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Elisa Piton Lovis

INVESTIGAÇÃO DO PRÉ-CONDICIONAMENTO INFLAMATÓRIO EM CAMUNDONGOS SUBMETIDOS AO ESTRESSE AGUDO E CRÔNICO POR CONTENÇÃO

Santa Maria, RS
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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Farmacologia, Área de Concentração em Neuropsicofarmacologia e Imunofarmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de Mestre em Farmacologia.

Orientador: Prof. Dr. Guilherme Vargas Bochi

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Aprovado em 23 de abril de 2024.

Guilherme Vargas Bochi, Dr. (UFSM)
(Presidente/ Orientador)

Cristiani Folharini Bortolatto, Dra. (UFPel)

Sara Marchesan de Oliveira, Dra. (UFSM)

Santa Maria, RS
2024

Dedico esse trabalho aos meus pais, Jandir e Elaine e a minha irmã Clara, que apesar da distância sempre se fizeram presentes, me incentivando, incansavelmente, na busca pela minha felicidade e realização.

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"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota."

Madre Teresa de Calcutá

RESUMO

INVESTIGAÇÃO DO PRÉ-CONDICIONAMENTO INFLAMATÓRIO EM CAMUNDONGOS SUBMETIDOS AO ESTRESSE AGUDO E CRÔNICO POR CONTENÇÃO

AUTORA: Elisa Piton Lovis
ORIENTADOR: Prof. Dr. Guilherme Vargas Bochi

O Transtorno Depressivo Maior (TDM) é um dos transtornos psiquiátricos de maior prevalência a nível mundial, atingindo aproximadamente 280 milhões de pessoas e causando cerca de 700 mil suicídios por ano. Esse transtorno de humor é uma das principais causas de incapacidade no mundo, podendo apresentar-se com diferentes características clínicas ou comorbidades, como a ansiedade. O TDM é um transtorno de humor heterogêneo, associado a diferentes fatores etiológicos, como fatores genéticos e/ou ambientais. Entretanto, sua fisiopatologia ainda não está complemente elucidada. Sabe-se que o estresse é um fator comum à praticamente todas as teorias propostas para explicar os mecanismos fisiopatológicos do TDM, uma vez que a exposição repetida à estímulos estressantes podem induzir mudanças comportamentais, emocionais e cognitivas mal adaptadas e disfuncionais. Além disso, existe uma relação entre o TDM, o estresse e o processo inflamatório. No que tange este último, sugere-se que um desequilíbrio imunológico, e, consequentemente, uma hiperativação inflamatória desencadeados pelo estresse, podem estar envolvidos na fisiopatologia do TDM. No entanto, ainda não há um amplo entendimento sobre o impacto do processo inflamatório sobre o risco de desenvolver o transtorno. Assim, o objetivo deste estudo foi investigar os efeitos do pré-condicionamento inflamatório (administração única de lipopolissacarídeo - LPS) em camundongos *Swiss* machos submetidos ao estresse agudo por contenção (EAC) ou ao estresse crônico por contenção (ECC), chamados de protocolo agudo e crônico, respectivamente. Os resultados indicaram que, no protocolo agudo, o pré-condicionamento inflamatório perturbou uma resposta fisiológica esperada após exposição de um estímulo estressante agudo. Neste protocolo, o LPS induziu prejuízos comportamentais relacionados à desmotivação, sem induzir os comportamentos tipo-depressivo e ansioso. No protocolo crônico, o pré-condicionamento inflamatório prejudicou os mecanismos adaptativos a exposições repetidas de um estímulo estressante previsível, desempenhando um papel importante no desenvolvimento dos comportamentos tipo-depressivo e ansioso. As alterações comportamentais induzidas pela administração de LPS no protocolo crônico foi relacionado com alterações bioquímicas e moleculares, indicando um desequilíbrio inflamatório e oxidativo, associado à neuroinflamação induzida pela microglia, atrofia dos astrócitos e redução do fator neurotrófico derivado do encéfalo (BDNF). Portanto, esse estudo sugere que o pré-condicionamento inflamatório perturba o comportamento fisiológica e regulado após um estímulo estressante agudo (protocolo agudo) e potencializa alterações comportamentais, bioquímicas e moleculares induzidas por um protocolo de estresse físico repetido (protocolo crônico). Através destes achados, a modulação do processo inflamatório pode ser uma potencial estratégia terapêutica para reduzir o risco de desenvolver comportamentos do tipo depressivo e ansioso.

Palavras-chave: Comportamento tipo-depressivo. Modelos animais. Alterações comportamentais. Neuroinflamação. Desequilíbrio oxidativo.

ABSTRACT

INVESTIGATION OF INFLAMMATORY PRECONDITIONING IN MICE SUBJECT TO ACUTE AND CHRONIC RESTRAINT STRESS

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Major Depressive Disorder (MDD) is one of the most prevalent psychiatric disorders worldwide, affecting approximately 280 million people and causing around 700,000 suicides per year. This mood disorder is one of the main causes of disability in the world, and may present several clinical characteristics or comorbidities, such as anxiety. MDD is a heterogeneous mood disorder, associated with different etiological factors, such as genetic and/or environmental. However, its pathophysiology has not yet been fully elucidated. It is known that stress is a common factor in practically all theories proposed to explain the pathophysiological mechanisms of MDD, since repeated exposure to stressful stimuli can induce maladaptive and dysfunctional behavioral, emotional and cognitive changes. Furthermore, there is a relationship between MDD, stress and the inflammatory process. Regarding the latter, it is suggested that an immunological imbalance, and, consequently, an inflammatory hyperactivation triggered by stress, may be involved in the pathophysiology of MDD. However, there is still no broad understanding of the impact of the inflammatory process on the risk of developing the disorder. Thus, the objective of this study was to investigate the effects of inflammatory preconditioning (single administration of lipopolysaccharide - LPS) in male Swiss mice subjected to acute restraint stress (ARS) or chronic restraint stress (CRS), called acute and chronic protocol, respectively. The results indicated that, in the acute protocol, inflammatory preconditioning disturbed an expected physiological response after exposure to an acute stressful stimulus. In this protocol, LPS induced behavioral impairments related to demotivation, without inducing depressive- and anxiety-like behaviors. In the chronic protocol, inflammatory preconditioning impaired adaptive mechanisms to repeated exposure to a predictable stressful stimulus, playing an important role in the development of depressive- and anxiety-like behaviors. The behavioral changes induced by LPS administration in the chronic protocol were related to biochemical and molecular changes, indicating an inflammatory and oxidative imbalance, associated with microglia-induced neuroinflammation, astrocyte atrophy and reduction in brain-derived neurotrophic factor (BDNF). Therefore, this study suggests that inflammatory preconditioning disrupts physiological and regulated behavior after an acute stressful stimulus (acute protocol) and potentiates behavioral, biochemical and molecular changes induced by a repeated physical stress protocol (chronic protocol). Through these findings, modulation of the inflammatory process may be a potential therapeutic strategy to reduce the risk of developing depressive and anxious behaviors.

Keywords: Depressive-like behavior. Animal models. Behavioral changes. Neuroinflammation. Oxidative imbalance.

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LISTA DE ABREVIATURAS E SIGLAS

ACTH	Hormônio adrenocorticotrófico (do inglês, *adrenocorticotropic hormone*)
BDNF	Fator neurotrófico derivado do encéfalo (do inglês, *brain-derived neurotrophic factor*)
BHE	Barreira-hematoencefálica
CORT	Corticosterona
CRH	Hormônio liberador de corticotrofina (do inglês, *corticotropin-releasing hormone*)
EAC	Estresse agudo por contenção
ECC	Estresse crônico por contenção
GFAP	Proteína ácida fibrilar glial (do inglês, *glial-fibrillary acid protein*)
HPA	Hipotálamo-pituitária-adrenal
H_2O_2	Peróxido de hidrogênio
HOCl	Ácido hipocloroso
IBA-1	Molécula adaptadora de ligação de cálcio ionizado 1 (do inglês, *ionized calcium-binding adapter molecule 1*)
IL-1β	Interleucina 1 beta
IL-6	Interleucina 6
LCR	Líquido cefalorraquidiano
LPS	Lipopolissacarídeo
MPO	Mieloperoxidase
SAM	Simpático Adrenomedular
SNC	Sistema nervoso central
TDM	Transtorno depressivo maior
TNF-α	Fator de necrose tumoral-alfa (do inglês, *tumor necrosis factor-alpha*)

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte dessa dissertação foram apresentados na forma de manuscrito científico. As seções Materiais e Métodos, Resultados, Discussão, Conclusão e Referências encontram-se no próprio manuscrito, no item **MANUSCRITO CIENTÍFICO** e representam a íntegra desse estudo. A seção **CONSIDERAÇÕES FINAIS** contém as principais conclusões obtidas com esse estudo. A sessão **REFERÊNCIAS** compreende apenas as citações presentes nos itens **INTRODUÇÃO** e **REVISÃO BIBLIOGRÁFICA** dessa dissertação.

1 INTRODUÇÃO

O Transtorno Depressivo Maior (TDM) representa um importante problema de saúde no mundo, atingindo aproximadamente 280 milhões de pessoas, sendo identificado como uma das principais causas de incapacidade no mundo (VOS, et al., 2020; ORGANIZAÇÃO MUNDIAL DA SAÚDE, 2023). Os sintomas que caracterizam esse transtorno podem ser psicológicos e físicos, incluindo: humor deprimido, perda de prazer ou interesse nas atividades (anedonia), perda de peso e apetite, falta de motivação, comprometimento cognitivo, além de outros sintomas (CUI et al., 2024). O TDM é um transtorno de humor complexo e heterogêneo, que pode ser desencadeado por diversos fatores, sejam eles psicológicos, biológicos, genéticos ou sociais (BOAS et al., 2019). Considerando o caráter heterogêneo do transtorno, o TDM deve ser especificado com base em diferentes características, como, por exemplo, a presença de comorbidades. Dentre essas, a ansiedade é uma das mais importantes. Estima-se que cerca de 54-78% dos pacientes com TDM apresentem a ansiedade como comorbidade (GASPERSZ et al., 2017a, GASPERSZ et al., 2017b; MCINTYRE et al., 2016; ZIMMERMAN et al., 2017).

Apesar de sua ampla prevalência, a fisiopatologia do TDM ainda não está totalmente elucidada, visto que nenhuma das hipóteses ou mecanismos propostos até hoje (hipótese monoaminérgica, neurotrófica, hiperativação do eixo hipotálamo-pituitária-adrenal (HPA), estresse oxidativo), são capazes de explicar esse transtorno neuropsiquiátrico em sua totalidade (HUANG et al., 2021; MALHI; MANN, 2018). Entretanto, já foi demonstrado que o estresse é um fator comum à praticamente todas as teorias, atingindo vários sistemas, incluindo o Sistema Nervoso Central (SNC), o sistema imunológico e o sistema endócrino (HALL et al., 2012; JANGRA et al., 2016; KHAN; KHAN, 2017). Além disso, a inflamação também é relacionada ao TDM, já que uma hiperativação imunológica, desencadeada pelo estresse, pode estar envolvida no desenvolvimento do TDM (HASSAMAL et al., 2023). No entanto, não está totalmente definido o papel do processo inflamatório como fato de risco para o TDM.

Modelos animais com roedores são amplamente utilizados na pesquisa básica para estudar o TDM. Muitos modelos utilizam o estresse como fator indutor do comportamento tipo-depressivo, como o estresse agudo por contenção (EAC) e o estresse crônico por contenção (ECC) (JIANG et al., 2018; PENG et al., 2022; MISZTAK et al., 2021; KWATRA et al., 2021, TRIPATHI et al., 2021; BETTIO et al., 2014; DOMINGUES et al., 2019; CASARIL et al., 2019; SOUSA et al., 2018). Também existem modelos animais que utilizam

a administração de lipopolissacarídeo (LPS) para induzir a inflamação em roedores e estudar algumas doenças, como o TDM (WANG et al., 2020; ALI et al., 2020; YANG et al., 2020).

Dessa forma, tanto o estresse, agudo ou crônico, como a administração de LPS são modelos utilizados para estudar o TDM em roedores. Entretanto, ainda não está elucidado o impacto de um pré-condicionamento inflamatório sobre alterações comportamentais/bioquímicas/moleculares induzidas por um modelo de estresse por contenção. Assim, é de grande relevância que seja estudado se um estímulo inflamatório aumenta a suscetibilidade ao estresse.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Avaliar os efeitos comportamentais, bioquímicos e moleculares do pré-condicionamento inflamatório (administração única de LPS) em camundongos submetidos ao estresse agudo por contenção (EAC) e ao estresse crônico por contenção (ECC).

1.1.2 Objetivos Específicos

Os objetivos específicos estão divididos de acordo com cada protocolo experimental:

Protocolo Agudo:

- Padronizar o modelo de pré-condicionamento inflamatório associado ao EAC;
- Avaliar se o pré-condicionamento inflamatório associado ao EAC induz alterações relacionadas ao comportamento tipo-depressivo, através dos testes comportamentais de nado forçado e suspensão da cauda;
- Verificar se o pré-condicionamento inflamatório associado ao EAC induz alterações relacionadas ao comportamento tipo-ansioso, por meio do teste de labirinto em cruz elevado;
- Averiguar se o pré-condicionamento inflamatório associado ao EAC causa alterações na atividade locomotora e exploratória dos animais, através do teste comportamental de campo aberto.

Protocolo crônico:

- Padronizar o modelo de pré-condicionamento inflamatório associado ao ECC;

- Avaliar as alterações comportamentais induzidas pelo pré-condicionamento inflamatório associado ao ECC, através dos testes comportamentais de preferência pela sacarose, campo aberto, labirinto em cruz elevado, suspensão da cauda e nado forçado;
- Averiguar no decorrer do protocolo de pré-condicionamento inflamatório associado ao ECC, as alterações comportamentais relacionadas ao estado da pelagem e peso corporal;
- Verificar o efeito do modelo experimental de pré-condicionamento inflamatório associado ao ECC sobre os níveis de corticosterona plasmática;
- Analisar os parâmetros moleculares por meio da expressão gênica do fator neurotrófico derivado do encéfalo (BDNF), da molécula adaptadora de ligação de cálcio ionizado 1 (IBA-1) e da proteína ácida fibrilar glial (GFAP) nas estruturas cerebrais de córtex pré-frontal e hipocampo;
- Investigar as alterações nos parâmetros inflamatórios centrais (córtex pré-frontal e hipocampo) e periféricos (plasma) dos animais submetidos ao pré-condicionamento inflamatório associado ao ECC;
- Analisar as alterações nos parâmetros oxidativos nas estruturas cerebrais de córtex pré-frontal e hipocampo dos animais submetidos ao pré-condicionamento inflamatório associado ao ECC.

1.2 JUSTIFICATIVA E RELEVÂNCIA

Apesar da ampla incidência e prevalência, o TDM ainda não tem sua fisiopatologia completamente elucidada. Por isso, o estudo desse transtorno de humor na pesquisa básica pode contribuir para desvendar seus mecanismos fisiopatológicos e descobrir novos alvos com potencial terapêutico. Até os dias atuais, o achado mais robusto sobre o TDM consiste em sua estrita relação com anormalidades na resposta frente ao estresse (JURUENA et al., 2018). Devido a isso, a maioria dos modelos animais que induzem o comportamento tipo-depressivo e tentam mimetizar algumas condições observadas em pacientes, são baseados na exposição à agentes estressores, como os modelos de EAC e ECC, ou à exposição a agentes relacionados a etiologia do TDM (administração de LPS) (PENG et al., 2022; MISZTAK et al., 2021; KWATRA et al., 2021, TRIPATHI et al., 2021; DOMINGUES et al., 2019; CASARIL et al., 2019; SOUSA et al., 2018; WANG et al., 2020; ALI et al., 2020; YANG et al., 2020).

Estudos com camundongos submetidos aos modelos citados acima, relataram alterações comportamentais, aumento nos níveis de marcadores inflamatórios e oxidativos,

além de alterações a nível de eixo HPA (LIU et al., 2018; SOUSA et al., 2018; ZHU et al., 2019). Esses achados são frequentemente visualizados em pacientes com TDM. Desta forma, tais protocolos consistem em bons modelos para a indução de comportamento tipo-depressivo (ROMAN; IRWIN, 2020; KOWALSKA et al., 2020; CAO et al., 2022; CORREIA; CARDOSO; VALE, 2023a). Nesse contexto, considerando que tanto a inflamação quanto o estresse podem participar na fisiopatologia do TDM, os modelos de EAC ou ECC, após exposição única ao LPS, podem consistir em um novo modelo de indução ao comportamento tipo-depressivo e ansioso. Essa abordagem combinada pretende mimetizar de maneira mais consistente o que é observado em pacientes, fornecendo uma perspectiva mais translacional entre a pesquisa básica e a clínica.

2 REVISÃO BIBLIOGRÁFICA

2.1 TRANSTORNO DEPRESSIVO MAIOR (TDM)

O Transtorno Depressivo Maior (TDM) representa um importante problema de saúde, identificado como uma das principais causas de incapacidade no mundo (VOS, et al., 2020). Sua prevalência leva a projeções preocupantes, atingindo aproximadamente 280 milhões de pessoas e causando cerca de 700 mil suicídios por ano, sendo que esta é a quarta principal causa de morte entre jovens-adultos de 15 a 29 anos (ORGANIZAÇÃO MUNDIAL DA SAÚDE, 2023). O TDM é um transtorno heterogêneo, que pode ser desencadeado por diversos fatores, sejam eles psicológicos, biológicos, genéticos ou sociais (BOAS et al., 2019). Por isso, sua fisiopatologia ainda não está completamente elucidada. Considerado um transtorno de humor complexo, o TDM pode gerar prejuízos afetivos, cognitivos e fisiológicos, o que somado à ampla gama de sintomas, dificulta o seu diagnóstico (ATHIRA et al., 2018; BOAS et al., 2019).

Dentre os sintomas que caracterizam esse transtorno, podem ser incluídos: humor deprimido, irritação, perda de prazer ou interesse nas atividades (anedonia), perda de peso e apetite, falta de motivação, problemas de sono, sentimento de culpa, comprometimento cognitivo, além de outros sintomas psicológicos e físicos (CUI et al., 2024). Ainda, diferentes características cínicas ou comorbidades, como a ansiedade, podem acompanhar o TDM, tornando o perfil neurobiológico do paciente com TDM e ansiedade (ou depressão ansiosa) distinto daquele com TDM sem ansiedade (GASPERSZ et al., 2018). O TDM com ansiedade comórbida apresenta uma maior desregulação do sistema imunológico e do eixo HPA, assim como alteração no funcionamento das vias corticolímbicas (GASPERSZ et al., 2018; YE et al., 2023). Além disso, o TMD com ansiedade comórbida está associado a maior gravidade desse transtorno, aumento na tendência suicida e pior resultado com o tratamento farmacológico, resultando em uma pior qualidade de vida (GASPERSZ et al., 2018; HASIN et al., 2018; HURLEY et al., 2022).

Devido à complexidade da sua fisiopatologia, o diagnóstico e consequentemente o tratamento do TDM ainda permanecem um desafio, pois nenhuma das teorias ou mecanismos propostos até o momento conseguem explicar em sua totalidade esse transtorno neuropsiquiátrico (HUANG et al., 2021; MALHI; MANN, 2018). Além disso, o TDM apresenta curso altamente variável, com possibilidade de diferentes respostas aos tratamentos farmacológicos disponíveis. Após a primeira tentativa de tratamento com antidepressivos, somente um terço dos pacientes alcançam a remissão, 20% apresentam resposta terapêutica e

50% não obtém nenhuma resposta (ANDREWS et al, 2012; LEE et al., 2019; LI, 2019; TRIVEDI et al., 2006).

2.2 FISIOPATOLOGIA DO TDM

Várias hipóteses buscam explicar os mecanismos envolvidos no desenvolvimento do TDM. Dentre elas, a hipótese monoaminérgica é a pioneira, sugerindo que alterações nos níveis dos neurotransmissores monoaminérgicos, como serotonina, noradrenalina e dopamina, estejam relacionadas aos sintomas depressivos (DEAN; KESHAVAN, 2017). Entretanto, existem algumas limitações nessa hipótese, pois ela não explica todos os sintomas e, mesmo após o tratamento com antidepressivos que atuam no sistema monoaminérgico, não ocorre a completa recuperação dos pacientes. Além disso, estudos sugerem que há o envolvimento de outras substâncias biológicas na etiologia do TDM, além desse sistema (DREVETS; PRICE; FUREY, 2008; FILATOVA; SHADRINA; SLOMINSKY, 2021; VILLANUEVA, 2013).

A hipótese neurotrófica relaciona o TDM a uma redução da atividade das neurotrofinas, como o BDNF (LI et al., 2021). O BDNF é uma neurotrofina envolvida em diversos processos fisiológicos, como neurogênese, neuroplasticidade, neuroproteção, aprendizagem, memória e regulação do humor (SCHIRÒ et al., 2022; OH; LEWIS; SIBILLE, 2016). Os efeitos neuroprotetores do BDNF também podem ser associados à sua ação de prevenir a morte neuronal, reduzir o dano oxidativo e inibir a autofagia (CHEN et al., 2017). A via de sinalização dessa neurotrofina é amplamente estudada devido ao seu envolvimento em diversas doenças do SNC, como o TDM (CORREIA; CARDOSO; VALE, 2023b; YOU; LU, 2023). Níveis reduzidos de BDNF estão associados a atrofia cerebral, declínio cognitivo, maior risco para o desenvolvimento de distúrbios psiquiátricos e aumento da frequência de sintomas depressivos (OH; LEWIS; SIBILLE, 2016; YOU; LU, 2023; CORREIA; CARDOSO; VALE, 2023b). Ainda, já foi demonstrado em estudos anteriores, que os níveis reduzidos de BDNF podem ser restaurados após o tratamento com antidepressivos (AROSIO et al., 2021; MIRANDA et al., 2019; KISHI et al., 2018).

A hiperativação do eixo HPA é outra hipótese que busca explicar o TDM. Quando o eixo HPA é ativado, ocorre a liberação do hormônio liberador de corticotrofina (CRH) pelo hipotálamo, que estimula a pituitária a liberar o hormônio adrenocorticotrófico (ACTH), o qual estimula o córtex da adrenal a produzir os glicocorticoides (KHAN; KHAN, 2017). Indivíduos com TDM podem apresentar uma hiperativação desse eixo, em decorrência do déficit no seu processo de retroalimentação ou *feedback* negativo, resultando em aumento dos níveis de glicocorticoides (cortisol em humanos e corticosterona em roedores), podendo

causar vários danos no hipocampo, como atrofia, redução do volume e da neurogênese (ANACKER et al., 2011; MCEWEN, 2007; WU et al., 2013). Entretanto, nem todos os pacientes com TDM apresentam hipercortisolemia, uma vez que há casos em que é relatada uma hipocortisolemia, que ainda não tem seu mecanismo de ação bem definido (FILATOVA; SHADRINA; SLOMINSKY, 2021; KUNUGI; HORI; OGAWA, 2015). Isso indica que há diferentes subtipos de TDM, com perfis bioquímicos distintos.

O envolvimento do estresse oxidativo no TDM também é alvo de investigação. Vários estudos já demonstraram que o estresse oxidativo contribui para o desenvolvimento e progressão do TDM, interagindo e desregulando vários sistemas, como o eixo HPA, as vias serotoninérgicas e o BDNF (BHATT; NAGAPPA; PATIL, 2020; KELLER et al., 2017; CORREIA; CARDOSO; VALE, 2023b; LESCHIK et al., 2022; RUMAJOGEE et al., 2004). Ainda, o estresse oxidativo e a inflamação podem intensificar um ao outro, resultando em um estado patológico encontrado em vários transtornos psiquiátricos (CAO et al., 2022). As reações de oxidação e redução são básicas no processo de metabolismo celular, porém, a oxidação excessiva ou a resposta antioxidante insuficiente, podem levar ao acúmulo de espécies reativas ao oxigênio, o chamado estresse oxidativo (JI et al., 2023). O estresse oxidativo perturba a homeostase, desencadeando o processo neuroinflamatório, a neurodegeneração, o dano tecidual, a disfunção mitocondrial e a morte celular (BAJPAI et al., 2014; SANI et al., 2023). No SNC a enzima mieloperoxidase (MPO) pode ser secretada pela microglia, astrócitos e neurônios, podendo estar associada a neuroinflamação e ao TDM (SIRAKI et al., 2021; RAY; KATYAL, 2016). Além disso, na presença de peróxido de hidrogênio (H_2O_2) e haletos (Cl, Br), a MPO catalisa reações de formação de agentes oxidantes/clorantes reativos, como o ácido hipocloroso (HOCl), causando modificações celulares, nas proteínas, nos lipídios e no DNA (ODOBASIC et al., 2016; VAN DER VEEN; DE WINTHER; HEERINGA, 2009; VACCARINO et al., 2008).

O estresse é um fator comum à praticamente todas as hipóteses citadas acima (ANISMAN; MATHESON, 2005; HUANG et al., 2021). É definido como estresse qualquer evento ou experiência que ameace a capacidade de um indivíduo de lidar e se adaptar a uma situação considerada estressante (BOAS et al., 2019). O estresse pode atingir vários sistemas do organismo, como o SNC, o sistema imunológico e o sistema endócrino (HALL et al., 2012; HUANG et al., 2021; JANGRA et al., 2016; KHAN; KHAN, 2017). Já foi verificado que o estresse perturba a homeostase de dois sistemas interligados: o eixo HPA e o sistema Simpático Adrenomedular (SAM). Com a desregulação do eixo HPA, ocorre a hipercortisolemia. Em decorrência da ativação do sistema SAM, ocorre a produção de

catecolaminas (noradrenalina e adrenalina) pela medula adrenal, promovendo o instinto de luta ou fuga (KHAN; KHAN, 2017). Além disso, muitos modelos animais utilizam o estresse como fator indutor do comportamento tipo-depressivo, visto que o estresse está envolvido no início e na exacerbação desse transtorno (KHAN, KHAN, 2017; TONG et al., 2023).

2.3 RELAÇÃO ENTRE O TDM E A INFLAMAÇÃO

Existe uma relação entre o TDM, o estresse e a inflamação, pois já foi proposto que uma hiperativação imunológica desencadeada pelo estresse pode estar envolvida no desenvolvimento do TDM (HASSAMAL et al., 2023). Sabe-se que o aumento de biomarcadores inflamatórios está associado a sintomatologia do TDM e da ansiedade (ROMAN; IRWIN, 2020; KOWALSKA et al., 2020; MICHOPOULOS et al.,2017). Já foi evidenciado, em estudos pré-clínicos e clínicos de TDM, elevados níveis de citocinas pró-inflamatórias, como TNF-α, IL-1β e IL-6, a nível periférico e central (PANDEY et al., 2018; YOUNG; BRUNO; POMARA, 2014; PENG et al., 2022). A atividade neuronal do cérebro também é afetada pela inflamação, pois as citocinas inflamatórias alteram a função dos neurotransmissores, reduzindo os níveis de serotonina, afetando o humor e o comportamento (DANTZER, 2017; KARIMI et al., 2022; TONG et al., 2023). Além disso, evidências demonstraram que existe uma relação entre os níveis elevados de citocinas pró-inflamatórias, no soro e líquido cefalorraquidiano (LCR), com uma maior gravidade e resistência ao tratamento do TDM (OSIMO et al., 2019).

A resposta exacerbada do sistema imunológico pode comprometer o funcionamento adequado da barreira hematoencefálica (BHE), favorecendo a passagem de citocinas pró-inflamatórias periféricas para o SNC de forma bidirecional (MEDINA-RODRIGUEZ, BEUREL, 2022). Essa troca de citocinas pró-inflamatória entre a periferia e o SNC, leva a ativação imunológica central exacerbada, causando um processo inflamatório central denominado de neuroinflamação (PENG et al., 2022; RAHIMIAN et al., 2022). É sugerido uma associação entre a neuroinflamação e os transtornos neuropsiquiátricos, uma vez que ela contribui significativamente para o início e a progressão do TDM (RAHIMIAN et al., 2021; WANG; JIN, 2015; ADZIC et al., 2018; WANG et al., 2015).

A regulação da neuroinflamação ocorre principalmente pelas células microgliais, modulando a resposta a diferentes estressores, através da comunicação entre o sistema nervoso e imunológico, a fim de manter a homeostase cerebral (RAHIMIAN et al., 2022). As células microgliais são consideradas os macrófagos residentes do SNC, que atuam como primeira linha de defesa imunológica dessa região, representando cerca de 15% de todas as

células presentes no SNC (MÜLLER et al. 2015; STRATOULIAS et al. 2019). Quando a homeostase é ameaçada, por meio de infecção, insulto inflamatório ou lesão tecidual, o microambiente do SNC se altera, levando a ativação microglial, que resulta em aumento da liberação de citocinas pró-inflamatórias e redução das anti-inflamatórias (RAHIMIAN et al., 2022; FELGER; LOTRICH, 2013; HE et al., 2020). A sensibilização de longa duração da microglia ativada está associada ao desenvolvimento de transtornos neuropsiquiátricos, como o TDM (RAHIMIAN et al., 2021; MECHAWAR; SAVITZ, 2016; MONDELLI et al., 2017). Essa exacerbação da ativação microglial é capaz de causar morte neuronal, diminuição da produção de neurotransmissores, de fatores neurotróficos e da neuroplasticidade (SINGHAL; BAUNE, 2017; MILLER; MALETIC; RAISON, 2009). A ativação microglial é verificada em estudos animais através do marcador chamado molécula adaptadora de ligação de cálcio ionizado 1 (IBA-1) (KWATRA et al., 2021; PENG et al., 2022).

Em condições normais, o SNC é protegido de patógenos e substâncias tóxicas pela BHE, constituída por células endoteliais, pericitos, junções oclusivas e astrócitos (SHI et al., 2018). Entretanto, em condições de estresse, ocorre o aumento do perfil inflamatório, podendo levar ao dano da BHE e a infiltração de citocinas pró-inflamatórias (PENG et al., 2022). Além disso, astrócitos e microglia são células gliais que respostem rapidamente a estímulos nocivos, podendo alterar sua morfologia e função, exacerbando ainda mais a neuroinflamação (SHI et al., 2018; PENG et al., 2021). Os astrócitos são o tipo celular mais abundante no SNC e desempenham diversas funções como a reciclagem de neurotransmissores e a sinalização imunológica (GIOVANNONI; QUINTANA, 2020). Vários estudos demonstraram uma redução de astrócitos em cérebros de pacientes (*post-mortem*) com TDM e em modelos animais de estresse crônico, assim como uma redução nos níveis de proteína ácida fibrilar glial (GFAP), um marcador de astrócitos (MURPHY-ROYAL; GORDON; BAINS, 2020; NASKAR; CHATTARJI, 2019; WANG; XU, 2020; LIU et al., 2022; PENG et al., 2015; KIM et al., 2018). A disfunção astrocitária, bem como as alterações relacionadas ao GFAP, têm sido relacionadas aos transtornos de humor, como o TDM e a ansiedade (KIM et al., 2018; HE et al., 2023). A redução desse marcador sugere a atrofia/degeneração, a perda da funcionalidade e o remodelamento patológico dos astrócitos, uma vez que astrócitos atróficos, com expressão reduzida de GFAP, são menores e apresentam capacidade funcional prejudicada (KIM et al., 2018; PEKNY et al., 2016).

Ademais, existem modelos animais que induzem a inflamação, por meio da administração de LPS em roedores, para estudar algumas doenças que tenham o envolvimento

da inflamação na sua etiologia, como o TDM (WANG et al., 2020; ALI et al., 2020; YANG et al., 2020). Também já foi evidenciado, em roedores, que a administração de LPS ou de citocinas pró-inflamatórias é capaz de reduzir os níveis de BDNF no hipocampo e córtex, indicando que a inflamação afeta a expressão de BDNF, contribuindo para o desenvolvimento de TDM (CALABRESE et al., 2014).

2.4 MODELOS ANIMAIS UTILIZADOS PARA ESTUDAR O TDM

Na pesquisa básica, modelos pré-clínicos com roedores são amplamente utilizados para estudar os aspectos e mecanismos moleculares/celulares envolvidos em doenças humanas. Os modelos animais constituem uma importante ferramenta para a compreensão da fisiopatologia do TDM e investigação de novos alvos terapêuticos, embora seja um desafio desenvolver modelos animais que traduzam o comportamento humano (VAZQUEZ-MATIAS et al., 2023; WANG et al., 2017). Existem três principais parâmetros para avaliar a confiabilidade dos modelos, a validade de face, validade de construto e validade preditiva. A validade de face representa a similaridade entre a sintomatologia humana e o comportamento animal; a validade de construto está relacionada aos mecanismos envolvidos na fisiopatologia; por fim, a validade preditiva se refere a eficácia do tratamento farmacológico, sendo equivalente em humanos e animais (PLANCHEZ; SURGET; BELZUNG, 2019).

Além disso, devem ser levados em considerações alguns critérios essenciais para a escolha de um modelo experimental. Dentre esses critérios, deve ser observado se a complexidade neurológica do animal é suficiente para mimetizar o que acontece na clínica, bem como outros fatores, como a facilidade e acessibilidade do uso experimental do animal e a sua manutenção (ITALIA et al., 2020). Existem diferentes modelos animais para estudar o TDM, como os modelos baseados na aplicação de estressores, modelos baseados na causalidade biológica e modelos baseados na manipulação genética (Figura 1).

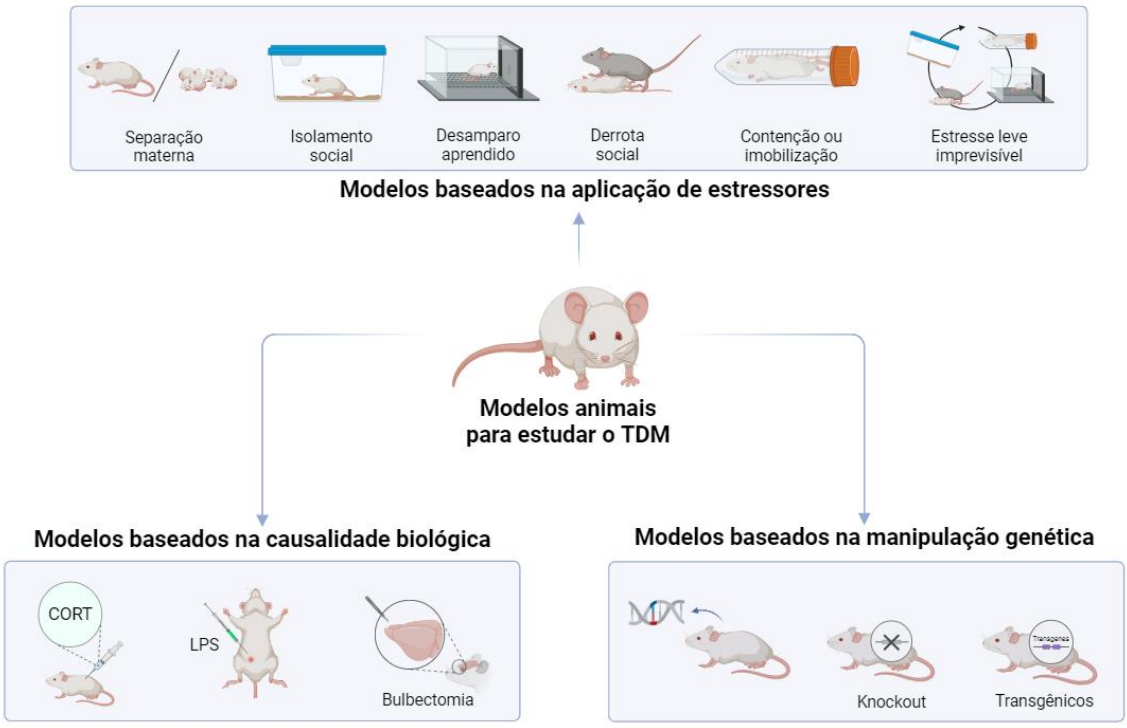
2.4.1 Modelos baseados na aplicação de estressores

O estresse é um forte fator de risco para o desenvolvimento do TDM (TONG et al., 2023). Por isso, os modelos baseados na aplicação de estressores são muito utilizados, seja na fase de desenvolvimento ou na idade adulta dos animais (PLANCHEZ; SURGET; BELZUNG, 2019; VAZQUEZ-MATIAS et al., 2023). Alguns exemplos de modelos pertencentes a esse subgrupo são: a separação materna, o isolamento social, o desamparo aprendido, o estresse de derrota social, o estresse leve imprevisível e o estresse por contenção (Figura 1). Nesse trabalho foram utilizados os modelos de estresse agudo e crônico por

contenção. O estresse por contenção consiste na imobilização do animal dentro de um cilindro estreito e transparente, realizado em tempos variados, em uma única sessão ou em repetidas sessões (ITALIA et al., 2020).

Alguns trabalhos já relataram que uma única sessão de estresse por contenção (estresse agudo) é capaz de induzir o comportamento tipo-depressivo (BETTIO et al., 2014; DOMINGUES et al., 2019; CASARIL et al., 2019; SOUSA et al., 2018). Ainda, outros trabalhos indicaram que sessões repetidas de contenção (estresse crônico) levam ao comportamento tipo-depressivo, simulando um estresse psicológico humano, repetitivo e previsível, mas inevitável, como o estresse de um trabalho que é repetido todos os dias, o estresse social ou financeiro (JIANG et al., 2018; PENG et al., 2022; MISZTAK et al., 2021; WANG et al., 2017; ITALIA et al., 2020; MAO; XU; YUAN, 2022).

Figura 1 – Modelos animais para o estudo do TDM



Fonte: autora. Criado em BioRender.com.

2.4.2 Modelos baseados na causalidade biológica

Os modelos baseados na causalidade biológica, visam recapitular outros fatores que estejam associados a etiologia do TDM, como as alterações no eixo HPA, no sistema imunológico e em circuitos cerebrais (PLANCHEZ; SURGET; BELZUNG, 2019). Esses

modelos se baseiam em induzir nos animais alterações fisiológicas que conferem a suscetibilidade dos humanos ao TDM, para moldar e estudar as causas envolvidas no desenvolvimento do TDM (PLANCHEZ; SURGET; BELZUNG, 2019).

Se enquadram nesse subgrupo de modelo: a administração de corticosterona (CORT), a administração de LPS e a bulbectomia olfatória (Figura 1). Nesse trabalho foi utilizada uma única administração de LPS, no protocolo agudo e crônico. Vários trabalhos utilizam o LPS, uma endotoxina bacteriana, para induzir inflamação em roedores e estudar algumas doenças, como no estudo do TMD (WANG et al., 2020; ALI et al., 2020; YANG et al., 2020). Frequentemente os estudos apontam que uma única exposição ao LPS é capaz de induzir o comportamento tipo-depressivo, com uma única dose de 500 a 830 µg/Kg (ADEBESIN et al., 2017; CASARIL et al., 2019; LUDUVICO et al., 2020).

2.4.3 Modelos baseados na manipulação genética

Existem ainda modelos animais desenvolvidos a partir de alterações genéticas, que induzem o comportamento tipo-depressivo, auxiliando no estudo do TDM e de possíveis agentes antidepressivos (BECKER; PINHASOV; ORNOY, 2021). Visto que o TDM apresenta uma correlação com componentes genéticos, foram desenvolvidas linhagens transgênicas de animais, a fim de modificar a expressão de genes ou tornar linhagens mais sensíveis ao estresse, como os ratos *Wistar-Kyoto* (BECKER; PINHASOV; ORNOY, 2021; PLANCHEZ; SURGET; BELZUNG, 2019). Outra estratégia utilizada na manipulação genética, é a geração de animais *knockout* para determinado gene, a fim de investigar alterações alélicas específicas, envolvidas na suscetibilidade ou resiliência ao estresse (ITALIA et al., 2020; PLANCHEZ; SURGET; BELZUNG, 2019).

Alterações genéticas são consideradas um fator de suscetibilidade, quando associada a outros fatores. Entretanto, isoladamente não causa a maioria dos transtornos neuropsiquiátricos (ITALIA et al., 2020). Além disso, mutações específicas auxiliam no estudo do TDM em animais, mas não são capazes de evocar a etiologia genética do TDM (PLANCHEZ; SURGET; BELZUNG, 2019).

Dessa forma, embora exista uma ampla gama de modelos animais que consigam mimetizar alguns aspectos encontrados no TDM, não existe um modelo animal ideal ou que consiga representar esse transtorno de humor em sua totalidade (BECKER; PINHASOV; ORNOY, 2021). Alguns sintomas apresentados por pacientes com TDM, não podem ser detectados/mensurados em modelos animais, como o sentimento de culpa ou inutilidade e a ideação suicida, representando parcialmente o quadro clínico do TDM (PLANCHEZ;

SURGET; BELZUNG, 2019). Dessa forma, a combinação de modelos, que utilizam diferentes fatores para induzir o comportamento tipo-depressivo, pode ser uma estratégia interessante para estudar o TDM e aumentar a validade do modelo experimental.

3 MANUSCRITO CIENTÍFICO

3.1 MANUSCRITO: Lipopolysaccharide administration disrupts acute restraint stress-induced physiological response and aggravates chronic restraint stress-induced behavioral and molecular changes

O manuscrito apresentado a seguir foi submetido ao periódico *Inflammopharmacology - Experimental and Therapeutic Studies* (Qualis A2 – Ciências Biológicas II), por isso segue as normas desse periódico.

Lipopolysaccharide administration disrupts acute restraint stress-induced physiological response and aggravates chronic restraint stress-induced behavioral and molecular changes

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Author Contributions

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Abstract

Major Depressive Disorder (MDD) is one of the main causes of disability in the world. MDD is a heterogeneous mood disorder, associated with different etiological factors and clinical characteristics, such as anxiety. The pathophysiology of MDD has not yet been fully elucidated, but it is known that stress is involved in the development of MDD. Furthermore, it has been proposed that stress-triggered immune hyperactivation may be involved in MDD. Therefore, the objective of this study was to investigate the effects of inflammatory preconditioning, through a single injection of lipopolysaccharide (LPS), in adult male Swiss mice subjected to acute restraint stress (ARS) or chronic restraint stress (CRS). For this, in the acute protocol, the animals received LPS 24 hours before the behavioral tests and were subjected to ARS for 4 hours. In the chronic protocol, LPS was injected 24 hours before the first CRS, performed for 6 hours daily for 28 days. Our results indicated that the acute protocol (LPS+ARS) induced behavioral impairments related to demotivation, indicating the participation of the inflammatory process in these changes. The chronic protocol (LPS+CRS) demonstrated that inflammatory preconditioning plays an important role in the development of depressive and anxious-like behaviors, associated with inflammatory/oxidative imbalance, neuroinflammation, astrocyte atrophy and BDNF reduction. Thus, this study indicates that the inflammatory process acts as an important risk factor for the development of depressive-like behaviors. Furthermore, modulation of this inflammatory stimulus may be a potential therapeutic target to reduce the risks of developing this disorder.

Keywords: Major Depressive Disorder. Inflammatory preconditioning. Oxidative imbalance. Depressive-like behavior. Anxiety-like behavior. Neuroinflammation.

1. Introduction

Major Depressive Disorder (MDD) is one of the leading causes of disability in the world, affecting approximately 280 million people and causing around 700,000 suicides per year, making it the fourth leading cause of death among people aged 15 to 29 (Vos et al. 2020; World Health Organization, 2023). This psychiatric disorder is characterized by a set of symptoms that may include the following: depressed mood, irritation, loss of pleasure or interest in activities (anhedonia), loss of weight and appetite, lack of motivation, sleep problems, feelings of guilt, cognitive impairment, and other psychological and physical symptoms (Cui et al. 2024). MDD is a heterogeneous mood disorder associated with different etiological factors, such as psychological, biological, genetic, and social. Furthermore, its pathophysiology is not fully elucidated (Huang et al. 2021; Villas Boas et al. 2019) . Different clinical characteristics or comorbidities, such as anxiety, can present themselves in MDD, making the neurobiological profile of a patient with MDD and anxiety (or anxious depression) different from that with MDD without anxiety (Ye et al. 2023). The first situation, in general, is associated with a more significant dysregulation of the immune system and the Hypothalamus-Pituitary-Adrenal (HPA) axis (Gaspersz et al. 2018). Furthermore, MDD with anxiety comorbidity is associated with greater severity of this disorder, increased suicidality, worse outcomes with pharmacological treatment and worse quality of life (Gaspersz et al. 2018; Hasin et al. 2018).

Rodent animal models are widely used in basic research to investigate depression-like and anxiety-like behavior and their implications at a biochemical and molecular level. Many of these models use some stressful stimulus. An acute stressful stimulus is expected to provoke physiological and adaptive responses. It is consistent to consider that normal physiological stress has necessary and beneficial effects in daily life (Dhabhar 2018; James et al. 2023). Conversely, chronic stress induces maladaptive changes in the brain via the cumulative action of glucocorticoids, which is associated with mood disorders (James et al. 2023; McEwen and Akil 2020). Among the models that use stress as a stimulus, acute restraint stress (ARS) and chronic restraint stress (CRS) are widely used (Casaril et al. 2019; Domingues et al. 2019; Jiang et al. 2018; Misztak et al. 2022; Peng et al. 2022; Sousa et al. 2018).

There is also a relationship between MDD, stress and inflammation. It has been proposed that immunological hyperactivation triggered by stress may be involved in the development of MDD (Hassamal 2023). The exacerbated immune system response can compromise the optimal functioning of the blood-brain barrier, favoring the passage of

peripheral pro-inflammatory cytokines to the central nervous system (CNS) in a bidirectional manner (Medina-Rodriguez and Beurel 2022). This exchange of pro-inflammatory cytokines between the periphery and the CNS leads to exacerbated central immune activation, causing neuroinflammation(Peng et al. 2022). Evidence shows that there is also an association between the high levels of pro-inflammatory cytokines in serum and cerebrospinal fluid with severity and resistance to treatment of MDD (Osimo et al. 2019). Additionally, inflammation and oxidative stress can intensify each other, resulting in a pathological state found in several psychiatric disorders (Cao et al. 2022). Microglia is the fundamental cell type in the regulation of neuroinflammation, modulating immune responses and maintaining brain homeostasis. In conjunction with astrocytes, microglia respond quickly to a pathological stimulus, with both cell types changing their morphology and function, favoring the neuroinflammatory process (Peng et al. 2022; Rahimian et al. 2022). Furthermore, there are animal models that use Lipopolysaccharide (LPS) to induce inflammation and depressive-like behavior (Ali et al. 2020; Wang et al. 2020). However, the impact of LPS-induced inflammatory preconditioning on the behavioral responses expected after an acute stressful stimulus and on the biochemical and molecular changes related to depressive- and anxiety-like behavior induced by a chronic stress model is not fully understood. Therefore, the present study aims to investigate the effects of inflammatory preconditioning in mice subjected to ARS or CRS.

2. Material and Methods

2.1 Animals

Eighty male Swiss mice (20–30 g; 6–8 weeks old) purchased from the Federal University of Santa Maria were maintained in standard clear plastic cages under controlled temperature (22 ± 2 °C) and 12 h light-dark cycle, with free access to food and water, except during periods of stress protocols. Initially, the animals were acclimated for one week to the new environment, and then they were randomly distributed into experimental groups. All procedures were performed according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978), approved by Institutional Animal Care and Use Committee of the Federal University of Santa Maria (Process number 9619250121/2021 and 3601271021/2022) and performed between 8:00 a.m. and 5:00 p.m.

2.2 Chemicals and reagents preparation

Lipopolysaccharide (LPS, from Escherichia coli O55:B5, catalog number L2880, Sigma Aldrich, USA) was dissolved in hypotonic saline solution (0.9% sodium chloride) and administered in a single intraperitoneal (i.p.) injection at a dose of 830 µg/Kg/10 mL (Adebesin et al. 2017; Luduvico et al. 2020). For groups not exposed to LPS, hypotonic saline solution (10 mL/Kg i.p.) was used as a vehicle (VEH).

2.3 Experimental design

This study was divided into two different experimental protocols: acute and chronic. The experiment timelines of the protocols are shown in Fig 1.

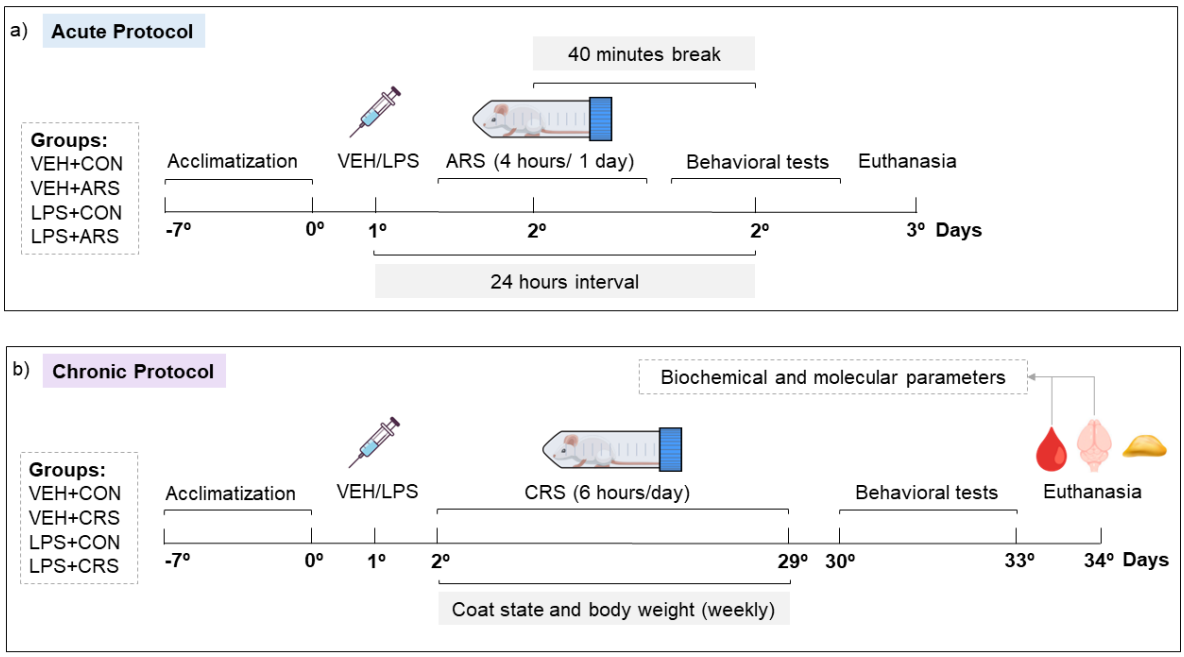

Fig. 1 Experiment timelines. **a)** Acute protocol. The VEH+CON group received an intraperitoneal (i.p.) injection of saline and remained in their box at the time of ARS, without water and food. The VEH+ARS group received an i.p. injection of saline solution and was subjected to ARS for 4 hours in a single day. The LPS+CON group was administered an i.p. of LPS and remained in its box at the time of the ARS, without water and food. The LPS+ARS group was administered an i.p. injection of LPS and was subjected to ARS for 4 hours in a single day. Behavioral tests (open field, elevated plus maze, tail suspension and forced swim) occurred 24 hours after administration of saline or LPS and 40 minutes after ARS. The saline solution was administered in a volume of (10mL/Kg) and the LPS was administered at a dose of 830 µg/Kg/10 mL. Euthanasia was performed 24 hours after behavioral tests. **b)** Chronic protocol. The i.p. injection of saline or LPS was performed 24 hours before the first CRS, at the same dose and volume as the acute protocol. In this protocol, the VEH+CRS or LPS+CRS group was subjected to CRS for 6 hours daily for 28 days. Throughout the protocol, assessments of coat state and body weight were carried out. 24 hours after the last CRS, behavioral tests (sucrose preference, open field, elevated plus maze, tail suspension and forced swim) were performed for 4 days. The animals were euthanized 24 hours after the last day of testing, with blood collection, removal of brain structures (PFC and HIP) and adrenal glands. Vehicle (VEH), control (CON), lipopolysaccharide (LPS), acute restraint stress (ARS), chronic restraint stress (CRS)

2.3.1 Acute protocol

After a week of acclimatization, the animals were randomly segregated into 4 groups (n=8-10 for each group): vehicle plus control (VEH+CON), vehicle plus acute restraint stress (VEH+ARS), lipopolysaccharide plus control (LPS+CON) and lipopolysaccharide plus acute restraint stress (LPS+ARS). For inflammatory pre-exposure, a single i.p. injection of LPS (830μg/Kg/10mL) occurred 24 hours before behavioral tests (12:10 p.m.). Animals not exposed to inflammatory pre-exposure received a single i.p. injection of VEH (10 mL/Kg). The ARS was performed in cylindrical polypropylene tubes (50 mL Falcon tubes, with dimensions of 3.2 cm in diameter x 11.6 cm in length) adequately ventilated for 4 hours (7:30 am – 11:30 am) (Casaril et al. 2019; Domingues et al. 2019; Sousa et al. 2018). Meanwhile, animals not subjected to restraint stress remained in their boxes and were called control (CON). All groups had restricted access to water and food during restraint stress (Chotiwat and Harris 2006). At the end of the ARS, the animals returned to their boxes for a 40-minute break. After this period, behavioral tests were performed (12:10 p.m.), including open field test (OFT), elevated plus maze test (EPMT), tail suspension test (TST) and forced swim test (FST). 24 hours after the behavioral tests, euthanasia was performed.

2.3.2 Chronic protocol

After a week of acclimatization, the animals were randomly segregated into 4 groups (n=8-10 for each group): vehicle plus control (VEH+CON), vehicle plus chronic restraint stress (VEH+CRS), lipopolysaccharide plus control (LPS+CON) and lipopolysaccharide plus chronic restraint stress (LPS+CRS). For inflammatory pre-exposure, a single i.p. injection of LPS (830 μg/Kg/10mL) occurred 24 hours before the first CRS session. Animals not exposed to inflammatory pre-exposure received a single injection of VEH (10 mL/Kg). All groups had restricted access to water and food during restraint stress (Chotiwat and Harris 2006). The CRS was performed in cylindrical polypropylene tubes (50 mL Falcon tubes, with dimensions of 3.2 cm in diameter x 11.6 cm in length) adequately ventilated for 6 h daily from 7:30 a.m. to 1:30 p.m., and this procedure was repeated for 28 days (Jiang et al. 2018; Misztak et al. 2022; Peng et al. 2022). Due to the long period of exposure to stress, these animals were evaluated weekly regarding body weight and coat condition to observe the general condition of these animals (Du Preez et al. 2021; Nollet 2021). Behavioral tests occurred 24 hours after the last CRS, for 4 days, in order from least stressful to most stressful (days 1 and 2: sucrose preference test (SPT); day 3: OFT and EPMT; day 4: TST and FST). Euthanasia occurred 24 hours after the last behavioral test. The animals were anesthetized with isoflurane to perform a

cardiac puncture for blood collection (with heparin anticoagulant), followed by the collection of brain structures (prefrontal cortex - PFC and hippocampus - HIP) and adrenal glands. The blood samples were centrifuged at 3000 xg, at room temperature, for 10 minutes, and the plasma was separated and stored in a freezer at -80ºC, as well as the brain structures, for subsequent biochemical analyses.

2.4 Assessment of the general state of the animals: body weight, coat state, corticosterone levels and adrenal/body weight ratio

Body weight and coat state were parameters assessed during the chronic protocol. The animals were also evaluated weekly for body weight. To estimate the impact of the experimental protocol on body weight, weight gain was calculated by subtracting the final weight from the initial weight for each animal. The coat state was assessed weekly, as it reflects the animal's auto-grooming behavior and may be associated with motivation and self-care, which can be affected by stress (Nollet 2021). For this, two areas of the body were observed, including the head/neck and back, assigning increasing scores to each of them, varying between good state (score 0.0), with smooth and clean coat, in animals without changes in behavior, moderately bad state (score 0.5), the coat is a little bristly and slightly dirty and bad state (score 1.0), with bristly and dirty coat, in animals with impaired behavior (Nollet 2021). At the end of the experiment, the condition of each animal's coat was expressed by the sum of the scores of the areas evaluated weekly; thus, the higher the sum of the scores, the worse the animal's condition (Nollet 2021). After the euthanasia of the animals, blood was collected to analyze plasma corticosterone levels, as well as the removal of the adrenal glands, to establish the relationship between the adrenal glands and body weight.

2.5 Behavioral Tests

2.5.1 Tail Suspension Test (TST)

The TST was performed according to the methodology of Steru et al. (1985) with minor modifications. This test aims to evaluate the immobility time in seconds, which reflects depressive-like behavior in animals (Wang et al. 2020). For this, each animal was suspended by the tail, fixed by an adhesive tape in the apparatus, 60 cm above a flat surface. The duration of the test is 6 minutes. However, only the final 4 minutes were considered for the immobility parameter, allocating the initial 2 minutes for the adaptation of the animals to the test (Bai et al. 2018). In the chronic protocol, the latency to the first episode of immobility was also verified.

2.5.2 Forced Swim Test (FST)

The FST was carried out according to Porsolt et al. (1977). Each animal was placed individually in a colorless cylindrical tank (45 cm high x 35 cm diameter) containing 40 cm of water at 25 ± 2°C. The test was recorded for 6 minutes, but immobility and climbing behavior (fight or flight) were recorded in the last 4 minutes (Bai et al. 2018). Immobility was detected when the animals remained floating passively, reflecting a behavioral state of demotivation and depressive-like behavior (Wang et al. 2020). In the chronic protocol, the latency to the first episode of immobility was also evaluated, as well as the average speed and total distance travelled; the latter two were verified using the ANY-maze Software (version 7.20, Stoelting Co).

2.5.3 Elevated Plus Maze Test (EPMT)

The EPMT was used to verify anxiety-like behavior, carried out according to Donatti et al. (2017). The apparatus is elevated 50 cm from the floor and consists of two open arms and two closed arms opposite each other and the central platform. Initially, the animals were placed on the central platform, facing the open arm. During the test, the number of entries and the time spent in the open and closed arms were evaluated and used to calculate the anxiety index: 1 - [(time in open arms/ test duration) + (entries into open arms/ entries into open and closed arms) / 2]. The number of immersions of the animal's head below the surface of the maze (also called head-dipping) was also evaluated, and the total distance travelled was evaluated using the ANY-maze Software (version 7.20). The test was recorded and lasted 5 minutes.

2.5.4 Open Field Test (OFT)

The locomotor and exploratory activity of the animals was evaluated using the OFT (Prut and Belzung 2003). Initially, each animal was placed in the center of the apparatus (60 cm x 60 cm), surrounded by 60 cm high walls, in which the floor was divided into 12 equal squares. The test was filmed and lasted 5 minutes to evaluate the number of crossings (evaluation of locomotion), number of rearings (evaluation of exploratory capacity) and time of grooming (chronic protocol). The average speed and total distance travelled (chronic protocol) were evaluated using the ANY-maze Software (version 7.20, Stoelting Co).

2.5.5 Sucrose Preference Test (SPT)

The SPT was used as a behavioral measure of anhedonia, a symptom related to depressive-like behavior in rodents (Kim YH et al. 2018). For this, each mice was isolated in a box at 7 p.m. and had free access to two bottles, one containing filtered water and the other containing 2% sucrose solution, for 24 hours. After 12 hours of testing (7 a.m.), the bottles were inverted to avoid a preference for the side on which the bottle was positioned. Subsequently, the preference for sucrose was calculated based on the animals' consumption of the solutions, after 12 and 24 hours, following the calculation (Couch et al. 2016): Sucrose preference (%) = sucrose intake (ml) / water intake (ml) + sucrose intake (ml) * 100.

2.5.6 Emotionality Z-Score

The calculation for the Z-score was carried out according to Guilloux et al. (2011). To verify the emotionality score for depressive-like behaviors, immobility data from TST and FST were used. Initially, the Z-score for immobility was calculated in each test separately, as follows: Z-score (TST or FST) = (immobility time of each animal – mean immobility time of the VEH+CON group) / Standard deviation of the VEH+CON group. Next, the emotionality score was calculated, combining the Z-score results from each of the tests (TST and FST): Emotionality score= (TST Z-score + FST Z-score) / number of tests (2).

2.6 Biochemical assessments

2.6.1 Determination of protein levels in tissues

The samples were homogenized, under refrigeration, in Tris buffer (50 mM, pH 7.4) and then centrifuged at 3,000 rpm, at 4 ºC, for 20 minutes to obtain the supernatant. After collecting the supernatant, the protein was determined in the samples using the Bradford method (Bradford 1976).

2.6.2 Assessment of oxidative parameters

The oxidative parameters evaluated in this study include hydrogen peroxide (H_2O_2) levels, activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) and activity of the enzyme myeloperoxidase (MPO). These parameters were analyzed in the PFC and HIP, previously prepared for analysis, through homogenization in Tris buffer (50 mM, pH 7.4) and centrifugation (3,000 rpm, at 4 ºC, for 20 minutes) to obtain the supernatant, stored at -20ºC until further analysis. To evaluate H_2O_2 levels, the samples were centrifuged (12,000 g, at 4 ºC, for 20 minutes) with sodium azide (25 mM, ratio 1:5) to inhibit antioxidant

enzymes present in the samples. With the new supernatant collected, H_2O_2 levels were measured using the phenol red method described by Trevisan et al. (2013). Its concentration was expressed in nmol of H_2O_2/mg of protein from each sample.

To evaluate the activity of the SOD involved in the H_2O_2 formation reaction, the initial supernatant was used according to the technique described by Misra and Fridovich (1972) and its activity was expressed as IU of SOD/mg of protein. To assess the activity of the CAT, involved in the H_2O_2 degradation reaction, was performed according to Aebi (1984) and was expressed in μmol of CAT/mg of protein. The analysis of the activity of the MPO used as a marker of inflammatory cell infiltration in brain tissues was performed according to the technique previously described by Klafke et al. (2016), and the result was expressed as OD of MPO/mg of protein, in 3 minutes of reaction.

2.6.3 Central and peripheral cytokines

To investigate the involvement of the central inflammatory markers after the chronic protocol, the levels of IL-10, IL-6 and TNF-α were evaluated in PFC and HIP brain tissues using a Mini TMB Enzyme-linked immunosorbent assay (ELISA) kit (IL-10, catalog number: 900-TM53, IL-6, catalog number: 900-TM50, TNF-α, catalog number: 900-TM54) (Peprotech, USA) by the manufacturer's instructions. The absorbance was read on a plate reader at 450 nm and 620 nm. All incubations were at room temperature. Cytokine concentrations were expressed as pg/mg of protein for each sample.

To investigate the involvement of the peripheral inflammatory process after the chronic protocol, plasma levels of the cytokines IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and INF-γ were evaluated, using a commercial kit for flow cytometry (BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit), following the manufacturer's instructions. Sample analyzes were performed on a flow cytometer (BD FACSVerse) and the channel mean fluorescence intensity (MFI) derived from the fluorescence graph, was used to perform the following calculations. Each cytokine's control was analyzed to determine sample gates using FlowJo Software (version 10.8.1, Becton, Dickinson and Company - BD). The data was presented in pg/mL.

2.6.4 Molecular parameters

Total mRNA from brain structures (PFC and HIP) was extracted using Trizol and purified with chloroform, 75% ethanol and isopropyl alcohol, stored after completion of the procedure in a -80 ºC freezer until the technique was completed. For the synthesis of

complementary DNA (cDNA), DNase I (Sigma-Aldrich, St. Louis, MO) was used to remove genomic DNA, while reverse transcription was performed using iScript Reverse Transcription Supermix (Bio-Rad, California, USA) and the product was stored at -20 ºC. For amplification, the SYBR Green I mix (GoTaq qPCR Master Mix 2X, Promega, Madison, WI, USA) and oligonucleotides obtained from Extend Biotechnology (Campinas, Brazil) were used. The cycling conditions were: 3 min at 95 ºC, followed by 40 cycles of 10 s at 95 ºC and 1 min at 60 ºC. The Quantitative Real-Time Polymerase Chain Reaction (q-PCR) were performed in duplicate. The analyzed genes and their respective primer sequences were described in Table 1. The relative gene expression levels of the target genes were normalized using β-Actin as a reference gene (housekeeping). The results were visualized in the CFX Manager Software (version 3.0, Bio-Rad Laboratories) and analyzed by the $2^{-\Delta\Delta CT}$ method.

Table 1 Analyzed genes and their respective primer sequences

Gene	Primer sequence
BDNF	Forward: TCA TAC TTC GGT TGC ATG AAG G Reverse: AGA CCT CTC GAA CCT GCC C
GFAP	Forward: TGC AGG AGT ACC AGG ATC TAC Reverse: GAT CTG GAG GTT GGA GAA AGT C
IBA-1	Forward: AGG AGA AAA ACA AAG AAC ACA AGA Reverse: CAA TCA GGG CAG CTC GGA GAT AGC
β-Actin	Forward: CAT TGC TGA CAG GAT GCA GAA GG Reverse: TGC TGG AAG GTG GAC AGT GAG G

Brain-Derived Neurotrophic Factor (BDNF); Glial Fibrillary Acid Protein (GFAP); Ionized Calcium Binding Adaptor Molecule 1 (IBA-1).

2.6.5 Corticosterone levels

Plasma was obtained after blood collection during euthanasia, through cardiac puncture with heparinized syringes and subsequent centrifugation (3,000 xg at room temperature, for 10 min) of the blood. An ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) was used to check circulating plasma corticosterone levels, following the manufacturer's instructions. Data were expressed in pg/mL, using logarithmic transformation.

2.7 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). After evaluating the normality of the data, a two-way analysis of variance (Two-way ANOVA) was performed, followed by Tukey's multiple comparisons post hoc test when appropriate. The ANOVA results were only presented in the text when the p values were significant, described as follows: main effect of inflammatory preconditioning (LPS); main effect of stress (ARS or CRS); and interaction of factors (LPS+ARS or LPS+CRS). Additional ANOVA results were detailed in Table 1 of the supplementary material (Tab. S1). The statistical program used was GraphPad Prism Software (version 8.00). Results were considered statistically significant when $p < 0.05$.

3. Results

3.1 The acute protocol induces behavioral changes related to demotivation, while the chronic protocol induces depressive-like behavior

To assess depressive-like behavior, the TST and FST were performed. In the TST, the two-way ANOVA detected in the acute protocol only a main effect of inflammatory preconditioning ($F_{(1,28)}= 23.72$, $p< 0.0001$) on the immobility time. The LPS+CON group showed a longer time of immobility than the VEH+CON ($p= 0.0471$) and VEH+ARS ($p= 0.0005$) groups, as well as the LPS+ARS group, which exhibited greater immobility than the VEH+ARS group ($p= 0.0016$). In the chronic protocol, an effect of inflammatory preconditioning ($F_{(1,32)}= 7.558$, $p= 0.0097$) and stress ($F_{(1,32)}= 8.351$, $p= 0.0069$) on immobility time was detected. An increased immobility time was found in the LPS+CRS group compared to the VEH+CON group ($p= 0.0026$). Still in the chronic protocol, in this same test (TST), a main effect of inflammatory preconditioning was observed on the latency time for immobility ($F_{(1,34)}= 9.984$, $p= 0.0033$). A shorter latency time for immobility was observed in the LPS+CRS group compared to the VEH+CON ($p= 0.0299$) and VEH+CRS ($p= 0.0455$) groups (Fig 2).

In the FST, no significant effect of factors was identified in relation to immobility time in the acute protocol. However, in climbing time there was a main effect of inflammatory preconditioning ($F_{(1,30)}= 14.59$, $p= 0.0006$) and a tendency towards a stress effect ($F_{(1,30)}= 3.830$, $p=0.0597$). A reduction in climbing time was found in the LPS+ARS group compared to the VEH+CON group ($p= 0.0012$) and VEH+ARS ($p= 0.0147$). In the chronic protocol, a main effect of stress ($F_{(1,32)}= 6.565$, $p= 0.0153$) on immobility time was identified. There was an increase in immobility time in the LPS+CRS group compared to the VEH+CON ($p =$

0.0476) and LPS+CON (p= 0.0373) groups. In the latency to immobility, there was an effect of inflammatory preconditioning ($F_{(1,28)}$= 5.106, p= 0.0318), however Tukey's post-hoc showed no significant difference between the groups. Regarding climbing time, an effect of inflammatory preconditioning ($F_{(1,36)}$= 4.444, p= 0.0420) and stress ($F_{(1,36)}$= 11.65, p= 0.0016) was detected (Fig 3).

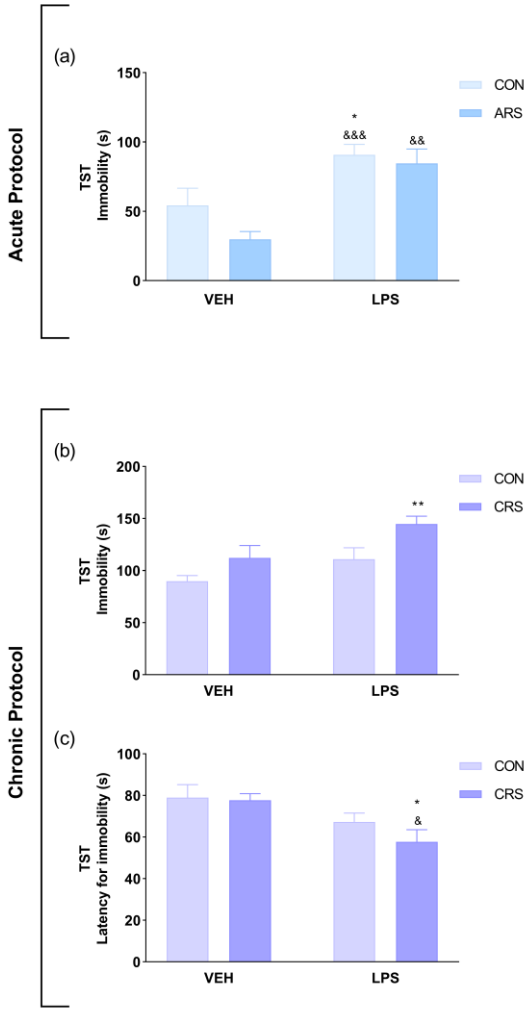

Fig. 2 Tail Suspension Test. Acute protocol: **a)** Immobility time. Chronic protocol: **b)** Immobility time, **c)** Latency for immobility time. Data are expressed as mean ± SEM (n = 8-10/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test, * P < 0.05; ** P < 0.01 when compared to VEH+CON group, & P < 0.05; && P < 0.01; &&& P < 0.001 when compared to VEH+ARS or VEH+CRS groups

A reduction in climbing time was observed in the LPS+CRS (p= 0.0022) and VEH+CRS (p= 0.0207) groups compared to the VEH+CON group (Fig. 2E). No significant effects were detected on the animals' average swimming speed, nor on the total distance travelled. In the emotionality Z score, used to evaluate the set of depressive behaviors, the acute protocol showed no significant differences. In the chronic protocol an effect of inflammatory preconditioning was identified ($F_{(1,31)}$= 7.251, p= 0.0113) and stress ($F_{(1,31)}$=

9.299, p= 0.0047). An increase in the z-score was found in the LPS+CRS group compared to the VEH+CON (p= 0.0020), VEH+CRS (p = 0.0201) and LPS+CON (p= 0.0106) (Fig. 4). In SPT, no effect of the factors was observed at 12 and 24 hours (figure 1 in supplementary material - Fig. S1). Together, these results suggest that the acute protocol induced only demotivational behavior, while the chronic protocol induced depressive-like behavior, accompanied by demotivation, without changes in locomotor activity.

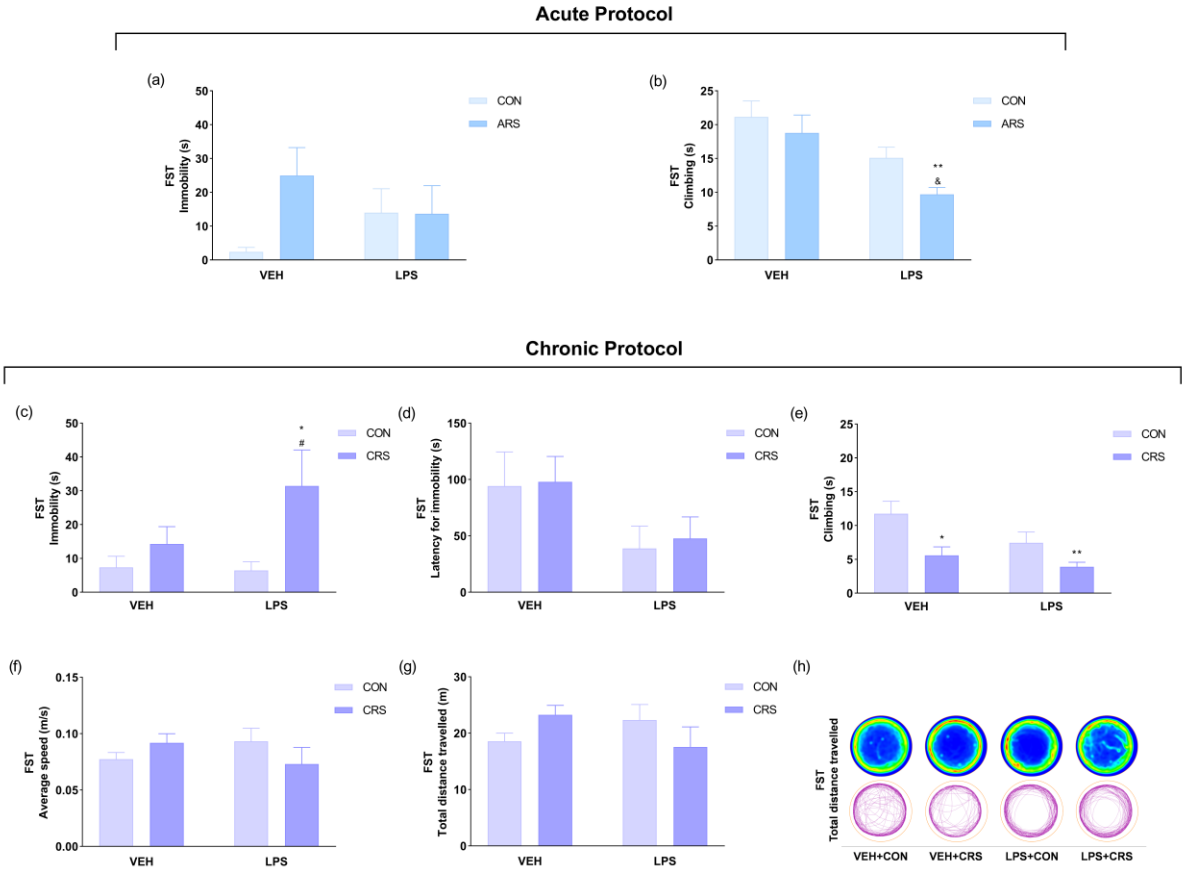

Fig. 3 Forced Swimming Test. Acute protocol: **a)** Immobility time, **b)** Climbing time. Chronic protocol: **c)** Immobility time, **d)** Latency for immobility time, **e)** Climbing time. **f)** Average speed, **g)** Total distance travelled, **h)** Heat map and track plot of total distance travelled. Data are expressed as mean ± SEM (n = 8-10/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test, * P < 0.05; ** P < 0.01 when compared to VEH+CON group, & P < 0.05 when compared to VEH+ARS or VEH+CRS groups, # P < 0.05 when compared to LPS+CON group

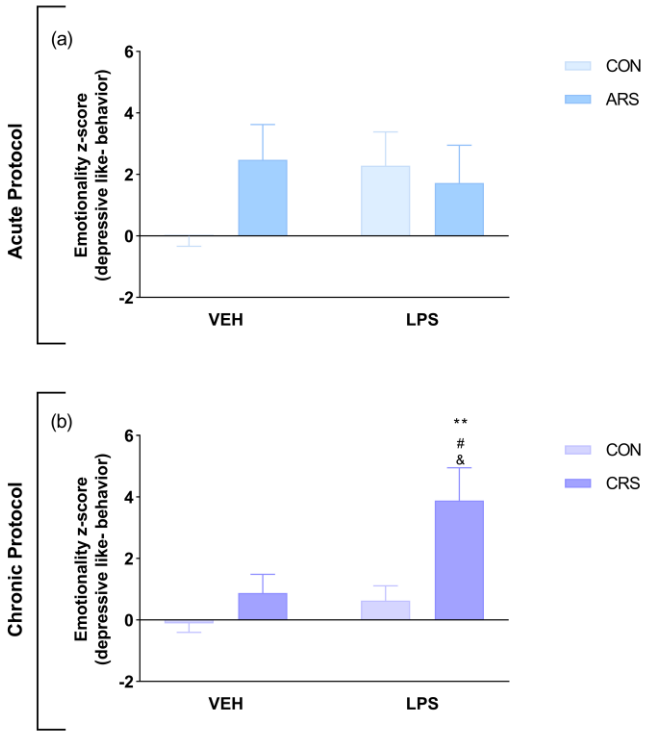

Fig. 4 Emotionality Z-Score for depressive-like behavior. **a)** Acute protocol. **b)** Chronic protocol. Data are expressed as mean ± SEM (n = 8-10/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test, ** P < 0.01 when compared to VEH+CON group, & P < 0.05 when compared to VEH+CRS group, # P < 0.05 when compared to LPS+CON group

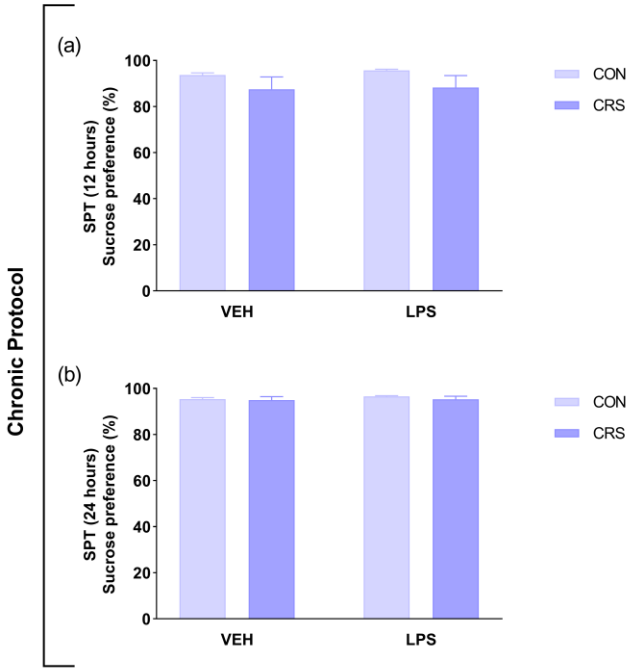

Fig. S1 Sucrose Preference Test in chronic protocol. **a)** Sucrose preference in 12 hours. **b)** Sucrose preference in 24 hours. Data are expressed as mean ± SEM (n = 8-10/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test

3.2 The chronic but not acute protocol induces anxiety-like behavior

To assess anxiety-like behavior, the EPMT was performed. In the acute protocol, a main effect of inflammatory preconditioning on the number of entries into the open arms was identified ($F_{(1,34)}$= 14.27, p= 0.0006). There was a reduction in the number of entries into the open arms in CON+LPS compared to the VEH+ARS group (p= 0.0238). In the number of entries in the closed arms there was an effect of inflammatory preconditioning ($F_{(1,35)}$= 27.63, p< 0. 0001) and stress ($F_{(1,35)}$= 6.244, p= 0.0173). A reduction in the number of entries into the closed arms was observed in CON+LPS compared to the VEH+CON (p= 0.0104) and VEH+ARS (p< 0.0001) groups, as well as in LPS+ARS when compared to VEH+ARS (p= 0.0013) group. The time spent in the open arms was not changed by the factors, however, the time spent in the closed arms indicated an interaction effect of the factors ($F_{(1,33)}$= 4.353, p= 0.0447). A reduction in time in the closed arms was found in VEH+ARS compared to the VEH+CON (p= 0.0018), LPS+CON (p= 0.0019) and LPS+ARS (p= 0.0408) groups (Fig. 3D). A significant effect of stress ($F_{(1,36)}$= 7.700, p= 0.0087) on the number of head-dipping was also identified, but Tukey's post-hoc didn't identify any significant difference between groups. Finally, the anxiety index was calculated, but no significant effect was found (Fig. 5).

In the chronic protocol, two-way ANOVA found no significant effect of factors on the number of entries into the open or closed arms. Regarding the time spent in the open arm, an interaction effect of factors was identified ($F_{(1,31)}$= 9.316, p= 0.0046). There was a less time spent in the open arms in the LPS+CRS group compared to the VEH+CON (p= 0.0031), VEH+CRS (p= 0.0172) and LPS+CON (p= 0.0002) groups. An interaction effect of the factors ($F_{(1,33)}$= 11.48, p= 0.0018) was also found on the time spent in the closed arms. An increase in the time spent in the closed arms was detected in the LPS+CRS group compared to the VEH+CON (p= 0.0455), VEH+CRS (p= 0.0033) and LPS+CON (p= 0.0019) groups, supporting the previous result. A main effect of stress was detected in the number of head-dipping ($F_{(1,32)}$= 15.65, p= 0.0004), with a reduction in the number of head-dipping in the LPS+CRS group compared to the VEH+CON (p= 0.0022) and LPS+CON (p= 0.0020) group. The calculation of the anxiety index also demonstrated a main effect of stress ($F_{(1,34)}$= 10.45, p= 0.0027), with a higher anxiety index in the LPS+CRS group compared to the VEH+CON (p= 0.0465) and LPS+CON (p= 0.0196) groups. Furthermore, no effect of the factors on the total distance travelled in the EPMT apparatus was found (Fig. 6). Thus, it was demonstrated that the acute protocol didn't cause behavioral changes related to anxiety-like

behavior, unlike the chronic protocol, which was able to induce anxiety-like behavior associated with demotivation, without compromising the animals' locomotor activity.

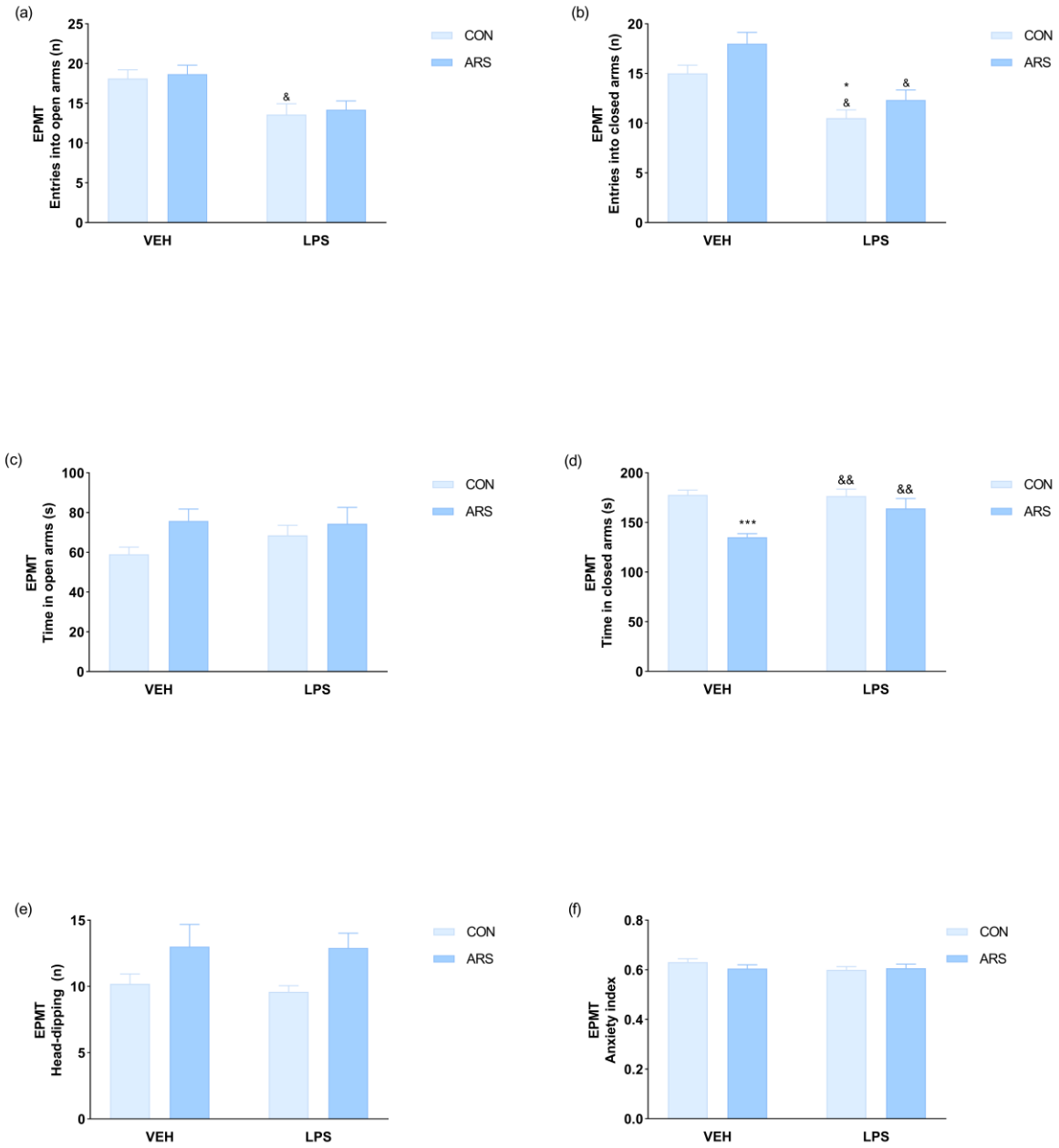

Fig. 5 Elevated Plus Maze Test for the acute protocol. **a)** Number of entries into open arms. **b)** Number of entries into closed arms. **c)** Time in open arms. **d)** Time in closed arms. **e)** Number of head-dipping. **f)** Anxiety index. Data are expressed as mean ± SEM (n = 8-10/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test, * $P < 0.05$; *** $P < 0.001$ when compared to VEH+CON, & $P < 0.05$; && $P < 0.01$ when compared to VEH+ARS group

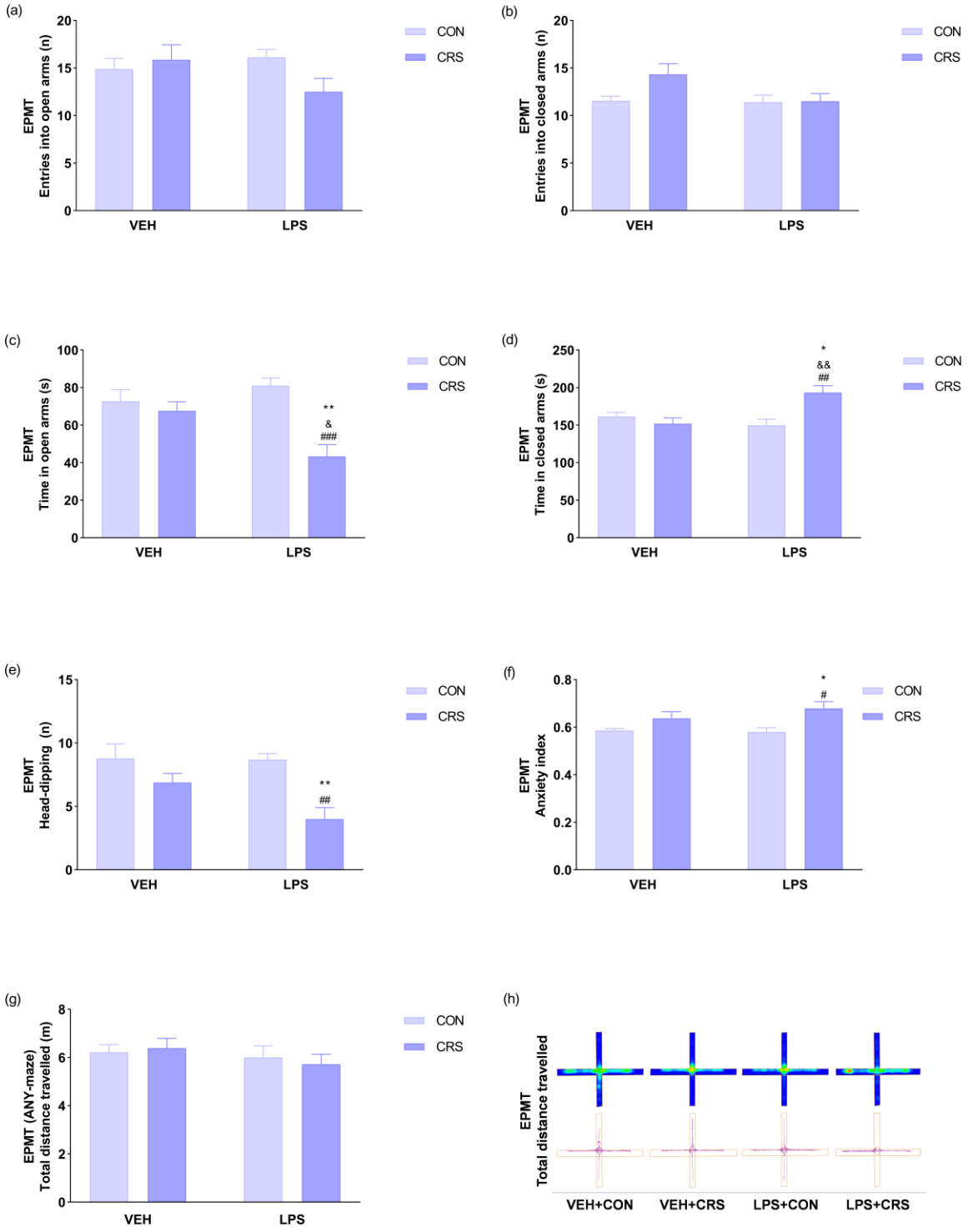

Fig. 6 Elevated Plus Maze Test for the chronic protocol. **a)** Number of entries into open arms. **b)** Number of entries into closed arms. **c)** Time in open arms. **d)** Time in closed arms. **e)** Number of head-dipping. **f)** Anxiety index. **g)** Total distance travelled. **h)** Heat map and track plot of total distance travelled. Data are expressed as mean ± SEM (n = 8-10/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test, * $P < 0.05$; ** $P < 0.01$ when compared to VEH+CON group, & $P < 0.05$; &&; $P < 0.01$ when compared to VEH+CRS group, # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ when compared to LPS+CON group

3.3 The acute and chronic protocols cause demotivational behavior without impairing the animals' locomotor activity

Locomotor activity was assessed through the number of crossings in the OFT. In the acute protocol an effect of inflammatory preconditioning ($F_{(1,30)}$= 14.14, p= 0.0007) and stress ($F_{(1,30)}$= 17.54, p= 0.0002) was identified. There was an increased in number of crossings in the VEH+ARS group compared to the VEH+CON (p= 0.0020), LPS+CON (p< 0, 0001) and LPS+ARS (p= 0.0060) groups. Exploratory activity, observed through the number of rearings, showed a main effect of inflammatory preconditioning ($F_{(1,29)}$= 32.69, p< 0.0001).

A reduction in the number of rearings in LPS+CON was observed compared to the VEH+CON (p= 0.0020) and VEH+ARS (p= 0.0001) groups, as well as the LPS+ARS compared to the VEH+CON (p= 0.0257) and VEH+ARS (p= 0.0019) groups. In the chronic protocol, two-way ANOVA identified an interaction effect between the factors ($F_{(1,31)}$= 18.08, p= 0.0002) on the number of crossings. An increase in the number of crossings was found in VEH+CRS compared to the VEH+CON (p= 0.0104) and LPS+CRS (p<0.0001) groups. Regarding the number of rearings, an interaction effect was observed ($F_{(1,33)}$= 9.802, p= 0.0036). A reduction in the number of rearings was found in the LPS+CRS group compared to the VEH+CON (p< 0.0001), VEH+CRS (p= 0.0011) and LPS+CON (p< 0.0001) groups.

Grooming time was also evaluated to verify the demotivating behavior regarding self-care and an interaction of factors was verified ($F_{(1,29)}$= 5.214, p= 0.0299), with a reduction in grooming time in the VEH+CRS group compared to the VEH+CON (p= 0.0002) and CON+LPS (p= 0.0274) groups, as well as the LPS+CRS group compared to the VEH+CON group (p= 0.0052). An interaction effect between the factors was identified in the average speed travelled in the OFT ($F_{(1,30)}$= 5.376, p= 0.0274), with a reduction in LPS+CRS compared only to the VEH+CRS group (p= 0.0100). In the total distance travelled, an interaction effect was also detected ($F_{(1,30)}$= 5.346, p= 0.0278), again a reduction in the total distance travelled was observed in the LPS+CRS group only when compared to the VEH+CRS group (p= 0.0098) (Fig. 7). Therefore, it can be suggested that the two protocols didn’t induce damage to locomotor activity, but were capable of inducing demotivation in the animals, reducing exploratory activity in both and, additionally in the case of the chronic protocol, self-care time.

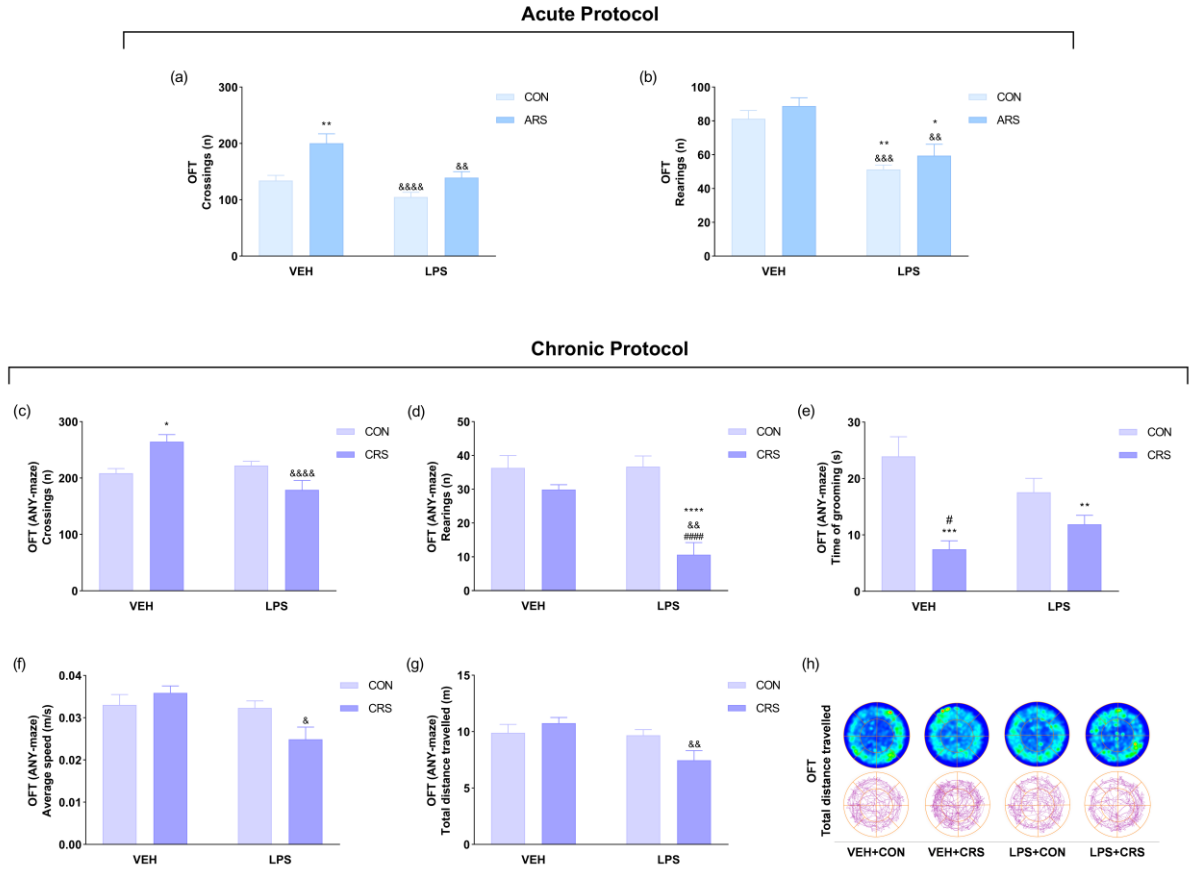

Fig. 7 Open Field Test. Acute protocol: **a)** Number of crossings, **b)** Number of rearings. Chronic Protocol: **c)** Number of crossings, **d)** Number of rearings, **e)** Time of grooming, **f)** Average speed, **g)** Total distance travelled, **h)** Heat map and track plot of total distance travelled. Data are expressed as mean ± SEM (n = 8-10/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test, * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001 when compared to VEH+CON group, & P < 0.05; && P < 0.01; &&& P < 0.001; &&&& P < 0.0001 when compared to VEH+ARS or VEH+CRS group, # P < 0.05; #### P < 0.0001 when compared to LPS+CON group

3.4 Stress is the main factor in changes in the general state of animals, especially in the parameters of coat state and body weight, resulting in reduced self-care and demotivation.

As the chronic protocol has a prolonged duration, throughout its execution the animals were evaluated weekly regarding some parameters, such as coat state and body weight. Along with these parameters, the adrenal/body weight ratio and plasma corticosterone levels, checked at the end of the protocol, make up the general state of these animals. In coat state, only a main effect of stress was detected ($F_{(1,33)}$= 56.84, p< 0.0001). LPS+CRS group showed an increase in coat score compared to the VEH+CON (p= 0.0007) and LPS+CON (p= 0.0013) groups. The VEH+CRS group also showed an increase in score compared to the VEH+CON (p< 0.0001) and LPS+CON (p< 0.0001) groups.

In body weight gain, the main effect of stress was identified ($F_{(1,36)}$= 23.79, p< 0.0001). A reduction in weight gain in the LPS+CRS was found compared to the VEH+CON

(p= 0.0006) and LPS+CON (p= 0.0046) groups, as well as in the VEH+CRS compared to the VEH+CON group (p= 0.0122). There was no effect of any factor on the adrenal/body weight ratio, as well as on plasma corticosterone levels (Fig. 8). Thus, it can be observed that both the LPS+CRS group and the VEH+CRS group showed changes in the general condition of the animals, mainly in the parameters of coat state and body weight, indicating a reduction in self-care and demotivation.

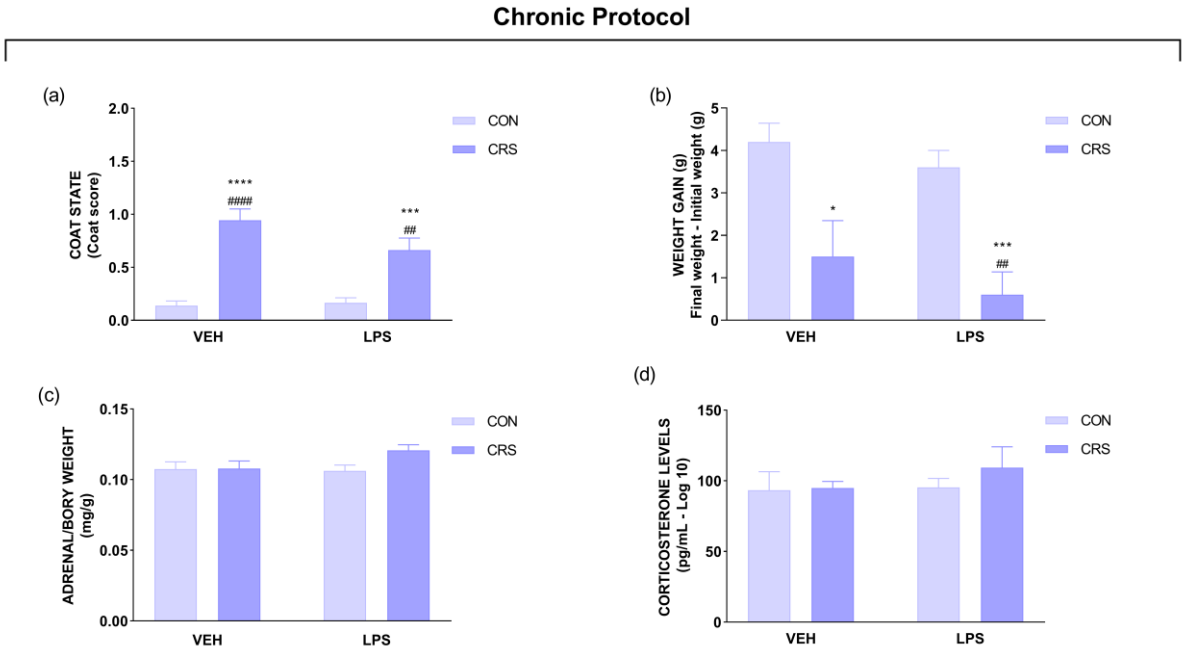

Fig. 8 General state of animals in chronic protocol. **a)** Coat score. **b)** Weight gain. **c)** Adrenal/body weight. **d)** Corticosterone levels. Data are expressed as mean ± SEM (n = 8-10/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test, * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$ when compared to VEH+CON group, ## $P < 0.01$; #### $P < 0.0001$ when compared to LPS+CON group

3.5 Biochemical and molecular assessments

3.5.1 Oxidative imbalance resulting from the association of LPS+CRS, generated by excess H_2O_2 and increased MPO activity, mainly in HIP.

Among the oxidative parameters evaluated, regarding the activity of the SOD enzyme, a main effect of inflammatory preconditioning ($F_{(1,18)}$= 11.22, p= 0.0036) was identified in the PFC. A reduction in SOD was only found in LPS+CON compared to the VEH+CON group (p= 0.0183). In HIP, two-way ANOVA did not identify any effect of factors on SOD. Regarding H_2O_2 levels, no differences were found in PFC. However, in HIP an effect of inflammatory preconditioning ($F_{(1,14)}$= 10.67, p= 0.0056) and stress ($F_{(1,14)}$= 11.32, p= 0.0046) was identified in H_2O_2 levels. An increased H_2O_2 levels was found in the LPS+CRS

compared to the VEH+CON (p= 0.0018), VEH+CRS (p= 0, 0153) and LPS+CON (p= 0.0088) groups.

CAT activity was also altered, in which a main effect of inflammatory preconditioning was observed in the PFC ($F_{(1,15)}$= 16.80, p= 0.0009). A reduction in CAT activity was observed in the LPS+CRS compared to the VEH+CON group (p= 0.0345), as well as in the LPS+CON group compared to the VEH+CON group (p= 0.0189). In the HIP, an interaction effect ($F_{(1,15)}$= 5.462, p= 0.0337) on CAT was identified, with an increase in its activity in the LPS+CRS group compared to the LPS+CON group (p= 0.0399).

The activity of the MPO enzyme in the PFC did not undergo any significant change. In the HIP there was an effect of preconditioning ($F_{(1,14)}$= 6.018, p= 0.0279) and stress ($F_{(1,14)}$= 6.370, p= 0.0243), with increased MPO activity in the LPS+CRS group compared to the VEH+CON group (p= 0.0223) (Fig. 9). Therefore, these results suggest an oxidative imbalance in the LPS+CRS group, as there is an increase in H_2O_2 levels and MPO activity in the HIP, accompanied by a reduction in CAT activity in the PFC.

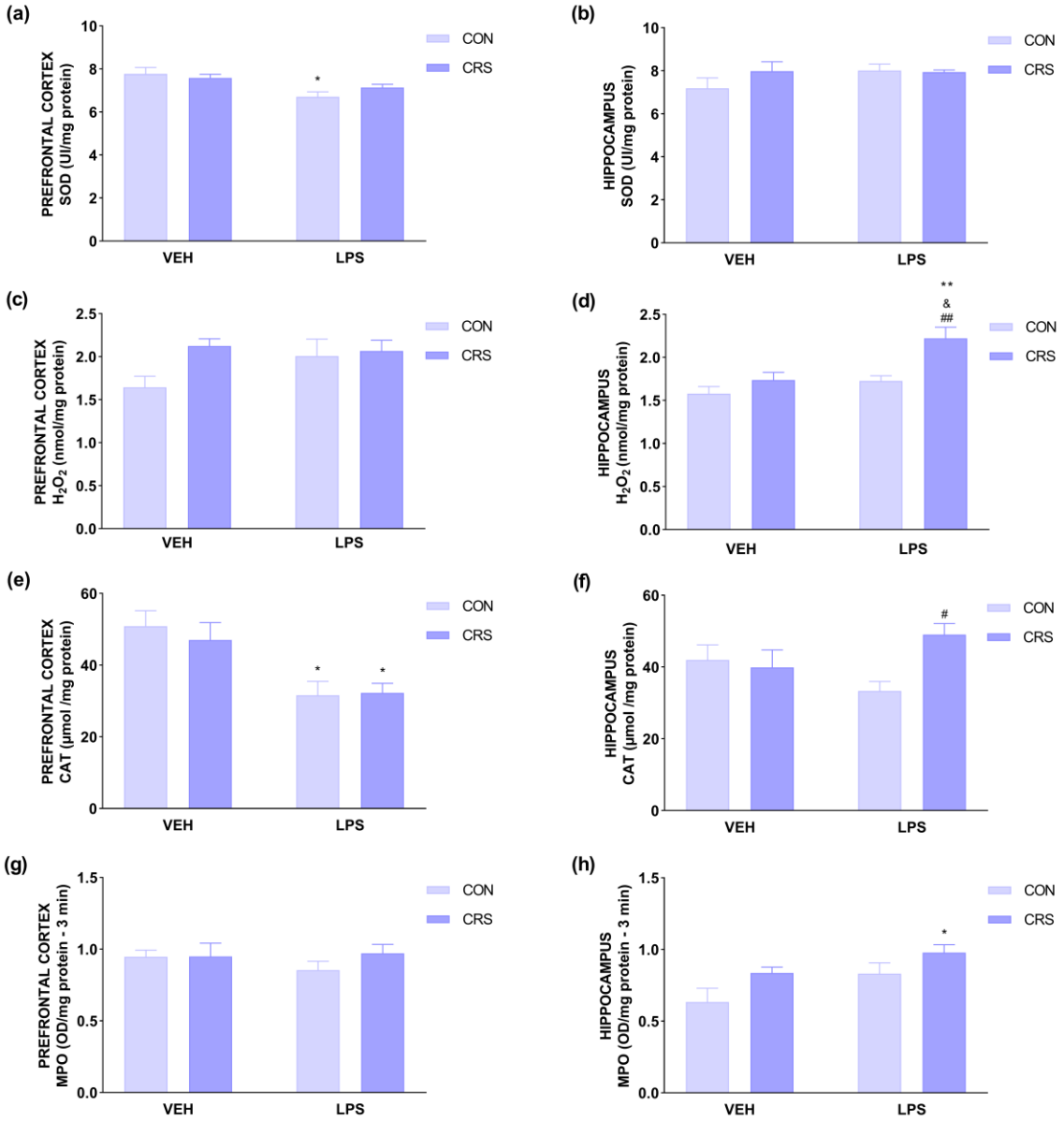

Fig. 9 Oxidative Stress of chronic protocol. Superoxide dismutase (SOD) activity in **a)** prefrontal cortex and **b)** hippocampus. Hydrogen peroxide (H_2O_2) levels in **c)** prefrontal cortex and **d)** hippocampus. Catalase (CAT) activity in **e)** prefrontal cortex and **f)** hippocampus. Myeloperoxidase (MPO) activity in **g)** prefrontal cortex and **h)** hippocampus. Data are expressed as mean ± SEM (n = 4-6/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test, * P < 0.05; ** P < 0.01 when compared to VEH+CON group, & P < 0.05 when compared to VEH+CRS group, # P < 0.05; ## P < 0.01 when compared to LPS+CON group

3.5.2 Stress can induce an increase in the level of pro-inflammatory interleukin (IL-17)

In relation to levels of peripheral pro- and anti-inflammatory cytokines, an interaction of factors was detected on IL-6 ($F_{(1,14)}$= 22.16, p= 0.0003), with an increase in its concentration in the LPS+CON group compared to the VEH+CON group (p= 0.0247) and reduction in the LPS+CRS group compared to the VEH+CRS (p= 0.0211) and LPS+CON (p= 0.0004) groups. Another pro-inflammatory interleukin that showed changes was IL-17, in

which a main effect of stress was detected ($F_{(1,12)}$= 14.45, p= 0.0025), with an increase in IL-17 levels in the VEH+CRS group compared to the VEH+CON group (p= 0.0130), furthermore, a tendency to increase the levels of this cytokine was observed in the LPS+CRS group compared to the VEH+CON group (p= 0.0751). As for the other cytokines, no changes were detected in the peripheral levels of IL-2, IL-4, INF-γ, TNF-α and IL-10 (Fig. 10). The results of this analysis suggest that stress induce an increase in peripheral pro-inflammatory cytokine level (IL-17). In addition, the association of factors (LPS+CRS) demonstrated a tendency to increase IL-17 levels, which is quite related to MDD.

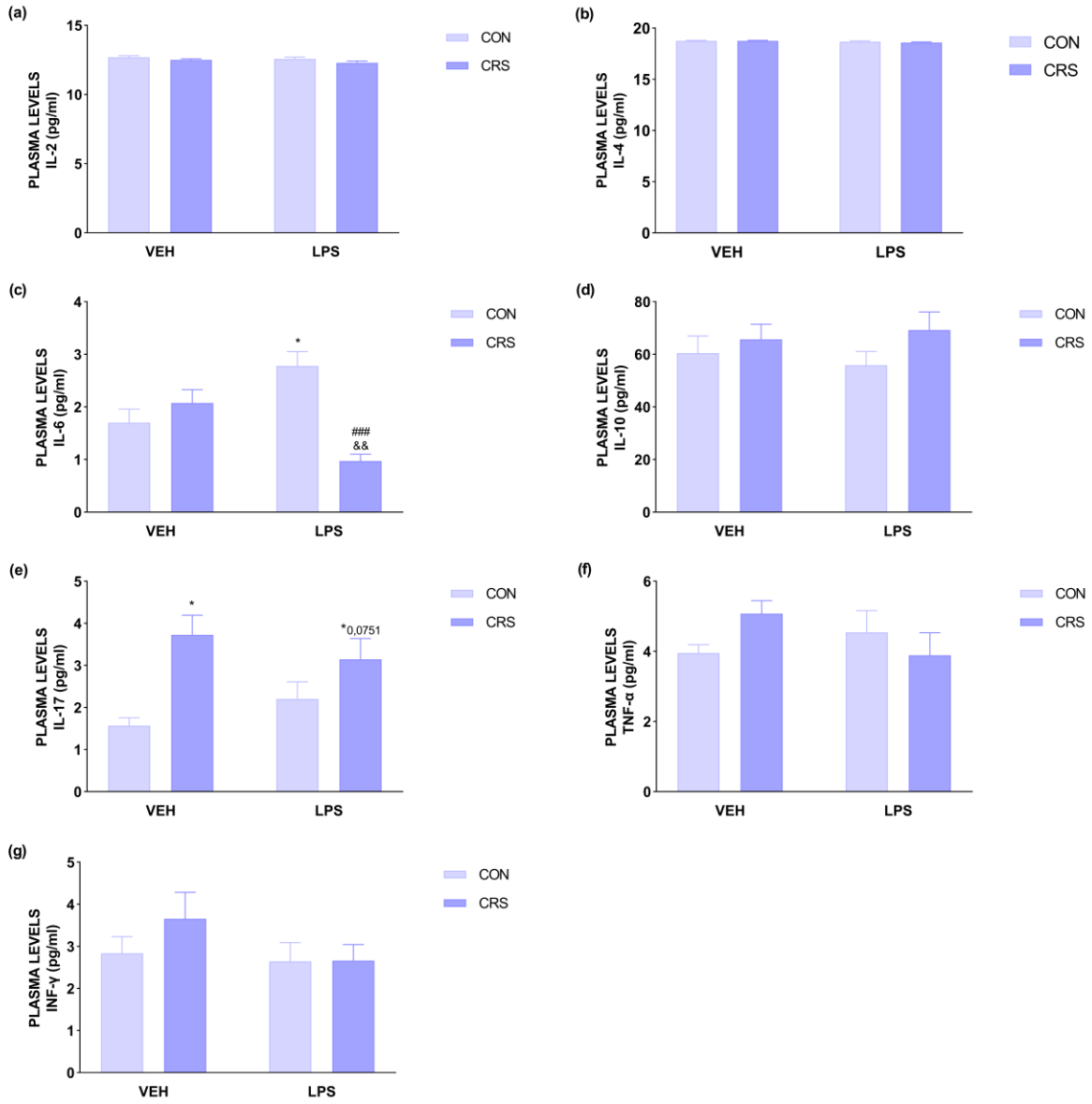

Fig. 10 Inflammatory peripheral parameters in chronic protocol. **a)** Plasma levels of IL-2. **b)** Plasma levels of IL-4. **c)** Plasma levels of IL-6. **d)** Plasma levels of IL-10. **e)** Plasma levels of IL-17. **f)** Plasma levels of TNF-α. **g)** Plasma levels of INF-γ. Data are expressed as mean ± SEM (n = 4-5/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test, * P < 0.05; *0.0751 (tendency) when compared to VEH+CON group, && P < 0.01 when compared to VEH+CRS group, ### P < 0.001; when compared to LPS+CON group

3.5.3 LPS+CRS generates a reduction in anti-inflammatory interleukin (IL-10) in the PFC, suggesting a central inflammatory imbalance.

When evaluating the levels of pro- and anti-inflammatory cytokines, an effect of inflammatory preconditioning ($F_{(1,12)}$= 5.710, p= 0.0342) and an effect of stress ($F_{(1,12)}$= 7.502, p= 0.0180) was identified on IL-10 levels, only in the PFC. The post hoc test demonstrated a reduction in IL-10 levels in the LPS+CRS group compared to the VEH+CON group (p= 0.0159). Two-way ANOVA found no effect of factors on IL-6 and TNF-α levels in both structures, CPF and HIP (Fig. 11). These results indicate that the combination of LPS+CRS reduced the levels of IL-10, an anti-inflammatory interleukin, suggesting a central inflammatory imbalance.

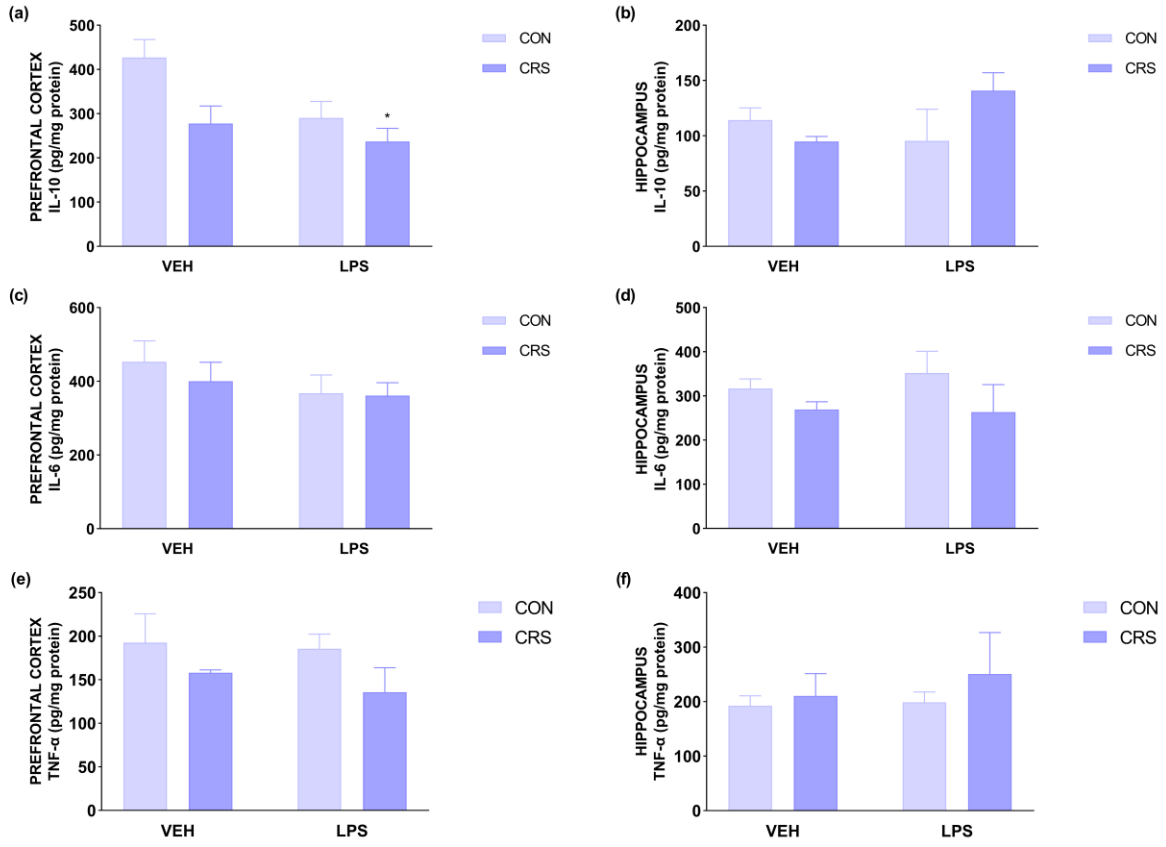

Fig. 11 Inflammatory central parameters of chronic protocol. IL-10 levels in **a)** prefrontal cortex and **b)** hippocampus. IL-6 levels in **c)** prefrontal cortex and **d)** hippocampus. TNF-α in **e)** prefrontal cortex and **f)** hippocampus. Data are expressed as mean ± SEM (n = 3-4/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test, * P < 0.05 when compared to VEH+CON group

3.5.4 The association of LPS+CRS can alter the gene expression levels of BDNF, GFAP and IBA-1, mainly in the PFC.

When evaluating BDNF gene expression levels, two-way ANOVA identified an interaction effect between factors ($F_{(1,8)}$= 7.776, p=0.0236) on PFC. A reduction in these

levels was observed in the LPS+CRS group compared to the VEH+CON (p= 0.0120), LPS+CON (p= 0.0062) and VEH+CRS (p= 0.0367) groups. In the HIP, although an interaction effect was identified ($F_{(1,8)}$= 5.466, p= 0 .0476), the multiple comparisons test did not identify significant differences between the groups.

Regarding GFAP gene expression levels in the PFC, only a main effect of stress was detected ($F_{(1,12)}$= 50.99, p< 0.0001). There was a reduction in GFAP gene expression levels in the LPS+CRS compared to the VEH+CON (p= 0.0004) and LPS+CON (p= 0.0006) groups. The VEH+CRS group also reduction in GFAP compared to the VEH+CON (p= 0.0031) and LPS+CON groups (p= 0.0071). In HIP, no changes were detected in the levels of this marker.

About the expression levels of the IBA-1 gene, an interaction effect was found between the factors ($F_{(1,8)}$= 13.48, p= 0.0063), with an increase in the expression of the IBA-1 gene in the LPS+CRS (p= 0.0224), VEH+CRS (p= 0.0040) and LPS+CON (p= 0.0204) compared to the VEH+CON group, without changes in HIP (Fig. 12). It can be observed that the effects on the gene expression of these markers are predominantly related to the brain structure of the PFC, mainly in the LPS+CRS group. These results suggesting that the increase in IBA-1 corresponds to an exacerbated central immune response. The reduction in GFAP may indicate atrophy or loss of functional capacity of astrocytes, accompanied by a reduction in BDNF, neurotrophin associated with neuroplasticity and neuronal survival.

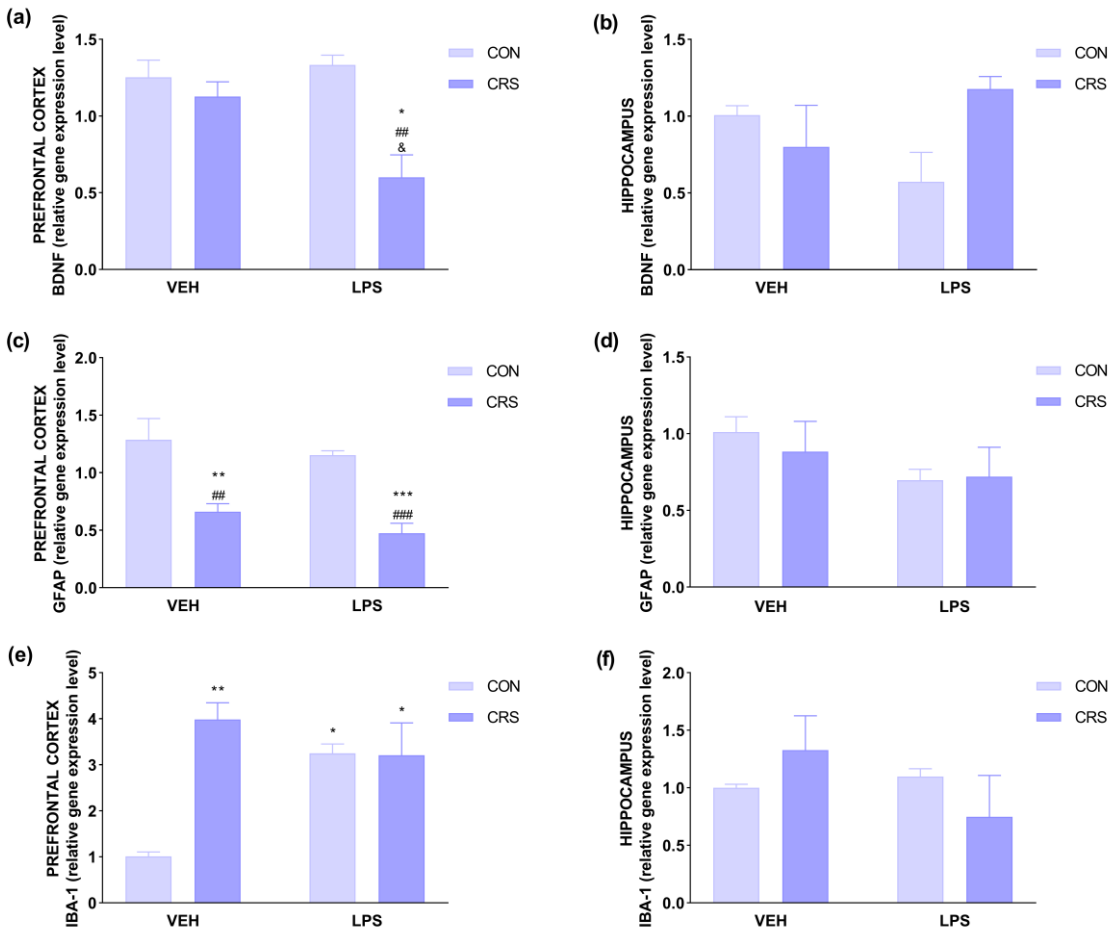

Fig. 12 Molecular parameters in chronic protocol. Relative gene expression levels of BDNF in **a)** prefrontal cortex and **b)** hippocampus. Relative gene expression levels of GFAP in **c)** prefrontal cortex and **d)** hippocampus. Relative gene expression levels of IBA-1 in **e)** prefrontal cortex and **f)** hippocampus. Data are expressed as mean ± SEM (n = 3-5/group) and were evaluated by two-way ANOVA, followed by Tukey's post hoc test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared to VEH+CON group, & $P < 0.05$ when compared to VEH+CRS group, ## $P < 0.01$; ### $P < 0.001$ when compared to LPS+CON group

4. Discussion

Although many studies have investigated depressive and anxiety-like behavior using the restraint stress model (Jiang et al. 2018; Peng et al. 2022; Misztak et al. 2022) or the effect of the isolated inflammatory inducer (Wang et al. 2020; Ali et al. 2020), little is known about how animals can respond to stress after being subjected to inflammatory preconditioning. A large body of literature on risk for MDD has identified environmental (e.g., family environment, socioeconomic status, lifestyle) and individual (e.g., gender, psychiatric comorbidity, personality) risk factors (Ye et al. 2023; Peng et al. 2022; Remes et al. 2021). Stressful life events are associated with the first onset, as well as subsequent recurrence, of MDD (Tong et al. 2023). However, the impact of an inflammatory stimulus on this risk is poorly addressed and elucidated. Here, we investigated whether inflammatory preconditioning

disrupts an expected physiological response following an acute stress protocol and whether it increases susceptibility to the development of depressive and anxiety-like behaviors in animals subjected to CRS protocols. Our results indicated that only the animals that received LPS and that were subsequently subjected to the chronic protocol (CRS) showed depressive- and anxiety-like behaviors (as can be observed in TST, FST and Z-Score). Both protocols (acute and chronic) induced demotivational behavior observed through the reduction of climbing (as can be observed in FST) and exploratory activity (as can be observed in OFT). In the chronic protocol, changes were found in the general health condition of the animals, indicated by a reduction in self-care due to demotivation/apathy and a possible reduction in appetite. In the biochemical and molecular evaluation, the LPS+CRS group showed an oxidative imbalance in the HIP (increased H_2O_2 levels and MPO activity). In the PFC of these animals, we found a central inflammatory imbalance (reduction in IL-10 levels) and an exacerbated immune response was suggested (elevated gene expression of IBA1), accompanied by possible atrophy/loss of astrocyte functionality and compromised neuroplasticity (reduced gene expression of GFAP and BDNF). At the peripheral level, a tendency to increase levels of pro-inflammatory interleukin (IL-17) was observed. A summary of these results can be seen in Fig 13.

Behavioral responses resulting from acute or short-term stress are considered a fundamental psychophysiological defense mechanism for survival and well-being (Dhabhar 2018). This short-term stress prepares the organism for an immediate reaction or response to a stressor, disappearing after the extinction of this stimulus (James et al. 2023). Therefore, acute stress can trigger a beneficial response in the body to combat or escape a specific stressor. Our results demonstrated that the acute protocol didn't induce depressive and anxiety-like behavior. Although there are divergences in the literature, it has also been demonstrated that protocols using restraint stress for less than 2 weeks generally do not induce significant behavioral changes, nor anxiety-like behavior, which typically appears after 1 week of restraint (Wang et al. 2017; Codeluppi et al. 2021). Nonetheless, the LPS+ARS group showed shorter climbing time in the FST compared to the VEH+ARS group. This suggests that inflammatory preconditioning reduces the climbing response, disturbing a protective adaptive strategy in the face of an acute stressful stimulus. In addition, inflammatory preconditioning significantly reduced the number of rearings in the LPS+ARS group compared to VEH+ARS, reflecting impaired exploratory behavior. The behavioral measure of rearings, verified when the animal rests on its hind legs, constitutes rodents' exploration behavior and vertical mobility (Tanaka et al. 2012). Exploratory behavior is intrinsic to rodents, which have an

innate curiosity for new stimuli and functions as an important behavior for survival, as it performs an adaptive response to a new environment (Adelöf et al. 2021). Thus, we propose that inflammatory preconditioning impaired exploratory behavior and may be associated with demotivational behavior.

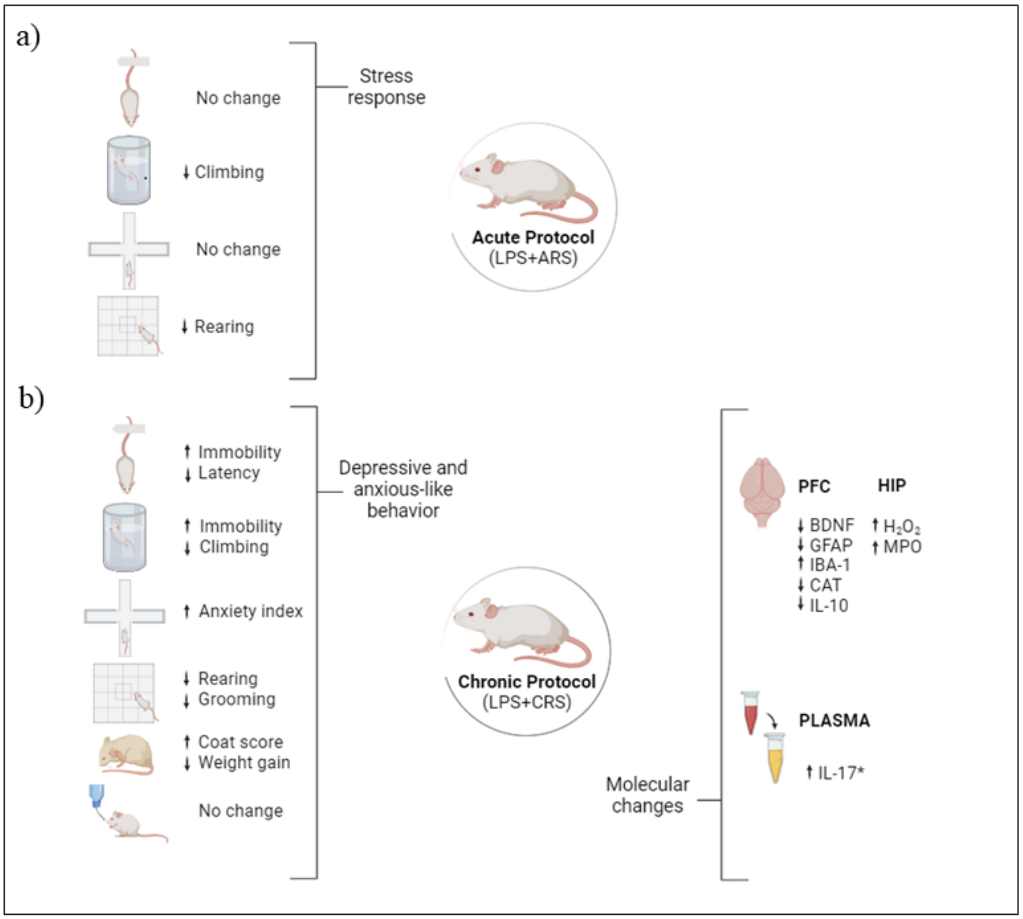

Fig. 13 Schematic diagram illustrating the summary of the main conclusions of the study when comparing the LPS+ARS and LPS+CRS groups with the VEH+CON group of each protocol. **a)** The acute protocol (LPS+ARS) induced a stress response, related to demotivation. **b)** The chronic protocol (LPS+CRS) induced depressive- and anxiety-like behaviors. These behaviors were related to oxidative imbalance, due to a reduction in CAT in the PFC and an increase in H2O2 and MPO in the HIP. The inflammatory imbalance may also have contributed to behavioral changes, due to the reduction of an anti-inflammatory interleukin (IL-10) and increased microglial activation (IBA-1) in the PFC. Furthermore, a tendency towards an increase in a pro-inflammatory interleukin (IL-17) was found at the peripheral level, closely related to MDD. Astrocyte atrophy (GFAP) and reduction of a neurotrophin important for synaptic plasticity (BDNF) can also be suggested as contributors to this behavioral outcome. Figure created with BioRender.com

According to our results, acute stress can generate a beneficial responses and resolution skills. However, when this stress becomes prolonged and repetitive (chronic), unwanted effects on physical and mental health occur, reducing resistance to the stressor and leading to possibly irreversible physiological damage (James et al. 2023). This maladaptive response to persistent stress impacts neural circuits that would normally regulate stress reactivity, contributing to the development of psychiatric disorders such as anxiety and MDD

(McEwen and Akil 2020). Thus, restraint stress simulates human psychological stress, which is repetitive and predictable but inevitable, such as repetitive stress caused by work or, social or financial stress (Peng et al. 2022; Wang et al. 2017). In addition to the stressful factor, it is known that the inflammatory process plays an important role in the development and progression of neuropsychiatric diseases, including anxiety and MDD. There is a relationship between MDD, stress and inflammation. Acute stress can temporarily increase inflammation, which is reversed by the anti-inflammatory effect of glucocorticoids (Steptoe et al. 2018). Chronic stress causes glucocorticoid resistance, impairing the anti-inflammatory response and consequently increasing the inflammatory profile (Barnes and Adcock 2009). The pathogenesis of MDD developed by stress may be associated with a dysregulation in the inflammatory response in some individuals, in the periphery and the CNS, with different sources providing inflammatory factors (Berk et al. 2013; Tong et al. 2023). Furthermore, inflammatory processes cause symptoms like MDD, such as sickness behavior, anhedonia, lack of motivation, reduced social interaction and altered sleep (Tong et al. 2023). Our behavioral results indicated that only the LPS+CRS group showed depressive and anxiety-like behavior, indicating that inflammatory preconditioning was fundamental for the development of this behavior. Although anhedonia is involved in this disorder, in this study it was not possible to observe significant differences. We suggest that this result may be related to the duration of our stress protocol (4 weeks), as this behavior usually appears after 5 weeks of stress (Codeluppi et al. 2021). Interestingly, only the VEH+CRS group showed an increase in the number of crossings in the OFT, probably due to the stress model used, as restraint attenuates fear learning and generates locomotor hyperactivity (Ito et al. 2010). The LPS+CRS group showed a reduction in rearings, as in the acute protocol, demonstrating again that the damage to the animals' exploratory activity is generated from inflammatory preconditioning and didn't depend on the duration of the subsequent stress. The assessment of the coat state is used to verify apathy in animal models of MDD, perceived when the animal is stressed and presents a reduction in coat care (Planchez et al. 2019). We found that stress is the factor involved in reducing self-care behavior, representing a state of demotivation and apathy in animals, as both VEH+CRS and LPS+CRS groups showed an increase in coat scores. In the OFT, deficits in grooming behavior were also observed in these same groups. In these same groups, a reduction in the animals' body weight was also shown. This finding is in line with what may occur in patients with MDD, since changes in body weight and appetite are frequent symptoms of this disorder (Cui et al. 2024). Regarding plasma corticosterone levels, we found no difference, possibly due to the duration of the protocol. It has been

demonstrated that in the first weeks of CRS there is hyperactivation of the HPA axis. However, after 3 weeks this hyperactivation disappears and there are no significant changes in corticosterone levels (Chiba et al. 2012; Voorhees et al. 2013). Together, these results suggest that chronic stress is the factor involved in the changes observed in the general state.

It is known that the increase in inflammatory biomarkers is associated with MDD and anxiety symptoms (Roman and Irwin 2020; Michopoulos et al. 2017). In pre-clinical and clinical studies involving MDD, high levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, have already been demonstrated at peripheral and central levels (Peng et al. 2022; Young et al. 2014). Neuronal activity in the brain is also affected by inflammation, as these inflammatory cytokines alter the function of neurotransmitters, reducing serotonin levels, affecting mood and behavior (Tong et al. 2023; Karimi et al. 2022; Dantzer 2017). Furthermore, studies have shown that the use of anti-inflammatory agents associated with antidepressants can favor the response to MDD treatment, especially in those patients who present exacerbated immune activation (Bai et al. 2020; Arteaga-Henríquez et al. 2019; Simon et al. 2023). The set of data on the inflammatory profile obtained in this study indicated a main effect of stress on the peripheral increase in IL-17 observed in the VEH+CRS group. In contrast, the LPS+CRS group showed a tendency to increase this pro-inflammatory cytokine. At a central level, we found an effect of inflammatory preconditioning and stress on IL-10 levels, reduced only in the LPS+CRS group. On the other hand, it has already been described in the literature that the administration of IL-10 attenuates the depressive-like behavior in mice, corroborating our data (Worthen et al. 2020; Laumet et al. 2018). In addition, there is an association between neuroinflammation and neuropsychiatric disorders, suggesting that it contributes significantly to the onset and progression of MDD (Adzic et al. 2017; Troubat et al. 2021). The regulation of neuroinflammation occurs mainly by microglial cells, modulating the response to different stressors through communication between the nervous and immune systems to maintain brain homeostasis (Rahimian et al. 2022). When homeostasis is threatened through an infection, inflammatory insult or tissue injury, the CNS microenvironment changes, and microglial activation occurs, resulting in increased release of pro-inflammatory cytokines and reduced anti-inflammatory cytokines (Rahimian et al. 2022; Felger and Lotrich 2013; He et al. 2020). Long-lasting sensitization of activated microglia is associated with the development of neuropsychiatric disorders such as MDD (Rahimian et al. 2021; Mechawar and Savitz 2016; Mondelli et al. 2017). This microglial activation can cause neuronal death, decreased production of neurotransmitters, neuroplasticity and neurotrophic factors (Singhal and Baune

2017; Miller et al. 2009). Our results demonstrated that LPS+CRS and VEH+CRS induced microglial activation, detected by IBA-1, suggesting an increase in the central immune response, possibly generating neuroinflammation in both groups.

Under stress conditions, the increase in the inflammatory profile can damage the blood-brain barrier (BBB), causing the infiltration of pro-inflammatory cytokines (Peng et al. 2022). Furthermore, astrocytes and microglia rapidly respond to noxious stimuli, potentially altering their morphology and function, further exacerbating neuroinflammation (Shi et al. 2018; Peng et al. 2021). Astrocytes are the most abundant cell type in the CNS and perform several functions, such as recycling neurotransmitters and immunological signaling (Giovannoni and Quintana 2020). Several studies have demonstrated a reduction in astrocytes in the brains of patients (post-mortem) with MDD and animal models of chronic stress, as well as a reduction in the levels of GFAP, an astrocyte marker (Murphy-Royal et al. 2019; Liu et al. 2022; Kim R et al. 2018) . It has been proposed that reduced GFAP may indicate atrophy/degeneration, loss of functionality and pathological remodeling of astrocytes (Kim R et al. 2018; Pekny et al. 2016). We demonstrate here that the LPS+CRS and VEH+CRS groups showed a reduction in GFAP gene expression in the PFC. This result may suggest atrophy or functional loss of astrocytes caused by stress, which may cause damage to the BBB, further contributing to neuroinflammation. BDNF is a neurotrophin involved in several physiological processes, such as neurogenesis, neuroplasticity, neuroprotection and mood regulation (Schirò et al. 2022; Oh et al. 2016). The neuroprotective effects of BDNF are also associated with preventing neuronal death and reducing oxidative damage (Chen et al. 2017). Reduced BDNF levels are associated with cognitive decline and increased frequency of depressive symptoms, which are restored after antidepressant use (You and Lu 2023; Correia et al. 2023b). It has already been demonstrated that the administration of LPS or pro-inflammatory cytokines in rodents reduces BDNF levels in the HIP and cortex, indicating that inflammation affects the expression of BDNF, contributing to the development of MDD (Calabrese et al. 2014). Our study demonstrated that LPS+CRS reduced BDNF gene expression in the PFC, compared to the VEH+CON and VEH+CRS groups, indicating that inflammatory pre-exposure exerts an effect that was possibly enhanced by subsequent stress.

The involvement of oxidative stress in the development of MDD is also the subject of investigation. Oxidative stress interacts and deregulates several systems, such as the HPA axis, serotonergic pathways and BDNF(Keller et al. 2017; Correia et al. 2023a). Excessive oxidation or insufficient antioxidant response can lead to the accumulation of reactive oxygen species, called oxidative stress (Ji et al. 2023). This oxidative stress disrupts homeostasis and

exacerbates neuroinflammation, neurodegeneration and tissue damage (Bajpai et al. 2014; Sani et al. 2023). Our results also indicated an increase in H_2O_2 levels in the LPS+CRS group compared to the VEH+CON and VEH+CRS groups in the HIP. We also found in the LPS+CRS group a reduction in CAT activity and an increase in MPO activity compared to VEH+CON, in the PFC and in the HIP, respectively. This suggests that inflammatory pre-exposure followed by stress generates greater oxidative imbalance, especially in HIP. In the CNS, MPO is secreted by microglia, astrocytes and neurons, associated with neuroinflammation and MDD(Siraki 2021). In the presence of H_2O_2 and halide (Cl, Br), MPO catalyzes reactions that form reactive oxidizing/chlorinating agents, such as hypochlorous acid (HOCl), causing cytotoxic effects(Ray and Katyal 2016). Thus, the results of this study indicate the involvement of oxidative stress in depressive-like behavior, in line with what occurs in the clinic, as patients with MDD present increased oxidative stress (Bhatt et al. 2020).

5. Conclusion

To our knowledge, our study is the first to evaluate the impact of inflammatory preconditioning followed by restraint stress. The acute protocol (LPS+ARS) induced behavioral impairments related to demotivation, indicating the participation of the inflammatory process in these changes. The chronic protocol (LPS+CRS), on the other hand, demonstrated that inflammatory preconditioning plays an important role in the development of depressive and anxious-like behaviors, associated with inflammatory/oxidative imbalance, neuroinflammation, astrocyte atrophy and BDNF reduction. Our results also indicate that the inflammatory process may be an important risk factor for the development of behaviors phenotypically related to MDD. From this perspective, modulation of the inflammatory stimulus may be a potential therapeutic target for a possible reduction in the risk of developing the disorder. Despite the limitations found in animal models and the complexity involved in MDD, any advance in preclinical research can contribute to improving the understanding of the pathophysiological mechanisms of MDD. Thus, considering the different aspects or symptoms of MDD, it is interesting to use different behavioral, biochemical and molecular parameters to identify changes that complement each other, helping to deepen knowledge about this serious and prevalent mood disorder, as well as in the search for new therapeutic strategies.

Table S1 ANOVA results

Readout	Main effect of inflammatory preconditioning (LPS)	Main effect of Restraint Stress (ARS or CRS)	Interaction effects (inflammatory preconditioning * restraint Stress) (LPS+ARS or LPS+CRS)
ACUTE PROTOCOL			
Behavioral Tests			
TST			
Immobility (s)	$F_{(1,28)} = 23.72$, $p < 0.0001$	$F_{(1,28)} = 2.664$, $p = 0.1138$	$F_{(1,28)} = 0.9419$, $p = 0.3401$
FST			
Immobility (s)	$F_{(1,29)} = 0.0003$, $p = 0.9863$	$F_{(1,29)} = 2.597$, $p = 0,1179$	$F_{(1,29)} = 2.756$, $p = 0.1077$
Climbing (s)	$F_{(1,30)} = 14.59$, $p = 0.0006$	$F_{(1,30)} = 3.830$, $p = 0.0597$	$F_{(1,30)} = 0.5769$, $p = 0.4534$
EPMT			
Entries into open arms (n)	$F_{(1,34)} = 14.27$, $p = 0.0006$	$F_{(1,34)} = 0.2364$, $p = 0.6300$	$F_{(1,34)} = 0.0003$, $p = 0.9852$
Entries into closed arms (n)	$F_{(1,35)} = 27.63$, $p < 0.0001$	$F_{(1,35)} = 6.244$, $p = 0.0173$	$F_{(1,35)} = 0.3638$, $p = 0.5503$
Time in open arms (s)	$F_{(1,32)} = 0.4609$, $p = 0.5021$	$F_{(1,32)} = 3.611$, $p = 0.0664$	$F_{(1,32)} = 0.8377$, $p = 0.3669$
Time in closed arms (s)	$F_{(1,33)} = 3.711$, $p = 0.0627$	$F_{(1,33)} = 14.42$, $p = 0.0006$	$F_{(1,33)} = 4.353$, $p = 0.0447$
Head-dipping (n)	$F_{(1,36)} = 0.1014$, $p = 0.7520$	$F_{(1,36)} = 7.700$, $p = 0.0087$	$F_{(1,36)} = 0.05174$, $p = 0.8214$
Anxiety index	$F_{(1,34)} = 0.9982$, $p = 0.3248$	$F_{(1,34)} = 0.4353$, $p = 0.5138$	$F_{(1,34)} = 1.237$, $p = 0.2738$
OFT			
Crossings (n)	$F_{(1,30)} = 14.14$, $p = 0.0007$	$F_{(1,30)} = 17.54$, $p = 0.0002$	$F_{(1,30)} = 1.740$, $p = 0.1971$
Rearings (n)	$F_{(1,29)} = 32.69$, $p < 0.0001$	$F_{(1,29)} = 2.289$, $p = 0.1411$	$F_{(1,29)} = 0.004482$, $p = 0.9471$
Emotionality Z-Score (TST+FST)	$F_{(1,28)} = 0.5716$, $p = 0.4559$	$F_{(1,28)} = 0.8864$, $p = 0.3545$	$F_{(1,28)} = 2.225$, $p = 0.1469$
CHRONIC PROTOCOL			
Behavioral Tests			
TST			
Immobility (s)	$F_{(1,32)} = 7.558$, $p = 0.0097$	$F_{(1,32)} = 8.351$, $p = 0.0069$	$F_{(1,32)} = 0.3412$, $p = 0.5633$
Latency for immobility (s)	$F_{(1,34)} = 9.984$, $p = 0.0033$	$F_{(1,34)} = 1.188$, $p = 0.2834$	$F_{(1,34)} = 0.6910$, $p = 0.4116$
FST			
Immobility (s)	$F_{(1,32)} = 1.692$, $p = 0.2026$	$F_{(1,32)} = 6.565$, $p = 0.0153$	$F_{(1,32)} = 2.107$, $p = 0.1564$
Latency for immobility (s)	$F_{(1,28)} = 5.106$, $p = 0.0318$	$F_{(1,28)} = 0.07367$, $p = 0.788$	$F_{(1,28)} = 0.01195$, $p = 0.9137$
Climbing (s)	$F_{(1,36)} = 4.444$, $p = 0.0420$	$F_{(1,36)} = 11.65$, $p = 0.0016$	$F_{(1,36)} = 0.8364$, $p = 0.3665$
Average Speed (m/s)	$F_{(1,31)} = 0.01711$, $p = 0.8968$	$F_{(1,31)} = 0.07056$, $p = 0.7923$	$F_{(1,31)} = 2.582$, $p = 0.1182$
Total distance travelled (m)	$F_{(1,30)} = 0.1454$, $p = 0.7057$	$F_{(1,30)} = 9.980e\text{-}005$, $p = 0.9921$	$F_{(1,30)} = 3.444$, $p = 0.0733$
SPT			
Sucrose preference (%) (12 hours)	$F_{(1,32)} = 0.1650$, $p = 0.6873$	$F_{(1,32)} = 4.106$, $p = 0.0511$	$F_{(1,32)} = 0.03369$, $p = 0.8555$
Sucrose preference (%) (24 hours)	$F_{(1,33)} = 0.5114$, $p = 0.4796$	$F_{(1,33)} = 0.7565$, $p = 0.3907$	$F_{(1,33)} = 0.1568$, $p = 0.6947$
EPMT			
Entries into open arms (n)	$F_{(1,31)} = 0.6848$, $p = 0.4143$	$F_{(1,31)} = 1.042$, $p = 0.3152$	$F_{(1,31)} = 3.183$, $p = 0.0842$
Entries into closed arms (n)	$F_{(1,34)} = 3.344$, $p = 0.0762$	$F_{(1,34)} = 3.100$, $p = 0.0873$	$F_{(1,34)} = 2.684$, $p = 0.1106$
Time in open arms (s)	$F_{(1,31)} = 2.238$, $p = 0.1448$	$F_{(1,31)} = 16.02$, $p = 0.0004$	$F_{(1,31)} = 9.316$, $p = 0.0046$
Time in closed arms (s)	$F_{(1,33)} = 3.535$, $p = 0.0690$	$F_{(1,33)} = 4.669$, $p = 0.0381$	$F_{(1,33)} = 11.48$, $p = 0.0018$
Head-dipping (n)	$F_{(1,32)} = 3.172$, $p = 0.0844$	$F_{(1,32)} = 15.65$, $p = 0.0004$	$F_{(1,32)} = 2.848$, $p = 0.1012$
Anxiety index	$F_{(1,34)} = 0.5551$, $p = 0.4614$	$F_{(1,34)} = 10.45$, $p = 0.0027$	$F_{(1,34)} = 1.026$, $p = 0.3182$

OFT			
Crossings (n)	$F_{(1,31)} = 9.556$, p= 0.0042	$F_{(1,31)} = 0.3209$, p= 0.5752	$F_{(1,31)} = 18.08$, p= 0.0002
Rearings (n)	$F_{(1,33)} = 9.021$, p=0.0051	$F_{(1,33)} = 26.75$, p < 0.0001	$F_{(1,33)} = 9.802$, p= 0.0036
Time of grooming (s)	$F_{(1,29)} = 0.1661$, p= 0.6866	$F_{(1,29)} = 22.03$, p<0,0001	$F_{(1,29)} = 5.214$, p= 0.0299
Average speed (m/s)	$F_{(1,30)} = 6.853$, p= 0.0137	$F_{(1,30)} = 1.058$, p= 0.3120	$F_{(1,30)} = 5.376$, p= 0.0274
Total distance travelled (m)	$F_{(1,30)} = 6.953$, p= 0.0131	$F_{(1,30)} = 1.027$, p= 0.3191	$F_{(1,30)} = 5.346$, p= 0.0278
General state			
Coat state (coat score)	$F_{(1,33)} = 2.168$, p= 0.1504	$F_{(1,33)} = 56.84$, p < 0.0001	$F_{(1,33)} = 3.220$, p= 0.0819
Weight gain (g)	$F_{(1,36)} = 1.648$, p= 0.2075	$F_{(1,36)} = 23.79$, p< 0.0001	$F_{(1,36)} = 0.06591$, p= 0.7989
Adrenal/ body weight (mg/g)	$F_{(1,34)} = 1.559$, p= 0.2203	$F_{(1,34)} = 2.558$, p= 0.1190	$F_{(1,34)} = 2.270$, p= 0.1411
Corticosterone levels (pg/mL)	$F_{(1,14)} = 0.7023$, p= 0.4161	$F_{(1,14)} = 0.6450$, p= 0.4353	$F_{(1,14)} = 0.4006$, p= 0.5370
Emotionality Z-Score (TST+FST)	$F_{(1,31)} = 7.251$, p= 0.0113	$F_{(1,31)} = 9.299$, p= 0.0047	$F_{(1,31)} = 2.654$, p= 0.1134
Biochemical assessments			
Oxidative parameters			
SOD (UI/mg protein) - PFC	$F_{(1,18)} = 11.22$, p= 0.0036	$F_{(1,18)} = 0.3175$, p= 0.5801	$F_{(1,18)} = 1.798$, p= 0.1967
SOD (UI/mg protein) - HIP	$F_{(1,18)} = 1.165$, p= 0.2947	$F_{(1,18)} = 0.9670$, p= 0.3385	$F_{(1,18)} = 1.433$, p= 0.2468
H2O2 (nmol/mg protein) - PFC	$F_{(1,14)} = 1.334$, p= 0.2674	$F_{(1,14)} = 4.123$, p= 0.0618	$F_{(1,14)} = 2.537$, p= 0.1335
H2O2 (nmol/mg protein) - HIP	$F_{(1,14)} = 10.67$, p= 0.0056	$F_{(1,14)} = 11.32$, p= 0.0046	$F_{(1,14)} = 3.031$, p= 0.1036
CAT (µmol /mg protein) - PFC	$F_{(1,15)} = 16.80$, p= 0.0009	$F_{(1,15)} = 0.1450$, p= 0.7087	$F_{(1,15)} = 0.3026$, p= 0.5904
CAT (µmol /mg protein) – HIP	$F_{(1,15)} = 0.003581$, p= 0.9531	$F_{(1,15)} = 3.172$, p= 0.0952	$F_{(1,15)} = 5.462$, p= 0.0337
MPO (OD/mg protein) - PFC	$F_{(1,20)} = 0.2703$, p= 0.6088	$F_{(1,20)} = 0.7725$, p= 0.3899	$F_{(1,20)} = 0.6969$, p= 0.4137
MPO (OD/mg protein) - HIP	$F_{(1,14)} = 6.018$, p= 0.0279	$F_{(1,14)} = 6.370$, p= 0.0243	$F_{(1,14)} = 0.1626$, p= 0.6928
Inflammatory peripheral parameters			
IL-2 (pg/ml)	$F_{(1,16)} = 2.084$, p= 0.1681	$F_{(1,16)} = 3.987$, p= 0.0631	$F_{(1,16)} = 0.1023$, p= 0.7532
IL-4 (pg/ml)	$F_{(1,15)} = 3.697$, p= 0.0737	$F_{(1,15)} = 0.2723$, p= 0.6094	$F_{(1,15)} = 0.4611$, p= 0.5075
IL-6 (pg/ml)	$F_{(1,14)} = 0.003530$, p= 0.9535	$F_{(1,14)} = 9.580$, p= 0.0079	$F_{(1,14)} = 22.16$, p= 0.0003
IL-10 (pg/ml)	$F_{(1,13)} = 0.008765$, p= 0.9268	$F_{(1,13)} = 2.236$, p= 0.1587	$F_{(1,13)} = 0.4254$, p= 0.5256
IL-17 (pg/ml)	$F_{(1,12)} = 0.003759$, p= 0.9521	$F_{(1,12)} = 14.45$, p= 0.0025	$F_{(1,12)} = 2.220$, p= 0.1621
TNF-a (pg/ml)	$F_{(1,13)} = 0.3269$, p= 0.5772	$F_{(1,13)} = 0.2006$, p= 0.6616	$F_{(1,13)} = 2.861$, p= 0.1145
INF-g (pg/ml)	$F_{(1,14)} = 1.447$, p= 0.2490	$F_{(1,14)} = 0.7161$, p= 0.4117	$F_{(1,14)} = 0.6655$, p= 0.4283
Inflammatory central parameters			
IL-10 (pg/mg protein) - PFC	$F_{(1,12)} = 5.710$, p= 0.0342	$F_{(1,12)} = 7.502$, p= 0.0180	$F_{(1,12)} = 1.701$, p= 0.2166
IL-10 (pg/mg protein) - HIP	$F_{(1,8)} = 0.5912$, p= 0.4640	$F_{(1,8)} = 0.5556$, p= 0.4774	$F_{(1,8)} = 3.424$, p= 0.1014
IL-6 (pg/mg protein) - PFC	$F_{(1,9)} = 1.562$, p= 0.2429	$F_{(1,9)} = 0.3576$, p=0.5646	$F_{(1,9)} = 0.2162$, p= 0.6530
IL-6 (pg/mg protein) - HIP	$F_{(1,10)} = 0.09337$, p= 0.7662	$F_{(1,10)} = 1.988$, p= 0.1889	$F_{(1,10)} = 0.1804$, p= 0.6800
TNF-a (pg/mg protein) - PFC	$F_{(1,11)} = 0.3542$, p= 0.5638	$F_{(1,11)} = 2.924$, p= 0.1153	$F_{(1,11)} = 0.09656$, p= 0.7618
TNF-a (pg/mg protein) - HIP	$F_{(1,11)} = 0.2308$, p= 0.6403	$F_{(1,11)} = 0.5162$, p= 0.4874	$F_{(1,11)} = 0.1230$, p= 0.7324
Molecular parameters			
BDNF (relative gene expression level) - PFC	$F_{(1,8)} = 4.215$, p= 0.0742	$F_{(1,8)} = 15.63$, p= 0.0042	$F_{(1,8)} = 7.776$, p= 0.0236
BDNF (relative gene expression level) – HIP	$F_{(1,8)} = 0.02675$, p= 0.8741	$F_{(1,8)} = 1.311$, p= 0.2853	$F_{(1,8)} = 5.466$, p= 0.0476
GFAP (relative gene expression level) – PFC	$F_{(1,12)} = 3.066$, p= 0.1054	$F_{(1,12)} = 50.99$, p< 0.0001	$F_{(1,12)} = 0.07601$, p= 0.7875
GFAP (relative gene expression level) – HIP	$F_{(1,8)} = 2.502$, p= 0.1524	$F_{(1,8)} = 0.1176$, p= 0.7405	$F_{(1,8)} = 0.2477$, p= 0.6321
IBA-1 (relative gene expression level) – PFC	$F_{(1,8)} = 3.143$, p= 0.1142	$F_{(1,8)} = 12.78$, p= 0.0072	$F_{(1,8)} = 13.48$, p= 0.0063
IBA-1 (relative gene expression level) - HIP	$F_{(1,8)} = 1.038$, p= 0.3381	$F_{(1,8)} = 0.002419$, p= 0.9620	$F_{(1,8)} = 2.034$, p= 0.1916

Detailed two-way ANOVA results, with main effect of inflammatory preconditioning (LPS), main effect of restraint stress (ARS or CRS), and interaction effects between factors (LPS * ARS or CRS)

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Statements and Declarations

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Competing Interests

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4 CONSIDERAÇÕES FINAIS

- O pré-condicionamento inflamatório induziu uma resposta ao estresse alterada após exposição ao EAC, sem induzir os comportamentos tipo-depressivo e ansioso.
- No protocolo agudo, nos animais submetidos ao EAC, o pré-condicionamento inflamatório induziu alterações comportamentais relacionadas a desmotivação, indicando a participação do processo inflamatório nessa alteração comportamental.
- Os animais que receberam uma administração de LPS, antes do protocolo de ECC, apresentaram comportamentos tipo-depressivo e ansioso, demonstrando a participação do processo inflamatório sobre a resposta ao estresse.
- No protocolo crônico, a administração de LPS promoveu desequilíbrio oxidativo e inflamatório central nos animais submetidos ao ECC.
- O grupo LPS+ECC apresentaram alterações moleculares, que podem ser interpretadas como uma possível neuroinflamação induzida pela ativação microglial, atrofia ou perda da atividade dos astrócitos e redução gênica de BDNF.

Portanto, com esse estudo foi possível observar que o pré-condicionamento inflamatório induziu comportamento tipo-depressivo e ansioso apenas nos animais submetidos ao protocolo crônico. No protocolo agudo, nossos achados sugerem que há apenas uma perturbação da resposta comportamental, esperada após um estímulo agudo estressor. Uma vez que o TDM apresenta características de doença crônica, a aplicação de um estímulo agudo seria insuficiente para induzir comportamentos compatíveis com o transtorno. Desta forma, de acordo com esta perspectiva, o presente estudo indica que um protocolo crônico de estresse seria o mais adequado para a investigação de mecanismos fisiopatológicos relacionados ao TDM. Além disso, os achados deste estudo apontam que o processo inflamatório pode ser um importante fator de risco para o desenvolvimento de comportamentos fenotipicamente relacionados ao TDM. Nesse sentido, a modulação do estímulo inflamatório, pode ser um potencial alvo terapêutico para uma possível redução do risco de desenvolver o transtorno. Outro aspecto interessante desses resultados, é que o LPS pode ter prejudicado o processo de adaptabilidade ao estresse, nos animais expostos ao protocolo com estímulo de estresse repetido e previsível. Também, é importante mencionar que a associação dos modelos de pré-exposição inflamatória e ECC, indicou ser uma ferramenta interessante e que contribui para o estudo do TDM, uma vez que esse transtorno

tem origem multifatorial. Por fim, considerando que existem vários aspectos ou sintomas relacionados ao TDM, é relevante a utilização de diferentes parâmetros comportamentais, para que sejam identificadas alterações que se complementam, juntamente às avaliações bioquímicas e moleculares.

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ANEXO A – CERTIFICADO DE APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA UNIVERSIDADE FEDERAL DE SANTA MARIA – PROTOCOLO AGUDO

Universidade Federal de Santa Maria
Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "PADRONIZAÇÃO DO MODELO DE ESTRESSE FÍSICO AGUDO POR CONTENÇÃO (EAC), MODELO DE ESTRESSE INFLAMATÓRIO POR LIPOPOLISSACARÍDEO (LPS) E ASSOCIAÇÃO DOS MODELOS", protocolada sob o CEUA nº 9619250121 (ID 003357), sob a responsabilidade de **Guilherme Vargas Bochi** *e equipe; Gabriele Cheiran Pereira; Elisa Piton; Brenda Moreira* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 23/03/2021.

We certify that the proposal "STANDARDIZATION OF THE MODEL OF ACUTE PHYSICAL STRESS BY RESTRICTION (EAC), MODEL OF INFLAMMATORY STRESS BY LIPOPOLISACARID (LPS) AND MODEL ASSOCIATION", utilizing 40 Heterogenics mice (40 males), protocol number CEUA 9619250121 (ID 003357), under the responsibility of **Guilherme Vargas Bochi** *and team; Gabriele Cheiran Pereira; Elisa Piton; Brenda Moreira* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 03/23/2021.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 03/2021 a 12/2023 Área: Departamento de Fisiologia E Farmacologia

Origem:	Biotério Central UFSM			
Espécie:	Camundongos heterogênicos	sexo: Machos	idade: 6 a 8 semanas	Quantidade: 40
Linhagem:	Swiss		Peso: 25 a 30 g	

Santa Maria, 22 de março de 2024

Dra. Patrícia Bräunig
Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Profa. Dra. Vania Lucia Loro
Vice-Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

ANEXO B – CERTIFICADO DE APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA UNIVERSIDADE FEDERAL DE SANTA MARIA – PROTOCOLO CRÔNICO

Universidade Federal de Santa Maria
Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "PADRONIZAÇÃO DO MODELO DE ESTRESSE FÍSICO CRÔNICO POR CONTENÇÃO ASSOCIADO OU NÃO À PRÉ-EXPOSIÇÃO INFLAMATÓRIA AO LPS", protocolada sob o CEUA nº 3601271021 (ID 003671), sob a responsabilidade de **Guilherme Vargas Bochi** *e equipe; Gabriele Cheiran Pereira; Elisa Piton; Jaime Sardá Aramburú Júnior* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 01/02/2022.

We certify that the proposal "STANDARDIZATION OF THE CHRONIC RESTRAINT STRESS MODEL ASSOCIATED OR NOT WITH INFLAMMATORY PRE-EXPOSURE TO LPS", utilizing 80 Heterogenics mice (80 males), protocol number CEUA 3601271021 (ID 003671), under the responsibility of **Guilherme Vargas Bochi** *and team; Gabriele Cheiran Pereira; Elisa Piton; Jaime Sardá Aramburú Júnior* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 02/01/2022.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 01/2022 a 12/2023 Área: Departamento de Fisiologia E Farmacologia

Origem:	Biotério Central UFSM			
Espécie:	Camundongos heterogênicos	sexo: Machos	idade: 6 a 8 semanas	Quantidade: 40
Linhagem:	Swiss		Peso: 20 a 30 g	

Santa Maria, 26 de junho de 2023

Dra. Patrícia Bräunig
Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Profa. Dra. Vania Lucia Loro
Vice-Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria