016).

Peroxiredoxins (PRDX), in turn, are antioxidant enzymes that act by decreasing the substrates used in hydrogen peroxide (H_2O_2) formation and modulating intracellular signaling (Antunes and Brito, 2017; Sies et al., 2017; Won et al., 2012). Findings showed decreased PRDX-6 levels in rodents exposed to corticosterone treatment, and this reduction was associated with impairments in cell morphology and neuronal death in the cerebral cortex (Skynner et al., 2006). In addition to modulation in the antioxidant enzymes activity, a postmortem study has reported lower GSH concentrations in the PFC of individuals with MDD (Gawryluk et al., 2011). A recent meta-analysis conducted by Liu and colleagues identified reduced levels of total antioxidant capacity (TAC) and increased levels of free radicals as well as oxidative damage products in depressive patients when compared to controls (Liu et al., 2015).

Beyond to damage biomolecules, ROS act as mediators in signal transduction of inflammatory pathways involving nuclear factor kappa B (NF-κB). Furthermore, immune system activation and increased oxidative stress induce a cascade of events orchestrated by transcription factors such as the nuclear factor derived from erythroid 2 (Nrf2) and NF-κB, revealing synergistic effects on the pathogenesis of MDD. At higher levels of oxidative stress, NF-*κ*B is activated and depending on the cellular context inflammatory cascades, pro-oxidant or antioxidant genes can be triggered. At low concentrations, ROS activate Nrf2 and stimulate the transcription of genes with antioxidant function, including the expression of antioxidant enzymes (Bakunina et al., 2015). BDNF is a neurotrophin that induces complex neuronal signaling cascades critical for cellular changes underlying synaptic plasticity. Also, a recent study showed that BDNF play a crucial role as an inducer of neuronal antioxidant responses inducing Nrf2 nuclear translocation (Bruna et al., 2018).

An increase in oxidative stress and inflammation are usually associated with cognitive impairment and are considered to be one of the potential mechanisms involved with neuroprogression and early aging in mood disorders (Maurya et al., 2016). A crescent body of evidence indicates that the excessive production of ROS and reduced antioxidant capacity lead to an increase in oxidative stress and may be responsible for neuronal changes inducing cell death, thus promoting the atrophy of specific regions involved in behavior (Michel et al., 2012). Therefore, we aim to evaluate central measures of BDNF, oxidative damage and antioxidant capacity of rats submitted to chronic unpredictable mild stress (CUMS), and investigate the relationship between BDNF levels and differentially expressed proteins involved in oxidative stress and inflammatory biological processes according to the different patterns of stress-susceptibility.

2. Materials and methods

2.1. Animals

Thirty-five male Wistar rats (45-day-old, 220-250g) were obtained from the Central Animal House of the Universidade Federal do Rio Grande do Sul, Porto Alegre, state of Rio Grande do Sul, Brazil. They were housed singly in standard polycarbonate rat cages under standardized environmental conditions: 12h light/dark cycle (lights on between 7:00 a.m. and 7:00 p.m.), controlled temperature (22 ± 1ºC), and food and water available ad libitum. All experimental procedures were approved by the ethics committee for animal research of the Hospital de Clínicas de Porto Alegre (protocol 150353) and were carried out following the eighth edition of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals and their suffering.

2.2. Sucrose preference test (SPT)

After two weeks of laboratory habituation and housing conditions, the animals were exposed to water and 1% sucrose solution in eight baseline tests conducted twice a week in their home cage. After 12h of food and water deprivation, two bottles, one with 1% sucrose solution and another with water, stayed available for animals during 1h. Bottles were weighed before and after the test to evaluate sucrose intake. All analyses were performed half an hour after the beginning of the dark cycle. Based on sucrose preference rates in the final baseline test, animals with unstable and/or low basal sucrose preference (under 60%) were excluded. The remaining animals were divided into two paired groups: control (n= 7) and "to-be-stressed" (n= 16). The reduced sucrose preference, which is used as an index of anhedonia, was calculated according to the following formula: sucrose consumption/[water consumption + sucrose consumption] ×100%. Subsequently, sucrose preference was monitored once a week, under similar conditions, until the end of the study.

2.3. CUMS

CUMS protocol was based on previous studies (Willner, 2017). The control animals were housed and handled in a separate room and had no contact with the stressed group. They were deprived of food and water during 12 h before each sucrose test, but otherwise, food and water were freely available. The CUMS group was daily exposed to different stressors during six weeks. Based on most common components of the CUMS regime, the stress protocol consisted in food and water deprivation (24h), tail pinch (1min), cage tilt (24h), the light-dark reversal (24h), overcrowding (2h), immobilization (2h) and wet bedding (24h) (Fig. 1). Also, all animals were weighted on the first day of CUMS and after the last stressor, at the end of the protocol.

2.4. Anhedonic and non-anhedonic clusters

After six weeks of CUMS, sucrose preference test was the first behavioral outcome evaluated. Based on Nasca and colleagues, following statistical approach was used for the assignment of animals subjected to CUMS in anhedonic and non-anhedonic clusters, according to their sucrose preference. Animals which fell into the standard deviation from the mean of the control group were considered non-anhedonic since they showed a behavior similar to unstressed rats. Animals that fell outside the standard deviation of the mean of the control group were designated as anhedonic (Nasca et al., 2015).

2.5. Open field test (OFT)

An open-field test was used to assess locomotor activity. The test was performed in a 77 cm diameter field enclosed by a 50 cm high acrylic wall with an open top. The dark floor of the arena was divided into quadrants, and its internal space was empty. Individually and randomly, the animals were placed at the center of the apparatus, without previous habituation, to explore the open field for 5 min. The device was continuously cleaned with alcohol between the tests. We used a video camera connected to a computer to record the

behavior of each animal. The videos were analyzed using the ANYmaze behavioral tracking software, which provided the number of crossings and total distance traveled by each animal.

2.6. Forced swim test (FST)

FST test was performed as described previously by Porsolt, with minor modifications (Porsolt et al., 1977). In brief, rats were placed individually in a black cylinder (height: 50 cm, diameter: 30 cm) filled with 30 cm of water at 25°C. In this cylinder, rats may not touch the bottom or escape. In the first exposure, the training session, rats were placed on the water for 15 min of forced swimming. Then, 24h later rats were placed on the cylinder again for 5 min, to perform the test session. We used a video camera connected to a computer to record the behavior of each animal. The videos were analyzed using the Boris software (Friard and Gamba, 2016), which provide total duration (seconds) of immobility. Water in the tank was changed after every five sessions.

2.7. Euthanasia and tissue collection

Post-behavioral analysis, rats were euthanized by decapitation and brain were dissected. The brain tissue were immediately frozen on dry ice in eppendorf tubes and then stored at −80 °C for further analyses. PFC and one hemisphere of the hippocampus were used for determination of oxidative lipid and protein damage and trolox equivalent antioxidant capacity (TEAC). Furthermore, BDNF measurement was performed using the other hippocampus hemisphere, and adrenal glands were collected and weighted nearly after euthanasia.

2.8. Determination of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was monitored by the formation of TBARS during an acid-heating reaction, according to a protocol adapted from Wills (Wills, 1966). Specifically, 400 µL of supernatant from each sample was combined with 600 µL of 15% trichloroacetic acid and

0,67% thiobarbituric acid. The mixture was heated at 100°C for 15 min. After cooling to room temperature (RT), the samples were centrifuged at 5200 xg for 5 min. The supernatants were isolated, and their absorbance was measured at 530 nm. Hydrolyzed 1,1,3,3-tetramethoxypropane (TMP) was used as a standard, and the results were expressed as nmol MDA/mg. Total protein levels were evaluated using the Bradford method (Bradford, 1976).

2.9. Determination of protein carbonyl

Oxidative damage to proteins was measured based on the reaction of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1990). For the assay, 200 µL of DNPH (10 mM) or 200 µL of HCl (2 M) were added to 50 µL of supernatants. The reaction mixture was incubated in the dark for 30 min, and vortexed every 10 min. 250 µL of 20 % trichloroacetic acid was added to each reaction mixture and centrifuged at 3300 xg for 10 min. The supernatants from each sample were discarded, and the pellets were washed three times with ethanol-ethyl acetate (1:1) to remove free reagents. The samples were centrifuged, and the pellets were resuspended in 1000 µL of urea solution (8 M) at 37 °C, during 15 min. Absorbance was read at 365 nm, and the results were expressed as nmol DNPH/mg. Total protein levels were evaluated using the Bradford method (Bradford, 1976).

2.10. Determination of TEAC

The screening of TAC was performed by TEAC method, through the ability of the sample to scavenging the radical $ABTS^{+}$ [2,2-azino-bis (3-etilbenzotiazolin) -6-sulfonic acid]. The $ABTS^{+}$ solution is formed from the reaction of 7 mM ABTS with 2.45 mM potassium persulphate. This solution was kept in the dark at RT for 12-16 h before use. The solution ABTS • + was diluted with 5mM phosphate buffer saline (PBS pH 7.4) until an absorbance of 0.700 ± 0.35 at 734 nm. Then, 1.0 mL of ABTS • + diluted solution was added to 10 µL of sample (Re et al., 1999). The absorbance was read exactly 6 minutes after the initial mixture. For quantification, a standard curve was used with Trolox solution, and the results were

expressed in µmol TEAC/mg. Total protein levels were evaluated using the Bradford method (Bradford, 1976).

2.11. Determination of BDNF protein levels

BDNF serum levels were determined by sandwich-ELISA using monoclonal antibodies specific for BDNF (R&D Systems, USA). Briefly, microtiter plates (96-well flat-bottom) were coated overnight at RT with monoclonal anti-BDNF antibody at 4 µg/mL in phosphate-buffered saline (PBS). After that, plates were washed three times with wash buffer and blocked with PBS containing 5 % nonfat milk powder for 1 hour at RT. After washing, plates were incubated two hours at RT with the samples diluted 1:5 in sample diluent (PBS with 1 % bovine serum albumin - BSA) and the standard curve ranged from 15.63 to 1,000 pg/mL of BDNF. Plates were washed, and biotinylated anti-BDNF antibody at 0.2 µg/mL in PBS was added, which was incubated for 2 hours at RT. After washing, incubation with streptavidin-peroxidase conjugate (diluted 1:1,000 in sample diluent) for 1 hour at RT was performed, and subsequently, plates were rewashed and incubated with the substrate (3,3′,5,5′-Tetramethylbenzidine) for 20 minutes at room temperature. Finally, the stop solution was added, and the amount of BDNF was determined by measuring absorbance at 450 nm. Total protein levels were evaluated using the Bradford method (Bradford, 1976). The standard curve demonstrates a direct relation between optical density and BDNF concentration and the results were expressed in pg/mg of total protein.

2.12. Proteomics analyses: a bioinformatic approach

Studies on chronic stress were selected based on their methodology similarity and ability to identifying groups of animals that were susceptible to stress. The following process was performed: (1) "chronic mild stress" AND "proteomics" keywords retrieved 39 articles on PubMed; (2) only 7 studies performed proteomic evaluation in the hippocampus of rats submitted to chronic mild stress; and finally (3) 3 studies had similar methodology and used

bioinformatic approaches to evaluate their data. All studies used sucrose consumption to classify animals in anhedonic and non-anhedonic, using the same rationale and methods for this outcome. Input data was prepared using all differentially expressed proteins (DEP) in the three works (Han et al., 2015; Henningsen et al., 2012; Zhang et al., 2018).

2.13. Statistical analyses

Statistical analyses of experimental outcomes were performed using SPSS 18.0 version and *p*-values < 0.05 were considered statistically significant. The results were checked for normality by Shapiro-Wilk test and analyzed with one-way ANOVA followed by Bonferroni's test for post-hoc comparisons. In proteomic analyses, to all DEP, we assigned the fold change values of +/- 1 and *p*-value < 0.05, only to use them in the enrichment analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) results and Gene Ontology Biological Process pathways were rechecked and plotted using the R software environment v.3.4.4 (Team, 2013) and the R package pathfindR v.1.2.1 (Ulgen et al., 2018) considering as significantly enriched pathways those with FDR < 0.05 in the function run_pathfindR.

3. Results

3.1. Effects of CUMS in behavioral assessment

Behavior outcomes are exposed in Fig. 2. Anhedonia is a hallmark symptom of MDD that can be evaluated in rodents through a decrease in the sucrose preference compared to water in the SPT. Remarkably, anhedonic rats showed a significant reduction in sucrose preference after CUMS compared to non-anhedonic and control groups ($p< 0.001$). On the other hand, locomotor activity in OFT and total immobility time in FST remained unchanged between all groups.

3.2. Body weight and adrenal glands relative weight

The following morphometric variables are described in Fig. 3. Body weight was recorded in the beginning and in the end of the CUMS period. The control group presented a higher weight gain compared to animals exposed to stress, both anhedonic (p= 0.004) and non-anhedonic (p= 0.001). Furthermore, anhedonic rats had significant higher adrenal relative weight in comparison to the control group (p= 0.014).

3.3. Oxidative parameters

Assessment of TBARS, protein carbonyl and TEAC in PFC and hippocampus of anhedonic, non-anhedonic and control groups are given in Table 1. Lipid peroxidation and protein carbonylation did not present differences between groups. However, lower TEAC levels were found in the PFC and hippocampus, respectively, of anhedonic (p= 0.00 and p= 0.006) and non-anhedonic (p= 0.026 and p= 0.005) animals compared to controls.

3.4. BDNF levels

Hippocampus BDNF protein levels among groups are reported in Fig. 4. One-way ANOVA test revealed significant differences between CUMS exposed animals and control group. In particular, the anhedonic group showed an augmentation of 1,4-fold (p= 0. 001) and the non-anhedonic group presented an increase of 1,2-fold (p= 0.009) in BDNF protein compared to controls.

3.5. Proteomics findings

Interestingly, differently expressed groups of proteins were observed when incorporating all data, with no intersection among studies (Han et al., 2015; Henningsen et al., 2012; Zhang et al., 2018). The biological processes enriched in the studies are indicated in Fig. 5. Thus, Map3k5 (apoptosis signal-regulating kinase 1(ASK-1)), Map3k7 (transforming growth factor beta-activated kinase 1 (TAK-1)) and RELA (transcriptional factor p65 known as NF-κB p65

subunit) were down-regulated, while PRDX1 was up-regulated in the hippocampus of animals presenting anhedonic behavior (see Table 2).

4. Discussion

The findings of our study indicate that CUMS protocol was associated with an increase in hippocampus BDNF concentrations accompanied by a decrease in TEAC, despite the absence of oxidative damage to lipids and proteins. Withal, bioinformatics proteomic approach indicated that anhedonic animals show a PRDX-1 up-regulation and RELA, ASK-1 and TAK-1 down-regulation in the hippocampus. Taken together, these data suggest that, mainly in animals with anhedonic behavior, BDNF may have a compensatory role, attenuating neuronal damage through the modulation of enzymatic antioxidant activity.

A same stressful event may be more or less significant for an individual according to previous experiences and with their vulnerability, even in identical twins or isogenic rodents (Fraga et al., 2005; Freund et al., 2013). It is generally thought that chronic stress can lead to depressive-like behavior in animals. Indeed, reduced sucrose preference is an indicator of anhedonic-like behavioral change, the core symptom of MDD (Antoniuk et al., 2019; Freund et al., 2013). Our findings demonstrate that CUMS decreased sucrose preference in rats susceptible to stress-induced anhedonic behavior, in association to a lower body weight gain and an increase of relative adrenal glands weight compared to controls, which is in accordance with previous findings (Lucca et al., 2008; Vollmayr and Henn, 2003; Zhang et al., 2014). In contrast, no changes in immobility time and locomotor activity were found.

Ulrich and colleagues have shown that an increased adrenal weight after chronic stress is due to hyperplasia and hypertrophy in specific adrenal subregions, which is associated with increased maximal corticosterone responses to HPA axis (Ulrich-Lai et al., 2006). GCs are the crucial hormones involved in stress adaptation because of their role in feedback regulation on the functioning of the HPA axis. Therefore, corticosterone expression can be regarded as a

signal of stress injury during exposure to a stressor. Usually, chronic stress is associated with impairment in HPA axis response, leading to persistent higher GCs levels and decreased levels of BDNF (McEwen, 2006; Zhang et al., 2014).

Interestingly, although our findings indicate that stress-induced hypertrophy in adrenal glands - mainly in anhedonic rats - an increase in hippocampal BDNF was found. Previous studies showed that acute stress with increased plasma corticosterone levels was accompanied by a high expression of BDNF and tropomyosin receptor kinase (TrkB) in the hippocampus (Marmigère et al., 2003; Shi et al., 2010). Furthermore, Adlard and colleagues reported that a chronic immobilization protocol resulted in a significant increase in hippocampal BDNF, as well as intermittent immobilization and chronic cold stress also demonstrated the same trend (Adlard et al., 2004). Taken together, these findings suggest that augmented BDNF levels may be part of a compensatory response to preserve hippocampal homeostasis, or a way of neuronal plasticity to cope with stressor stimuli.

Moreover, in addition to the stressed animals present increased levels of BDNF compared to controls, the anhedonic rats showed slightly higher levels than the non-anhedonic ones, which may point a more pronounced biological response in these animals, even though there was no statistic difference between groups. BDNF is a neurotrophin involved in central nervous system (CNS) homeostasis, as well as in synaptic transmission and plasticity, playing an essential role in survival, maintenance, and growth of neurons (Bathina and Das, 2015; Lipsky and Marini, 2007; Ninan, 2014; Yamada et al., 2002). In addition, BDNF modulates the expression of a range of other genes, interacting with neurotransmitter systems such as glutamatergic (Carvalho et al., 2008), dopaminergic (Berton et al., 2006) and serotonergic (Martinowich and Lu, 2008; Pezawas et al., 2008).

Neurotrophins such as BDNF, can also contribute to neuronal cell protection against oxidative stress (Ichim et al., 2012). As mentioned before, BDNF can induce Nfr2 nuclear

translocation, promoting the transcription of antioxidant genes and leading to protective effects against ROS (Bruna et al., 2018). Using differentiated rat pheochromocytoma cells (PC12) with neuron-like characteristics, Ogura and colleagues reported that subtoxic levels of oxidative stress induced by H_2O_2 stimulated BDNF expression, supporting the involvement of a sensitive mechanism to ROS underlying BDNF neuroprotective effects (Ogura et al., 2014). In this line, our results identified the absence of oxidative lipid and protein damage in animals exposed to CUMS. Although, a decrease in TEAC was detected in the PFC and hippocampus of these animals, which is in accordance with clinical findings that showed reduced TAC in acute episodes of depressed patients (Liu et al., 2015), probably indicating a rapid response against modifications in the redox state. Nevertheless, the TEAC assay measures the antioxidant capacity provided by non-enzymatic compounds (e.g.., glutathione), based on redox chemical reactions, usually excluding the evaluation of enzymatic activities (Bartosz, 2010).

Interestingly, based on bioinformatics approach, it was evidenced that some enriched biological processes include H_2O_2 pathways (Fig. 5). At the same time, up-regulation of PRDX-1 was found in anhedonic animals (Table 2). In accordance, Palmfeldt and colleagues recently demonstrated that PRDX-1 and PRDX-2 were down-regulated in resilient animals compared to anhedonic animals, possibly indicating that resilient animals were submitted to lower oxidative stress (Palmfeldt et al., 2016). It has been proposed that PRDX reduce more than 90% of cellular H_2O_2 (Adimora et al., 2010; Cox et al., 2009). Thus, their central role as peroxide scavenging enzymes in the cellular arsenal of antioxidant enzymes, such as CAT and glutathione peroxidase (GPx), has been recently recognized (Knoops et al., 2016; Perkins et al., 2015). Considering that PRDX-1 is highly sensitive to H_2O_2 and that BDNF may also mediate antioxidant effects, it may be suggested that up-regulation of PRDX-1 along with the increase of BDNF, might represent an adjacent mechanism in the modulation of moderate

levels of oxidative stress, preventing oxidative damages such as lipid peroxidation and protein carbonylation. According to STICH: chemical association networks ("STITCH network view," n.d.), the combined score for interactions between H_2O_2 and BDNF is high, meaning that these two biomolecules have a great biological interaction (Fig. 6).

In addition to up-regulation of PDRX-1 indicated by proteomic analyses, it was found downregulation of ASK-1 and TAK-1, kinases involved in signaling for cell apoptosis, and RELA, a subunit of NF-κB, in anhedonic animals (Table 2). At low levels of oxidative stress, Nrf2 is activated (even through BDNF) and initiates transcription of antioxidative genes (e.g., antioxidant enzymes), leading to cytoprotective effects. At higher levels of oxidative stress, NF-κB is activated, and depending on the cellular context, can activate inflammatory cascades, pro-oxidant or antioxidant genes (Bakunina et al., 2015). Anti-apoptotic proteins are also suggested to be necessary for physiological functioning of neurons and synapses as well as for resilience to stress. Shishkina and colleagues found that both short-term stress and acute glucocorticoid exposures induce anti-apoptotic proteins expression (e.g., Bcl-2) and may reflect an adaptive attempt of the brain to reduce potential apoptotic effects of these treatments on neuronal cells (Shishkina et al., 2015).

Based on our results, we may suggest that CUMS might have induced the generation of ROS in low levels, activating sensitive antioxidant responses through increased BDNF levels and PRDX-1 up-regulation instead of activating apoptotic pathways, which is supported by down-regulation of ASK-1, TAK-1, and RELA. It has been reported that unpredictable or acute stress tends to provide an excited state with augmented expression of BDNF, contributing to protection against stress (Marmigère et al., 2003; McEwen, 2006). Thus, BDNF and GCs appear to oppose each other, with BDNF reversing the excitability in hippocampal neurons induced by stress levels of corticosterone. However, it does not mean that these pathways would remain inactive if exposure to stress were more persistent. With

stress' maintenance, if the excitation is not attenuated, a decrease in BDNF expression may occur (Shi et al., 2010). Moreover, Hiroshi and collegues showed that glucocorticoid receptor (GR) interacts directly with TrkB and persistent exposure to GCs reduces this interaction, decreasing BDNF/TrkB signaling and, consequently, collaborating to the impairment of neurotrophic effects (Kunugi et al., 2010).

5. Conclusion

To sum up, the absence of oxidative damage to lipids and proteins, as well as the decreased TEAC concentration and down-regulation of ASK1, TAK1 and RELA may suggest that even in animals with an anhedonic behavior, structural damage exerted by oxidative stress and inflammation in response to activation of HPA axis and increased corticosterone, may be attenuated by a compensatory response via increased BDNF and up-regulation of PRDX-1.

BDNF and PRDX-1 seem to be sensitive biomarkers to ROS (e.g., H_2O_2), and they might represent an initial response to stress to the maintenance of redox homeostasis, preventing oxidative damage to lipids and proteins, mainly in anhedonic rats. Nonetheless, further investigation is needed to elucidate the relationship among the stress response and biological compensatory mechanisms involving coping to chronic stress.

6. Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgements

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Figures

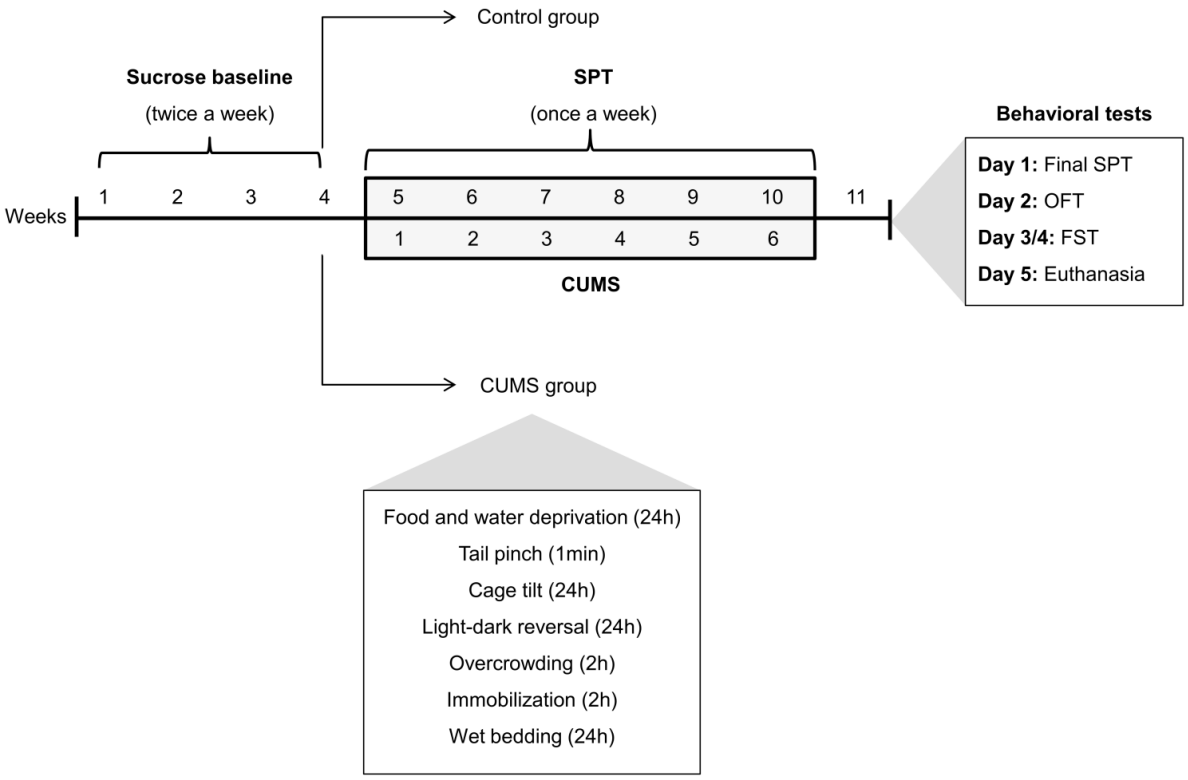

Fig.1. Schematic representation of the experimental procedures. CUMS, chronic unpredictable mild stress; FST, forced swim test; OFT, open field test; SPT, sucrose preference test.

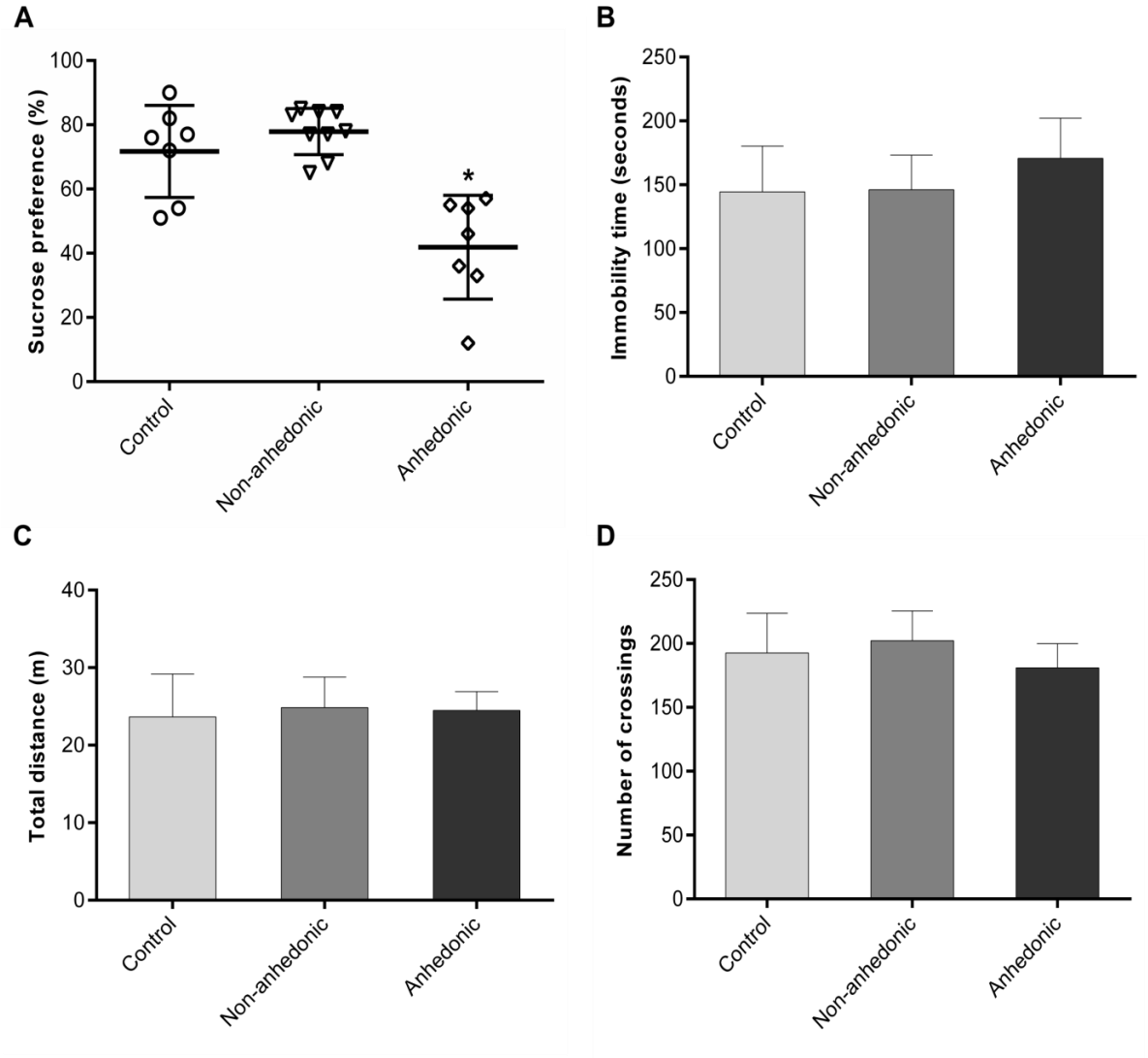

Fig. 2. Effects of CUMS on behavior assessments. (A) Sucrose preference test was performed to assign anhedonic and non-anhedonic clusters after six weeks of CUMS, according to sucrose preference rate. (B) Total immobility time was assessed by forced swim test, and the open-field test indicates (C) total distance traveled and (D) number of crossings. All values are shown as mean ± SD. Groups: anhedonic (n= 7), non-anhedonic (n= 9) and control (n= 7). *p≤ 0.001 according to one-way ANOVA followed by Bonferroni's post-hoc test. CUMS, chronic unpredictable mild stress; SD, standard deviation.

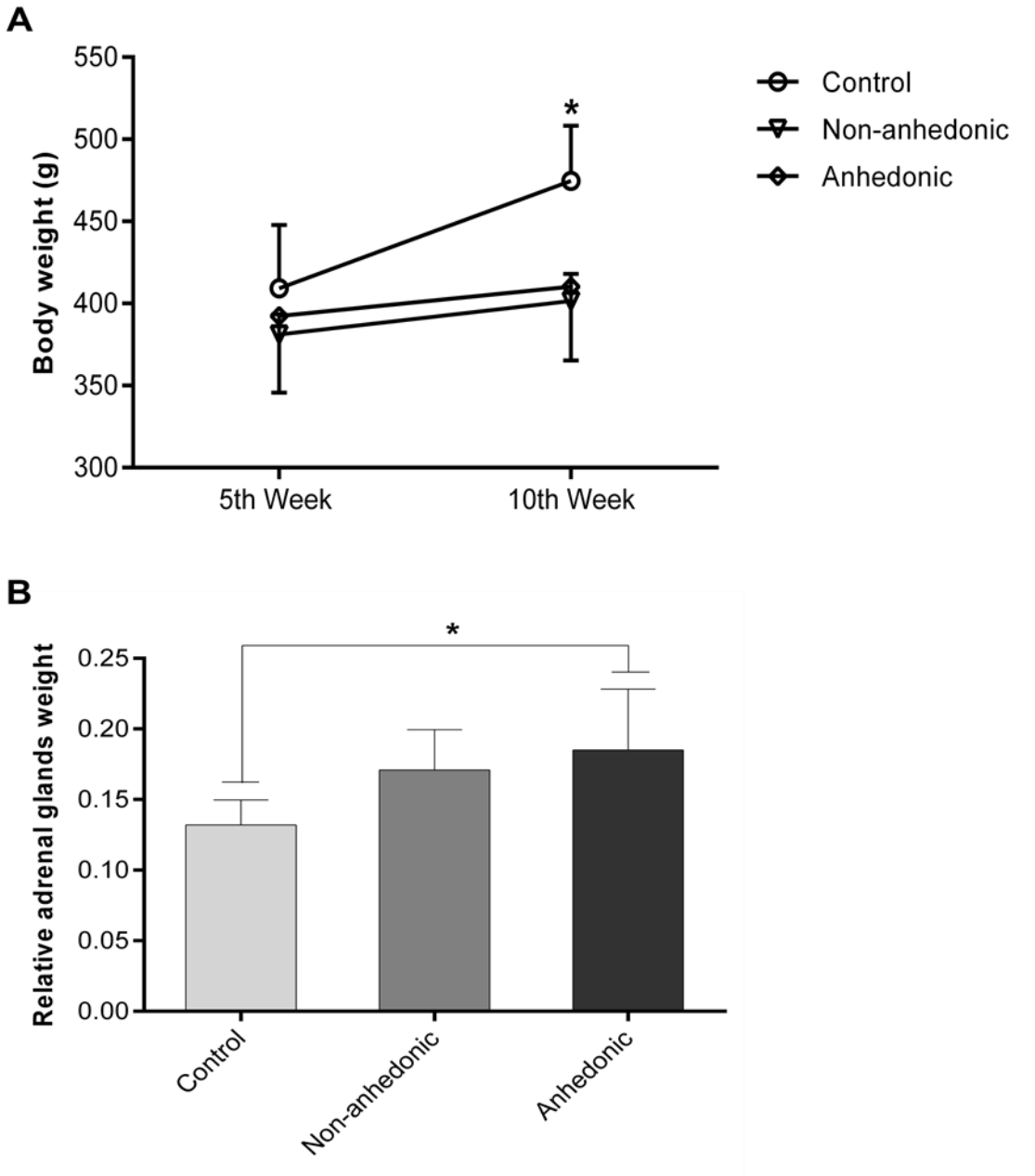

Fig. 3. Effects of CUMS in morphometric variables. (A) Animals' body weight before and after CUMS protocol and (B) relative adrenal glands weight (adrenals weight/body weight) among the groups. All values are shown as mean ± SD. Groups: anhedonic (n= 7), non-anhedonic (n= 9) and control (n= 7). *p≤ 0.01 or **p≤ 0.001 according to one-way ANOVA followed by Bonferroni's post-hoc test. CUMS, chronic unpredictable mild stress; SD, standard deviation.

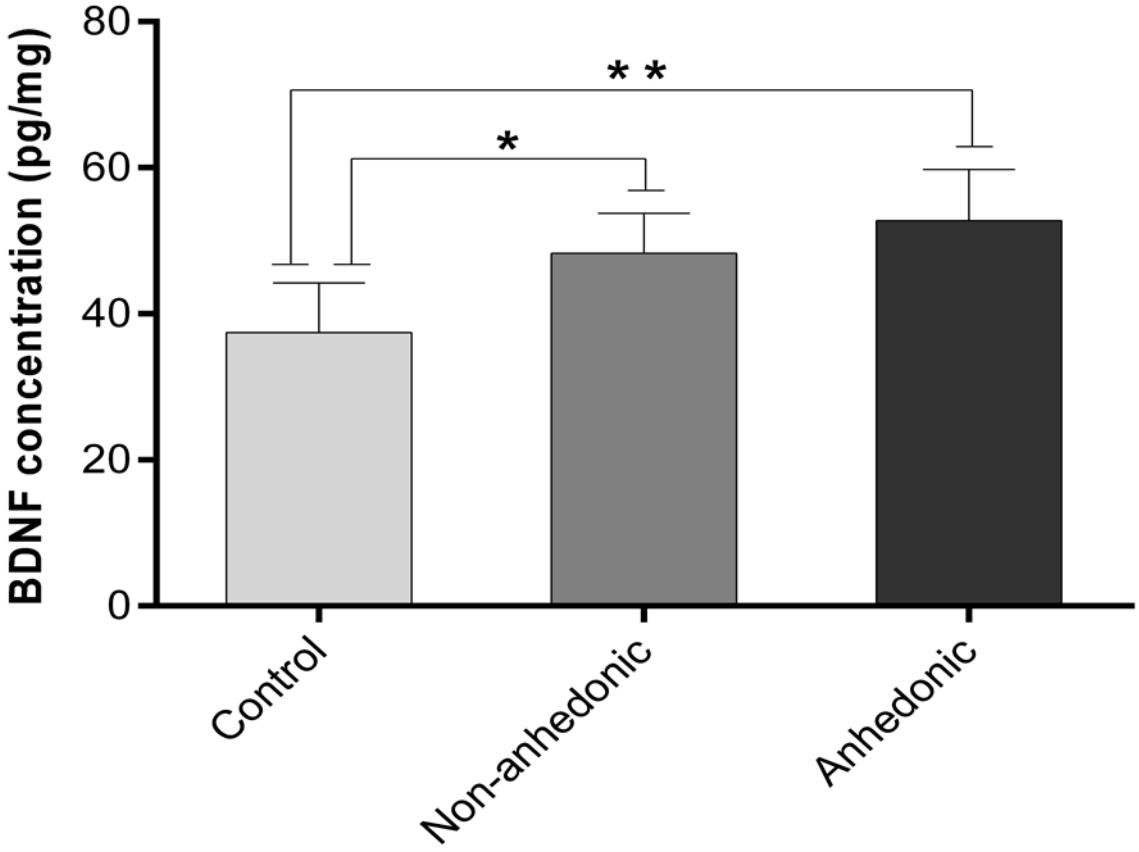

Fig. 4. Effects of CUMS in BDNF protein levels in the hippocampus. All values are shown as mean ± SD. Groups: anhedonic (n= 7), non-anhedonic (n= 9) and control (n= 7). *p≤ 0.01 or **p≤ 0.001 according to one-way ANOVA followed by Bonferroni's post-hoc test. CUMS, chronic unpredictable mild stress; SD, standard deviation.

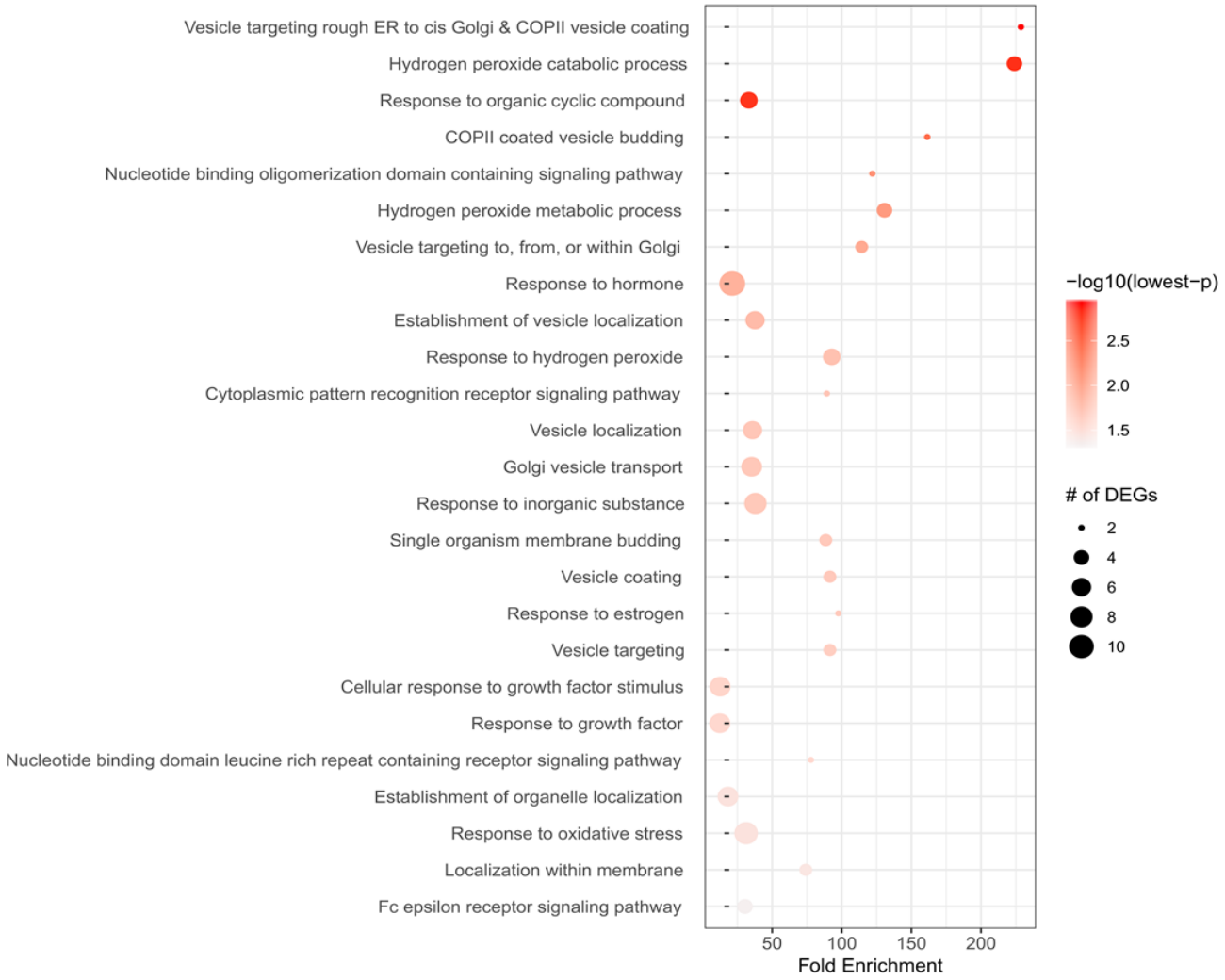

Fig. 5. Enrichment analysis by pathfindR - all pathways-GO-BP. DEGs, differentially expressed genes.

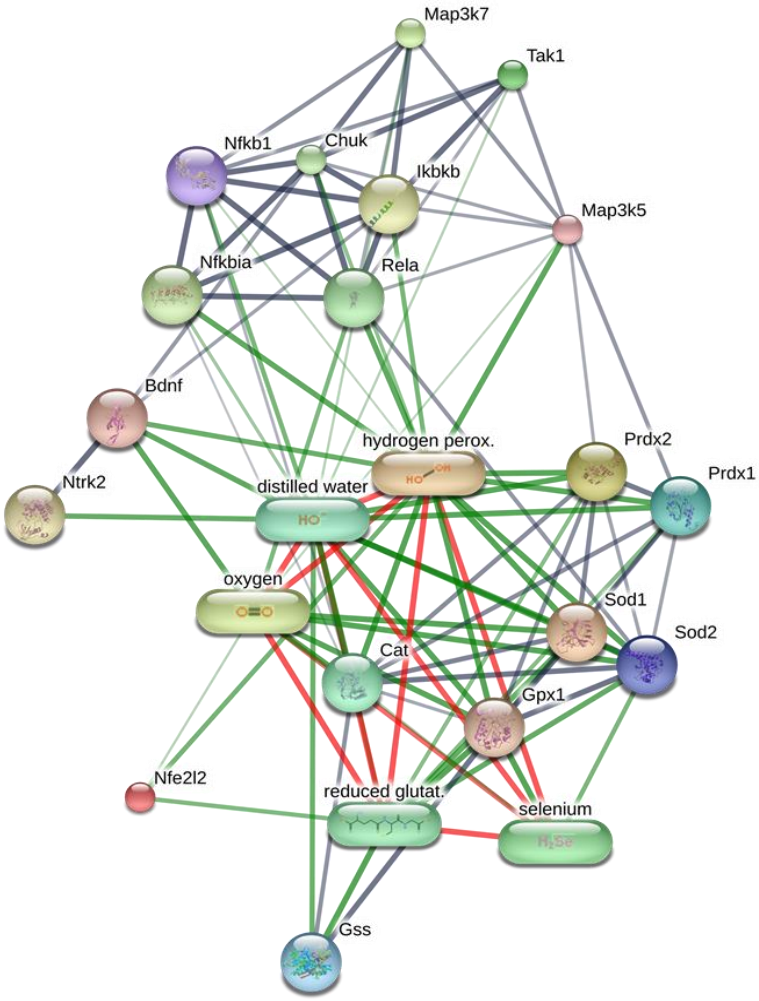

Fig. 6. Chemical association networks by STITCH - proteins involved with BDNF signaling, oxidative stress and inflammation (BDNF x H2O2 Combined Score = 0.891). Bdnf, brain-derived neurotrophic factor; Cat, catalase; Chuk, conserved helix-loop-helix ubiquitous kinase; Gpx, glutathione peroxidase 1; Gss, glutathione synthase; Ikbkb, inhibitor of nuclear factor kappa b kinase subunit beta; Map3k5 = Ask1, apoptosis signal-regulating kinase 1; Map3k7 = Tak1, transforming growth factor beta-activated kinase 1; Nfe2l2 = Nrf2, nuclear factor (erythroid-derived 2)-like 2; NF-κB, factor nuclear kappa b; NFκB1, nuclear factor kappa b subunit 1; Prdx1, peroxirredoxin 1; Prdx2, peroxirredoxin 2; Rela, transcription factor p65; Sod1, superoxide dismutase-1; Sod2, superoxide dismutase-2. Link to access complete results: http://stitch.embl.de/cgi/network.pl?taskId=h07KR7iXK0cu.

Table 1. Effects of CUMS in central TBARS, Carbonyl Protein, and TEAC.

	PC (nmol DNPH/mg)		**TBARS (nmol MDA/mg)**		**TEAC (µmol of TEAC/mg)**	
	PFC	Hippocampus	PFC	Hippocampus	PFC	Hippocampus
Control	0.21 ± 0.09 (n= 7)	0.25 ± 0.14 (n= 7)	0.44 ± 0.21 (n= 7)	0.33 ± 0.08 (n= 7)	15.89 ± 5.45 (n= 6)	36.09 ± 24.34 (n= 7)
Non-anhedonic	0.32 ± 0.15 (n= 9)	0.17 ± 0.07 (n= 9)	0.39 ± 0.08 (n= 9)	0.22 ± 0.09 (n= 8)	9.04 ± 4.97* (n= 9)	9.71 ± 5.58* (n= 9)
Anhedonic	0.28 ± 0.12 (n= 7)	0.16 ± 0.09 (n= 7)	0.45 ± 0.21 (n= 7)	0.28 ± 0.08 (n= 7)	7.14 ± 0.73* (n= 6)	7.95 ± 2.47* (n= 6)

All values are shown as mean ± SD. Outliers were identified by GraphPad quick calc: outlier calculator, and removed from the analyses. *$p \leq 0.01$ compared to the control group, according to one-way ANOVA followed by Bonferroni's post-hoc test. DNPH, 2,4-dinitrophenylhydrazine; PC, protein carbonyl; PFC, prefrontal cortex; SD, standard deviation; TBARS, thiobarbituric acid reactive substances; TEAC, Trolox equivalent antioxidant capacity.

Table 2. Enrichment Analysis of Gene Ontology Biological Process (GO-BP).

Pathway ID	Pathway description	Up-regulated	Down-regulated
GO:0042744	Hydrogen peroxide catabolic process	Prdx-1	Hba1, Hba2, Hbb
GO:0042743	Hydrogen peroxide metabolic process	Prdx-1	Hba1, Hba2, Hbb
GO:0042542	Response to hydrogen peroxide		Hba1, Hba2, Hbb Map3k5, Rela
GO:0002753	Cytoplasmatic pattern recognition receptor signaling pathway		Map3k7, Rela
GO:0010035	Response to inorganic substance	mt3	Cdkn1b, Hba1, Hba2, Hbb, Map3k5, Pef1, Rela
GO:0006979	Response to oxidative stress	Cpeb2, Mt3, Prdx-1	Hba1, Hba2, Hbb, Map3k5, Naprt, Rela

Cdnk1b, cyclin-dependent kinase inhibitor 1; Hba1, hemoglobin alpha 1; Hba2, hemoglobin alpha 2; Hbb, hemoglobin beta; Map3k5 = Ask1, apoptosis signal-regulating kinase 1; Map3k7 = Tak1, transforming growth factor beta-activated kinase 1 p65; Naprt, nicotinate phosphoribosyltransferase; Pef1, penta-ef-hand domain containing 1; Prdx-1, peroxirredoxin 1; Rela, transcription factor.

PARTE III

5 CONSIDERAÇÕES FINAIS

Os achados deste estudo indicam que o CUMS esteve associado a um aumento nas concentrações de BDNF. Neurotrofinas são biomoléculas associadas à neurogênese, podendo também contribuir para a proteção das células neuronais contra o estresse oxidativo (168). O BDNF, por sua vez, pode induzir a translocação nuclear do fator Nrf2, ativando a transcrição de genes antioxidantes e resultando em efeitos protetores contra as EROs (169). Um estudo com células tipo-neuronais indicou que níveis sub-tóxicos de estresse oxidativo induzido por H_2O_2 induziram a expressão de BDNF, sustentando o envolvimento de um mecanismo sensível às EROs, subjacente aos efeitos neuroprotetores dessa neurotrofina (170).

Curiosamente, a análise dos bancos de dados de proteômica evidenciou que alguns processos biológicos enriquecidos se referem a vias relacionadas ao H_2O_2. Ao mesmo tempo, a u*p-regulation* de PRDX-1 foi identificada em animais anedônicos. Considerando que a PRDX-1 é altamente sensível ao H_2O_2 e que o BDNF também pode mediar efeitos antioxidantes, sugere-se que, em conjunto, o aumento desses marcadores pode representar um mecanismo modulatório frente a níveis moderados de estresse oxidativos em animais anedônicos.

Ainda, a ausência de dano oxidativo, bem como a diminuição da TEAC e a *down-regulation* de ASK-1, TAK-1 e RELA, sugerem que o dano estrutural que poderia ser provocado por alterações no estresse oxidativo e inflamação em animais com comportamento anedônico, pode estar sendo atenuado por uma resposta compensatória via aumento de BDNF e *up-regulation* de PRDX-1. Nesse sentido, sugere-se que o CUMS possa ter induzido a geração de EROs em níveis moderados, ativando respostas antioxidantes sensíveis através via BDNF e PRDX-1 em detrimento da ativação da sinalização apoptótica e inflamatória.

O presente estudo apresenta limitações, como: I) o modelo experimental de CUMS está em fase de implementação em nosso grupo de pesquisa, sendo necessárias adaptações no

protocolo para adequá-lo às nossas investigações; II) a eutanásia e coleta de amostras biológicas foi realizada cinco dias após o último estressor, devido a realização dos testes comportamentais; III) as quantidades de amostra biológica limitaram o número de dosagens bioquímicas a serem realizadas, não tendo sido possível realizar a avaliação proteômica experimental. É importante destacar que as proteínas diferencialmente expressas, identificadas pela análise dos bancos de dados de proteômica, não foram validadas no presente estudo. Nesse sentido, a discussão dos resultados levanta hipóteses acerca de um possível mecanismo reunindo os achados experimentais e os resultados evidenciados pela bioinformática, uma vez que a patogênese da DM é altamente complexa e envolve alterações em vários processos biológicos.

É importante citar que, de forma geral, os experimentos com modelo animal avaliam fatores associados à uma janela temporal. Em contrapartida, a DM apresenta características desenvolvimentais que são moduladas em diferentes estágios da vida, frente aos mais diversos estímulos, além do componente genético associado à herdabilidade da doença. Por esse motivo, torna-se difícil mimetizar ao mesmo tempo todos os possíveis gatilhos envolvidos com o desenvolvimento da DM. Desse modo, mais investigações são necessárias para elucidar a relação entre a resposta ao estresse e os mecanismos biológicos compensatórios envolvendo o enfrentamento ao estresse crônico.

6 PERSPECTIVAS

Considerando as variações do protocolo experimental recentemente descritas na literatura, pretendemos adaptar o modelo, a fim de promover diariamente a exposição a dois estressores, com duração de 12 horas cada, durante as seis semanas de protocolo. Sugere-se que uma adaptação do protocolo de CUMS possa promover a ativação exacerbada do eixo HPA, além de induzir um estado pró-inflamatório, com comprometimento neurotrófico e alterações do estado redox em animais suscetíveis. Nesse contexto será possível estudar os mecanismos envolvidos com essas alterações já descritas e classicamente associadas à patogênese da DM, relacionando-as com o comportamento anedônico.

Ainda, pretendemos realizar a avaliação proteômica de animais submetidos ao CUMS e validar, via *Western blott*, as proteínas diferencialmente expressas nos processos biológicos enriquecidos que forem identificados. Dessa forma, além de avaliar parâmetros neurotróficos, oxidativos e inflamatórios, temos a perspectiva de estudar possíveis alterações na neurotransmissão que possam estar envolvidas com a manifestação do comportamento anedônico, incluindo o sistema serotoninérgico, glutamatérgico e seus receptores.

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ANEXO A

Carta de aprovação do projeto sob número150353 pela Comissão de Ética no Uso de Animais do Hospital de Clínicas de Porto Alegre (CEUA/HCPA).

HOSPITAL DE CLÍNICAS PORTO ALEGRE RS

UFRGS UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

GRUPO DE PESQUISA E PÓS GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificamos que o projeto abaixo, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) e pelas áreas de apoio indicadas pelo pesquisador.

Projeto: 150353 **Data de Aprovação do Projeto:** 21/10/2016

Título: AVALIAÇÃO PROTEÔMICA NO MODELO EXPERIMENTAL DE DEPRESSÃO UNIPOLAR INDUZIDO POR ESTRESSE CRÔNICO MODERADO E IMPREVISÍVEL **Data de Término:** 01/03/2018

Pesquisador Responsável: ADRIANE RIBEIRO ROSA

Equipe de pesquisa:

BRUNA MARIA ASCOLI ELLEN SCOTTON LUIZA PAUL GÊA RAFAEL COLOMBO

Submissão	Documento	Espécie/Linhagem	Sexo/Idade	Qtd.	Data Reunião	Situação
24/07/2015	APROVAÇÃO	RATO - WISTAR	M/2meses	95	27/09/2016	APROVADO
22/12/2016	EMENDA	N/A	-/	0	10/01/2017	APROVADO
15/04/2018	EMENDA		/	0	24/04/2018	APROVADO

Total de Animais: 95

ANA HELENA DA ROSA PAZ
Coordenadora
Comissão de Ética no Uso de Animais

- Os membros da CEUA/HCPA não participaram do processo de avaliação onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

ANEXO B

Instruções para a submissão de manuscritos na revista Brain, Behavior, and Immunity.

BRAIN, BEHAVIOR, AND IMMUNITY

AUTHOR INFORMATION PACK

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DESCRIPTION

Brain, Behavior, and Immunity, founded in 1987, is the official journal of the Psychoneuroimmunology Research Society (PNIRS). This innovative journal publishes peer-reviewed basic, experimental, and clinical studies dealing with **behavioral**, **neural**, **endocrine**, and **immune system** interactions in humans and animals. It is an international, interdisciplinary journal devoted to original research in neuroscience, immunology, integrative physiology, behavioral biology, psychiatry, psychology, and clinical medicine and is inclusive of research at the molecular, cellular, social, and whole organism level. The journal features online submission and review. Manuscripts are typically peer-reviewed and returned to authors within 30 days of submission, leading to timely publication of experimental results. There are no submission fees or page charges for *Brain, Behavior, and Immunity*, which is published eight times a year. Detailed instructions for authors can be found at http://ees.elsevier.com/bbi/.

Research areas include: Physiological mechanisms that convey messages between the immune and nervous systems and regulate their functions Stress and immunity, including the role of stress-related hormones and neurotransmitters on the immune system. Actions of cytokines, growth factors and PAMP activation on neuronal and glial cells that regulate behavior, learning, memory and neurogenesis Role of hormones, growth factors and cytokines in the immune and central or peripheral nervous systems Interactions between the immune system and brain that are involved in development of neurological, psychiatric, and mental health disorders Role of immunological processes in neurodegenerative disorders The effects of psychotropic medications on immunological mechanisms and their potential relevance to therapeutic interventions Neuroimaging studies examining how immunological mechanisms affect brain structure and function Clinical trials and experimental studies testing the effects on both immune stimulation and immune suppression on brain and behaviorThe role of microglia in pain, psychological processes and in psychiatric disorders Immunological mechanisms involved in traumatic brain injury and its resolution Immunologic disorders, infection and behaviorRole of the immune system in development and maintenance of inflammatory and chronic pain Immune mechanisms that regulate the blood-brain-interface (BBI) Immune factors that affect health psychologySleep, exercise, immunity and healthImmune system interactions that affect behavior following use of psychotropic drugs, alcohol and other drugs of abuseHealthy aging of the immune system and brainRole of inflammation and stress during perinatal developmentCancer and its treatment, stem cells and their effects on brain behavior and immunity Reciprocal communication between the microbiome, immune and nervous systemsRegulation of nerve injury and repair by the immune systemPsychosocial, behavioral, and neuroendocrine influences on immunity and on the development and progression of immunologically-mediated diseasesNutrition, inflammation, obesity and behaviorGenomics of behavior and immunity

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INTRODUCTION

Brain, Behavior, and Immunity, founded in 1987, is the official journal of the Psychoneuroimmunology Research Society (PNIRS). This innovative journal publishes peer-reviewed basic, experimental, and clinical studies dealing with behavioral, neural, endocrine, and immune system interactions in humans and animals. It is an international, interdisciplinary journal devoted to original research in neuroscience, immunology, integrative physiology, behavioral biology, psychiatry, psychology, and clinical medicine and is inclusive of research at the molecular, cellular, social, and whole organism level. The journal features online submission and review. Manuscripts are typically peer-reviewed and returned to authors within 30 days of submission, leading to timely publication of experimental results. There are no submission fees or page charges for *Brain, Behavior, and Immunity*, which is published eight times a year. Detailed instructions for authors can be found at http://ees.elsevier.com/bbi/.

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