UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM PSIQUIATRIA E CIÊNCIAS DO COMPORTAMENTO

TESE DE DOUTORADO

ESTRESSE SOCIAL, RESILIÊNCIA E INFLAMAÇÃO: RELAÇÃO COM COMPORTAMENTO TIPO-DEPRESSIVO

Dirson João Stein

Orientadora: Profª Drª Keila Maria Mendes Ceresér

Co-orientadora: Profª Drª Rosa Maria Martins de Almeida

Porto Alegre, 2018.

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Tese apresentada ao Programa de Pós-Graduação em Psiquiatria e Ciências do Comportamento, como requisito parcial para obtenção do título de Doutor.

Orientadora: Profª Drª Keila Maria Mendes Ceresér

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"Education is not the learning of facts, but the training of the mind to think."

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SUMÁRIO

ABREVIATURAS E SIGLAS 7
RESUMO 9
ABSTRACT 11
1. APRESENTAÇÃO 13
2. INTRODUÇÃO 15
3. REVISÃO DA LITERATURA 17
3.1 O Transtorno Depressivo Maior (TDM) 17
3.2 Estresse social e comportamento tipo-depressivo 21
3.3 O Sistema Imunológico, Citocinas e Inflamação 23
3.4 Inflamação e TDM 25
3.5 *Coping*, Resiliência e Vulnerabilidade ao Estresse Social 27
4. REFERÊNCIAS BIBLIOGRÁFICAS 31
5. JUSTIFICATIVA 41
6. OBJETIVOS 42
6.1 Objetivo Geral 42
6.2 Objetivos Específicos 42
7. CONSIDERAÇÕES ÉTICAS 43
8. ARTIGOS 44
8.1 Artigo 1: 44
8.2 Artigo 2: 55
9. CONCLUSÃO E CONSIDERAÇÕES FINAIS 92
REFERÊNCIAS 94
10. ANEXOS 95
Anexo A: carta de aprovação do projeto de pesquisa pela Comissão de Ética no Uso de Animais (CEUA) do Hospital de Clínicas de Porto Alegre 95
Anexo B: artigo publicado na revista *Frontiers in Behavioral Neuroscience* 2017; 11:207. ... 96
Anexo C: artigo enviado para publicação na revista *Psychoneuroendocrinology*. PNEC_2018_113 97

ABREVIATURAS E SIGLAS

5-HT	Serotonina
ANOVA	*Analysis of Variance*
APA	*American Psychiatric Association*
BDNF	*Brain-Derived Neurotrophic Factor*
BLA	*Basolateral Amygdala*
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CCL2	*C-C Motif Ligand 2*
CD11b	*Cluster of Differentiation 11b*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CNS	*Central Nervous System*
COX-2	*Ciclo-Oxigenase-2*
CRF	*Corticotropin-Releasing Factor*
CRP	*C-reactive Protein*
CSD	*Chronic Social Defeat*
CSF	*Cerebrospinal Fluid*
CX_3CR_1	*CX_3 Chemokine Receptor 1*
DG	*Dentate Gyrus*
ECR	Ensaio Clínico Randomizado
FIPE	Fundo de Incentivo à Pesquisa e Eventos
FI	Fator de Impacto
FST	*Forced Swim Test*
HCPA	Hospital de Clínicas de Porto Alegre

HPA	Eixo Hipotálamo-Pituitária-Adrenal
Iba-1	*Ionized Calcium-Binding Adapter Molecule 1*
IFN	*Interferon*
IL	*Interleukin*
LPS	*Lipopolysaccharide*
MDD	*Major Depressive Disorder*
MWM	*Morris Water Maze*
NPY	**N**europeptídeo Y
OF	*Open Field*
OMS	Organização Mundial da Saúde
mPFC	*medial Prefrontal Cortex*
PAMPs	*Patogen-Associated Molecular Patterns*
RSD	*Repeated Social Defeat*
SNC	Sistema Nervoso Central
TDM	Transtorno Depressivo Maior
TLR	*Toll-Like Receptor*
TNF-α	*Tumor Necrosis Factor-alpha*
TSPO	*Translocator Protein*
VTA	*Ventral Tegmental Area*
WHO	*World Health Organization*

RESUMO

Transtornos de humor tais como a depressão e a ansiedade estão entre as desordens psiquiátricas mais comuns na atualidade, com tendência de aumento do número de casos, assim como vêm ocorrendo nas últimas décadas. O Transtorno Depressivo Maior (TDM), uma desordem psiquiátrica dispendiosa e ameaçadora da vida, afeta profunda e negativamente a qualidade de vida dos indivíduos afetados e irá atingir até 20% da população em algum momento ao longo de sua existência. Porém, a descrição de sua patofisiologia segue incompleta e o principal pré-requisito para controlar a doença é entender de forma detalhada as alterações moleculares e comportamentais que a acompanham. O estresse social, como um dos principais indutores da depressão, tem sido alvo de estudos tanto clínicos quanto pré-clínicos, servindo também como um mecanismo laboratorial que auxilia pesquisadores a rastrear alterações moleculares e comportamentais dessa doença. Recentemente, sem deixar de lado as demais hipóteses, o sistema imunológico através de respostas inflamatórias, tem recebido atenção crescente e é investigado por ser potencialmente um indutor e/ou facilitador de estados depressivos, contribuindo para a patofisiologia da depressão. Além disso, considerando que o estresse não afeta a todos os indivíduos da mesma maneira, a compreensão das diferenças individuais que podem resultar em resiliência pode auxiliar pesquisadores quanto aos fatores que afetam o desenvolvimento ou não do transtorno na população.

Esta tese é composta por dois artigos, e visa investigar em um modelo pré-clínico, a contribuição do estresse social por subordinação (do inglês, *social defeat – SD*) ao comportamento tipo-depressivo, relacionando-o com inflamação. No primeiro artigo foi revisada a literatura mais recente sobre a contribuição do SD para a ativação microglial, o principal elemento neuroinflamatório do sistema nervoso central (SNC), e sua relação com o desenvolvimento dos comportamentos tipo-ansioso e tipo-depressivo. No segundo artigo investigou-se o papel de um estressor social contínuo (21 dias consecutivos de derrota social crônica) em um grupo de ratos Wistar adultos, relacionando respostas comportamentais a alterações de marcadores imunológicos periféricos e a duas estratégias de *coping*.

O modelo de estresse por derrota social tem sido utilizado em diversos estudos de transtornos psiquiátricos e é uma das principais formas de indução de estados tipo-depressivos em animais de laboratório. Ademais, vêm demonstrando ser útil na indução de alterações do sistema

imunológico, tanto centrais quanto periféricas. Exposição ao estresse por derrota social induz em células microgliais um estado de hiperativação que, dependendo do tempo de exposição, pode levar ao desenvolvimento de desordens psiquiátricas como a ansiedade e a depressão. Além disso, o protocolo de 21 dias de derrota social contínua revelou dois estilos comportamentais em ratos Wistar. As estratégias de *coping* passivo e ativo observadas estão relacionadas a vulnerabilidade e a resiliência, respectivamente, e foram correlacionadas com distintos perfis imunológicos periféricos. Animais resilientes apresentam comportamentos e perfil imunológico periférico que os protegem do desenvolvimento de psicopatologias associadas ao estresse.

Palavras-chave: Depressão. Inflamação. Estresse Social. Desordens Neuropsiquiátricas. Resiliência. Derrota Social. Rato Wistar.

ABSTRACT

Humor disorders such as depression and anxiety are among the currently most common disorders, with a trend to an increasing number of cases, like in the past few decades. Major Depressive Disorder (MDD) is an expensive and life-threatening psychiatric disorder that profoundly and negatively affects individual´s quality of life and will affect up to 20% of the population at some point throughout life. However, the pathophysiology of MDD remains incompletely described, and a detailed description of its molecular and behavioral alterations is one of the core prerequisites for disease control. Social stress, one of the main inducers of depression, has been the subject of both clinical and preclinical studies, and has been used as a laboratory tool to help researchers track molecular and behavioral changes of this disease. Recently, without leaving other hypotheses aside, the immune system through inflammatory responses has received increasing attention and is investigated as a potential contributor in the pathophysiology of depression. Furthermore, considering that stress does not affect all individuals to de same extend, understanding individual differences that can turn into resilience may help researchers unravel the factors that influence the development of this disorder in the population.

The present dissertation is composed of two articles, aiming to investigate in a preclinical model the contribution of social defeat stress (SD) to depressive-like behavior and correlate it to inflammation. In the first article, we reviewed the most recent literature on the contribution of SD to microglial activation, the main neuroinflammatory element of the central nervous system (CNS), and its relation to the development of anxiety and depressive-like behaviors. In the second paper, we investigated the role of a continuous social stressor (21 consecutive days of chronic social defeat) in a group of adult male Wistar rats, relating behavioral responses and two different coping strategies to changes in peripheral immune markers.

The social defeat stress model has been used in several studies of psychiatric disorders and is one of the main forms to induce depressive-like states in laboratory animals. Additionally, it has been shown to be useful in inducing central and peripheral immune alterations. Exposure to stress due to social defeat induces microglial cells into a state of hyperactivation that, depending on the time of exposure, can lead to the development of psychiatric disorders such as anxiety and depression. Furthermore, the 21-day protocol of continuous social defeat revealed two behavioral

styles in Wistar rats. The observed passive and active coping strategies are related to vulnerability and resilience, respectively, and have been correlated with different peripheral immunological profiles. Resilient animals present behaviors and a peripheral immune profile that protect them from the development of stress-associated psychopathologies.

Keywords: Depression. Inflammation. Social Stress. Neuropsychiatric Disorders. Resilience. Social Defeat. Wistar Rat.

1. APRESENTAÇÃO

Este trabalho consiste na Tese de Doutorado intitulada “Estresse Social, Resiliência e Inflamação: Relação com Comportamento Tipo-Depressivo”, apresentada ao Programa de Pós-Graduação em Psiquiatria e Ciências do Comportamento da Universidade Federal do Rio Grande do Sul, em 06 de abril de 2018. O trabalho é apresentado em três partes, na seguinte ordem: (1) Introdução, Revisão da Literatura, Justificativa e Objetivos; (2) dois artigos científicos e (3) Conclusões e Considerações Finais.

Essa tese faz parte de um projeto maior intitulado "Atividade neuronal, marcadores inflamatórios e estratégias de *coping* após estresse agudo e crônico por derrota social em ratos Wistar". Além de um projeto de pesquisa, representa um projeto de vida na minha formação como biólogo e pesquisador. É motivado pelo desejo de compreender e contribuir com soluções para um dos problemas mais sérios enfrentados na atualidade que, conforme a Organização Mundial da Saúde (OMS) tem uma incidência crescente na população mundial e muito em breve será a principal causa de incapacidade humana: o Transtorno Depressivo Maior (TDM).

O projeto contou com a colaboração das equipes de dois laboratórios da UFRGS: Laboratório de Psicologia Experimental, Neurociências e Comportamento (LPNeC) e Laboratório de Psiquiatria Molecular/HCPA. Ambas as equipes buscam compreender melhor as causas dos transtornos neuropsiquiátricos e de humor e procuram colaborar com ideias e direcionar a atenção a novas alternativas de tratamento. Nesse sentido, através desse trabalho buscou-se trazer contribuições para o objetivo maior, com a produção de dois artigos científicos e colaboração em outras publicações.

O primeiro artigo, intitulado ***"Microglial Over-Activation by Social Defeat Stress Contributes to Anxiety- and Depressive-like Behaviors"*** (Hiperativação Microglial por Estresse por Derrota Social Contribui para os Comportamentos Tipo-Ansioso e Tipo-Depressivo) visou revisar a literatura mais recente sobre o modelo laboratorial de investigação pré-clínica de derrota social e as consequentes reações imunológicas associadas, com foco nas células microgliais do SNC, e sua contribuição na indução de estados tipo-ansioso e tipo-depressivo. Estas células têm sido alvo de investigação e são apontadas como potenciais indutoras de transtornos

neuropsiquiátricos. O artigo está em formato de *Mini-review* e se encontra publicado na revista *Frontiers in Behavioral Neuroscience* (outubro/2017).

O segundo artigo, intitulado ***"Proactive Coping during Chronic Social Defeat Stress is associated with an Anti-inflammatory response in male Wistar rats"*** (*Coping* proativo durante Estresse Crônico por Derrota Social está associado à resposta Anti-inflamatória em ratos Wistar), segue a linha de investigação do artigo anterior apresentando resultados que ligam o estressor social a reações inflamatórias, desta vez, porém, na circulação periférica. A aplicação de um protocolo de estresse por derrota social revelou que animais submetidos ao estresse crônico apresentam uma distribuição bimodal em relação a estilos de *coping*, caracterizado por diferenças comportamentais associadas a marcadores imunológicos periféricos. Para este trabalho foi utilizado um protocolo novo de estresse social crônico para ratos Wistar, que até o momento havia sido aplicado apenas em fêmeas, consistindo de 21 dias de exposição contínua a um agressor, durante o qual foram avaliadas reações comportamentais relacionados a estados tipo-depressivos, além de parâmetros moleculares periféricos. Esse artigo foi submetido para publicação na revista *Psychoneuroendocrinology* e encontra-se em análise pela equipe editora e revisora desde fevereiro de 2018.

Após a apresentação dos dois artigos são feitas uma conclusão geral e algumas considerações finais com perspectivas futuras. Em seguida, os anexos mais significativos são apresentados. Dessa forma, a tese procura contribuir para a compreensão da relação estresse social, resiliência, inflamação e depressão, além de testar um protocolo alternativo para o estudo laboratorial pré-clínico de comportamento tipo-depressivo em ratos Wistar.

2. INTRODUÇÃO

O Transtorno Depressivo Maior (TDM) é uma desordem psiquiátrica muito comum, porém séria, causando sintomas severos que afetam como o indivíduo se sente e como lida com atividades diárias, tais como dormir, alimentar-se e trabalhar. Com uma etiologia quase totalmente desconhecida, é uma das principais causas mundiais de incapacidade e irá afetar até 20% da população em algum momento ao longo de sua vida (Kessler et al., 2007; Menard et al., 2017; Whiteford et al., 2013). O maior desafio dos investigadores do TDM na atualidade é compreender sua patofisiologia, que provavelmente envolve múltiplos mecanismos interligados: seus sinais e sintomas são muito variados e não podem mais ser explicados através de uma única hipótese (Jesulola et al., 2018). Acredita-se que estressores ambientais e fatores genéticos hereditários, agindo através de respostas imunológicas e endócrinas, iniciem alterações estruturais e funcionais em diversas regiões encefálicas, resultando em neurogênese e neurotransmissão disfuncionais que se manifestam numa ampla gama de sintomas, finalmente apresentando-se como depressão. Dentre as hipóteses aceitas atualmente (aminérgica, genética, ambiental, imunológica, endócrina e neurogênica), a que mais tem recebido a atenção de pesquisadores nos últimos anos é a hipótese imunológica/inflamatória (Soskin et al., 2012).

A interferência entre as vias inflamatórias e os circuitos neurais no cérebro pode levar a respostas comportamentais, tais como evitação e alerta, que provavelmente conferiram aos primeiros humanos uma vantagem evolutiva nas suas interações com agentes patogênicos e predadores (Dantzer et al., 2008; Felger and Lotrich, 2013; Haroon et al., 2012). A inflamação induzida por estresse deriva da pressão evolutiva da interação humana com patógenos, predadores e outros rivais, que resultou num viés inflamatório, que inclui respostas imunológicas e comportamentais, com o objetivo de conservar energia, combater infecções e manter a vigilância contra novos ataques. Acredita-se que este viés inflamatório tenha sido mantido sob controle durante grande parte da evolução humana. Porém, nos tempos modernos, os desafios psicológicos instigam os repertórios imunológicos e comportamentais ancestrais que são capazes de induzir altas taxas de doenças relacionadas com inflamação, incluindo a depressão (Miller and Raison, 2016). Tais interações entre a inflamação e o cérebro parecem impulsionar o desenvolvimento da depressão e outros transtornos psiquiátricos e podem contribuir para a falta de resposta às terapias

antidepressivas convencionais. Pesquisas recentes elucidaram os mecanismos pelos quais os sistemas imunológicos inato e adaptativo interagem com neurotransmissores e neurocircuitos para influenciar o risco de depressão (Miller and Raison, 2016). Acredita-se que exista um ciclo bidirecional no qual a depressão facilita as respostas inflamatórias e os processos inflamatórios promovem a depressão, ambos fortemente afetados por estressores (Kiecolt-Glaser et al., 2015).

O papel do estresse no desenvolvimento de transtornos neuropsiquiátricos é bem descrito na literatura. Sabe-se também que nem todos os indivíduos são afetados por ele da mesma maneira, evidenciado pela grande variabilidade individual nas consequências de exposição a eventos estressores. Enquanto alguns são resilientes, outros são mais suscetíveis, especialmente quando se trata de exposição a estressores psicossociais (Sapolsky, 1994; Villada et al., 2016; Wood and Bhatnagar, 2015). De fato, a maioria dos indivíduos expostos a eventos traumáticos ou estressores sociais crônicos não desenvolvem patologias relacionadas ao estresse, o que pode ser explicado pela estratégia utilizada para lidar em tais situações (Wood, 2014). A inabilidade de manejar de forma adequada uma situação de enfrentamento a um evento estressor, *coping* inadequado, pode representar um risco ao desenvolvimento de desordens psiquiátricas como a depressão (Veenema et al., 2003). Traços de personalidade submissa ou estratégias de *coping* passivo durante estresse crônico estão associados com a vulnerabilidade a psicopatologias (Billings and Moos, 1984; Folkman and Lazarus, 1980), enquanto *coping* ativo está associado à resiliência (Southwick et al., 2005).

Estudos pré-clínicos são amplamente utilizados na investigação desta relação (estresse, inflamação, *coping* e comportamento tipo-depressivo), sendo que um dos estressores mais aceitos, por ser etologicamente relevante e ter alta validade de face, é o estressor social (Koolhaas et al., 2007; Krishnan and Nestler, 2011). Como um dos grandes responsáveis pelo desenvolvimento de desordens neuropsiquiátricas e afetivas em humanos, incluindo a indução de estados depressivos, o estresse psicossocial tem sido alvo de investigação por induzir a sinalização imunológica central e periférica, através da ativação repetida dos sistemas neuroendócrino e neurovegetativo (Glaser and Kiecolt-Glaser, 2005; Lehmann et al., 2016). Expondo-se um indivíduo repetidamente ao estresse social, o ambiente homeostático do seu cérebro se altera e pode dar origem a várias desordens cognitivas e de humor que prejudicam seu funcionamento diário e a qualidade de vida em geral (McKim et al., 2016).

3. REVISÃO DA LITERATURA

3.1 O Transtorno Depressivo Maior (TDM)

O TDM é comumente conhecido como depressão e de acordo com a descrição do Diagnóstico Manual e Estatístico de Transtornos Mentais (DSM-5, American Psychiatric Association - APA, 2013), os sintomas centrais incluem humor triste ou deprimido, anedonia (interpretada como a capacidade reduzida de sentir prazer com recompensas naturais), irritabilidade, dificuldade de concentração, e sintomas neurovegetativos como alterações de apetite e sono, acompanhados de mudanças somáticas e cognitivas que afetam significativamente a capacidade da pessoa de funcionar (Krishnan and Nestler, 2008).

O TDM é diagnosticado (DSM-5, APA, 2013, p. 160) através dos seguintes critérios, quando:

A. Cinco (ou mais) dos seguintes sintomas estiveram presentes durante o mesmo período de duas semanas e representam uma mudança em relação ao funcionamento anterior; pelo menos um dos sintomas é (1) humor deprimido ou (2) perda de interesse ou prazer.

Nota: Não incluir sintomas nitidamente devidos a outra condição médica.

1. Humor deprimido na maior parte do dia, quase todos os dias, conforme indicado por relato subjetivo (p. ex., sente-se triste, vazio, sem esperança) ou por observação feita por outras pessoas (p. ex., parece choroso). (**Nota:** Em crianças e adolescentes, pode ser humor irritável.)
2. Acentuada diminuição do interesse ou prazer em todas ou quase todas as atividades na maior parte do dia, quase todos os dias (indicada por relato subjetivo ou observação feita por outras pessoas).
3. Perda ou ganho significativo de peso sem estar fazendo dieta (p. ex., uma alteração de mais de 5% do peso corporal em um mês), ou redução ou aumento do apetite quase todos os dias. (**Nota:** Em crianças, considerar o insucesso em obter o ganho de peso esperado.)
4. Insônia ou hipersonia quase todos os dias.
5. Agitação ou retardo psicomotor quase todos os dias (observáveis por outras pessoas, não meramente sensações subjetivas de inquietação ou de estar mais lento).
6. Fadiga ou perda de energia quase todos os dias.
7. Sentimentos de inutilidade ou culpa excessiva ou inapropriada (que podem ser delirantes) quase todos os dias (não meramente auto recriminação ou culpa por estar doente).
8. Capacidade diminuída para pensar ou se concentrar, ou indecisão, quase todos os dias (por relato subjetivo ou observação feita por outras pessoas).
9. Pensamentos recorrentes de morte (não somente medo de morrer), ideação suicida recorrente sem um plano específico, uma tentativa de suicídio ou plano específico para cometer suicídio.

B. Os sintomas causam sofrimento clinicamente significativo ou prejuízo no funcionamento social, profissional ou em outras áreas importantes da vida do indivíduo.
C. O episódio não é atribuível aos efeitos fisiológicos de uma substância ou a outra condição médica.

Nota: Os Critérios A-C representam um episódio depressivo maior.

Nota: Respostas a uma perda significativa (p. ex., luto, ruína financeira, perdas por um desastre natural, uma doença médica grave ou incapacidade) podem incluir os sentimentos de tristeza intensos, ruminação acerca da perda, insônia, falta de apetite e perda de peso observados no Critério A, que podem se assemelhar a um episódio depressivo. Embora tais sintomas possam ser entendidos ou considerados apropriados à perda, a presença de um episódio depressivo maior, além da resposta normal a uma perda significativa, também deve ser cuidadosamente considerada. Essa decisão requer inevitavelmente o exercício do julgamento clínico baseado na história do indivíduo e nas normas culturais para a expressão de sofrimento no contexto de uma perda.

D. A ocorrência do episódio depressivo maior não é mais bem explicada por transtorno esquizoafetivo, esquizofrenia, transtorno esquizofreniforme, transtorno delirante, outro transtorno do espectro da esquizofrenia e outro transtorno psicótico especificado ou transtorno da esquizofrenia e outro transtorno psicótico não especificado.
E. Nunca houve um episódio maníaco ou um episódio hipomaníaco.

Nota: Essa exclusão não se aplica se todos os episódios do tipo maníaco ou do tipo hipomaníaco são induzidos por substância ou são atribuíveis aos efeitos psicológicos de outra condição médica.

Embora possa ocorrer apenas um episódio, geralmente é uma condição recorrente, que deve ser distinguida da tristeza ou luto, que apresentam sintomas muito semelhantes, porém passageiros e menos duradouros. Para diagnosticar uma pessoa com depressão, os sintomas precisam causar impacto significativo no convívio social, no trabalho ou em outras áreas importantes de sua vida (APA, 2013). O TDM é uma desordem psiquiátrica dispendiosa e ameaçadora e uma das principais causas mundiais de incapacidade. Com sua etiologia parcialmente desconhecida, o TDM irá afetar até 20% da população em algum momento ao longo da vida (Menard et al., 2017). Apesar de haverem antidepressivos disponíveis para tratamento, até 30% dos pacientes apresentam resistência aos medicamentos, indicando que ainda existem características desconhecidas da doença e que há necessidade de seguir investigando. Como nenhuma hipótese lançada até o momento cobre a explicação de todos os aspectos de sinais e sintomas do TDM, acredita-se que os mecanismos da doença sejam múltiplos, o que desafia os investigadores a avançar na compreensão e descrição de sua patofisiologia (Jesulola et al., 2018).

Desvendar a patofisiologia da depressão é um desafio, não somente pelo fato de as síndromes depressivas serem heterogêneas e possuírem diversas etiologias, mas também pelos sintomas como culpa e suicídio serem impossíveis de se reproduzir em modelos animais (Jesulola

et al., 2018). No entanto, outros sintomas já foram precisamente modelados e, juntamente com dados clínicos, estão fornecendo informações sobre a neurobiologia de depressão. Estudos recentes que combinam técnicas comportamentais, moleculares e eletrofisiológicas revelam que certos aspectos da depressão resultam de alterações neuroplásticas mal-adaptativas induzidas por estresse, em circuitos neurais específicos. Eles também mostram que compreender os mecanismos de resiliência ao estresse oferece uma nova dimensão, crucial para o desenvolvimento de novos tratamentos antidepressivos (Krishnan and Nestler, 2008).

Estudos têm sugerido que a herdabilidade da doença está em torno de 37% (Bouchard, 1994, Fernandez-Pujals et al., 2015; Flint and Kendler, 2014), porém está claro que a depressão não é causada por um único gene, apresentando características genéticas complexas. Múltiplos genes com efeitos modestos, interagindo uns com os outros e com fatores ambientais produzem vulnerabilidade a esta desordem (Lesch et al., 2002). Um dos genes associados à doença é o gene que codifica para o transportador de serotonina (5-HT), uma proteína envolvida no controle da função serotoninérgica, responsável pela receptação deste neurotransmissor da fenda sináptica de volta para o botão pré-sináptico. As variantes que causam redução da recaptação deste neurotransmissor nas células pré-sinápticas no SNC conferem uma predisposição ao desenvolvimento de depressão (Caspi et al., 2003; Lesch et al., 2002). Falha na receptação de 5-HT afeta a homeostasia deste neurotransmissor, dessensibilizando os receptores tanto pré quanto pós-sinápticos e impedindo a manutenção do estoque de 5-HT no botão pré-sináptico (Owens and Nemeroff, 1994). Outros estudos associam fatores ambientais (que afetam o genoma epigeneticamente) na predisposição à depressão, tais como o que indica que o aumento do cuidado maternal em roedores causa alterações epigenéticas na região promotora do gene que codifica para o receptor de glicocorticoide (Weaver et al., 2004).

As principais hipóteses indicam vários mecanismos envolvidos, incluindo alterações dos sistemas serotoninérgico, noradrenérgico, dopaminérgico e glutamatérgico, aumento da inflamação, anormalidades do eixo Hipotálamo-Pituitária-Adrenal (HPA), alterações vasculares e diminuição da neurogênese e neuroplasticidade, todos inter-relacionados (recentemente revisado em detalhes por Dean and Keshavan, 2017). A hipótese monoaminérgica postula que a depressão é causada pela alteração nos níveis de uma ou mais monoaminas. As evidências incluem achados de redução de metabólitos de 5-HT e NA em pacientes diagnosticados com TDM, tratados

eficazmente com antidepressivos tricíclicos e inibidores da recaptação de 5-HT/NA (Leonard, 2002; Richelson, 2001) e do mau funcionamento do sistema mesolímbico da DA, afetando os mecanismos de recompensa (Watt and Panksepp, 2009). Além disso, achados implicam o glutamato na regulação do humor, possivelmente através da manutenção da neuroplasticidade (Iadarola et al., 2015).

A hipótese de desregulação do eixo HPA origina-se de achados que relacionam estresse e depressão. O estresse pode induzir mudanças comportamentais em roedores associadas a sintomas depressivos, incluindo anedonia, diminuição da motivação e alterações nos padrões de sono (Willner, 2005). Além disso, o estresse pode induzir hiperativação do eixo HPA em pacientes com depressão (Pruessner et al., 2003). Estresse crônico altera as conexões cerebrais responsáveis pelo processamento de emoções e pela adaptação, incluindo projeções da amígdala basolateral (BLA) para o córtex pré-frontal medial (mPFC) e aumento da excitabilidade da amígdala (Duvarci and Pare, 2007). As conexões entre o mPFC e a amígdala permitem ajustar o comportamento emocional quando as condições no ambiente se alteram. Especialmente as conexões dos subnúcleos basomedial e basolateral da BLA com o córtex pré-límbico e com o córtex infralímbico são importantes no processamento emocional. Alterações nesse circuito, causando a perda de controle do mPFC sobre a amígdala, podem causar um descontrole sobre as emoções (Morgan et al., 1993; Lee et al., 2013). Adicionalmente, quando presente nas fases iniciais do desenvolvimento, o estresse promove alterações duradouras no eixo HPA, aumentando a sensibilidade a estressores posteriores.

A hipótese imunológica é mais recente e está baseada nas observações de níveis aumentados de marcadores inflamatórios em pacientes com depressão (Felger and Lotrich, 2013). Aumento da inflamação pode ser considerado como uma cicatriz biológica da exposição prévia a altos níveis de estresse e se caracteriza por anormalidades na saúde física e mental (Baumeister et al., 2016). Como essa hipótese está diretamente relacionada a esta tese, será revisada com mais detalhes em uma seção posterior.

O cérebro é um órgão reconhecidamente plástico capaz de criar e eliminar sinapses, assim como alterar circuitos funcionais durante adaptação e aprendizado (Dean and Keshavan, 2017). Para manter a neuroplasticidade funcional, fatores neurotróficos como o BDNF (do inglês: *Brain-Derived Neurotrophic Factor*) são fundamentais, por promoverem a sobrevivência dos neurônios

existentes e influenciarem crescimento e diferenciação de novos neurônios e novas sinapses (Calabrese et al., 2014). A redução dos níveis séricos de BDNF em pacientes diagnosticados com TDM envolve essa neurotrofina na patofisiologia da depressão. Adicionalmente, foi observado que o uso de antidepressivos pode aumentar a neurogênese hipocampal (Santarelli, 2003), o que liga a hipótese neurogênica / neuroplástica à patofisiologia desse transtorno.

3.2 Estresse social e comportamento tipo-depressivo

A depressão é uma desordem neuropsiquiátrica muitas vezes desencadeada por estresse (Müller, 2014). O cérebro, o órgão central do estresse e da adaptação a estressores físicos e sociais, determina o que é ameaçador, armazena memórias e regula as respostas fisiológicas e comportamentais que podem ser protetivas ou prejudiciais (McEwen et al., 2015). Grande parte do conhecimento atual sobre a patogênese das desordens de humor, incluindo o TDM, vem dos estudos conduzidos em modelos animais (Krishnan and Nestler, 2011). Os modelos de depressão popularmente utilizados em pesquisas biomédicas unem ensaios comportamentais etologicamente válidos com avanços tecnológicos em biologia molecular e rastreios comportamentais automatizados em vídeo. Embora os sintomas como a culpa, o suicídio e o humor triste sejam provavelmente características puramente humanas, outros aspectos da síndrome depressiva (desamparo, anedonia, desânimo comportamental e mudanças neurovegetativas tais como alterações no sono e padrões de apetite) têm sido replicados em animais de laboratório, e em diversos casos melhoram com tratamentos antidepressivos.

Há evidências consideráveis de que o estresse psicológico pode desencadear um aumento significativo na atividade inflamatória, mesmo com a ausência de injúria física (Kiecolt-Glaser et al., 2015). Aumento da inflamação pode, por sua vez, provocar mudanças profundas no comportamento, que incluem o início de sintomas depressivos, tais como: humor triste, anedonia, fadiga, retardo psicomotor e afastamento social. Há uma grande semelhança entre os sintomas do comportamento-doente (do inglês, *sickness-behavior*) e os sintomas da depressão. De uma perspectiva evolutiva, a depressão tem sido comparada à estratégia de derrota involuntária (EDI) que é desencadeada quando um animal percebe a derrota em uma luta hierárquica por recursos (Griffiths et al., 2014; Sloman, 2008). Características de retardo psicomotor, hipervigilância, anedonia e distúrbios do sono nesses casos de derrota conferem uma vantagem adaptativa na

medida em que servem para proteger os perdedores de novos ataques e os fazem focar no planejamento de maneiras de evitar problemas sociais complexos (Nesse, 2000; Watson and Andrews, 2002). A maioria, se não todos os modelos animais de depressão visam testar quantitativamente alguma forma de induzir experimentalmente "derrota" ou "aflição", mesmo que esse aspecto do comportamento dos mamíferos seja provavelmente fisiológico (adaptativo) e não patológico. Além disso, enquanto o comportamento de aflição é muitas vezes extrapolado como sendo tipo-depressivo, a aplicação do estresse em roedores também produz mudanças tipo-ansiosas que são manifestações da resposta de luta ou fuga (exploração reduzida, congelamento, hipertermia induzida pelo estresse, etc.).

Os modelos de depressão atuais são frequentemente avaliados por três critérios principais: (1) validade de face (quando o modelo apresenta homologia sintomática), (2) validade de construto ou validade etiológica (quando o modelo mede fatores causais similares) e (3) validade farmacológica (quando os sintomas depressivos induzidos pelo modelo podem ser revertidos com a utilização de antidepressivos clássicos) (Nestler and Hyman, 2010). Os modelos pré-clínicos de indução de estados tipo-depressivos em animais de laboratório podem ser divididos em dois grandes grupos: agudos e crônicos, sendo os do primeiro grupo mais eficazes na produção desses comportamentos. Dentre estes, os modelos de estresse psicossocial são particularmente interessantes, pois estão baseados inteiramente em comportamento social inato (Krishnan and Nestler, 2011; Kumar et al., 2013). A derrota social é caracterizada por um estressor natural, que fornece forte validade de face e farmacológica, ao contrário de outros estressores experimentais, que utilizam formas mais artificiais de estresse. Consiste em permitir encontros agonísticos entre dois ou mais sujeitos da mesma espécie, geralmente machos, de modo que um dos animais assume uma posição de dominância enquanto o(s) outro(s) permanece(m) subordinado(s) ao primeiro (Berton, 2006; Miczek et al., 2008). Além da imprevisibilidade do estressor, que é um fator importante na indução de comportamento tipo-depressivo, e o estresse físico intenso durante os encontros sociais agonísticos, alguns investigadores permitem coabitação, quando os animais subordinados permanecem residindo em uma gaiola ou em um compartimento protegido dentro da moradia do agressor, conferindo estresse psicológico constante sem contato físico permanente (Martinez et al., 1998; Shimamoto et al., 2011). Após diversos episódios de derrota, roedores apresentam redução da exploração social, diminuição da atividade locomotora e exploratória, anedonia, maior imobilidade induzida por estresse e alterações no funcionamento do eixo HPA

(Avgustinovich et al., 2005) e que podem ser revertidos pela administração crônica de antidepressivos (Becker et al., 2008; Rygula et al., 2008). O modelo de derrota social, porém, como qualquer outro modelo experimental, apresenta limitações: o comportamento agressivo do dominante pode ser interrompido por qualquer alteração no ambiente por ele dominado e, além disso, pesquisadores devem estar atentos à quantidade de agressão sofrida pelo animal subordinado, já que injúrias físicas em excesso, além de serem eticamente questionáveis, interferem nas medidas fisiológicas, especialmente relacionadas à inflamação (Krishnan and Nestler, 2011).

3.3 O Sistema Imunológico, Citocinas e Inflamação

Nas últimas décadas, cientistas têm reconhecido que a resposta inflamatória do corpo tem um papel fundamental nas doenças mentais, o que leva a crer que a próxima onda de antidepressivos deverá ter como alvo principal o sistema imunológico (Slavich and Irwin, 2014). Em um estudo publicado recentemente, observando amostras de sangue de 113 pacientes com depressão severa, foram encontrados 90 genes superexpressos, dos quais muitos estão relacionados ao sistema imunológico e à resposta corporal a infecções (Leday et al., 2018). Cientistas estão atentos a proteínas inflamatórias para utilizar como biomarcadores, e que possam ajudar a prever qual tratamento anti-inflamatório funcionará melhor para cada indivíduo. Em um ensaio clínico randomizado (ECR), Raison e colaboradores (2013) sugerem que o tratamento antidepressivo com antagonistas de proteínas inflamatórias, pelo menos observado para o TNF, não possui eficácia generalizada, mas pode melhorar os sintomas depressivos em pacientes cujos biomarcadores inflamatórios basais estão elevados.

O sistema imunológico é um componente crítico para o bem-estar e a saúde humana, pois ajuda a coordenar as respostas corporais a injúrias físicas e infecções que, se não forem combatidas, podem causar doenças ou até a morte. Esse sistema geralmente é descrito como uma composição de dois ramos interligados. O primeiro desses ramos, evolutivamente mais antigo, chamado de imunidade inata, é a primeira linha de defesa do corpo contra danos nos tecidos e infecção microbiana (Medzhitov, 2007). A imunidade inata é composta por células do sistema imunológico tais como monócitos / macrófagos e células dendríticas que circulam constantemente no corpo e usam diversos receptores para detectar uma grande variedade de agentes patogênicos. Essas células sinalizam a ocorrência de lesão ou infecção e iniciam uma cascata de processos inflamatórios que

ajudam a conter uma infecção promovendo a cicatrização e a recuperação (Medzhitov, 2007). Quando as defesas inatas do sistema imunológico são insuficientes, essas células ativam o segundo ramo do sistema imunológico, denominado imunidade adaptativa (Barton, 2008). Ao contrário da imunidade inata, que é inespecífica e não confere proteção duradoura para o hospedeiro, a imunidade adaptativa cria uma memória imunológica após uma resposta inicial e responde a um patógeno específico com base nesta memória. As células que realizam a resposta imune adaptativa são células brancas do sangue conhecidas como linfócitos (linfócotos B e T) (Atanackovic et al., 2006; Gruys et al., 2005). Enquanto a resposta imunológica inata é rápida, ocorrendo em minutos ou horas, a resposta imunológica adaptativa leva dias para se desenvolver completamente (Barton, 2008).

A ativação inicial do sistema imunológico inato é chamada de fase aguda e envolve um aumento da atividade inflamatória que pode ocorrer tanto localmente (no local de lesão ou infecção) quanto sistemicamente (Hennessy et al., 2009). Essa resposta é desencadeada quando os receptores das células do sistema imunológico inato reconhecem características altamente conservadas de micróbios ou padrões moleculares associados a patógenos (do inglês, *PAMPs*), que dá ao sistema a capacidade de detectar e responder a uma ampla diversidade microbiana (Barton, 2008; Medzhitov, 2007). Os receptores das células do sistema imunológico inato que reconhecem esses de padrões desempenham um papel importante ligando o reconhecimento de alvos microbianos à inflamação. Uma das famílias mais bem caracterizadas é a dos receptores tipo Toll (do inglês, TLR: *toll-like receptors*). Os TLRs reconhecem componentes conservados de micróbios (bactérias, vírus e fungos) e ativam respostas imunológicas inflamatórias e antimicrobianas inatas (Medzhitov, 2001). Quando os TLRs são ativados, inicia-se uma cascata de sinalização que resulta na ativação de dois principais fatores de transcrição intracelular: fator nuclear kappa B (NF-κB) e interferon (IFN), especialmente interferon do tipo I, IFN-α e IFN-β (Hertzog et al., 2003; Karin, 2006; Kawai and Akira, 2006). Esses fatores de transcrição, por sua vez, conduzem a expressão de genes de resposta imunológica pró-inflamatória, como o fator alfa de necrose tumoral (TNF-α) e interleucina-1 (IL-1) que produzem pequenas moléculas de proteína chamadas citocinas, os principais efetores da resposta inflamatória (Karin, 2006; Raison et al., 2006).

As citocinas desempenham um papel central no sistema imunológico e na resposta inflamatória. Além de coordenar a comunicação entre as células, elas podem alterar os processos

neuroquímicos e neuroendócrinos que têm amplos efeitos sobre a fisiologia e o comportamento (Curfs et al., 1997). Liberadas de células imunológicas tais como monócitos/macrófagos, células dendríticas e neutrófilos, as citocinas podem funcionar de forma semelhante a neurotransmissores e hormônios na medida em que medeiam respostas fisiológicas, dependem das interações receptor-ligante e tem efeitos locais e distais (Jain and Mills, 2007). Entre as citocinas que coordenam funções celulares relacionadas à inflamação, aqueles que aumentam a inflamação são referidas como pró-inflamatórias, enquanto as que diminuem a inflamação são conhecidas como anti-inflamatórias.

Citocinas têm efeitos específicos no corpo comumente reconhecido como sinais de inflamação. Nos locais de infecção, por exemplo, as citocinas causam vermelhidão, calor, inchaço e dor. A um nível mais sistêmico, certas citocinas induzem a produção da proteína de fase aguda e biomarcador chave da inflamação, proteína C-reativa (do inglês, CRP: *c-reactive protein*) que, juntamente com citocinas, pode levar ao aumento da temperatura corporal, frequência cardíaca, frequência respiratória e febre (Poon et al., 2013). Quando combinados, estes efeitos ajudam a acelerar a cicatrização de feridas e limitam a propagação de infecção no hospedeiro. Eles também promovem o comportamento de retraimento social que ajuda o organismo a se recuperar e esta recuperação reduz a probabilidade de que a infecção se espalhe para os demais indivíduos da espécie no ambiente circundante (Audet et al., 2014).

Pesquisas recentes têm demonstrado que a resposta do sistema imunológico inato também pode ser ativada no contexto social contemporâneo, quando, por exemplo, um indivíduo é exposto a condições adversas, como conflito social, rejeição, isolamento ou exclusão, talvez por causa das implicações históricas que essas condições representavam para o perigo físico (Slavich and Cole, 2013). Baseados nessas observações, pesquisadores voltaram sua atenção para os efeitos de processos inflamatórios na depressão (Soskin et al., 2012).

3.4 Inflamação e TDM

A teoria inflamatória da depressão, proposta há mais de duas décadas (Maes et al., 1995; Smith, 1991), foi influenciada por estudos em células do sistema imunológico e desde então tem sido o estímulo para inúmeros projetos de pesquisa com o objetivo de entender a relação que existe entre a função imunológica e a depressão. Baseados na observação de que pacientes com depressão

clínica severa apresentam aumento das concentrações de biomarcadores inflamatórios no sangue, estes pesquisadores propuseram que a depressão está associada com a resposta de fase aguda. Segundo esta teoria, as citocinas pró-inflamatórias que são responsáveis pela reação de fase aguda, também causam vários aspectos clínicos da depressão, incluindo hiperatividade do eixo HPA, metabolismo serotoninérgico alterado e sintomas neurovegetativos (Maes et al., 1995).

O sistema imunológico consiste de estruturas biológicas e processos que auxiliam o organismo a se adaptar a estressores psicológicos e fisiológicos (Haapakoski et al., 2016). Em humanos, o TDM desenvolve-se em praticamente 1/3 dos pacientes que são tratados com a citocinas recombinantes humanas IL-2 e IFN-α, que têm sido utilizadas para aumentar a responsividade do sistema imunológico na eliminação de tumores e eliminar o vírus da hepatite C (Lotrich, 2015).

A maioria das teorias adaptativas da depressão focou nos potenciais benefícios dos sintomas depressivos para relacionamentos com outros seres humanos (Watson and Andrews, 2002). No entanto, modelos recentes mudaram o foco das relações com pessoas para a relação com os patógenos (Raison and Miller, 2013). Essas teorias, que são apoiadas por evidências convergentes, postulam que os humanos modernos herdaram um viés genômico em relação à inflamação porque esta resposta, e os sintomas depressivos que ela promove, aumentaram a sobrevivência e reprodução do hospedeiro nos ambientes altamente patogênicos em que os seres humanos evoluíram. A partir desta perspectiva teórica, pelo menos parte da vulnerabilidade humana à depressão evoluiu a partir de um repertório comportamental, muitas vezes referido como comportamento-doente que promoveu a sobrevivência do hospedeiro em face da infecção (Dantzer et al., 2008). Na verdade, a hipótese de que a evasão social e a anedonia características da depressão servem para reduzir os recursos energéticos para combater a infecção e a cicatrização de feridas, enquanto que a hipervigilância característica dos transtornos de ansiedade, geralmente comórbida com depressão, é útil para a proteção do ataque e posterior exposição ao patógeno. Mesmo o estresse psicológico pode ser entendido a partir desta perspectiva teórica, uma vez que a grande maioria dos estressores enfrentados pelos mamíferos ao longo do tempo evolutivo reduziam-se a riscos inerentes à caça ou competição por *status* reprodutivo. Em todas estas circunstâncias, o risco de invasão de patógenos, e subsequente morte por infecção, aumentou bastante por conta de ferimentos. Em ambientes ancestrais, a associação entre a percepção do estresse e o risco de

ferimentos subsequentes foi suficientemente confiável para que a evolução favorecesse os organismos que ativavam facilmente sistemas inflamatórios em resposta a uma ampla gama de ameaças e desafios ambientais (incluindo estressores psicossociais), mesmo que essa ativação fosse, em muitas ocasiões, um alarme falso (Miller and Raison, 2016).

Pacientes com TDM exibem todas as principais características de uma resposta inflamatória, incluindo aumento da expressão de citocinas pró-inflamatórias e seus receptores e aumento dos níveis de proteínas de fase aguda, quimiocinas e moléculas de adesão solúveis no sangue periférico e no líquido cérebro-espinhal (CSF) (Maes, 1999; Miller et al., 2009). Desta forma a inflamação, especialmente no SNC, tem sido apontada por ter uma relação de proximidade com desordens psiquiátricas, incluindo a depressão (Dowlati et al., 2010; Passos et al., 2015; Stein et al., 2017; Stein et al., 2018). Acredita-se assim, que a inflamação e a depressão estejam interconectadas num ciclo bidirecional onde cada uma é responsável pela facilitação de ocorrência da outra (Kiecolt-Glaser et al., 2015), numa resposta que pode ser ativada por ameaças sociais da vida moderna (Slavich and Irwin, 2014) e pode ser replicada em modelos animais pré-clínicos de estudo da depressão (Hodes et al., 2015; Miller and Raison, 2016).

3.5 *Coping*, Resiliência e Vulnerabilidade ao Estresse Social

O cérebro, como órgão central do estresse e da adaptação a estressores, desempenha um papel central nas respostas comportamentais e fisiológicas que podem levar tanto à adaptação bem sucedida quanto à doença mental e física (McEwen, 1998). Respostas adequadas a estressores são geralmente traduzidas como "resiliência", que é definida por McEwen e colaboradores (2015) como a forma de "alcançar um resultado positivo ao enfrentar uma adversidade". Apesar desta ser uma definição adequada, resiliência é um constructo bem mais complexo. Ela ocorre desde funções celulares básicas até comportamentos complexos e não é apenas o resultado de ausência de um processo patológico, mas também o mecanismo ativo de adaptação com uma base biológica (Friedman et al., 2014; Russo et al., 2012). McEwen e colaboradores (2015) ainda propõem diversas questões que ainda faltam ser respondidas na busca pela compreensão deste traço natural: (1) há uma capacidade limitada de adaptação? (2) quando, durante o curso de vida de um indivíduo, o cérebro desenvolve esta flexibilidade de se adaptar positivamente a novos desafios? (3) esta

flexibilidade pode ser restabelecida em indivíduos nos quais as experiências de vida têm limitado a capacidade de adaptação? (McEwen et al., 2015).

A plasticidade cerebral, diferente do que se acreditava no passado, se estende por toda a vida. A capacidade flexível de se adaptar depende das experiências adquiridas durante a vida, especialmente nas fases iniciais, que promovem uma arquitetura cerebral saudável, dando suporte à flexibilidade cognitiva, que permite que o cérebro continue se adaptando às novas experiências. Identificar como o cérebro e o corpo se adaptam a novos desafios, comumente chamados de estressores, e as alterações biológicas que determinam resiliência ou vulnerabilidade (levando a desfechos negativos), são passos importantes para o avanço da compreensão e desenvolvimento de novos tratamentos para doenças relacionadas ao estresse. A reativação da plasticidade encontrada no início da vida em indivíduos que não apresentam resiliência apresenta-se como um desafio para a ciência moderna (McEwen et al., 2015).

A exposição ao estresse é um fator de risco independente no desenvolvimento de desordens psiquiátricas tais como ansiedade, depressão e estresse pós-traumático (Javidi and Yadollahie, 2012; Kendler et al., 1999; Kessler, 1997). Porém, o potencial patogênico de um estressor não depende somente da severidade de exposição ao estresse, mas também da estratégia utilizada para lidar com o evento, evidenciado pela grande variabilidade individual nas consequências da exposição a um evento estressor. Uma característica que pode estar relacionada à resistência ou vulnerabilidade ao estresse é o estilo de *coping* utilizado para lidar com o estressor, podendo ser tanto ativo quanto passivo (Veenema et al., 2003; Wood and Bhatnagar, 2015). *Coping* ativo é definido pela resposta comportamental que um indivíduo utiliza para minimizar o dano físico, psicológico e social em uma determinada situação, e está relacionado à resiliência ao estresse (Southwick et al., 2005).

O processo ativo de responder e de se adaptar a desafios para tentar manter a homeostase, conhecido como alostase, envolve mediadores autonômicos, inflamatórios, metabólicos e neuromodulatórios, que interagem uns com os outros, promovendo adaptação. Quando este mecanismo adaptativo é ligado e desligado de forma eficiente e não muito frequente, o corpo é capaz de lidar eficientemente com desafios. Porém, se em algumas circunstâncias o sistema alostático é superestimulado ou não responde de forma apropriada, ocorre carga alostática (McEwen, 1996). A carga alostática refere-se ao desequilíbrio dos sistemas que promovem a

adaptação e pode levar ao desenvolvimento de doenças. Esse desequilíbrio pode ser o resultado de estresse repetido, mas também pode ser o resultado de um sistema que falha em desligar ou falha em ligar quando necessário. Desta forma, as estratégias de *coping* comportamentais e fisiológicas apresentadas frente a um desafio vão determinar se a resposta vai resultar em estresse positivo, com desfecho satisfatório ou se haverá consequências negativas (McEwen, 1998).

Múltiplos mecanismos neurais são determinantes da resiliência e vulnerabilidade ao estresse. Peptídeos como o fator liberador de conticotropina (CRF, da sigla em inglês) (Elliott et al., 2010), o neuropeptídeo Y (NPY) (Cohen et al., 2012; Sajdyk et al., 2008) e a dopamina na área tegmental ventral (VTA, da sigla em inglês) (Isingrini et al., 2016; Wood and Bhatnagar, 2015) são bem caracterizados nesse sentido, enquanto outros mecanismos, como o sistema imunológico, foram menos estudados em relação ao seu papel como mediadores de resiliência e vulnerabilidade. Sugere-se que a resiliência não represente simplesmente o oposto da vulnerabilidade, pois há mecanismos que são dicotômicos em animais resilientes e vulneráveis. Outro desafio para a ciência é descrever como estes mecanismos interagem para gerar fenótipos resilientes e suscetíveis. Uma ideia forte é a de que os fenótipos associados à resiliência não são estressor-específicos, de modo que um indivíduo resiliente a um contexto estressor pode não ser resiliente em outro contexto (Bracha, 2004; Rutter, 2006). Manter o mesmo fenótipo resiliente quando o ambiente estressante muda pode não ser necessariamente adaptativo e o fenótipo resiliente pode ter que ser ajustado (Wood and Bhatnagar, 2015).

Em animais normalmente dois padrões de *coping* se apresentam em resposta ao estresse social (Koolhaas et al., 1999). O *coping* ativo ou proativo é caracterizado por agressão territorial e controle e foi originalmente descrito por Walter Cannon (1915). O *coping* passivo ou reativo é caracterizado por imobilidade e baixos níveis de agressividade (Engel and Schmale, 1972). As duas estratégias apresentam características comportamentais opostas e foram identificadas em diversas espécies, desde peixes até mamíferos como roedores e primatas humanos e não humanos (Koolhaas et al., 1999). As características comportamentais de cada uma das estratégias são acompanhadas por diferenças fisiológicas e neuroendócrinas. *Coping* passivo está associado com a alta reatividade do eixo HPA e é acompanhado por altas concentrações plasmáticas de corticosterona e baixas concentrações de norepinefrina (Korte et al., 1992), enquanto *coping* ativo distingue-se pela baixa reatividade do eixo HPA e alta reatividade do sistema simpático a situações estressantes (Fokkema

et al., 1995). Frente a isso, quando a resposta de coping é inadequada para atenuar o efeito do estresse no organismo, consequências fisiológicas e psicológicas induzidas pelo estresse podem ocorrer.

Mais recentemente tem surgido a ideia de que um perfil anti-inflamatório pode ser característico de um fenótipo resiliente, enquanto um perfil pró-inflamatório está mais associado à vulnerabilidade aos efeitos do estresse (Wood et al., 2015). Esta linha de investigação tem recebido grande atenção de pesquisadores que buscam por soluções para os transtornos psiquiátricos influenciados pelo estresse e está se transformando em uma das principais fontes de pesquisa pré-clínica, com foco em diferenças individuais.

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5. JUSTIFICATIVA

Considerando a importância do TDM e a relevância científica e clínica de uma compreensão mais profunda de sua patofisiologia, associada ao papel do estresse no desenvolvimento dessa desordem psiquiátrica, o presente trabalho é justificado por contribuir no entendimento destes aspectos. Utilizando um modelo de investigação pré-clínico de indução de comportamento tipo-depressivo como base e relacionando-o com reações imunológicas centrais e periféricas, procurou direcionar a atenção a alvos até pouco tempo negligenciados. Sendo assim, estudos que se propõe a investigar a relação do estresse social, do comportamento tipo-depressivo, das estratégias de *coping* e de reações imunológicas associadas, pode contribuir para o direcionamento de novas alternativas de tratamento, especialmente para uma parte da população que sofre deste transtorno, e aos quais as alternativas convencionais têm se mostrado ineficientes.

6. OBJETIVOS

6.1 Objetivo Geral

Essa tese tem por objetivo principal investigar a relação do estresse social com o comportamento tipo-depressivo utilizando um modelo laboratorial de investigação pré-clínica para roedores. Além disso, visa relacionar as características comportamentais com reações imunológicas, traduzidas como estratégias de *coping*, e seu papel na resiliência ao estresse.

6.2 Objetivos Específicos

Revisar a literatura recente de trabalhos que utilizaram o modelo de estresse por derrota social e seus efeitos na reatividade das células microgliais do sistema nervoso central e sumarizá-los em forma de texto.

Testar, utilizando um modelo crônico de estresse por derrota social em ratos Wistar, as reações comportamentais associadas à ativação imunológica periférica.

Investigar possíveis diferenças individuais em relação às estratégias de *coping* adotadas por animais submetidos à derrota social por subordinação contínua, e relacioná-las com resiliência ao desenvolvimento de comportamento tipo-depressivo.

Descrever comportamentos e reações imunológicas associadas como resposta à exposição de ratos ao protocolo de 21 dias consecutivos de estresse por derrota social contínua.

Colaborar para uma melhor compreensão da patofisiologia do TDM.

7. CONSIDERAÇÕES ÉTICAS

Este projeto (HCPA nº 14/0274) foi realizado de acordo com as recomendações da Lei Federal Brasileira nº 11.794/2008, que estabelece procedimentos para o uso científico de animais. O protocolo experimental deste projeto foi analisado e aprovado em seus aspectos éticos e metodológicos pela Comissão de Ética no Uso de Animais (CEUA) do Hospital de Clínicas de Porto Alegre (HCPA) (Anexo A) e todos os procedimentos foram supervisionados pela equipe médica veterinária da Unidade de Experimentação Animal da mesma instituição.

8. ARTIGOS

8.1 Artigo 1:

Microglial Over-Activation by Social Defeat Stress Contributes to Anxiety and Depressive-Like Behaviors

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Microglial Over-Activation by Social Defeat Stress Contributes to Anxiety- and Depressive-Like Behaviors

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Hyper activation of the neuroimmune system is strongly related to the development of neuropsychiatric disorders. Psychosocial stress has been postulated to play an important role in triggering anxiety and major depression. In preclinical models, there is mounting evidence that social defeat stress activates microglial cells in the central nervous system. This type of stress could be one of the major factors in the development of these psychopathologies. Here, we reviewed the most recent literature on social defeat and the associated immunological reactions. We focused our attention on microglial cells and kept the effect of social defeat over microglia separate from the effect of this stressor on other immune cells and the influence of peripheral immune components in priming central immune reactions. Furthermore, we considered how social defeat stress affects microglial cells and the consequent development of anxiety- and depressive-like states in preclinical studies. We highlighted evidence for the negative impact of the over-activation of the neuroimmune system, especially by the overproduction of pro-inflammatory mediators and cytotoxins. Overproduction of these molecules may cause cellular damage and loss or decreased function of neuronal activity by excessively pruning synaptic connections that ultimately contribute to the development of anxiety- and depressive-like states.

Keywords: microglia, neuroimmunity, immune cells, psychosocial stress, neuropsychiatric disorders, inflammatory processes

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INTRODUCTION

Neuropsychiatric disorders, such as anxiety and major depression (MD), are highly prevalent and contribute significantly to the worldwide burden of diseases (Ferrari et al., 2013; Whiteford et al., 2013). As a major contributor to the development of affective and neuropsychiatric disorders in humans, psychosocial stress has been reported to induce central and peripheral immune pathway signaling by repeated activation of the neuroendocrine and neurovegetative systems (Glaser and Kiecolt-Glaser, 2005; Lehmann et al., 2016). When the individual is repeatedly exposed to stress, the brain homeostatic environment alters and may give rise to various cognitive and mood disorders that impair everyday functioning and overall quality of life (McKim et al., 2016a). Within the central nervous system (CNS) immunological defense, microglia are the key immune players and acquire a reactive profile to cope with altered homeostasis (Hanisch and Kettenmann, 2007). When activated,

these cells are supposed to trigger anxiety- and depressive-like behaviors (Lehmann et al., 2016), mainly by increasing the expression of pro-inflammatory mediators and neurotoxins in stress-sensitive brain regions (Reader et al., 2015; Ramirez and Sheridan, 2016), and can ultimately influence the overall cellular functions and survival, from neurons to glial cells.

Brief and prolonged episodes of social defeat (SD) have been correlated with anxiety- and depressive-like behaviors, respectively. While brief episodes can increase self-grooming, locomotion in novel environments, risk assessment and binge-like cocaine self-administration, prolonged episodes induce anhedonic behaviors such as reduced sweet solution preference, reduced mounting in copulatory behavior, reduced climbing in the forced swimming test (FST), lower general activity and sociability and suppressed cocaine intake (Razzoli et al., 2009; Miczek et al., 2011; Hollis and Kabbaj, 2014; Vasconcelos et al., 2015). Despite the clear evidence of the role of social stress triggering mood disorder-related behaviors, to the best of our knowledge, the exclusive contribution of SD to microglial over-activation has never been reviewed. Here, we discuss the emerging field of social stress-induced microglial over-activation, providing an overview of how microglial reactions can lead to these mood disorders, and briefly discuss some relevant translational significance of the findings. We hypothesized that acute/repeated and chronic social defeat (CSD) stress can induce microglial activation and over-activation that can engender anxiety and depressive-like states, respectively. The repeated social defeat (RSD) paradigm reported in this review is characterized by the introduction of an aggressive intruder male into the cages of established male cohorts of mice for three or six consecutive nights, leading to the establishment of dominance over the original colony (Wohleb et al., 2014b). CSD varied from 14 to 20 days of a 24 h/day dyadic social housing, exposing the defeated animal to continuous psychological stress via sensory interaction with the aggressor, accompanied by a 5 min/day agonistic encounter between the aggressor and the defeated animal (Brachman et al., 2015; Lehmann et al., 2016; Tong et al., 2017).

Articles used in this mini-review were selected from the PubMed, Embase and ScienceDirect databases between March and April 2017. Search terms were "microglia" and "SD", without any time limitation. Of the 23 selected articles, 11 were excluded for the following reasons: not an original article, no clear effect of stress over microglia and the use of mixed stress protocols.

MICROGLIA: THE FIRST DEFENCE OF THE CNS

Microglia comprise about 10%–15% of all brain cells and are crucial players in normal development through the regulation of functional and structural processes, contributing to plasticity from individual synapses to neural circuits and behavior (Wake et al., 2013; Salter and Beggs, 2014; Verkhratsky et al., 2015). Microglial cells originate from extra-embryonic yolk sac progenitors, establish unique CNS cell populations and are maintained throughout life by local proliferation (Ginhoux et al., 2010, 2013). As tissue-resident macrophages in the CNS, along with other mononuclear phagocytes, microglia are critical effectors and regulators of changes in CNS homeostasis during development, in health and disease (Hanisch and Kettenmann, 2007; Prinz and Priller, 2014).

Some evidence points to new and fundamental roles for microglia in the control of neuronal proliferation and differentiation, as well as in the formation of synaptic connections (Kettenmann et al., 2011; Ginhoux et al., 2013). These cells are distributed in the brain parenchyma, have small delineated processes and actively screen the inter-neuronal space for incoming threats, exhibiting immune regulatory functions, from local surveillance to the removal of debris (Prinz and Priller, 2014). Microglial activation is the main neuroinflammatory element in the CNS, providing the front line defense whenever injury, disease or infection occurs (Lehnardt, 2010; Tang and Le, 2016).

Inflammatory processes are usually self-limited, culminating with tissue repair; damage to the CNS occurs when the system is over-activated for a long time, extending the release of pro-inflammatory mediators and neurotoxins. This process can worsen tissue damage and negatively impact disease outcome, leading to anxiety- and depressive-like states (Reader et al., 2015; Ramirez and Sheridan, 2016). Increasing evidence points to a heterogeneous status of microglial activation in the CNS. Although it is not a consensus, some authors categorize microglia into two opposite activation states, M1 and M2 phenotypes, which can produce either cytotoxic or neuroprotective effects (Tang and Le, 2016). M1-polarized microglia are associated with the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), superoxide, nitric oxide, reactive oxygen species and proteases (Ajmone-Cat et al., 2013), whereas M2-polarized microglia express cytokines and receptors that are implicated in the inhibition of inflammation and restoration of homeostasis by tissue repair and extracellular matrix reconstruction (Nakagawa and Chiba, 2014; Tang and Le, 2016). Nevertheless, as this nomenclature is not fully accepted and some authors consider microglia polarization to have derived from studying peripheral macrophages rather than microglia (Ransohoff, 2016), it is important to carefully use and interpret these terms to avoid misunderstandings.

THE SD PARADIGM AS A VALID STRESSOR

Most stressors in human life arise from interactions within the social environment. In fact, social stress encompasses various types of significant life events, ranging from maternal separation (Meaney, 2001; Nishi et al., 2014), brief episodes of social confrontations in adolescence and adulthood, to continuous subordination stress (Miczek et al., 2008). In preclinical studies, some models of stress are often criticized as being artificial and not representative of human stress (Björkqvist, 2001; Almeida et al., 2002).

The SD paradigm is recognized as an ethological valid method to engender social stress in rodents (Vasconcelos et al., 2015; Henriques-Alves and Queiroz, 2016; Koolhaas et al., 2017). RSD is a stressor that recapitulates key physiological, immunological and behavioral alterations observed in humans exposed to chronic psychosocial stress (McKim et al., 2016a). Models of psychosocial stress rely on innate social behavior among pairs or groups of male rodents allowing the formation of stable dominant-subordinate relationships (Krishnan and Nestler, 2011). Another strong point of these models is the lack of habituation; despite repeated exposures, animals continue to generate emotional stress responses (Tidey and Miczek, 1997).

SD stress activates the hypothalamic-pituitary-adrenal axis and sympathetic nervous system, increasing systemic glucocorticoids that trigger the release of catecholamines and pro-inflammatory cytokines (Avitsur et al., 2001; Herman et al., 2016). Although there are distinct models of social stress, this review will focus on the role of SD in the development of anxiety and MD, tracking the contribution of the over-activation of the main CNS immune component, microglia, in triggering these psychiatric diseases.

EFFECTS OF SD STRESS ON MICROGLIAL CELLS

One of the major advances in the field of the study of psychiatric disorders came from the notion that the immune system and inflammatory processes can be activated by psychosocial stressors (Miller and Raison, 2015). Despite the well-established evidence that the peripheral and central immune systems act in concert to promote the stress reaction, greater attention has been given to immune cells of the CNS, in particular, microglia. Social stress may activate microglial cells in a way different from other stressors (Glaser and Kiecolt-Glaser, 2005; Calcia et al., 2016) and seems to exert a direct effect over microglia activity through the activation of glucocorticoid and mineralocorticoid (Sierra et al., 2008) and β-adrenergic receptors (Walker et al., 2013; Calcia et al., 2016). Considering these factors, we directed our attention to microglial reactions induced by SD; the evidence is presented in **Table 1**.

Microglia present increased activation status after SD (Wohleb et al., 2014b; Ramirez and Sheridan, 2016) and the effects are mainly observed within brain regions associated with fear, anxiety and threat appraisal (Wohleb et al., 2015). From a ramified aspect found in the immunosurveillant state, microglia change robustly to a de-ramified state with shorter and thicker processes (Wohleb et al., 2011, 2012, 2013, 2014b), leading to increased soma size after acute, RSD and CSD (McKim et al., 2016a; **Figure 1**). Changes in soma and processes are usually analyzed by increases in ionised calcium-binding adapter molecule 1 (Iba-1) or cluster of differentiation 11b (CD11b) immunoreactivity. However, although the vast majority of studies report results similar to those described above, decreases in microglial Iba-1, CD11b and consequently soma areas were found by others in the dentate gyrus (DG), but not in the medial prefrontal cortex, in a stress protocol that consisted of 20 days of exposure to SD (Tong et al., 2017). These controversial data could be attributed to differences in stress chronicity.

One additional way to identify changes in microglia activity is through the analysis of activation markers such as the chemokine (C-C motif) ligand 2 (CCL_2), toll-like receptor 4 (TLR-4) or the CX_3 chemokine receptor 1 (CX_3CR_1) which are expressed by microglial cells. SD induces an increase in the gene expression of TLR-4, CCL_2 and CX_3CR_1 (Ramirez et al., 2015, 2016; Ramirez and Sheridan, 2016). However, decreases in CX_3CR_1 gene expression were also observed after SD, although in enriched brain $CD11b^+$ cells (Wohleb et al., 2014a). One of the most evident reactions to SD observed in microglial cells is the rise in gene expression and mRNA levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and expression of the surface activation marker CD14. Increases of these inflammatory mediators were observed after acute, RSD and CSD (Wohleb et al., 2011, 2012, 2014a; Brachman et al., 2015; Ramirez et al., 2015, 2016; McKim et al., 2016a; Ramirez and Sheridan, 2016), even 24 days after stress cessation (Ramirez et al., 2015). The importance of these findings is reinforced by the results obtained from either microglial cells analyzed in fresh CNS tissue, isolated from socially defeated animals (Wohleb et al., 2012) or in *ex vivo* SD-sensitized microglia stimulated with lipopolysaccharide (LPS; Wohleb et al., 2011). Additionally, reduced levels of glucocorticoid responsive genes (GILZ and FKBP51) are evident after exposure to SD (Wohleb et al., 2011). Chronically SD stress-activated microglial cells increase their phagocytic activity. This effect is achieved by increasing the expression of $CD68^{hi}$ (a marker for phagocytic activity; Lehmann et al., 2016). The increasing phagocytic activity of microglia from CSD animals suggests that cellular debris or cell damage or death may be a hallmark of chronic stress effects on the brain. SD can also change microglial cell numbers; while acute SD enhances the number of microglia (Lehmann et al., 2016), CSD diminishes these cells (Tong et al., 2017), mainly in the hippocampus. It seems that a crucial factor is the intensity of activation of microglia by stress, which can lead to different psychiatric disorder outcomes (**Figure 1**). Taken together, these data highlight the broad spectrum of effects that can be observed in microglial cells when activated by SD.

THE LINK BETWEEN MICROGLIAL ACTIVATION, ANXIETY- AND DEPRESSIVE-LIKE BEHAVIORS

It is now well known that disturbances in microglial functioning has an etiological role in mood disorders (Frick et al., 2013; Kreisel et al., 2014). However, if the effect of social stress on these deregulated behaviors can be mainly attributed to microglial over-activation or if the participation of other CNS immune cells and/or the peripheral immune system plays a major role remains controversial. While researchers have shown in some studies that SD stress-induced anxiety- and depressive-like states are mediated by the activation of

TABLE 1 | Microglial activation profile induced by SD stress and related behavioral outcomes.

Defeated subjects	Age	Stressor	Source of microglia	Microglia changes	Behavioral outcomes	Reference
♂ICR mice	8–10 w	CSD (20 days)	HPC (DG) mPFC	↓ hippocampal microglial numbers, process lengths and soma areas No changes in microglia numbers in PFC ↓ expression levels of Iba-1 and CD11b	↑ immobility in the TST and FST ↓ in sucrose consumption in the SPT ↓ time in the center in the OFT	Tong et al. (2017)
♂C57BL/6J mice ♂$CX_3CR_1^{wt/gfp}$ mice	8–10 w	Acute RSD (3 days) Chronic RSD (14 days)	Infralimbic ctx Prelimbic ctx Anterior cingulate ctx Piriform ctx Nucleus accu. Dorsal DG Basolateral AMY	Chronic SD microglia phagocytosed more labeled material ↑ numbers of $CD68^{hi}$ microglia in chronic SD mice *vs* HC and ASD ↑ microglial phagocytosis *ex vivo* ↑ microglial proliferation after ASD	↓ marking preference in the USM task in CSD $CX_3CR_1^{wt/gfp}$ mice ↓ SI in CSD mice	Lehmann et al. (2016)
♂C57BL/6 mice	6–8 w	RSD (6 days)	HPC (DG)	↑ soma size, shorter and thicker cell processes ↑ microglial Iba-1 immunoreactivity, augmented in caudal HPC ↑ IL-1β mRNA	↑ latency and distance to reach the platform of the MWM ↑ time in the outer annulus of the MWM ↓ time in the target quadrant of the MWM ↑ latency to find the escape hole in the BM ↑ number of errors to reach the escape hole in the BM	McKim et al. (2016a)
♂C57BL/6 mice	6–8 w	RSD (6 days)	Whole brain homogenates	↑ microglia gene expression of IL-1β, IL-6, TNF-α ↑ microglia activation markers TLR-4, CCL_2 and CX_3CR_1	↓ number of center entries in the OFT ↑ time to first enter the center in the OFT ↓ time spent in the interaction zone in the SAT	Ramirez and Sheridan (2016)
♂C57BL/6 mice	6–8 w	RSD (6 days)	HPC HPT Whole brain homogenates	↑ microglia gene expression and mRNA expression of IL-1β, IL-6, TNF-α ↑ microglia activation markers TLR-4, CCL_2 and CX_3CR_1	↓ number of center entries in the OFT ↑ time to first enter the center in the OFT ↓ time spent in the interaction zone in the SAT	Ramirez et al. (2016)
Donors: ♂C57BL/6 mice Recipients: ♂$Rag2^{-/-}$ mice	10–13 w	CSD–(14 days)	Whole brain homogenates (-cerebellum)	Stressed/lymphocytes donors: ↑ basal gene expression of the M1 microglia cytokines IL-1β and IL-6. ↓ basal expression of the M2 marker MRC1 and muted ARG response to IL-4 Non-stressed/lymphocytes recipients: skewed microglia to a M2-like phenotype. ↑ ARG expression (basal and IL-4 stimulated) and a generally muted response of IL-1β and IL-6 to LPS stimulation	SD → Rag mice on C57BL/6 background: ↑ transitions in the L/D box ↑ center time in the OFT ↑ interaction in the SI test ↑ time mobile in the TST SD → Rag mice on 129 background: ↑ transitions in the L/D box ↑ travel in the OFT ↑ center time in the OFT ↑ marking preference in the USM	Brachman et al. (2015)

(Continued)

TABLE 1 | Continued

Defeated subjects	Age	Stressor	Source of microglia	Microglia changes	Behavioral outcomes	Reference
♂C57BL/6 mice	6–8 w	RSD (6 days)	Whole brain homogenates	↑ microglia gene expression of IL-6 ↑ gene expression of IL-1β, IL-6, and TNF-α in *ex vivo* LPS stimulated microglia from RSD mice ↑ relative gene expression of IL-6 in microglia 24 days after stress cessation	↓ time spent in the interaction zone in the SAT ↑ time spent in the corners in the SAT	Ramirez et al. (2015)
♂C57BL/6 WT mice ♂IL-1R1^{Ko} mice ♂IL-1R1^{Kd} mice	6–10 w	RSD (6 days)	Whole brain homogenates	Robust change in the morphology of microglia in WT mice after RSD ↑ surface expression of the activation marker CD14 Altered gene expression of inflammatory-related genes in brain CD11b^{+} cells De-ramified morphology in WT and IL-1R1^{Kd} mice with increased Iba-1 proportional area	WT: ↑ thigmotaxis in the OFT ↑ latency to enter the center in the OFT ↓ time spent in the center in the OFT ↓ time to enter the dark zone in the L/D box ↑ time spent in the dark zone of the L/D box	Wohleb et al. (2014b)
♂C57BL/6 WT mice	6–10 w	ASD (1 day) RSD (6 days)	PFC AMY HPC (CA3 and DG) Whole brain homogenates	ASD altered microglia morphology in stress sensitized mice ↑ Iba-1 proportional area in PFC, AMY and HPC (CA3 and DG) in RSD mice ↑ mRNA levels of IL-1β, TNF-α and CD14, 5 days after RSD ↓ CX_3CR_1 after RSD	↑ latency to enter the center in the OFT at 0.5 and 8 days ↓ time spent in the center in the OFT at 0.5 days ↓ time spent in the interaction zone in the SAT at 0.5, 8 and 24 days ↑ time spent in the corners in the SAT at 0.5 and 24 days ↓ % time spent in the interaction zone in the SAT	Wohleb et al. (2014a)
♂C57BL/6 mice	6–10 w	SSD (1 day) RSD (3 or 6 days)	PFC HPC (DG)	↑ Iba-1 immunoreactivity after RSD ↑ microglial activation (de-ramified morphology) in PFC and DG after RSD Microglia recruit peripheral myeloid cells to the brain	↑ latency to enter the center in the OFT after RSD (3 and 6 days) ↓ time spent in the center in the OFT after RSD (3 and 6 days) ↓ time to enter the dark zone in the L/D box after RSD (6 days) ↓ time spent in the light zone in the L/D box after RSD (6 days)	Wohleb et al. (2013)

(Continued)

TABLE 1 | Continued

Defeated subjects	Age	Stressor	Source of microglia	Microglia changes	Behavioral outcomes	Reference
♂C57BL/6 mice	6 w	RSD (6 days)	PFC HPC PVN AMY Whole brain homogenates	↑ mRNA levels of IL-1β, TNF-α, iNOS and CD14 ↑ IL-1β and TNF-α mRNA levels after LPS injection Hypertrophic microglia with shorter and thicker processes ↑ inflammatory response of brain $CD11b^{+}$ cells following a peripheral LPS challenge ↑ activated morphology of microglia ($Iba\text{-}1^{+}$) in the PFC, AMY, PVN, and HPC Amplified surface expression of CD14 after LPS injection	↓ social exploratory behavior ↓ time spent in the center in the OFT ↑ latency to enter the center in the OFT	Wohleb et al. (2012)
♂C57BL/6 mice ♂$IL\text{-}1R1^{-/-}$ mice	6–8 w	SSD (1 day) RSD (3 days) RSD (6 days)	AMY PFC HPC	↑ de-ramified (shorter and thicker processes by Iba-1) microglia in MeAMY, PFC and HPC ↑ surface markers CD14, CD68 and TLR-4 after RSD (6d) ↑ mRNA expression of interleukin IL-1β ↓ levels of glucocorticoid responsive genes (GILZ and FKBP51) ↑ *ex vivo* levels of IL-6, TNF-α and MCP-1 following LPS stimulation	WT mice: ↓ time to enter the dark zone in the L/D box after RSD (6 days) ↑ time spent in the dark zone in the L/D box after RSD (6 days)	Wohleb et al. (2011)

Abbreviations: ASD, Acute Social Defeat; AMY, Amygdala; BM, Barnes Maze; CCL_2, Chemokine (C-C motif) ligand 2; CSD, Chronic Social Defeat; Ctx, cortex; $CXCL_2$, Chemokine (C-X-C motif) ligand 2; d, days; DG, Dentate Gyrus; FKBP51, FK506 binding protein 51; FST, Forced Swimming Test; GILZ, Glucocorticoid-induced leucine zipper; HPC, Hippocampus; Iba-1, Ionized Calcium-Binding Adapter Molecule 1; $IL\text{-}1R1^{Kd}$, IL-1 receptor type-1 knock-down; $IL\text{-}1R1^{Ko}$, IL-1 receptor type-1 knock-out; L/D, Light/Dark; LPS, Lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; mPFC, medial Pre-Frontal Cortex; MWM, Morris Water Maze; OFT, Open-Field Test; PFC, Pre-Frontal Cortex; PND, Post-Natal Day; PVN, Paraventricular Nucleus; RSD, Repeated Social Defeat; SAT, Social Avoidance Test; SI, Social Interaction; SSD, Single Social Defeat; Tg, Transgenic; TST, Tail Suspension Test; USM, Urine Scent Marking; w, weeks; WT, Wild-Type.

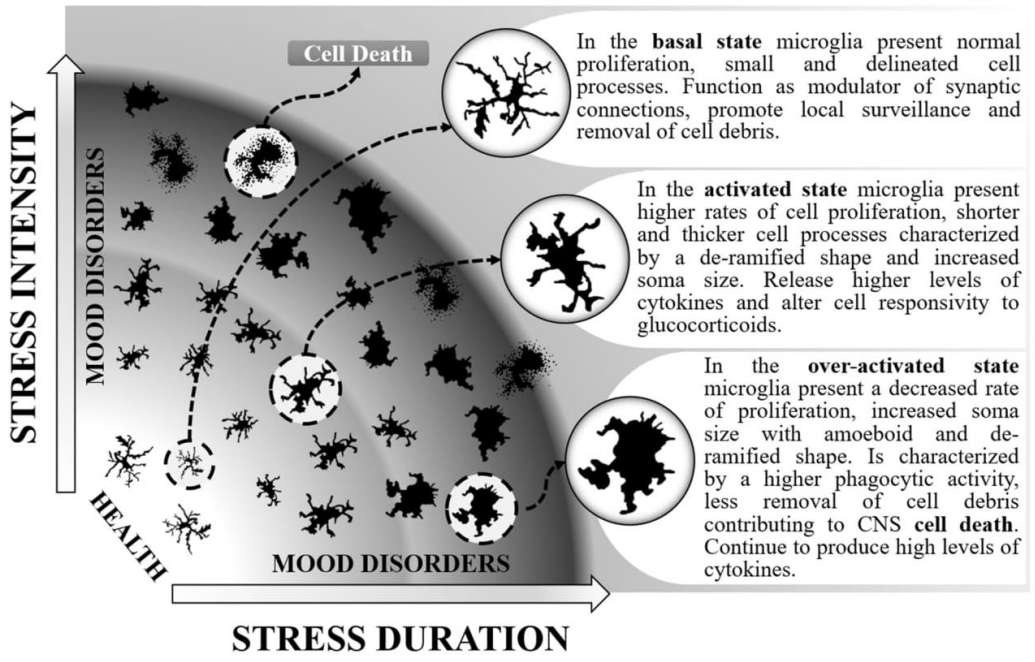

FIGURE 1 | Different stages of microglial activation caused by changes in the intensity and duration of social stressors that can maintain individuals in a healthy state or contribute to both anxiety and depressive-like behaviors. In the basal state, microglia can be distinguished by normal levels of immunoreactivity to ionized calcium-binding adapter molecule 1 (Iba-1) and CD11b. In this state, enable proper coping to stress situations. When activated, microglia proliferate, release higher levels of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), present higher expression of toll-like receptor 4 (TLR-4), chemokine (C-C motif) ligand 2 (CCL_2), CX_3CR_1) and decreased levels of glucocorticoid-induced leucine zipper (GILZ) and FK506 binding protein 51 (FKBP51). They can be distinguished by higher immunoreactivity to Iba-1, CD11b, CD14 and CD68. During the activated state, the release of pro-inflammatory mediators and the altered response to glucocorticoids may lead to anxiety. The activated state can also be protective by resuming stress effects. As a consequence, microglia return to their basal state. Otherwise, persistent stress shifts microglia to an over-activated state. Overactive microglia continue to release pro-inflammatory mediators (IL-1β and IL-6). They can be distinguished by higher immunoreactivity to $CD68^{hi}$ and possibly lower levels of CX_3CR_1 and iNOS antibodies. Along with the microglial phagocytic activity occurs a higher rate of cell death, including microglia as well as neuronal and other glial cells. This effect reduces their capacity to remove cell debris and surveillance of the inter-neuronal space, altogether leading to depression.

microglia with the involvement of peripheral macrophages and trafficking of monocytes to the brain (Wohleb et al., 2013, 2014b, 2015), other studies excluded the direct involvement of peripheral monocytes triggering these behaviors (Lehmann et al., 2016). Stress chronicity and/or peripheral wounds (triggers of peripheral immune reactions), which can usually be observed in defeated animals after confrontation with an aggressor, could be major determinants. This is one of the main reasons that led researchers to choose alternative stress protocols, such as variable unpredictable stress and foot shocks to study microglial activation in neuropsychiatric disorders, even though these procedures present lower ethological relevance.

Studies in humans have shown that microglial activation is positively correlated with psychiatric disorders. For example, individuals experiencing a major depressive episode present enhanced positron emission topography labeling of the translocator protein (TSPO), a putative marker of neuroinflammation and microglia activation (Setiawan et al., 2015). It has also been speculated that there is a causal link between microglial activation and suicidal behavior (Schnieder et al., 2014); neuroendocrine factors, cytokines and nitric oxide, which are released from microglial cells and are known to modulate noradrenergic or serotonergic neurotransmission, may trigger suicidal behavior (Steiner et al., 2008). Pro-inflammatory cytokines including IL-1β and TNF-α, can reduce the availability of serotonin, dopamine and noradrenaline by increasing the expression and function of reuptake transporters, reducing synthesis or decreasing monoamine precursors (Miller and Raison, 2015). Activated microglia can also act on the glutamate pathway and together with astrocytes stimulate the increased release of this neurotransmitter and decreased brain-derived neurotrophic factor, which ultimately leads to excitotoxicity (Steiner et al., 2012; Miller and Raison, 2015). Additionally, it has been shown that elevated pro-inflammatory cytokine levels caused by microglia activation associated with the recruitment

of monocytes to the brain contribute to the development and persistent anxiety-like behavior (Wohleb et al., 2014b, 2015). Moreover, chronic microglial activation in particular can result in neuronal apoptosis, neurogenesis inhibition, hippocampal volume reduction, lower neurotransmitters synthesis and cytotoxicity (Ascoli et al., 2016), which is ultimately related to depressive behavior.

Although microglia are not the only effectors of the immune system, it has been suggested that the anti-inflammatory effect of antidepressants may have protective effects by silencing RSD-induced priming and activation of microglia, thus down-regulating the biosynthesis of high levels of pro-inflammatory cytokines (Ramirez et al., 2015). Recently, microglia have been recognized as important targets for pharmaceutical research. Brain diseases, including depression and anxiety, could potentially be treated with drugs that are capable of inhibiting or restoring specific microglial functions (Biber et al., 2016). Anti-inflammatory drugs such as COX2 inhibitors or minocycline, aimed at inhibiting the pro-inflammatory status of microglia, have been suggested as therapeutics for inflammatory brain diseases (Biber et al., 2016). The CX_3CR_1, as an exclusive microglial marker, could also be a potential target. Since the activation of microglia is not consistent for all patients, it has been recently proposed that anti-inflammatory treatment targeting microglial activation could specifically be more effective in patients with increased microglial activation, leading to the idea that microglial activation may be a marker for severe and untreatable psychiatric disorders (Mondelli et al., 2017).

Social stress can alter the number of microglial cells (Lehmann et al., 2016; Tong et al., 2017), mainly dependent on the duration of stress exposure. While acute, but not CSD is supposed to increase microglial proliferation selectively in telencephalic stress-related brain areas (Lehmann et al., 2016), a loss of hippocampal microglia was observed and is supposed to promote the development of MD, indicating that the restoration of microglial functions and/or numbers may be beneficial for the therapy of MD (Tong et al., 2017). Since pro-inflammatory cytokines can also modify neurogenesis in the hippocampus (Koo and Duman, 2009), RSD has been shown to induce anxiety-like behavior by impairing the neuronal differentiation of neural progenitor cells in the hippocampus that proliferated during stress exposure. These data were positively correlated to an impairment in performance on working and spatial memory in the Morris water maze (MWM) and transiently disrupted short-term memory recall in the Barnes maze (BM; McKim et al., 2016a). Overall, these data highlight the magnitude of the microglial over-activation-induced deficits in monoamine neurotransmission, cytotoxicity, cellular loss and reduced neurogenesis, ultimately leading to memory impairment and behaviors that are observed in both, anxiety and depression.

CONCLUSION REMARKS

Exposure to SD induces microglial cells to assume an activated state, which initially may be considered beneficial. RSD and CSD can induce microglia to assume over-activated states that, by persistently releasing pro-inflammatory mediators, cytotoxins and reactive oxygen species, may cause cellular dystrophy and a loss or decreased function of neuronal activity through excessively pruned synaptic connections. All of these stress effects over microglia worsen memory and behaviors that are important factors in psychiatric disorders. The SD paradigm is an important tool to induce anxiety- and depressive-like states in laboratory animals for investigating stress-induced immunological and behavioral alterations.

It seems that the development of anxiety and MD is, besides microglial activation, dependent on peripheral monocyte recruitment to the brain (McKim et al., 2016b), attaching importance to the bidirectional communication between the brain and peripheral immune system. However, since the activation of microglia by psychosocial stress might be different from that of physical injury (Glaser and Kiecolt-Glaser, 2005), more attention must be given to peripheral wounds when studying SD stress effects over central immune reactions. SD protocols that allow physical injuries to the defeated animal during confrontations with an opponent may contribute to the participation of peripheral immune cells in the final outcome. Alternatively, stress protocols that do not involve physical injuries, such as chronic unpredictable stress, can be used to overcome this issue. Contradictory findings have shown that microglial over-activation, as well as microglial dystrophy and loss, can mediate the development of MD. Depression is considered to be a disorder that is associated with microglial over-activation. That leads to an interpretation that suppressed microglial hyperactivity should be the focus to treat depressive symptoms (Tong et al., 2017). However, since microglia in its basal state is also critical for brain normal function, microglial dystrophy and loss would also mediate the development of this disorder (Kreisel et al., 2014; Tong et al., 2017). Therefore, over-inhibition or over-down-regulation of microglial function will inevitably produce detrimental effects as well. Focusing on microglial cells as therapeutic targets for pharmacological interventions, especially by restoring functions and/or basal levels, may be a promising strategy for anxiety and depression therapy.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this study. All of them contributed to the conception and design of the work, literature analyses and interpretation, drafting the article, critical revision and final approval of the version to be published.

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8.2 Artigo 2:

Proactive Coping during Chronic Social Defeat Stress is associated with an Anti-inflammatory response in male Wistar rats

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Research Article

Title: Proactive Coping during Chronic Social Defeat Stress is associated with an Anti-inflammatory response in male Wistar rats

Título: *Coping* Proativo durante Estresse Crônico por Derrota Social está associado à resposta Anti-inflamatória em ratos Wistar

Título abreviado: *Coping* Proativo, Estresse Social e resposta Anti-inflamatória – Stein et al.

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DJS and MFV concept the study, designed the experiment, performed procedures, analyzed data, wrote and edited the manuscript.

LL, MGL, LB, LPG, EDR, LAS, RMMA and KMMC contributed equally to perform procedures and critically revise the manuscript.

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Conflict of Interest:

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Abbreviations

ANOVA	Analysis of Variance
BDNF	Brain Derived Neurotrophic Factor
BL	Baseline
BW	Body Weigh
CNS	Central Nervous System
CUMS	Chronic Unpredictable Mild Stress
IGF-1	Insulin-like Growth Factor 1
IL-10	Interleukin 10
LL	Long Latency
MDD	Major Depressive Disorder
OF	Open Field
OZ	Object Zone
PET	Positron Emission Tomography
PND	Post Natal Day
SD	Social Defeat
SEM	Standard Error of the Mean
SL	Short Latency
SP	Saccharin Preference
TNF-α	Tumor Necrosis Factor alpha
VR SD	Video Recorded Social Defeat
WG	Weight Gain

Abstract

Rationale – Chronic social stress is a common risk factor for affective disorders. In preclinical studies, depressive-like states are often induced by defeats using the resident-intruder paradigm. However, the extent to which different coping strategies determine vulnerability or resilience to develop stress-related psychiatric disorders remains unclear. While pro-inflammatory processes are increasingly recognized as key mediators in the etiology of psychopathologies, an anti-inflammatory profile could be responsible for resilience, characteristic of some individuals in the population.

Objectives – The present study tested a short length protocol of chronic social defeat stress in male rats, supposed to induce features of depressive-like symptoms. Further, we examined coping strategies adopted by defeated rats that were associated with circulating levels of growth factors and inflammatory proteins.

Methods – Adult male Wistar rats were continuously threatened by a dominant male rat and were daily defeated by another aggressor in a short encounter for 21 consecutive days. Non-stressed controls were handled for the same period throughout the experiment. Behaviors during confrontations, body weight, saccharin preference, spontaneous locomotor activity and reactivity to novelty in the open field were measured. Blood samples were collected for molecular analysis.

Results – 21 days of chronic social defeat stress produced two coping styles in male Wistar rats. Over the course of daily confrontations, two groups of stressed rats were identified, according to the latency do assume a submissive posture in confrontations with aggressors and were afterwards divided into short latency (SL) and long latency (LL) subpopulations. The two phenotypes were respectively associated with several behaviors related to vulnerability and resilience to stress that also correlated with altered levels of peripheral growth factors and inflammatory markers. Although both groups had reduced weight gain ($p<0.01$) and experienced similar frequency of attacks, LL rats showed increased latencies to being bitten ($p<0.01$), increasing upright postures ($p<0.05$), lower levels of IGF-1 ($p<0.01$) and higher levels of IL-10 ($p<0.01$), while SL rats had increased immobility ($p<0.05$) time during defeat, decreased locomotor activity in the open field ($p<0.01$) and no altered peripheral molecules compared to non-stressed controls.

Conclusions - Social defeat stress-induced behavioral changes are bimodally distributed in male Wistar rats and correlate with different phenotypes of peripheral inflammation. This study

highlighted that proactive coping with stress is closely associated with an anti-inflammatory response and may confer resilience to stress-related disorders.

Keywords: Resilience, Psychiatric Disorder, Inflammation, Animal Model, Rodent, Depressive-like Behavior.

Resumo

Introdução – Estresse social crônico é um fator de risco comum para desordens afetivas. Em estudos pré-clínicos, estados tipo-depressivos são frequentemente induzidos por derrotas utilizando o paradigma do residente-intruso. Porém, a dimensão com que estratégias de *coping* determinam vulnerabilidade ou resiliência no desenvolvimento de desordens psiquiátricas relacionadas ao estresse continua não muito clara. Enquanto processos pró-inflamatórios são cada vez mais reconhecidos como mediadores chave na etiologia de psicopatologias, um perfil anti-inflamatório poderia ser responsável pela resiliência, característica de alguns indivíduos na população.

Objetivos – No presente estudo, um protocolo curto de estresse crônico por derrota social em ratos foi testado, que supostamente induziria sintomas tipo-depressivos. Adicionalmente, nós examinamos estratégias de *coping* adotadas por ratos derrotados que foram associadas a fatores de crescimento e proteínas inflamatórias.

Métodos – Ratos Wistar adultos foram continuamente ameaçados por um rato macho dominante e sofreram derrotas diárias por outro agressor, em lutas curtas, por 21 dias consecutivos. Os comportamentos durante os confrontos, peso corporal, preferência por sacarina, atividade locomotora espontânea e reatividade à novidade no campo aberto foram medidos. Amostras de sangue foram coletadas para as análises moleculares.

Resultados – 21 dias de estresse crônico por derrota social revelou dois estilos de *coping* em ratos Wistar. Ao longo dos confrontos diários, dois grupos de ratos estressados foram identificados, de acordo com a latência para assumir uma postura submissa em confrontos com agressores e posteriormente foram divididos nas subpopulações latência curta (SL) e latência longa (LL). Os dois fenótipos foram associados, respectivamente, a vários comportamentos relacionados à vulnerabilidade e resiliência ao estresse, que também se correlacionaram com níveis alterados de fatores de crescimento e marcadores inflamatórios periféricos. Embora ambos os grupos tenham reduzido ganho de peso ($p < 0,01$) e tenham sofrido a mesma frequência de ataques, os ratos LL apresentaram latências aumentadas para mordidos ($p<0,01$), número aumentado de posturas verticais ($p<0,05$), menores níveis de IGF-1 ($p<0,01$) e níveis mais elevados de IL-10 ($p<0,01$), enquanto os ratos SL apresentaram aumento no tempo de imobilidade ($p<0,05$) durante a derrota, diminuição da atividade locomotora no campo aberto ($p<0,01$) e não apresentaram moléculas periféricas alteradas em comparação com os animais não estressados.

Conclusões – As alterações comportamentais induzidas pelo estresse por derrota social apresentam-se distribuídas de forma bimodal em ratos Wistar e estão correlacionadas com diferentes fenótipos de inflamação periférica. Este estudo ressaltou que o *coping* proativo em relação ao estresse está intimamente associado com uma resposta anti-inflamatória e pode conferir resiliência a desordens relacionadas ao estresse.

Palavras-chave: Resiliência, Desordem Psiquiátrica, Inflamação, Modelo Animal, Roedores, Comportamento Tipo-depressivo.

1. Introduction

Stressful life events are powerful inducers of physiological and behavioral deregulations that contribute to the development of several psychopathologies. In humans and other social species, the most common form of stress originates in the social environment (Kessler, 1997; Kessler et al., 1985). Psychosocial stress is one of the major risks factors for psychiatric disorders such as anxiety and major depression (Allen et al., 2014). However, not all individuals exposed to stress develop stress-induced illnesses, supposedly because of the individual differences in stress vulnerability or resilience (Krishnan et al., 2007). Resilience can be defined as "achieving a positive outcome in the face of adversity" (McEwen et al., 2015), leading different coping styles to represent important features in minimizing the impact of stress and determine the degree of vulnerability or resilience to stress-induced psychopathologies (Franklin et al., 2012; Wood et al., 2010).

Among the most common pathologies developed under chronic stress is major depressive disorder (MDD). MDD is a costly and life-threatening psychiatric disorder and one of the leading causes of worldwide disability. With a partly unknown etiology, MDD will affect up to 20% of the population at some point in the individual's lifetime (Menard et al., 2017). Despite the currently available antidepressants, up to 30% of patients are treatment-resistant, indicating hidden characteristics of MDD and the need to further investigate. As no single hypothesis covers the explanation of all aspects of signs and symptoms of depression, that likely involves multiple mechanisms, investigators are challenged to understand its pathophysiology (Jesulola et al., 2018). In the past few years, the inflammatory hypothesis has gained power (Soskin et al., 2012). Inflammation, especially in the central nervous system (CNS), has been indicated to have a close relationship to psychiatric disorders, including depression (Dowlati et al., 2010; Passos et al., 2015; Stein et al., 2017). Since the finding of evidences of immune activation in patients suffering from MDD (Maes et al., 1995) inflammation has been associated with the cognitive and emotional symptoms of depression, such as anhedonia, depressed mood, reduced feelings of self-worth and concentration, and suicidal ideation (Jokela et al., 2016).

Inflammation and depression are intertwined in a bidirectional loop, in which depression facilitates inflammatory responses and inflammation promotes depression (Kiecolt-Glaser et al., 2015) with inflammation having a role in at least some sub-population of patients (Haroon et al.,

2012). Higher levels of inflammation are particularly likely to underlie depression symptoms that, in an evolutionary perspective, have the beneficial effect of preserving energy resources for use in fighting infection and promoting healing processes (Dantzer et al., 2008; Haroon et al., 2012). However, this response can also be activated by modern-day social threats, leading to an increasingly proinflammatory phenotype that may be a key phenomenon driving depression pathogenesis and recurrence (Slavich and Irwin, 2014). Studies report higher levels of circulating pro-inflammatory cytokines in patients with MDD, a pattern that has been replicated in preclinical animal models of depression (Hodes et al., 2015; Miller and Raison, 2016).

In preclinical studies, alternative animal models of depression have begun to focus on exposure to social stressors. There are several available models of social stress, especially for rodents (Blanchard et al., 2001; Koolhaas et al., 1997), among which social defeat (SD) is particularly important. SD uses social conflict between members of the same species to generate emotional and psychological stress (Krishnan and Nestler, 2011). After some episodes rodents exhibit significantly decreased locomotor and exploratory activity (Koolhaas et al., 1997), reduced aggression and sexual behavior (Meerlo et al., 1996), and increased submissive behavior and anxiety (Ruis et al., 1999). Additionally, a number of depressive-like symptoms are induced by multiple exposures. In a recently released paper, using non-invasive positron emission tomography (PET) in male Wistar rats, Kopschina Feltes and colleagues (2017) showed that repeated SD induces transient glial activation and reduces brain glucose metabolism, findings that were associated with stress-induced behavioral changes and reinforces the hypothesis that social stress-induced neuroinflammation could be a contributing factor in the development of depression.

The present exploratory and correlational study was designed to test the hypothesis of induction of depressive-like states and correlate it to peripheral inflammation, applying an short length protocol of SD for male rats that for now was only used in female studies, with some similarities (Shimamoto et al., 2011). We aimed to investigate stress-induced depressive-like behaviors that further turned to the observation of different coping strategies in determine vulnerability or resilience to stress-related psychiatric disorders. Additionally, associated peripheral growth factors and inflammatory cytokines were analyzed because of their increasingly recognized importance as key mediators in the etiology of psychopathologies.

2. Material and Methods

2.1. Subjects

Adult male Wistar rats (n=38) were obtained at approximately postnatal day (PND) 55 weighing 220 – 250 g upon arrival at the Animal Experimental Unit of Hospital de Clínicas de Porto Alegre (Porto Alegre, RS, Brazil). Rats were group-housed (3 per cage) and adapted to the facility for at least 14 days. At PND 70, rats were singly housed in custom-built acrylic cages (15x25x20 cm) until the end of the experiment. Separate male Wistar rats (n=20), weighing 465±10 g, with a reliable history of aggressive behavior in confrontations with intruders, termed stimulus 'resident' rats, were housed in pairs with sterile female Wistar rats (n=20) in large custom-built acrylic cages (46×71×46 cm). Resident rats and intruders were kept in the same room during the stress protocol while control rats were maintained in a separate room, both with controlled environmental conditions: 21±1° C temperature, 40 - 60% humidity and 12/12 h light-dark cycle (lights on at 7:00 AM). All cages were lined with sterilized sawdust bedding and rats had free access to rodent chow and water. This study was carried out in accordance with the Brazilian Federal Law Nº 11.794/2008 (establishing procedures for the scientific use of animals) and the National Institutes of Health guide for the care and use of laboratory animals. The experimental protocol was analyzed and approved by the Ethics Committee on Animal Use from Hospital de Clínicas de Porto Alegre (project #140274) and all procedures were supervised by a veterinarian. Three out of 38 experimental subjects were excluded from the analysis due to poor health condition.

2.2. Experimental design

Research was conducted in four experimental blocks because of the need of several aggressors each day. All experimental rats were assigned into either a control or chronic social defeat stress group. During the stress protocol, rats were tested for saccharin preference (SP) and tested in the open field (OF) for spontaneous locomotor activity and reactivity to novelty. All procedures were carried out during the first three hours of the dark phase, between 7:00 and 10:00 PM. Rats were monitored daily for health conditions and weighed once a week. Twelve hours after the last defeat session, blood samples were collected via the caudal vein from stressed and non-

stressed controls and immediately after, animals from both groups were deeply anesthetized using isoflurane and euthanized (Fig. 1).

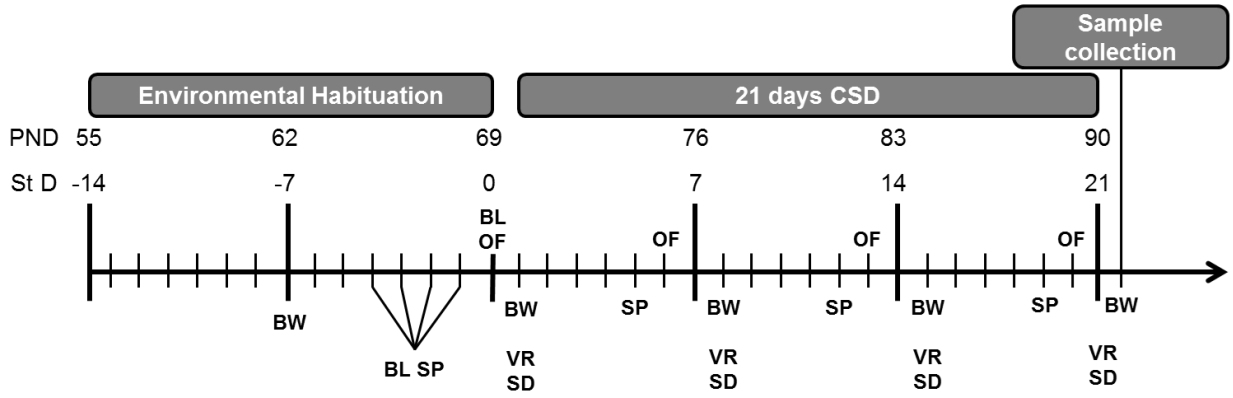


Fig. 1 – Experimental design: Experimental rats were habituated to the environment for 15 days, during which BL body weight, SP and behaviors in the OF were assessed. SD was applied to the stress group from St D 1 to 21, and sessions were video recorded on St D 1, 8, 15 and 21. Non-stressed controls were handled during the same period. At the same time, BW, SP, behaviors in the OF and during four episodes of SD were measured. Blood samples were collected for serum extraction on St D 22, 12 hours after the last defeat session. Right after, stressed and non-stressed controls were euthanized. BL = baseline; BW = body weight; CSD = chronic social defeat; OF = open field (activity and reactivity to novelty); PND = postnatal day; SD = social defeat; SP = saccharin preference test; St D = stress day; VR SD = video recorded social defeat.

2.3. Social Defeat Stress Procedure

The stress protocol was conducted following procedures established by Miczek and colleagues (Miczek, 1979; Shimamoto et al., 2011), with minor modifications. Briefly, stressed rats were kept in a 24h/day dyadic protected social housing with a dominant aggressor (resident male rat housed with a sterile female) and were subjected to daily agonistic confrontations with another aggressor in brief episodes of SD over the course of 21 days, or served as contemporary non-stressed home cage controls. Once a week, stressed rats were housed with a different resident couple. Confrontations took place on the first three hours of the dark phase, facing a different aggressor each day, while resident females were removed from the home cage. SD consisted of two phases: in the first (pre-defeat) phase, the intruder was placed inside the aggressor's home cage

protected in a perforated acrylic box (15x25x20 cm) for 10 min while threatened and investigated by the aggressor through the perforated walls; in the second (defeat) phase, the intruder was removed from the protective box and directly confronted the aggressive resident. The fight was terminated when the intruder displayed a supine posture for at least 5 consecutive seconds, or 5 min after being bitten the first time by the aggressor or after a total of 5 bites, whichever occurred first. Immediately after the end of the defeat phase, the intruder was replaced in his cage within the large resident's home cage. On stress days 1, 8, 15 and 21, confrontations were video recorded to measure the behavioral responses of the experimental rat to the aggressors, including frequencies of being threatened, defensive upright postures, escape from aggressors (flight), being bitten or nipped, and duration of immobility, supine posture, walking and sniffing (Shimamoto et al., 2011). Behaviors were blindly analyzed by a trained experimenter using "Behavioral Observation Research Interactive Software" (BORIS, v.5.1.3) (Friard and Gamba, 2016). Non-stressed controls were handled and weighed without being submitted to the stress protocol.

2.4. Preference for Saccharin

Before starting the stress protocol, on PND 63, rats were offered overnight a two-bottle choice (0.8% saccharin and sodium cyclamate solution - Zero·Cal, Hypermarcas; São Paulo, SP, Brazil) vs. unsweetened water). From PNDs 65 to 68, rats were screened for SP (baseline (BL)). Saccharin was used instead of sucrose to avoid excess calorie intake during tests across the experiment (Shimamoto et al., 2011). After collecting BL measurements of the SP, the experimental rats were randomly assigned to stressed or non-stressed control groups. Then, during the stress protocol, at PNDs 74, 81 and 88, stressed and non-stressed control rats were tested for SP. SP test consisted of a two-bottle choice method (0.8% saccharin solution vs. unsweetened water), offered during 1 h in a test cage (24×38×15 cm) in an appropriate room for behavioral testing, always in the dark period, between 8:00 and 9:00 PM. To counteract side preference, the position of the bottles was switched between trials. Water access was restricted for 1 h prior to the testing to ensure prompt fluid intake during the 1-h testing period. Fluid consumption was measured by weighing the bottles. Intakes for both saccharin solution and unsweetened water were measured, and the volume of saccharin divided by total fluid intake was calculated as SP (Rygula

et al., 2005). An empty “drip” cage served as control for evaporation and spillage due to handling of bottles.

2.5. Spontaneous Locomotor Activity and Reactivity to Novelty

On PND 69, one day before starting exposure to stress, intruders and non-stressed control rats were screened for BL spontaneous locomotor activity and reactivity to novelty (new object in the environment) in an activity box (OF). The activity box consisted of an acrylic circular arena (80 cm diameter, walls 37 cm high) divided into two circles/zones (inner/center and outer/periphery). The test was started by placing the animals in the center of the arena and lasted 5 min. Right after, a novel object was inserted in an area of the arena over the line between the periphery and the center zone, termed object zone (OZ), and the reactivity of the animal to the novelty was measured for 5 min. The locomotor activity was video recorded by a camera mounted on the ceiling and further analyzed using behavioral tracking software (ANY-maze 5.0 Video Tracking System; © 1999-2017 Stoelting Co., Wood Dale, IL, USA). Tests were conducted in an appropriate room for behavioral testing, always in the dark, between 7:00 and 10:00 PM. Objects were the following: green glass bottle 500 ml filled with sand (first/BL session); transparent plastic bottle 500 ml filled with water (second session); red soda can 350 ml filled with sand (third session); and orange plastic bottle 500 ml filled with water (fourth session). Behavioral measurements were total distance moved, percentage of time spent in each zone (center, periphery and OZ) and latency to enter the center and the OZ. Fecal boli were removed and the floor and the wall of the arena and the objects were cleaned after each testing with 70% alcohol to remove odors left by previous subjects. Intruders were tested always before the daily defeat session.

2.6. Blood Sampling and Serum Preparation

The day after the last defeat session, between 9:00 and 11:00 AM, experimental rats (stressed and non-stressed controls) were transferred to a separate room in veterinary incubators (model: C 186 TS, FANEM, São Paulo, Brazil) with oxygen and heat supply set at 30º C for 30 min to facilitate blood sampling. Right after, rats were manually immobilized, hold on a custom-built heating pad and 1.5 ml blood was drawn from the lateral tail vein, using 21g x ¾" IV butterfly

catheters (Venofix® Safety, B.Braun, Brazil), into 2.0 ml microcentrifuge safe lock tubes (without anticoagulant). Tubes were left set at room temperature for up to 30 min to enable blood clotting and centrifuged for 10 min at 2.000 x g. Serum was separated using a 200 µl micropipette and stored in aliquots at - 80º C.

2.7. Serum biomarker analysis by ELISA

Serum (1:2000) IGF-1 (Insulin-like Growth Factor 1) concentrations from stressed and non-stressed controls were measured by sandwich immunoassay using mouse/rat IGF-1 Quantikine® ELISA kit (Cat# MG100, R&D Systems, Minneapolis, MN, USA) as per manufacturer's instructions. Serum TNF-α (Tumor Necrosis Factor alpha) concentrations from stressed and non-stressed controls was measured by enzyme-linked immunosorbent assay using rat TNF-α ELISA kit (Cat# RAB0479, Sigma Aldrich, Saint Louis, MO, USA) as per manufacturer's instructions. Serum (1:2) IL-10 (Interleukin 10) concentrations from stressed and non-stressed controls was measured by enzyme-linked immunosorbent assay using rat IL-10 ELISA kit (Cat# RAB0246, Sigma Aldrich, Saint Louis, MO, USA) as per manufacturer's instructions. BDNF (Brain-Derived Neurotrophic Factor) serum (1:3) levels were determined by sandwich-ELISA using monoclonal antibodies specific for BDNF (R&D Systems, Minneapolis, MN, USA), in a protocol standardized in our lab that was previously published (Borba et al., 2016).

2.8. Statistical Analysis

Two-way repeated measures analysis of variance (ANOVA) (GraphPad Prism version 6.0 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com) was used to compare the main effect of stress and time and the interaction effect between stress and time on weight gain (WG) over time. To assess the effects of SD stress on SP, a two-way repeated measures ANOVA was performed using stress (SL and LL) vs. non-stress as the condition factor, and BL and three additional observation times as the session's factor. To assess the effect of SD stress on activity and reactivity to novelty in the OF (distance travelled, frequency of entries and time spent in each zone: central, periphery, and OZ), a two-way repeated measures ANOVA was performed using stress (SL and LL) vs. non-stress as the condition factor, and BL and three additional

observation times as the session's factor. To assess the effect of SD stress on behaviors during defeats (latency to supine, frequency of immobility, frequency of bites, latency to being bitten, frequency of lateral threats, duration of flight, frequency of upright posture and walk duration), a two-way repeated measures ANOVA was performed using SL vs. LL as the condition factor, and four observation times as the session's factor. To compare the effect of SD stress on serum levels of BDNF, IGF-1, TNF-α and IL-10, a one-way ANOVA was conducted using stress (SL and LL) vs. non-stress as conditions. *Pearson's* correlation was used to analyse the association between molecular and behavioral variables. When indicated by a significant effect, *Post hoc* comparisons were performed using the *Sidak's* test method. Missing data were imputed using mean substitution to avoid listwise deletion of cases in the two-way repeated measures ANOVA analysis. The statistical significance was set at $p \leq 0.05$ for all comparisons. Data are presented as mean ± SEM.

3. Results

3.1. Behavioral reactivity during SD

During SD, animals showed a naturally bimodal distribution with respect to average supine latency. Based on the analysis of their behavior over the course of four video recorded defeats (SD sessions 1, 8, 15 and 21), an average defeat latency of 60 sec was set as cutoff to define rats' behavioral response to SD, and stressed rats were divided into two subgroups termed short latency (SL) and long latency (LL) to present supine position (Wood et al., 2010). SL (n=8) exhibited mean short latencies to supine (<60 sec) whereas LL (n=9) exhibited mean long latencies to supine (>60 sec). Characteristically, the SL and LL populations had mean (± SEM) latencies to assume a submissive posture of 37.45±4.90 sec and 87.28±9.80 sec across sessions, respectively, $t_{(15)}$=4.40; p<0.01 (Fig. 2A). To determine whether differences developed over repeated exposures, we examined latency to be supined from the video recorded defeats. A two-way repeated measures ANOVA was performed that examined the effect of coping strategy (SL vs. LL) and time on latency to present submissive posture. There was no statistically significant interaction between these factors on latency to supine, $F_{(3,45)}$=0.72, p>0.05. Main effect analysis showed that SL and LL animals did not significantly differ across time, $F_{(3,45)}$=2.00; p>0.05, but were significantly different in regard to their coping strategy, $F_{(1,15)}$=16.00; p<0.01. *Post hoc* analysis revealed that this effect was due to an increasing duration of onset for LL rats to assume a submissive posture as the number of exposures to aggressive residents increased, and was significantly different from SL rats on stress day 21, $t_{(60)}$=2.80; p<0.05 (Fig. 2B). To identify other behavioral parameters potentially associated with regard to this bimodal distribution, recorded videos were blindly analyzed and revealed that, although all rats experienced similar frequency of attacks, SL rats exhibited shorter latencies to being bitten by the aggressors, lowered frequency of upright posture and increased frequency of immobility across defeat sessions. A two-way repeated measures ANOVA was performed to examine the effect of coping strategies and time on latency to being bitten by the aggressors during defeats. There was a statistically significant interaction between these factors, $F_{(3,45)}$=3.60; p<0.05. Main effect analysis showed that SL and LL animals did not significantly differ across time, $F_{(3,45)}$=1.80; p>0.05, but were significantly different regarding their coping strategy, $F_{(1,15)}$=4.80; p<0.05. *Post hoc* analysis revealed that this effect was due to an increasing latency for LL rats to being bitten over the course of defeat sessions, $t_{(45)}$=3.50; p<0.01 (1^{st} vs. 21^{st} session); $t_{(45)}$=3.60; p<0.01 (15^{th} vs. 21^{st} session) and that there was a significant

difference between SL and LL rats on stress day 21, $t_{(60)}$=3.90; p<0.01 (Fig. 2C). Afterwards, a two-way repeated measures ANOVA was performed to examine the effect of coping strategies and time on frequency to present upright posture (facing the aggressor) during defeat. There was no statistically significant interaction between these factors, $F_{(3,45)}$=0.85; p>0.05. However, main effect analysis showed that SL and LL animals did significantly differ across time, $F_{(3,45)}$=3.30; p<0.05, and were also significantly different regarding their coping strategy, $F_{(1,15)}$=5.30; p<0.05. *Post hoc* analysis failed to reveal how SL and LL rats differed in regard to upright frequency but showed that LL rats increased their frequency of upright posture over the course of defeat sessions, $t_{(45)}$=2.80; p<0.05 (1st vs. 8th session); $t_{(45)}$=2.90; p<0.05 (1st vs. 21st session) (Fig. 2D). A two-way repeated measures ANOVA was also performed that examined the effect of coping strategy and time on frequency to stay immobile during defeat. There was no statistically significant interaction between these factors, $F_{(3,45)}$=0.52; p>0.05. However, main effect analysis showed that SL and LL animals significantly differed across time, $F_{(3,45)}$=5.20; p<0.01, but were no significantly different regarding their coping strategy, $F_{(1,15)}$=0.07; p>0.05. *Post hoc* analysis revealed that only SL rats increased their immobility frequency comparing the 1st against the 21st defeat session, $t_{(45)}$=3.10; p<0.05 (Fig. 2E). Two-way ANOVA did not reveal statistically significant differences between groups in regard to flight duration, walk duration, frequency of bites and frequency of lateral threats (data not shown).

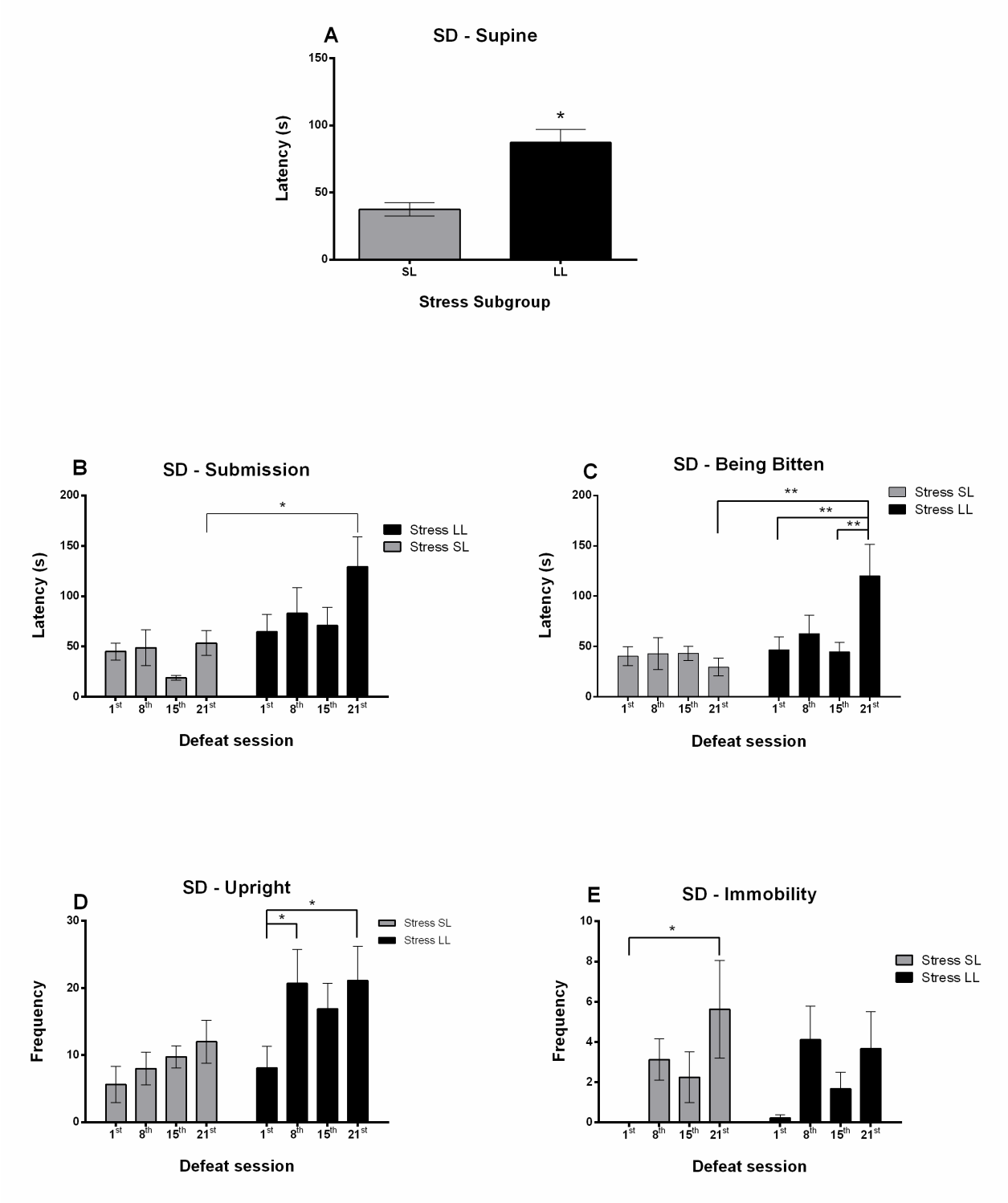


Fig. 2 – Behavioral reactivity of stressed rats during SD. Considering the average latency to be supined, a bimodal distribution of socially defeated animals was observed that was accompanied by other associated behavioral parameters. A: average latency (s) to supine over the course of four

video recorded defeats (1st, 8th, 15th and 21st sessions). B: SL (n=8) and LL (n=9) coping strategies on the onset to assume a submissive posture. C: latency (s) across sessions to being bitten by the aggressor. D: frequency of upright postures during video recorded defeats. E: frequency to present immobility during SD. Data are expressed as mean ± SEM; * $p<0.05$; ** $p<0.01$. SD = social defeat; SL = short latency; LL = long latency; s = seconds.

3.2. Effects of SD stress on body weight

Repeated exposures to SD stress altered body WG in both SL and LL rats. A two-way repeated measures ANOVA was performed that examined the effect of stress and time on WG over time, showing a statistically significant interaction between the effects of stress and time on WG, $F_{(8,128)}=7.10$; $p<0.01$. Simple main effect analysis showed that animals significantly increased WG over time $F_{(4,128)}=149.00$; $p<0.01$, and that WG over time differed between controls vs. both SL and LL rats $F_{(2,32)}=12.00$; $p<0.01$. Pairwise multiple comparisons revealed a significantly lower WG in both SL and LL rats, that started earlier for SL rats, $t_{(160)}=4.70$; $p<0.01$ (day 8), $t_{(160)}=6.20$; $p<0.01$ (day 15) and $t_{(160)}=4.20$; $p<0.01$ (day 22). LL rats, $t_{(160)}=4.50$; $p<0.01$ (day 15) and $t_{(160)}=4.10$; $p<0.01$ (day 22), always compared to the non-stressed control group (Fig. 3).

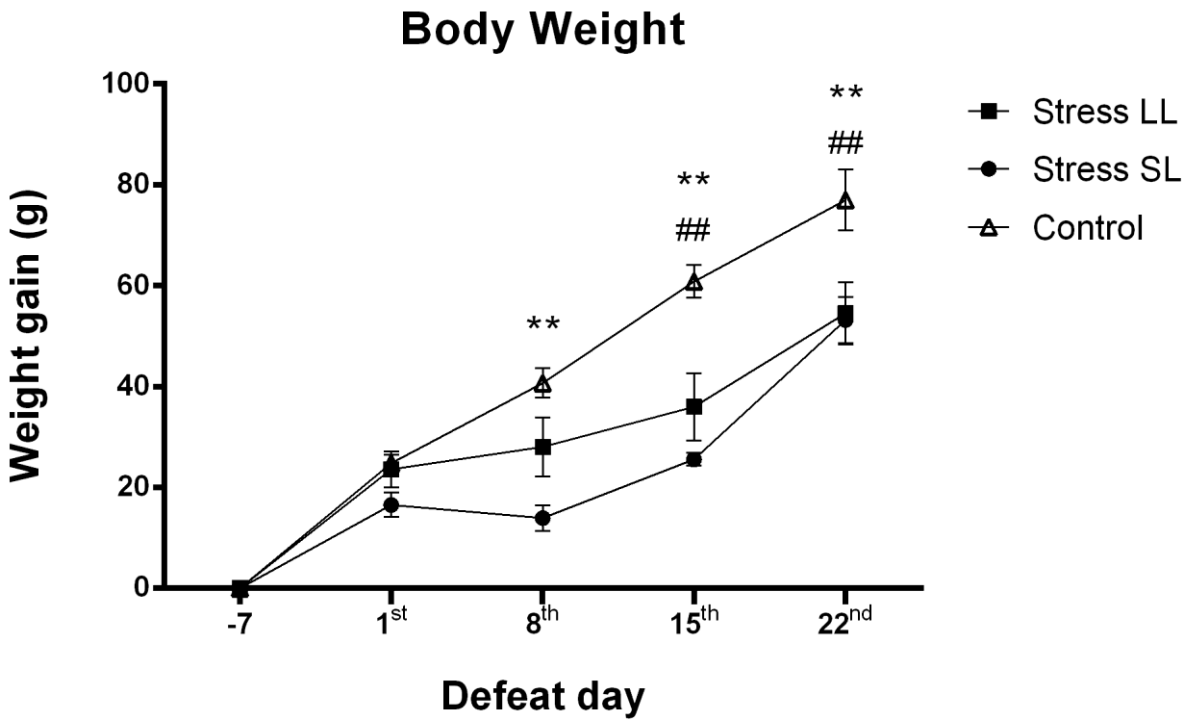


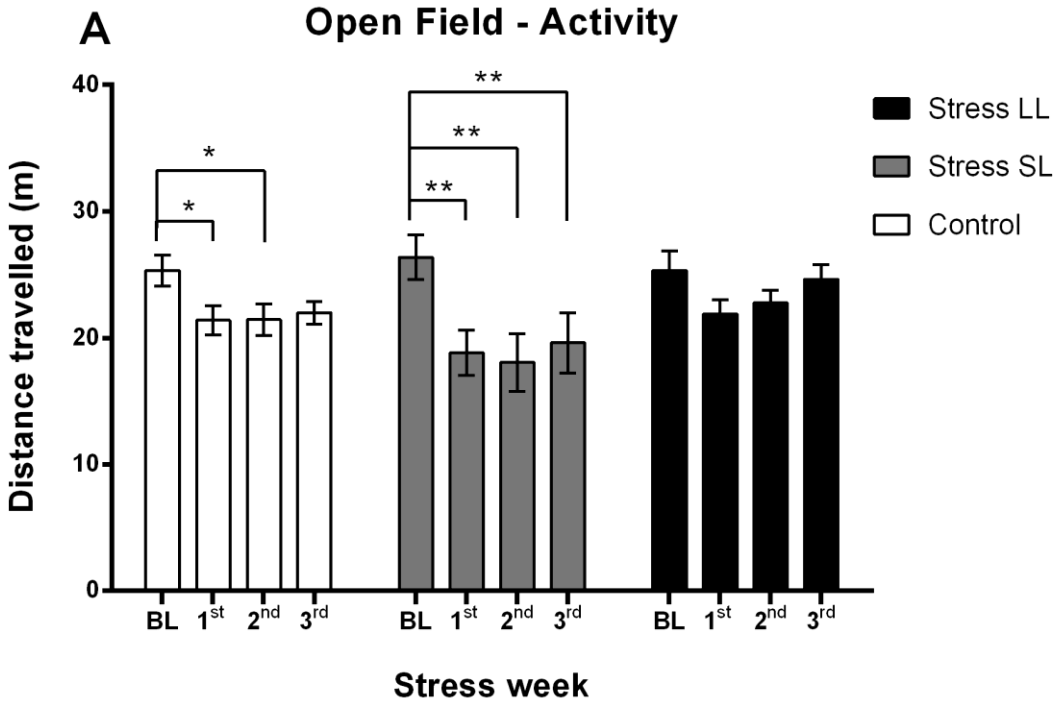
Fig. 3 – Effects of chronic SD stress on body weight gain over time for stressed SL (n=8), LL (n=9) and non-stressed home-cage controls (n=18), from day -7 (before the onset of SD) to day 22 (one day after the end of SD). Data are expressed as mean ± SEM; ** $p<0.01$ (controls vs. SL); ## $p<0.01$ (controls vs. LL). SL = short latency; LL = long latency; g = grams.

3.3. Effects of SD stress on spontaneous locomotor activity and reactivity to novelty in the OF

During tests of activity in the OF, animals from both control and SL groups showed decreased locomotor behavior, whereas rats from the LL group did not decrease locomotor activity across sessions. A two-way repeated measures ANOVA was performed that examined the effect of stress and time on spontaneous locomotor activity that revealed no statistically significant interaction between these factors on total distance travelled, $F_{(6,96)}=0.26$; $p>0.05$. However, main effect analysis showed that controls and SL animals significantly differed across time, $F_{(3,96)}=16.00$; $p<0.01$. *Post hoc* comparisons revealed that controls and SL rats reduced the distance travelled from the second session on, compared to BL. Controls, $t_{(96)}=3.00$; $p<0.05$ (BL vs. 1st w; BL vs. 2nd w). SL, $t_{(96)}=3.90$; $p<0.01$ (BL vs. 1st w), $t_{(96)}=4.30$; $p<0.01$ (BL vs. 2nd w) and $t_{(96)}=3.50$;

$p<0.01$ (BL vs. 3rd w) (Fig. 4A). Two-way ANOVA did not reveal statistically significant differences between groups in regard to the latency to enter the center and percentage of time in each zone (data not shown).

During tests of reactivity to novelty, animals from both control and SL groups showed increased time spent in the OZ, whereas rats from the LL group did not change time in this zone across sessions. A two-way repeated measures ANOVA was performed that examined the effect of stress and time on time spent in the OZ. There was no statistically significant interaction between these factors on time in the OZ, $F_{(6,96)}=0.92$; $p>0.05$. However, main effect analysis showed that controls and SL animals significantly differed across time, $F_{(3,96)}=8.10$; $p<0.01$. *Post hoc* comparisons revealed that controls and SL rats spent more time in the OZ in the last session compared to previous sessions. Controls, $t_{(96)}=4.10$; $p<0.01$ (3rd w vs. BL); $t_{(96)}=3.80$; $p<0.01$ (3rd w vs. 1st and 2nd w). SL, $t_{(96)}=2.80$; $p<0.05$ (3rd w vs. BL) (Fig. 4B). There were no statistically significant differences between groups in regard to the latency and number of entries in the OZ (data not shown).


A
Open Field - Activity
Distance travelled (m)
40
30
20
10
0
*
*
**
**
**
BL
1st
2nd
3rd
Stress week
Stress LL
Stress SL
Control

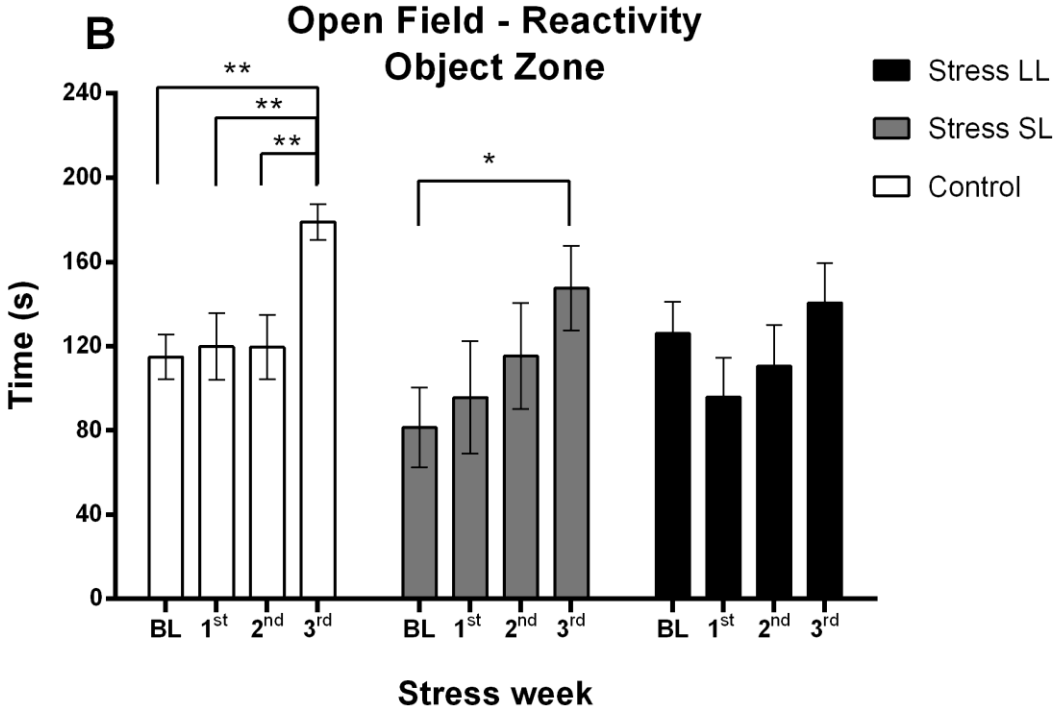
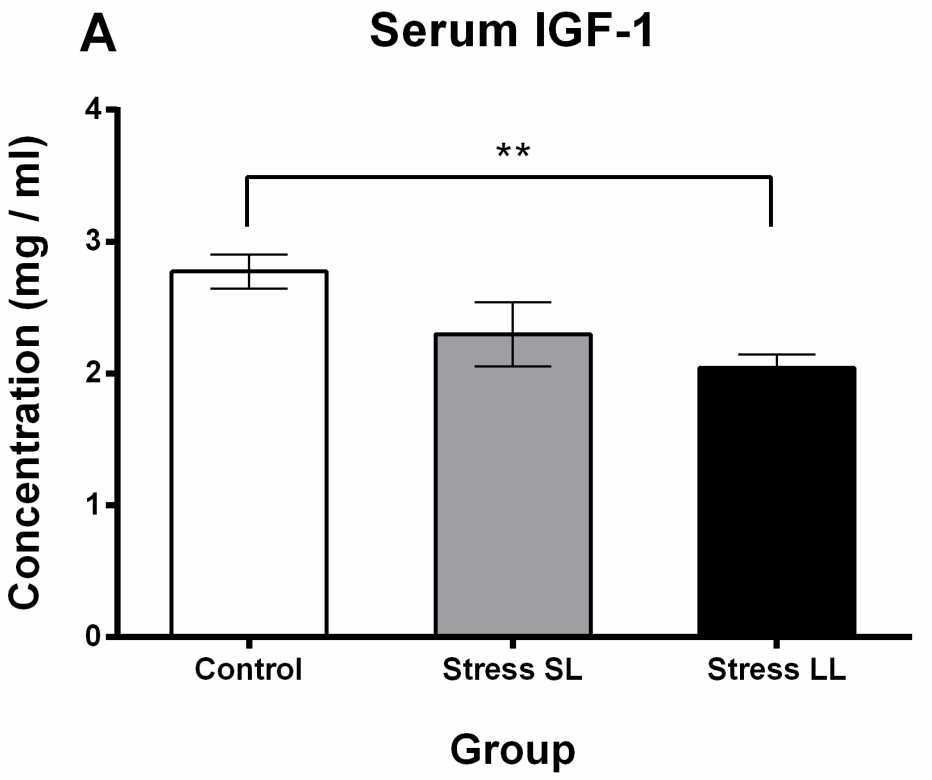

B
Open Field - Reactivity
Object Zone
Time (s)
240
200
160
120
80
40
0
**
**
**
*
BL
1st
2nd
3rd
Stress week
Stress LL
Stress SL
Control

Fig. 4 – Effects of chronic SD stress on spontaneous locomotor activity (4A) and reactivity to novelty (4B) in the OF for stressed SL (n=8), LL (n=9) and non-stressed home-cage controls (n=18), over the course of four sessions (BL, 1st, 2nd and 3rd week). Data are expressed as mean ± SEM; * $p<0.05$; ** $p<0.01$. BL = baseline; SL = short latency; LL = long latency; OF = open field; m = meters; s = seconds.

3.4. Effects of SD stress on peripheral growth factors and inflammatory markers

Serum samples were used to evaluate BDNF, IGF-1, TNF-α and IL-10 concentrations by sandwich immunoassay ELISA. Among these molecules, IL-10 from some animals was undetectable, indicated by the reduced sample size in each group. Using one-way ANOVA to determine de effect of stress and coping style on circulating IGF-1 levels, statistically significant differences were found between groups $F_{(2,32)}=6.19$; $p<0.01$. *Post hoc* analysis revealed that LL rats had significant lower levels of circulating IGF-1 compared to controls, $t_{(32)}=3.35$; $p<0.01$, while SL rats did not differ from both LL and controls (Fig. 5A). One-way ANOVA was also performed to determine the effect of stress and coping style on circulating concentrations of IL-10. Statistically significant differences were found between groups $F_{(2,16)}=7.09$; $p<0.01$, and a *Post hoc* analysis revealed that again, only LL rats differed from controls in regard to serum IL-10 levels, this time with higher concentrations, $t_{(16)}=4.00$; $p<0.01$, while SL rats did not differ from both LL and controls (Fig. 5B). One-way ANOVA showed no statistically significant differences between groups for serum BDNF levels, $F_{(2,32)}=2.00$; $p>0.05$ and TNF-α, $F_{(2,31)}=0.13$; $p>0.05$.


A
Serum IGF-1
**
Concentration (mg / ml)
4
3
2
1
0
Control
Stress SL
Stress LL
Group

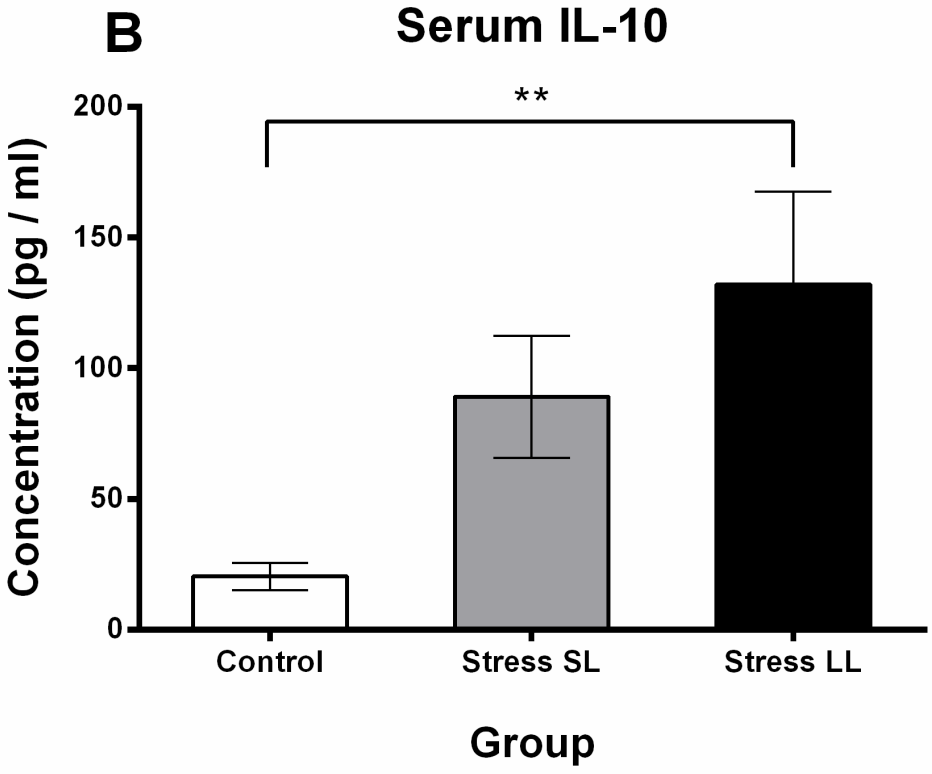

B
Serum IL-10
**
Concentration (pg / ml)
200
150
100
50
0
Control
Stress SL
Stress LL
Group

Fig. 5 – Effects of chronic SD stress on circulating molecules IGF-1 and IL-10. A: serum IFG-1 concentrations (mg / ml) for stressed SL (n=8), LL (n=9) and non-stressed home-cage controls (n=18). B: serum IL-10 concentrations (pg/ml) for stressed SL (n=5), LL (n=6) and non-stressed home-cage controls (n=8). Data are expressed as mean ± SEM; ** $p<0.01$. SL = short latency; LL = long latency; IGF-1 = insulin-like growth factor-1; IL-10 = interleukin-10; mg = milligram; pg = picogram.

3.5. Effects of chronic SD on preference for saccharin

Variations in sweet solution intake and/or preference, usually saccharin or sucrose, are used in preclinical studies to identify stress effects on anhedonic behavior. A two-way repeated measures ANOVA was performed that examined the effect of stress and time on SP. There was no statistically significant interaction between these factors on SP, $F_{(6,96)}=0.91$; $p>0.05$ nor did groups significantly differ across time, $F_{(3,96)}=1.07$; $p>0.05$ and coping style, $F_{(2,32)}=0.16$; $p>0.05$ (Table 1).

Associated behavioral and molecular profiles for each experimental group (control, SL and LL) are summarized in Table 1.

Table 1 – Behavioral and molecular profile of each experimental group.

Variable		**Experimental Group**		
		Control	**Stress SL**	**Stress LL**
Saccharin Preference (%, means ± SEM)	Baseline	0.78 ± 0.05	0.72 ± 0.11	0.83 ± 0.04
	1st week	0.89 ± 0.03	0.87 ± 0.04	0.77 ± 0.07
	2nd week	0.86 ± 0.04	0.79 ± 0.10	0.80 ± 0.08
	3rd week	0.86 ± 0.06	0.92 ± 0.03	0.85 ± 0.08
Weight gain during stress (vs. controls)		↔	↓↓**	↓↓**
Social Defeat	Latency to supine (mean)	-	< 60 sec	> 60 sec
	Latency to supine (across SD sessions)	-	↔	↑*
	Latency to being bitten (across SD sessions)	-	↔	↑↑**
	Frequency of upright postures (across SD sessions)	-	↔	↑*
	Frequency of immobility (across SD sessions)	-	↑*	↔
Open Field	Distance travelled (across sessions) vs. BL	↓*	↓↓**	↔
	Time in the OZ (interaction with novelty) vs. BL	↑↑**	↑*	↔
Molecular	Serum IGF-1 (vs. controls)	↔	↔	↓↓**
	Serum IL-10 (vs. controls)	↔	↔	↑↑**

For saccharin preference, BL values represent an average of four tests. ↔, ↑ and ↓ indicate no change or significantly greater than or less than, respectively. BL = baseline; SL = short latency; LL = long latency; SD = social defeat; OF = open field; OZ = object zone; SEM = standard error of the mean. Significant *p* value was set at 0.05. * $p<0.05$; ** $p<0.01$.

4. Discussion

In this study, a short length method of chronic SD for male rats was employed. We chose the chronic SD stress model because rodent males typically attack invaders of their territories and because prolonged threat by continuous housing maximizes the effects of defeat without additional physical damage to intruders, while exposed to psychological stress via sensory interaction (Covington and Miczek, 2001; Tidey and Miczek, 1996; Vasconcelos et al., 2015). Compared to other stressors, SD is recognized by its construct validity because of the analogy to what humans experience in modern society (Fuchs and Flügge, 2002). For the current experiment, intruders resided protected inside aggressor's home cage 24 hours a day during 21 consecutive days. Daily, intruders confronted a different male and once a week, intruder's cage was moved to another aggressor's home to avoid habituation. To the best of our knowledge, this is the first study to employ this protocol in male rats that was previously used in females (Shimamoto et al., 2011) with minor differences. The 21 day continuous defeat protocol was tested as an alternative to other conventional longer stress protocols, such as the 5 weeks continuous defeat without cohabitation (Rygula et al., 2005) and the 5 to 9 weeks chronic unpredictable mild stress (CUMS) (Katz, 1982; Willner, 2017).

The main finding of the present study was a bimodal distribution of socially defeated rats initially based on the latency to present submissive posture in confrontations with aggressors, and their typical behavioral and molecular phenotypes. An apparently homogeneous population of rats was divided into two subpopulations, termed SL and LL rats. In addition to the differences related to subordination, SL and LL rats presented several associated behavioral and molecular singularities that are respectively relevant to vulnerability and resilience. While SL rats behaved with passivity during defeats, LL rats exhibited behaviors that were associated with active coping, making the latter less vulnerable to the effects of stress (Wood and Bhatnagar, 2015). The combination of chronic defeat and continuous threat did not produce detectable depressive-like symptoms such as reduced SP, decreased activity in the OF and lowered interest to novelty. Comparing to the study of Shimamoto and colleagues (2011) in which a similar protocol induced such behavior in females, we believe that the outcome could be explained by gender differences. Females are more susceptible than males to the effect of stress-induced psychopathologies, a finding that has also been seen in humans, where women are twice as likely as men to develop

MDD (Albert, 2015). Differences could also be attributed to rat strain. Similarly however, we observed the emergence of two subpopulations of stressed rats that differed regarding WG, latency to supine, ability to delay bites, frequency of upright postures and duration of immobility during confrontations. Additionally, SL and LL rats did not resemble the behaviors in the OF nor peripheral circulating IL-10 and IGF-1 levels (summarized in Table 1).

Reduced WG was observed in both groups of stressed rats. Statistically significant differences from controls started earlier in the SL group, and may be a hallmark of vulnerable individuals (Beery and Kaufer, 2015). Body weight changes in socially defeated animals has been consistently observed in other studies, where SD stress usually produces potent effects in terms of lower WG (Krishnan et al., 2007; Liu et al., 2017; Shimamoto et al., 2011), with greater impact in vulnerable individuals (Wood and Bhatnagar, 2015). Lower WG cannot be fully explained by changes in food intake and might be a result of decreased caloric efficiency in animals repeatedly exposed to SD (Bhatnagar et al., 2006) that does not affect vulnerable and resilient animals to the same extend (Krishnan et al., 2007).

From the bimodal distribution of stressed rats into SL and LL phenotypes, several behaviors during SD linked animals to passive and proactive coping respectively. Animals who cope appropriately minimize the impact of stress and determine the degree of vulnerability or resilience to stress-induced psychopathologies (Franklin et al., 2012). Active coping is characterized by an individual's attempt to deal with a challenge, whereas passive coping is manifested by avoidance of conflicts and acceptance of defeat or submission (Wood and Bhatnagar, 2015). The current results show that SL's behaviors associated with passive coping were reduced latency to assume a submissive posture and increasing time in immobility during confrontations. LL's behaviors associated with proactive coping were the higher frequency of upright postures and the ability to delay bites while facing aggressors. Similarities to this bimodal distribution of socially stressed rats were reported by Wood and colleagues (2010). According to their study, rats that consistently exhibited more rapid subordination developed a neuroendocrine and behavioral phenotype that resembled melancholic depression, whereas rats resisting or delaying defeat exhibited proactive behaviors, such as upright postures, and were resistant to the development of neuroendocrine and behavioral depression-related signs.

Chronic SD stress also affects locomotor and exploratory activity. Reduced locomotor and exploratory activity represents a loss of interest in new stimulating situations, implying a deficit in motivation (Koolhaas et al., 1997; Meerlo et al., 1996). In this study, rats from control, SL and LL groups did not differ regarding distance travelled or exploration of novel objects in the OF. However, reduced locomotor activity and increasing interaction with new objects was observed across sessions for controls and SL rats, but not LL rats. Since the effects were similar for SL rats and controls, we believe that reduction of locomotor activity indicates habituation learning to the test, which is typical after repeated exposures to the same apparatus and increasing exploration of new objects represents decreasing levels of anxiety. Similarly, differences in locomotor activity between susceptible and resilient animals were reported after SD by Huang and colleagues (2013), and may indicate individual variations in response to the same challenge.

Stress can either enhance or suppress immune functions depending on a variety of factors, including the duration of the stressful event. While inflammatory processes are increasingly recognized as key mediators in the etiology of psychopathologies (Dowlati et al., 2010), an anti-inflammatory profile could thus be responsible for resilience (Wood et al., 2015), characteristic of some individuals in the population. Inflammation is associated with the cognitive and emotional symptoms of depression such as anhedonia, depressed mood, reduced feelings of self-worth and concentration, and suicidal ideation (Jokela et al., 2016). In the current study, defeated rats from the LL group differed from non-stressed controls, presenting reduced serum levels of IGF-1 and increased levels of IL-10, while SL rats did not exhibit such difference. Besides other functions, IGF-1 exerts immunomodulatory effects and may act as a proinflammatory factor with positive effects on the onset of inflammation through stimulation of inflammatory cytokines and chemokines, such as TNF-α (Renier et al., 1996) and IL-8 (Kooijman et al., 2002), respectively. IL-10, enhanced both at transcriptional and protein levels in stressed mice (Hu et al., 2014), has been recognized as a strong anti-inflammatory cytokine that has a critical role in limiting inflammatory responses and preventing tissue damage (Murray and Smale, 2012). The striking difference between the two stress reactivity phenotypes found in the present study was the effect of stress on IGF-1 levels and the protective cytokine IL-10. While SL rats do not differentiate from controls, LL rats presented lower serum levels of IGF-1 and higher levels of IL-10, indicating that both proteins could be potential biomarkers for stress resilience. It seems that anti-inflammation is associated with the resolution of a stress state that, at least to this time point, is more salient in

resilient animals. According to Selye (1936), adaptability and resistance to stress are fundamental prerequisites for life. Selye's "General Adaptation Syndrome" points that it develops in three stages (alarm, resistance and exhaustion) and most of the characteristic manifestations in the alarm stage disappear or are reversed during the stage of resistance, but reappear in the exhaustion stage. It is tempting to consider that animals from the resilient phenotype (LL) in the present study reach the stress upward curve (resistance stage) earlier than susceptible animals, a characteristic that may be related or is influenced by the anticipated anti-inflammatory immune state. These findings imply proactive coping strategies and anti-inflammatory phenotype as important characteristics in conferring resilience to stress-induced psychopathologies, such as depression. A detailed immunological and endocrine characterization for each phenotype at various stages of SD stress (before, during and after), one of the core limitations of this study, will help to better elucidate differences in reactivity to social stressors and the development of new therapeutic strategies.

5. Conclusions and Future Directions

Chronic SD stress in male Wistar rats proves to be an efficient protocol to identify subpopulations of stressed individuals, relevant to the study of individual differences related to resilience or susceptibility. The observed emergence of two different behavioral phenotypes that correlated with peripheral growth factors and inflammatory molecules might be an important start point for future studies that should focus on different behavioral measurements of aspects related to depression, such as forced swimming test, adding longitudinal molecular measurements during and after the end of stress. It would be also important to characterize the HPA-axis reactivity to this kind of stress and investigate central immune reactions, especially microglial cells activation.

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9. CONCLUSÃO E CONSIDERAÇÕES FINAIS

Modelos animais de estresse social são um recurso importante para a compreensão das reações comportamentais e fisiológicas da exposição ao estresse. Esses modelos têm auxiliado pesquisadores a descrever de forma cada vez mais detalhada os eventos relacionados às reações ao estresse, tais como os comportamentos e as alterações endócrinas e imunológicas a eles associados. Essa tese, composta de dois artigos científicos, procurou contribuir para a compreensão de algumas reações ao estresse e sua relação com desordens psiquiátricas, focando especialmente na depressão maior, que em animais de laboratório é interpretada através da expressão de comportamentos tipo-depressivos.

O primeiro artigo (Stein et al., 2017) revisou a literatura mais recente sobre os efeitos do estresse por derrota social em roedores e seu papel sobre ativação de células microgliais. As células microgliais são as principais responsáveis pelas reações imunológicas do SNC e têm sido consideradas peças-chave na indução de desordens neuropsiquiátricas. No artigo, foram destacadas evidências do impacto negativo da hiperativação do sistema neuroimunológico. Exposição ao estresse por derrota social induz as células da microglia a assumirem um estado de hiperativação, que inicialmente pode ser considerado benéfico. Porém, com exposição repetida ao estresse social, a microglia assume um estado de hiperativação e de forma persistente libera mediadores pró-inflamatórios, citotoxinas e espécies reativas de oxigênio, que podem causar dano e/ou perda celular ou ainda, afetar negativamente a atividade neuronal pela poda excessiva de conexões sinápticas. Todos esses efeitos do estresse sobre a microglia afetam a memória e os comportamentos, fatores que ao final contribuem para o desenvolvimento de desordens psiquiátricas como a ansiedade e a depressão. Além disso, ficou evidente que a comunicação bidirecional entre o cérebro e o sistema imunológico periférico tem um papel central no desenvolvimento da ansiedade e do TDM. A compreensão das reações imunológicas no desenvolvimento destas doenças tem sido fundamental para propor novas alternativas de tratamento, tendo a microglia como alvo terapêutico para intervenções farmacológicas. Associar antidepressivos convencionais a medicamentos que combatem a inflamação, e ainda assim manter ou restaurar as funções basais da microglia, mantendo-as funcionais pelo seu papel fundamental na

homeostase do SNC, parece ser uma estratégia promissora na terapia da ansiedade e da depressão maior.

O segundo artigo (Stein et al., (não publicado) - Anexo 3), seguindo a linha de investigação do primeiro artigo quanto à utilização do estressor social na investigação pré-clínica, utilizou um modelo de estresse crônico por derrota social em ratos Wistar como forma de induzir comportamentos tipo-depressivos. Apesar de não terem sido observadas alterações comportamentais significativas em relação a este comportamento, observou-se que o modelo produziu dois estilos de *coping* nos animais submetidos ao estresse social. Estes fenótipos, baseados inicialmente na latência para assumir uma posição de subordinação frente a um agressor, chamados de SL (*short latency*) e LL (*long latency*), foram associados respectivamente a diversos comportamentos relacionados à vulnerabilidade e à resiliência ao estresse. Além disso, os dois grupos de animais correlacionaram-se de forma distinta com níveis alterados de fatores de crescimento e marcadores inflamatórios periféricos. Uma população que inicialmente parecia homogênea foi dividida em duas subpopulações, cada uma com suas singularidades comportamentais e moleculares. Enquanto os animais SL comportaram-se de forma passiva durante os episódios de derrota, animais LL exibiram comportamentos que foram associados com *coping* ativo. *Coping* ativo é citado na literatura como uma forma de resiliência ao estresse. Animais resilientes apresentaram menores níveis séricos de IGF-1 e maiores níveis de IL-10, resultados que foram interpretados como um perfil imunológico de resolução de um estado de estresse. Parece que o perfil anti-inflamatório relacionado ao grupo resiliente tornou os animais menos suscetíveis aos efeitos do estresse social, talvez antecipando o estágio de resistência, descrito na "Síndrome Geral de Adaptação" de Selye (1936). Os achados implicaram estratégias de *coping* pró-ativo e um fenótipo anti-inflamatório como características importantes a conferir resiliência a psicopatologias induzidas por estresse.

Em suma, os resultados dos trabalhos aqui apresentados contribuem para a compreensão da relação estresse social, inflamação, resiliência e comportamento tipo-depressivo. Corroborando dados da literatura, o modelo pré-clínico de investigação de estresse por derrota social demonstrou ser um mecanismo útil para o estudo da depressão, enquanto um perfil inflamatório em pacientes com TDM pode indicar a necessidade de associar tratamentos convencionais à farmacologia anti-inflamatória, dependendo do seu grau de resiliência.

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Stein, D.J., Vasconcelos, M.F., Landau, L., Gallas-Lopes, M., Behrens, L., Géa, L.P., da Rosa, E.D., Albrechet-Souza, L., Ceresér, K.M.M., de Almeida, R.M.M., (unpublished). Proactive Coping during Chronic Social Defeat Stress is associated with an Anti-inflammatory response in male Wistar rats

10. ANEXOS

Anexo A: carta de aprovação do projeto de pesquisa pela Comissão de Ética no Uso de Animais (CEUA) do Hospital de Clínicas de Porto Alegre

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

A Comissão de Ética no Uso de Animais (CEUA/HCPA) analisou o projeto:

Projeto: 140274
Data da Versão do Projeto: 20/08/2014

Pesquisadores:
KEILA MARIA MENDES CERESER
DIRSON JOÃO STEIN
ROSA MARIA MARTINS DE ALMEIDA

Título: DERROTA SOCIAL POR SUBORDINAÇÃO CONTÍNUA EM RATOS WISTAR MACHOS: ESTRATÉGIAS DE COPING, ATIVIDADE NEURONAL E MARCADORES INFLAMATÓRIOS

Este projeto foi APROVADO em seus aspectos éticos e metodológicos de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08/10/2008, que estabelece procedimentos para o uso científico de animais.

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

Porto Alegre, 10 de outubro de 2014.

Profª Iraci Lucena da Silva Torres
Coordenadora CEUA/HCPA

Anexo B: artigo publicado na revista *Frontiers in Behavioral Neuroscience* 2017; 11:207.

Título: Microglial Over-Activation by Social Defeat Stress Contributes to Anxiety- and Depressive-Like Behaviors.

Autores: Dirson J. Stein, Mailton F. Vasconcelos, Lucas Albrechet-Souza, Keila M. M. Ceresér e Rosa M. M. de Almeida.

Abstract

Hyper activation of the neuroimmune system is strongly related to the development of neuropsychiatric disorders. Psychosocial stress has been postulated to play an important role in triggering anxiety and major depression. In preclinical models, there is mounting evidence that social defeat stress activates microglial cells in the central nervous system. This type of stress could be one of the major factors in the development of these psychopathologies. Here, we reviewed the most recent literature on social defeat and the associated immunological reactions. We focused our attention on microglial cells and kept the effect of social defeat over microglia separate from the effect of this stressor on other immune cells and the influence of peripheral immune components in priming central immune reactions. Furthermore, we considered how social defeat stress affects microglial cells and the consequent development of anxiety- and depressive-like states in preclinical studies. We highlighted evidence for the negative impact of the over-activation of the neuroimmune system, especially by the overproduction of pro-inflammatory mediators and cytotoxins. Overproduction of these molecules may cause cellular damage and loss or decreased function of neuronal activity by excessively pruning synaptic connections that ultimately contribute to the development of anxiety- and depressive-like states.

Anexo C: artigo enviado para publicação na revista *Psychoneuroendocrinology*. PNEC_2018_113

Título: Proactive Coping during Chronic Social Defeat Stress is associated with an Anti-inflammatory response in male Wistar rats.

Autores: Dirson João Stein, Mailton França de Vasconcelos, Luane Landau, Matheus Gallas-Lopes, Luiza Behrens, Lucas Albrechet-Souza, Luiza Paul Géa, Eduarda Dias da Rosa, Keila Maria Mendes Ceresér and Rosa Maria Martins de Almeida.

Abstract

Rationale – Chronic social stress is a common risk factor for affective disorders. In preclinical studies, depressive-like states are often induced by defeats using the resident-intruder paradigm. However, the extent to which different coping strategies determine vulnerability or resilience to develop stress-related psychiatric disorders remains unclear. While pro-inflammatory processes are increasingly recognized as key mediators in the etiology of psychopathologies, an anti-inflammatory profile could be responsible for resilience, characteristic of some individuals in the population.

Objectives – The present study tested a short length protocol of chronic social defeat stress in male rats, supposed to induce features of depressive-like symptoms. Further, we examined coping strategies adopted by defeated rats that were associated with circulating levels of growth factors and inflammatory proteins.

Methods – Adult male Wistar rats were continuously threatened by a dominant male rat and were daily defeated by another aggressor in a short encounter for 21 consecutive days. Non-stressed controls were handled for the same period throughout the experiment. Behaviors during confrontations, body weight, saccharin preference, spontaneous locomotor activity and reactivity to novelty in the open field were measured. Blood samples were collected for molecular analysis.

Results – 21 days of chronic social defeat stress produced two coping styles in male Wistar rats. Over the course of daily confrontations, two groups of stressed rats were identified, according to the latency do assume a submissive posture in confrontations with aggressors and were afterwards divided into short latency (SL) and long latency (LL) subpopulations. The two phenotypes were respectively associated with several behaviors related to vulnerability and resilience to stress that also correlated with altered levels of peripheral growth factors and inflammatory markers.

Although both groups had reduced weight gain ($p<0.01$) and experienced similar frequency of attacks, LL rats showed increased latencies to being bitten ($p<0.01$), increasing upright postures ($p<0.05$), lower levels of IGF-1 ($p<0.01$) and higher levels of IL-10 ($p<0.01$), while SL rats had increased immobility ($p<0.05$) time during defeat, decreased locomotor activity in the open field ($p<0.01$) and no altered peripheral molecules compared to non-stressed controls.

Conclusions: Social defeat stress-induced behavioral changes are bimodally distributed in male Wistar rats and correlate with different phenotypes of peripheral inflammation. This study highlighted that proactive coping with stress is closely associated with an anti-inflammatory response and may confer resilience to stress-related disorders.