PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

ANDRÉA WIECK

O PAPEL DO ESTRESSE PSICOSSOCIAL NA ATIVAÇÃO IMUNE DURANTE O DESENVOLVIMENTO E NA VIDA ADULTA

Porto Alegre, Março de 2013.

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul como requisito para obtenção do título de doutor.

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Porto Alegre, Março de 2013.

“Always account for variable changes...”

“NEVER LOSE YOUR COURAGE!
NEVER LOSE HOPE!
THERE IS ALWAYS A WAY!
HOPE DIES LAST!”

Do meu pai...

Agradecimentos

A gente entra no doutorado achando que vai mudar o mundo. Que vamos fazer uma super pesquisa em quatro anos e ser super pesquisadores. A gente sabe que tem um monte de dificuldades, mas acha que conosco vai ser diferente. A gente acredita que sozinhos somos capazes de mudar o mundo. Que temos a coragem e a força suficiente de encarar o mundo de peito aberto e fazer a diferença. Até o dia em que saímos da nossa zona de conforto e somos obrigados a olhar pra nós mesmos, desconstruir nossa própria imagem e idealização e começar a remontar da forma mais real possível.

Essa tese é dedicada a várias pessoas. Algumas me ajudaram cientificamente e fizeram experimentos comigo, outras nem entraram no lab...mas se não fosse cada uma delas, acho que eu não teria chegado ao fim com força, felicidade e certa de que fiz a escolha certa.

Maria de Lourdes Z. Wieck e Ricardo J. Wieck, muito obrigada por tudo, não existem palavras que sirvam pra agradecer a tudo o que vocês fizeram por mim, amo muito vocês. Felipe K. Ricachenvesky, te amo. Sem tu eu acreditaria muito menos em mim. Talvez ninguém entenda meus agradecimentos, mas não importa.

The Best of PhD soundtrack

1. Skyfall (Adele) - PhD Theme
2. Eu preciso dizer que te amo (Cazuza) - Felipe K. Ricachenevsky
3. The Best of you (Foo Fighters) - Susan L. Andersen, Moisés E. Bauer & Rodrigo Grassi-Oliveira
4. At your side (The corrs) - Priscila Salvato dos Santos, Sabrina Pozzati Moure, Luiza Heberle, Betânia L. Fonseca and Carine H. do Prado.
5. Gangnam Style (Psy) – laboratórios de imunologia e de Imunologia do Envelhecimento
6. Father and Son (Cat Stevens) – Ricardo Wieck & Maria de L. Z. Wieck (Pai e mãe)
7. Let it be (Beatles) – Ricardo Wieck & Maria de L. Z. Wieck (Pai e mãe)
8. With a little help from my friends (Beatles) – Andreia E. Vargas, Paula Rohr, Lucas B. Rizzo & Lucas Tortorelli
9. Blackbird (Beatles) – eu mesma…

1. Lista de Abreviaturas

SNC: Sistema Nervoso Central
HPA: Hipotálamo-Pituitária-Adrenal
IL-1β: Interleucina 1-Beta
TNF-α: Fator de Necrose Tumoral – alfa (Tumour necrose fator-alpha)
GC: Glicocorticoides
TB: Transtorno Bipolar
IL-2: Interleucina 2
IL-4: Interleucina 4
IL-1: Interleucina 1
NK: células *Natural Killers*
IL-6: Interleucina 6
IL-8: Interleucina 8
PCR: Proteína C-reativa
IFN- γ: Interferon - gama
sIL-6R: Receptor solúvel de Interleucina 6
sTNF-R1: Receptor Solúvel tipo 1 do Fator de Necrose Tumoral
Treg: Células T regulatórias
TGF: Fator de Crescimento Tumoral
TGF- β1: Fator de Crescimento Tumoral Beta 1
CD: Cluster de diferenciação
NF-κB: Fator de Transcrição Nuclear Kappa-Beta
TSST: Trier Social Stress Test – Protocolo de estresse psicossocial
DEX: Dexametasona
CREB: Proteína ligadora a elementos responsivos a cAMP.
cAMP: Adenosina Monofosfato Cíclico
MAPK: Proteína Cinase responsiva à mitogenos
ERK: Proteínas cinase regulada por fatores extracelulares
LPS: Lipopolisacarídeo bacteriano
BDNF: Fator neurotrófico derivado do cérebro
DNA: Ácido desoxirribonucléico
TEPT: Transtorno de estresse pós-traumático
NMDA: N-metil D-aspartato
p38: Proteína cinase ativada por mitogenos p38
HIV: Vírus da imunodeficiência humana
ANS: Sistema Nervoso Autônomo
IL-10: Interleucina 10
ACTH: Hormônio Adrenocorticotrófico
CRH: Hormônio Liberador de Corticotrofina
RANTES: ou CCL5 – motivo de ligação à citocinas tipo 5
BHE: Barreira Hemato-encefálica
IDO: Indoleamina 2,3-dioxigenase
COX2: Ciclo-oxigenase 2
PGE: Prostaglandina- E
PVB: Parvalbumina
NMDAr: Receptores de N-metil D-aspartato

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Resumo

O estresse psicossocial é um importante mecanismo de ativação dos sistemas nervoso, endócrino e imune muitas vezes levando à exacerbação de diversas doenças inflamatórias crônicas, assim como é um fator de risco importante para diversos transtornos de humor. A exposição ao estresse pode ser mais danosa quando esta ocorre cedo no desenvolvimento e pode resultar em alterações na reatividade/responsividade ao estresse na vida adulta. Diversos estudos vêm demonstrando alterações neuroimunoendócrinas importantes na patofisiologia dos transtornos de humor. Estudos prévios do nosso grupo e outros têm observado um perfil pró-inflamatório periférico e uma maior ativação linfocitária em pacientes com transtorno bipolar (TB). Os objetivos da tese são: 1) analisar os efeitos do estresse no desenvolvimento através do protocolo de separação materna em modelo animal; 2) analisar parâmetros neuroimunoendócrinos em resposta ao estresse em pacientes com TB tipo 1 eutímicos, utilizando-se um protocolo de estresse laboratorial, o Trier Social Stress Test (TSST). Dados do presente trabalho demonstram que a exposição ao estresse na infância (em modelo animal) resulta em ativação imune, caracterizada por aumento nos níveis plasmáticos de citocinas pró-inflamatórias (interleucina 1-β) perifericamente. Uma possível consequência dessa ativação imune é a perda de neurônios contendo parvalbumina, importantes para o desenvolvimento de comunicação serotoninérgica. A inflamação periférica serviu como marcador para os danos neuronais observados, pois quando houve a administração de interleucina-10 (IL-10, principal citocina anti-inflamatória) foi possível reverter os danos neuronais e a inflamação periférica. Neste trabalho, os pacientes com TB apresentaram respostas simpáticas e neuroendócrinas (cortisol) muito reduzidas após o estresse agudo quando comparados aos controles saudáveis. Ao nível basal, os pacientes com TB apresentaram uma redução na porcentagem de células T regulatórias (Treg), aumento na porcentagem das células T ativadas (CD4+CD25+) e aumento na sinalização celular através de uma maior fosforilação de ERK1/2 e NF-κB – corroborando para um estado de ativação celular. Além disso, observamos uma incapacidade em reduzir a ativação imune em resposta ao estresse, caracterizada pelo aumento ainda maior na porcentagem de células T ativadas e concomitante redução nas células Treg em pacientes TB. Tal incapacidade em controlar a resposta imune ao estresse pode ser explicada não apenas pelos baixos níveis de cortisol secretado, mas também a uma maior insensibilidade aos glicocorticoides no TB. Concluímos que os indivíduos com TB possuem alterações no eixo HPA que resultam em reduzida reatividade endócrina ao estresse assim como incapacidade de modular corretamente as respostas imunes.

Palavras-chave: Transtorno Bipolar, TSST, inflamação, MAPKs, NF-κB, ativação imunológica.

Abstract

Psychosocial stress has important role in activating endocrine, immune and central nervous systems. Stress exacerbates many chronic inflammatory conditions and is an important risk factor for several mood disorders. Early exposure to stress can be even more detrimental as it may lead to alterations in stress reactivity/responsivity later in life. Several studies have shown important neuroimmune changes associated with the pathophysiology of mood disorders. Previous studies from our group and others reported a pro-inflammatory profile and increased cellular activation in patients with bipolar disorder (BD). The objectives of the thesis are: 1) to analyze the stress effects on development using animal model of early life stress (maternal separation); 2) to analyze the neuroimmunendocrine responses to acute stress exposure (Trier Social Stress Test) in BD patients. Data presented here suggests that early life stress results in immune activation, characterized by increased pro-inflammatory serum levels (specifically IL1-β). As a consequence of this inflammation, a reduction of parvalbumin containing interneurons, substantial for serotonergic branches development, was also observed. Peripheral inflammation is a biological marker of neuronal damage observed, as interleukin-10 (IL-10, main anti-inflammatory cytokine) central administration overturned the neuronal damages as well as peripheral inflammation per se. In the second study, patients with BD showed blunted sympathetic and neuroendocrine (cortisol) stress responses following acute stress compared to healthy controls. Basal data corroborates the presence of increased cellular activation in BD patients as observed by reduced T regulatory (Treg) cells, increased activated T cells (CD4+CD25+) as well as increased intracellular signaling through increased ERK1/2 and NF-κB phosphorylation. Furthermore, an inability in reducing immune activation in response to stress was observed as increased percentage of activated T cells and concomitantly reduction in regulatory T cells in BD patients. Such inability in controlling immune response after stress exposure may be explained not only by reduced cortisol levels but also by reduced glucocorticoid sensitivity observed in BD patients. Given that, we conclude that BD patients have important HPA axis alterations that may lead to reduced endocrine reactivity to stress as well as inability to duly modulate immune responses.

Keywords: Bipolar Disorder, TSST, inflammation, MAPKs, NF-κB, immune activation.

SUMÁRIO

1. Introdução

1.1. Estresse

A definição de estresse foi estabelecida há aaproximadamente 60 anos atrás pelo médico e pesquisador Hans Seyle. Em seu importante trabalho ele descreve o estresse como um conjunto de alterações fisiológicas inespecíficas em resposta a demandas ambientais (1). Quando Seyle utiliza a definição "alterações fisiológicas inespecíficas" , ele quer dizer um conjunto de alterações que ocorrem em resposta a qualquer estresse, independente da natureza do mesmo (1). O conceito de estresse vem atrelado a outros dois importantes conceitos: alostase e carga alostática (2). Alostase é definida como a soma das alterações fisiológicas necessárias para a manutenção da correta funcionalidade de um organismo diante das demandas do ambiente (2, 3). A alostase de um organismo é mantida por diferentes sistemas do organismo (sitema nervoso central – CNS, sistema nervoso autômo – CNA, sistema endócrino, sistema imune) que interagem entre si de forma a permitir que o organismo funcione corretamente diante das demandas diárias do ambiente. Quando algum destes sistemas se encontra em desbalanço, levando a uma resposta inadequada as demandas diárias, temos, então, uma carga alostática (2, 3).

Diante desta definição, qualquer tipo de estressor, físico ou psicológico, desencadeia uma cascata de processos biológicos que irá modular precisamente uma resposta adaptativa de acordo com a demanda. As sinapses são ativadas de forma a aumentar a atenção e o alerta enquanto funções vegetativas, como comer e dormir são diminuídas. Além disso, os batimentos cardíacos, pressão sanguínea, taxa respiratória e gliconeogênese são elevadas. Exposições excessivas ou repetidas a eventos estressores podem contribuir para perda

neuronal em regiões chave como o sistema límbico, o hipocampo e a amígdala (Figura 1) (4, 5). Se o organismo é incapaz de cessar a resposta ao estresse ao final da exposição ao mesmo, ou ainda, se o organismo for exposto ao estresse crônico, os mecanismos adaptativos que respondem a este estresse podem resultar em condições patológicas.

1.2. Estresse e períodos de sensibilidade do desenvolvimento

A exposição ao estresse durante os chamados períodos de sensibilidade do desenvolvimento pode apresentar resultados ainda mais prejudiciais ao indivíduo. Períodos de sensibilidade do desenvolvimento são momentos em que o SNC e o organismo como um todo estão mais suscetíveis aos estímulos externos. Dependendo da natureza do estímulo (seja ele positivo ou negativo) as consequências podem ser duradouras e detrimentais para a saúde do indivíduo (6-10). Tais períodos estão associados ao desenvolvimento sinaptogênico e eventos maturacionais importantes para o correto desenvolvimento do SNC. A expressão de diferentes moléculas, como neurotransmissores e neurotrofinas, é finamente regulada de forma a adaptar o SNC imaturo às diferentes alterações no ambiente que cerca o indivíduo (11). O cérebro é um órgão altamente sensível a perturbações no delicado balanço de neurotransmissores, hormônios, citocinas e neurotrofinas irão romper este balanço resultando em maior vulnerabilidade ao estresse e, consequentemente, aumentando o risco de desenvolvimento de transtornos de humor (12).

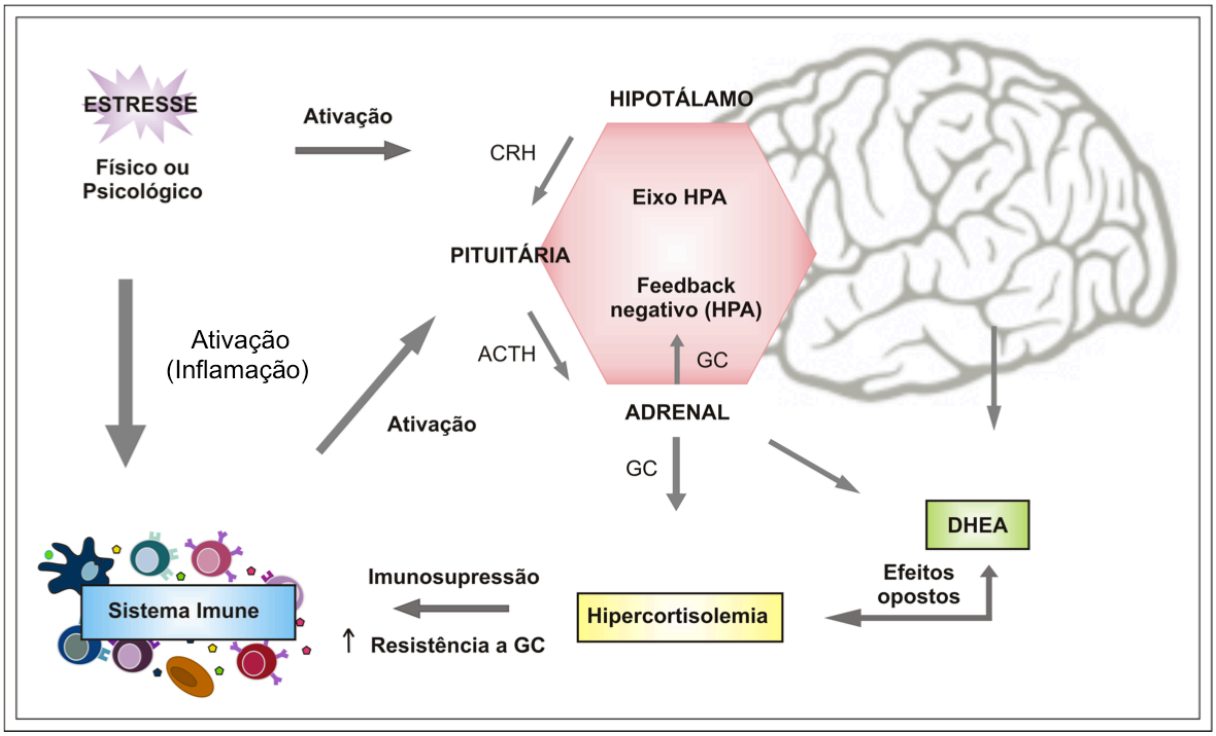

Figura 1: Efeitos no organismo da exposição ao estresse agudo. Exposição ao estresse agudo produz uma série de efeitos detrimentais ao organismo. Inicialmente, resulta em ativação do eixo HPA (aumento na secreção de cortisol) e do sistema imune (inflamação – aumento na secreção de citocinas pró-inflamatórias). A ativação do eixo HPA devido ao aumento de citocinas pró-inflamatórias em decorrência da exposição ao estresse resulta no aumento na secreção de GC (p.e. cortisol) com intuito de reduzir a ativação imune (imunossupressão). Tais efeitos se tornam ainda mais detrimentais quando há exposição crônica ao estresse (quadro amarelo, abaixo, à esquerda). A constante exposição a níveis elevados de GC resulta em redução da sensibilidade a GC em linfócitos e, consequentemente, linfócitos hiporesponsivos. O resultado final acaba por ser uma constante ativação do sistema imune que, por sua vez, leva à neuroinflamação e neurotoxicidade.

O eixo HPA é o responsável por desencadear as alterações fisiológicas características a resposta ao estresse. A exposição a eventos estressores durante os períodos de sensibilidade pode resultar em uma reprogramação desse eixo, alterando a resposta a estresses futuros (12).

Várias evidências demonstram que a exposição a eventos estressores durante os períodos do desenvolvimento possui impacto negativo na saúde mental (6-8, 10, 13-15). Um estudo prévio demonstrou uma relação entre maus-tratos e desenvolvimento precoce, gravidade da doença e número de episódios maníacos de indivíduos com TB (16). Históricos de experiências traumáticas são frequentes em pacientes com distúrbios mentais graves. Diversos relatos de pacientes com transtorno de personalidade demonstram que estes indivíduos sofreram maus-tratos infantis recorrentes e que o tipo de abuso varia desde abuso emocional, físico até negligência (17, 18), sugerindo um possível papel importante da exposição ao estresse na infância e desenvolvimento de transtorno de humor**.** Além disso, os maus-tratos são fatores de risco para a manifestação de comportamentos suicidas no adulto (19).

1.3. O Transtorno Bipolar

O transtorno bipolar (TB) é uma doença crônica com incidência de cerca de 0,4% na população mundial, sendo a incidência entre adolescentes maior, se aproximando de 3-4% (20, 21). De forma geral, este transtorno é caracterizado pela variação extrema do humor entre uma fase maníaca ou hipomania, hiperatividade e grande imaginação, e uma fase de depressão de inibição, lentidão para conceber idéias e realizá-las, e ansiedade ou tristeza.

Segundo a 10ª revisão da Classificação internacional de Doenças (CID-10), elaborado pela Organização Mundial de Saúde (OMS), o Transtorno Afetivo Bipolar ou Transtorno Bipolar (TB) é classificado como um transtorno de humor. O critério diagnóstico de TB consiste no aparecimento de no mínimo um ou mais episódios considerados maníacos, nos quais o humor e o nível de atividade do sujeito estão profundamente perturbados, caracterizado por elevação de humor, aumento da energia e da atividade. Episódios depressivos não são obrigatoriamente necessários para o diganóstico de TB, porém, dada a característica cíclica do transtorno, em algumas ocasiões pode ocorrer um rebaixamento do humor e de redução da energia e da atividade (depressão)(22).

Estes períodos de alteração no humor podem ou não ser intercalados por períodos de normalização do humor (eutimia ou remissão). Usualmente, a eutimia é - do ponto de vista operacional - definida como o estado em que o paciente não preenche critérios para episódio maníaco, hipomaníaco ou depressivo (humor neutro) e re-integrado funcionalmente a sua atividades rotineiras. Já a remissão implica no indivíduo manter o estado de eutimia, a ponto de a doença ser considerada controlada.

Um episódio hipomaníaco é definido como um período em que ocorre moderada alteração do humor, com aumento da energia e atividade, aumento da sociabilidade, energia sexual e diminuição da necessidade de dormir. As alterações de humor e comportamento não são acompanhadas de alucinações. O episódio maníaco apresenta sintomas semelhantes, mas ainda mais salientes. Dificuldade em manter a atenção, fugacidade do pensamento, excesso de confiança e ideias de grandiosidade são comuns e podem levar a comportamentos imprudentes. O episódio maníaco pode ou não ser acompanhado de sintomas psicóticos. O episódio depressivo é caracterizado por redução na

energia, tristeza e diminuição da atividade motora. Ocorrem alterações no sono e apetite e cansaço ao mínimo esforço. A baixa autoestima e autoconfiança, bem como ideias de culpa e inutilidade são comuns. Dependendo do grau de depressão, pode haver também ideário suicida (22, 23).

O TB é classificado de acordo com o Manual Diagnóstico e Estatístico de Transtornos Mentais, 4ª edição (DSM-IV) (23). Existem duas formas distintas de transtorno bipolar: o tipo I e tipo II. O TB tipo I (transtorno bipolar clássico), a forma mais severa da doença, é caracterizado principalmente por episódios maníacos ou mistos enquanto o TB tipo II inclui ao menos um episódio de hipomania (uma forma menos grave de mania). A prevalência do TB tipo I é estimada na faixa de 1%, e não há diferenças entre o gênero masculino e feminino. Já o TB tipo II é mais prevalente no sexo feminino, afetando entre 0,5- 3% (24-26). Ambos os pacientes com TB tipo I e tipo II podem apresentar quadros depressivos ao longo do curso do transtorno (27). Os episódios são de diferente gravidade, frequência e duração, e ocorrem ainda episódios mistos, com características de mania, hipomania e de depressão (28). A natureza e duração dos episódios variam grandemente de um indivíduo para outro, tanto em intensidade quanto em duração. Nos casos muito graves, pode haver risco pessoal e material. Normalmente o primeiro episódio ocorre no início da idade adulta, quando os indivíduos estão se estabelecendo longe da família de origem e, ao contrário de outras doenças mentais como esquizofrenia, os episódios maníaco-depressivos tendem a se tornar mais frequentes e mais graves ao longo da vida do indivíduo (29, 30). O transtorno bipolar possui uma etiologia complexa, envolvendo fatores genéticos, biológicos e psicossociais. Normalmente está associada a altas taxas de mortalidade e custos de saúde significantes (31).

A sintomatologia do TB, bem como suas comorbidades associadas, determina um prejuízo na capacidade de o indivíduo realizar seu papel funcional. Ambos os episódios maníacos e depressivos levam ao prejuízo funcional. De 75 a 87% dos pacientes relataram um comprometimento severo no desempenho de seus papéis durante episódios depressivos, enquanto isso foi relatado por aproximadamente 50-57% dos pacientes durante episódios maníacos (32, 33).

Os indivíduos com TB possuem um risco estimado de suicídio em 15%, e este risco aumenta consideravelmente durante a fase depressiva da doença (34). Além dos sintomas de depressão e mania (ou hipomania) e o risco de suicídio, os pacientes com TB enfrentam múltiplas comorbidades psiquiátricas e físicas, como o transtorno de ansiedade, altas taxas de suicídio, abuso de substâncias, obesidade, diabetes tipo II, doenças cardiovasculares, entre outras (27, 35).

Atualmente têm surgido diversas evidências de que o sistema imune, em interação direta com o sistema nervoso central, tem papel importante na patofisiologia do transtorno bipolar (31, 36-40). Acredita-se que as citocinas podem modular e modificar os mecanismos associados à serotonina e catecolaminas no cérebro e vice-versa (31, 41). Além disso, estudos relatam que os neurotransmissores, hormônios e citocinas agem no eixo HPA formando um circuito regulatório que mantém a homeostase em resposta ao estresse (42).

1.4. Alterações Imunológicas, Estresse e Transtornos de Humor

Num primeiro momento, a resposta imune ao estresse é caracterizada por ativação do sistema imune. Durante a resposta saudável ao estresse, a ativação do eixo HPA resulta em aumento na secreção de cortisol e, consequentemente, supressão daquela resposta

imune primeiramente ativada (Figura 1). O estresse crônico, entretanto, reduz vários componentes da resposta imune celular, aumentando desta maneira a morbidade e mortalidade das populações estressadas. Evidências da literatura têm demonstrado que o estresse crônico altera, particularmente, a imunidade celular, incluindo: diminuição na proliferação de células T e produção de interleucina (IL)-2 (43), diminuição da atividade NK (44, 45), e alterações de tráfego celular (46-51). O resultado é uma diminuição global da resposta imune celular do organismo.

A crescente prevalência de transtornos depressivos em doenças caracteristicamente inflamatórias (p.e. aterosclerose e síndrome coronariana aguda) ressalta a associação entre transtornos de humor e uma resposta imune inflamatória (52-54). Além disso, sabe-se que as citocinas estão envolvidas em funções do SNC que estão prejudicadas em indivíduos com tais transtornos, como sono, apetite, nível de atividade, comportamento de forma geral além de parâmetros neuroendócrinos. Entretanto, os mecanismos que ligam a resposta inflamatória aos transtornos de humor ainda são desconhecidos.

A depressão maior está associada a alterações na função linfocitária e ativação inflamatória sistêmica. Vários estudos verificaram que a depressão está associada com uma inibição da imunidade celular, incluindo uma redução da proliferação das células T, diminuição da atividade NK (44, 55-57) e redução da sensibilidade periférica a glicocorticoides (58). Além disso, estudos observaram elevação das citocinas pró-inflamatórias (TNF-α, IL-1, IL-6 e IL-8) séricas, assim como aumento da proteína C reativa (PCR), haptoglobina e antagonistas solúveis dos receptores de citocinas que podem estar associadas diretamente ao aparecimento de sintomas de depressão em pessoas predispostas (43, 52, 59-64). Esses dados sugerem que a depressão por si só é capaz de

alterar os níveis destes importantes mediadores imunes. Interessantemente, diferentes tratamentos antidepressivos são capazes de restabelecer os níveis normais de citocinas pró-inflamatórias e até mesmo estimular e produção de citocinas anti-inflamatórias (p.e IL10) (65) enfatizando a importância de um correto balanço entre citocinas pró/anti-inflamatórias na patofisiologia da depressão e resposta ao tratamento (66-70).

O estado do conhecimento sobre as alterações imunológicas no transtorno bipolar é bastante limitado. A grande maioria dos trabalhos enfoca a depressão maior. A dificuldade de estudar este transtorno reside no fato da bipolaridade estar associada com alterações imunes diferenciais ao longo dos ciclos de mania e depressão. Atualmente têm surgido diversas evidências de que o sistema imune, em interação direta com o sistema nervoso central, possui papel importante na patofisiologia do TB (31, 71).

Dados existentes na literatura relacionados às alterações imunes presentes em cada fase do TB ainda são bastante escassos e controversos. De forma geral, o TB é acompanhado de múltiplos sinais de ativação e alterações do sistema imunológico (52, 72), variando de acordo com a fase em que o paciente se encontra (fase maníaca, depressiva ou eutímica) (31), sugerindo um perfil pró-inflamatório no TB (72-77).

Durante a fase maníaca do transtorno, níveis aumentados das citocinas pró-inflamatórias tais como IL-6, IFN-γ (78), TNF-α (58, 79) e receptor solúvel IL-6 (sIL-6R) (79) foram relatados. Da mesma forma, os níveis plasmáticos do receptor solúvel TNF-α (sTNF-R1) eram significativamente maiores em pacientes bipolares em comparação com controles saudáveis (73, 80). Em contrapartida, alguns estudos têm proposto uma diminuição dos níveis de IL-6, IL-1 e IL-2 em pacientes durante a mesma fase (31). Além disso, estudos

indicam aumento significativo dos níveis de IL-4 nos pacientes em episódio de mania (31, 78). Os episódios depressivos têm sido associados com um aumento nos níveis de IL-6 (31, 58), IL-1 e diminuição dos níveis de IL-4. Além disso, os níveis séricos do sIL-2R foram encontrados elevados em pacientes bipolares quando comparados com indivíduos sem transtorno de humor, e o mesmo ocorreu quando comparando indivíduos maníacos com depressivos (74).

O desequilíbrio no balanço entre citocinas pró e anti-inflamatórias possivelmente reflete alterações na função e quantidade dos diferentes tipos linfocitários. Por exemplo, células T regulatórias (CD4+CD25+Foxp3+, Tregs) são indispensáveis para a manutenção da tolerância periférica e homeostase linfocitária em uma série de circunstâncias imunológicas (81, 82). Ao comparar indivíduos bipolares na fase maníaca e controles saudáveis, foi possível observar que os valores de TGF-β1 foram significativamente menores e a razão IFN-γ/TGF-β1 e IL-4/TGF-β1 foi maior nos pacientes bipolares (78) apontando para uma possível alteração no funcionamento das células Treg. Em estudo recente publicado por nosso grupo de pesquisa, a porcentagem desse subtipo linfocitário estava reduzida em pacientes TB comparados com indivíduos saudáveis, apoiando hipótese de desequilíbrio imunológico nesses indivíduos. Entretanto, ainda existem poucos estudos relacionando os diferentes subtipos linfocitários com TB. Breunis e colaboradores (2003) demonstraram que a porcentagem de células T ativadas (CD3+MHCII+, CD3+CD25+ e CD3+CD71+) e células B (CD19+CD20+) em indivíduos bipolares, independente da fase da doença em que se encontravam, foram maiores em comparação com indivíduos controles.

Os possíveis mecanismos da ativação da resposta imune inflamatória nestes indivíduos ainda são desconhecidos. Diferentes rotas de sinalização intracelular podem estar

envolvidas nesse processo. Um exemplo é a ativação do fator de transcrição Nuclear Kappa B (NF-κB), um dos principais fatores de transcrição da resposta imune inflamatória (83). NF-κB pode ser composto de homo e heterodímeros formado por diferentes subunidades: p50 (NF-κB 1), p52 (NF-κB 2), p65 (RelA), RelB, e c-Rel (84). A ativação deste fator de transcrição ocorés da fosforilação de suas subunidades, que, uma vez fosforiladas, possuem a capacidade de translocar ao núcleo. Uma vez no núcleo, estes fatores de transcrição se ligam às regiões promotoras de diferentes genes, entre eles genes relacionados à resposta imune como IL-1, IL-6, IL-8 e TNF-α. Estudos comparando as subunidade p50 e p65 demonstram que a subunidade p65 é a que possui a maior capacidade de tansativação, sendo uma das principais ativadoras da transcrição gênica (84). Estudos já demonstraram a translocação deste fator de transcrição para o núcleo diante de diferentes estímulos *in vitro* como por exemplo hipóxia, exposição à LPS, TNF-α e IL-6 (83). A análise de células mononucleares de pacientes acometidos por sepse e pacientes que sofreram trauma (diversos tipo de trauma, como acidentes de carro e queda de grandes alturas) demonstrou que o fator de transcrição NF-κB é o principal fator envolvido nas respostas imunes diante de tais desafios imunológicos (83, 84). Além disso, alguns estudos sugerem que o estresse agudo é capaz de aumentar a atividade do NF-κB, aumentando a ligação desta molécula a regiões promotoras de genes caracteristicamente inflamatórios (85-87). A importância funcional de NF-κB no processo inflamatório é sua capacidade de regular a transcrição de vários genes cujos produtos (citocinas, proteínas de fase aguda, entre outros) são críticos à esse processo (88).

1.5. Alterações neuroendócrinas, Estresse e Transtornos de Humor

Diversas teorias têm surgido para explicar o papel do estresse na patofisiologia dos transtornos do humor. Entre elas, temos a hipótese neurotrófica que explica o papel dos

neuropeptídeos na plasticidade e proteção neural em resposta ao estresse, e a hipótese do eixo HPA, a qual postula um desequilíbrio na função deste eixo que faz com que o estresse possa desencadear algum tipo de transtorno do humor (89). O eixo HPA é a maior rota de regulação das respostas ao estresse e estudos prévios demonstraram alterações funcionais deste eixo, tanto para mais quanto para menos (90). Estudos prévios demonstraram que o estresse reduz os níveis de expressão de neurotrofinas como o fator neurotrófico derivado do cérebro (*Brain Derived Neurotrophic Factor* – BDNF) em vários modelos animais e que os mecanismos pelos quais o estresse levaria a esta redução agem via receptores de mineralocorticoides, glicocorticoides e de NMDA (N-methyl-D-aspartic acid) (89, 91, 92).

O BDNF é um membro da família das neurotrofinas e influencia muitos aspectos do desenvolvimento do SNC. Está envolvido diretamente no crescimento e plasticidade neuronal no hipocampo e amígdala (89). Acredita-se que o BDNF regule a resposta do eixo HPA ao estresse ao mesmo tempo em que protege o cérebro contra danos neuronais gerados pelo estresse (90). Estudos recentes em humanos têm demonstrado uma redução nos níveis plasmáticos de BDNF em indivíduos acometidos por esquizofrenia, transtorno bipolar e depressão (93). Níveis reduzidos de BDNF também foram encontrados no hipocampo e córtex pré-frontal em estudos post-mortem no cérebro de suicidas (90). Resultados similares são observados em estudos feitos com modelos animais. Animais que são expostos a estresse crônico possuem níveis de BDNF reduzidos no hipocampo (13). Os autores sugerem que a separação materna repetidas vezes na infância pode reduzir os níveis de BDNF no hipocampo envolvendo o eixo HPA e a formação da memória durante o desenvolvimento (4).

Outra importante alteração neuroendócrina diz respeito aos níveis de cortisol. O cortisol é conhecido como principal hormônio do estresse. Sua secreção é estimulada através da ativação do eixo HPA diante de eventos estressores. A liberação de cortisol e outros neurotransmissores excitatórios devido à exposição ao estresse resultam em cascatas de ativação que acabariam levando à célula à morte por apoptose ou necrose (94). Uma das rotas mais comuns de ativação via BDNF é a MAPK/ERK. A ativação desta cascata leva à regulação transcricional do Elemento de ligação responsivo à cAMP (*cAMP Response Element Binding* – CREB) que irá aumentar a expressão de diversos genes necessários para plasticidade sináptica e sobrevivência neuronal (95). O aumento de cortisol e a concomitante diminuição de DHEA induzidos pelo estresse podem induzir várias alterações imunológicas já que os glicocorticoides endógenos são essenciais na regulação da atividade imunológica, principalmente a resposta inflamatória (96).

1.6. Interações neuroimunoendócrinas

Alterações imunológicas e transtornos de humor podem estar relacionadas através de interações neuroimunoendócrinas bidirecionais. Por um lado, o cérebro atua liberando glicocorticoides que tem função imunossupressora. Por outro, a inflamação reduz a liberação desses glicocorticoides, o que pode levar a uma desregulação do eixo HPA (97). Nessa intercomunicação, as citocinas, que são moléculas mediadoras da comunicação célula-célula do sistema imune, têm sido consideradas como fatores chave na interação entre o sistema imune e o Sistema Nervoso Central (SNC) (67). Citocinas pró-inflamatórias exercem profundos efeitos no SNC e no sistema endócrino alterando o metabolismo central das monoaminas e agindo como potentes ativadoras do eixo HPA, ambos os sistemas que apresentam alguma alteração nos transtornos de humor (54, 75). Além do perfil pró-

inflamatório observado em pacientes com transtorno de humor (31, 54, 64), estudos também encontraram associação entre a concentração plasmática de diversas citocinas pró-inflamatórias e a gravidade dos sintomas, assim como o aumento em determinadas citocinas de acordo com a fase em que o indivíduo com transtorno bipolar se encontrava (se mania ou depressão). Além disso, a administração terapêutica de interferon-α (IFN- α, uma citocina pró-inflamatória) leva ao desenvolvimento de depressão em aproximadamente 50% dos pacientes que utilizavam (54).

Estudos já desenvolvidos pelo nosso grupo sugerem que o estresse crônico está associado com uma importante desregulação neuroimunoendócrina. Em particular, foi demonstrado que cuidadores de pacientes com demência apresentam níveis mais elevados de cortisol salivar e linfócitos circulantes mais resistentes ao tratamento *in vitro* com glicocorticoides (98). Desta forma, além de causar imunossupressão, o estresse crônico altera a regulação linfocitária pelos glicocorticoides, trazendo consequências indesejadas para o sujeito. Por exemplo, esta desregulação neuroimunoendócrina pode contribuir para a etiologia ou curso clínico de doenças autoimunes, cuja terapêutica usual ainda é o uso de glicocorticoides sintéticos.

O mecanismo pelo qual as citocinas atuam no desenvolvimento dos transtornos de humor é uma questão intrigante. Dado o grande tamanho das moléculas de citocinas e sua resultante inabilidade de cruzar a barreira hemato-encefálica, algumas rotas de ação destas moléculas no cérebro têm sido sugeridas: a) entrada através de locais onde a barreira hemato-encefálica é mais permeável, como nos órgãos circumventriculares; b) ligação a receptores/transportadores específicos para as citocinas na barreira hemato-encefálica; c) ativação de fibras vagais aferentes que transmitiriam a sinalização das citocinas para pontos

específicos no cérebro (54, 64). Além disso, sabe–se que a resposta inflamatória leva ao aumento da permeabilidade da barreira hemato-encefálica, facilitando por si só a entrada de citocinas no cérebro. Estudos utilizando modelos animais demonstraram que as citocinas inflamatórias induzem uma síndrome conhecida como "*sickness behavior*", a qual possui muitas características semelhantes à depressão (problemas com sono, anorexia, e atividade motora reduzida entre outros) (54, 64). Assim como na depressão, estas modificações comportamentais induzidas pelas citocinas estão associadas à alterações no metabolismo monoaminérgico em regiões do cérebro essenciais para regulação das emoções e da função psicomotora. Além dos efeitos no metabolismo dos neurotransmissores, as citocinas inflamatórias possuem efeito estimulatório nos hormônios do eixo HPA como o Hormônio Liberador da Corticotrofina (CRH) em regiões do cérebro importantes para o controle do medo e ansiedade (54, 99).

De acordo com os diferentes mecanismos pelos quais as citocinas pró-inflamatórias agem no cérebro alterando metabolismo de moléculas importantes para a saúde mental, é perfeitamente compreensível que tais moléculas contribuam para o desenvolvimento de depressão no contexto de uma doença, o que é demonstrado pela prevalência de depressão em indivíduos acometidos por outras enfermidades. Porém, não é tão aparente o motivo pelo qual indivíduos sem algum tipo de doença física, mas com depressão ou transtorno bipolar, apresentariam um desbalanço das citocinas pró-inflamatórias. Uma possibilidade bastante aceita é o impacto do estresse na resposta imune (54). O estresse psicossocial é um fator de risco comum para o desenvolvimento de depressão e transtorno bipolar em diferentes populações, e a maioria dos episódios iniciais destas doenças são precedidos por um estressor (100). Dessa forma, acredita-se que o estresse ativaria as citocinas pró-

inflamatórias e suas rotas sinalizadoras no Sistema Nervoso Central (SNC). Tal efeito é geralmente demonstrado em modelos animais, onde se observou o aumento de citocinas pró-inflamatórias após exposição a diferentes tipos de estresses o que resultou em modificações comportamentais nestes animais (48). Em humanos, estresse agudo e crônico foram associados com aumento na produção e/ou liberação de citocinas pró-inflamatórias, além de redução das citocinas anti-inflamatórias (99). Entretanto, existem estudos que sugerem que, devido às alterações neuroquímicas induzidas por fatores inflamatórios serem semelhantes àquelas induzidas por estresse, é possível que o cérebro interprete a ativação imune como um estressor. Assim, devido aos efeitos semelhantes ao do estresse no SNC, é provável que a inflamação contribua para o desenvolvimento da depressão (54).

As técnicas de estresse aplicadas em laboratório são uma ferramenta importante para abordar os mecanismos fisiológicos relacionados à reatividade ao estresse. A reatividade ao estresse em pacientes TB já foi analisada através da indução de estresse químico. O estresse agudo induzido de forma química também é conhecido como teste combinado de DEX/CRH (Dexametasona/Hormônio Liberador da Corticotrofina). É um teste comumente usado cujo objetivo é induzir a resposta fisiológica ao estresse sem submeter os indivíduos ao mesmo. A dinâmica do teste consiste na ingestão de uma dose de 1,5 mg de dexametasona na noite anterior ao dia do estímulo com CRH (23:00h), no dia seguinte, às 13:00h inicia-se o procedimento de estímulo do eixo HPA. Estudos demonstraram alterações do eixo HPA em resposta ao teste da DEX/CRH, onde as alterações nos níveis de cortisol secretados em resposta ao teste variaram de acordo com a fase da doença em que paciente se encontra (101). Trabalho de Watson e colaboradores (2004) demonstrou alterações no funcionamento do eixo HPA de indivíduos com TB observado por aumento na secreção de

cortisol em resposta ao teste, comparado com indivíduos saudáveis (102). Entretanto, nenhum destes estudos analisou parâmetros imunológicos em resposta ao teste.

Outro método amplamente utilizado para estudo da reatividade ao estresse é o teste de estresse psicossocial (Trier Social Stress Test – TSST), desenvolvido para induzir estresse psicossocial moderado em condições laboratoriais. Este teste é capaz de ativar o eixo HPA e aumentar a produção de marcadores inflamatórios no plasma (103, 104). É amplamente aplicado em laboratório com intuito de analisar as alterações biológicas resultantes da exposição a uma situação controlada de estresse agudo (105). Com uso do TSST é possível avaliar diferentes sistemas que são acionados durante a exposição a um estresse agudo. A análise dos níveis de cortisol, principal hormônio do estresse, é considerada uma boa medida para análise de funcionamento do eixo HPA, um dos principais envolvidos na resposta ao estresse (106, 107). A verificação da frequência cardíaca ao longo do protocolo nos dá uma ideia do funcionamento do Sistema Nervoso Simpático (SNS) durante uma exposição ao estresse. Muitos outros parâmetros podem ser analisados através de coletas de sangue, no nosso caso parâmetros imunológicos, durante a resposta ao estresse.

A resposta ao estresse agudo com uso de TSST já foi estudada em diferentes transtornos de humor. Em indivíduos com depressão maior, submetidos ao TSST, houve ativação imune observada por aumento nos níveis circulantes de IL-6, aumento na capacidade de ligação ao DNA do fator de transcrição de NF-κB e aumento na porcentagem de células NK em resposta ao estresse (108). Já a resposta neuroendócrina variou de acordo com o gênero. Mulheres com depressão maior apresentaram uma maior secreção de cortisol comparado com voluntários saudáveis, enquanto homens não diferem entre pacientes com depressão maior e voluntários saudáveis (109-111). A resposta

neuroendócrina também foi observada reduzida em indivíduos que sofreram trauma na infância, ou pacientes com TEPT (112-116) enquanto não foram observadas diferenças relativas aos níveis de cortisol em resposta ao estresse em indivíduo com Transtorno de déficit de atenção e hiperatividade (TDAH) quando comparados com controles saudáveis (117). O mesmo pôde ser observado para indivíduos com transtorno de ansiedade social (115) e esquizofrenia (118).

Diante de diversos estudos utilizando TSST como ferramenta para análise da reatividade ao estresse em diferentes transtornos psiquiátricos (em que o estresse possui um importante papel na etiologia dos mesmos) chama a atenção o fato de não haver nenhum estudo analisando reatividade ao estresse através do TSST em pacientes com TB, justificando a presente tese. Além disso, os poucos testes que analisaram a reatividade ao estresse em pacientes com TB levaram em conta apenas parâmetros neuroendócrinos (medidas de função do eixo HPA).

2. Justificativa

Os dados da literatura demonstram um papel importante do estresse para a suscetibilidade e desenvolvimento de transtornos de humor. Mecanisticamente, alterações no sistema imune e endócrino (representado pelo eixo HPA) parecem possuir importante papel nos efeitos do estresse e suas complicações. Entretanto, existem ainda muitas lacunas a serem preenchidas como, por exemplo, quais alterações específicas ocorrem imunologicamente falando. Diante destes dados torna-se relevante analisar duas diferentes situações onde o estresse parece apresentar um papel relevante para a suscetibilidade e desenvolvimento de transtornos de humor: os efeitos da exposição ao estresse crônico durante o desenvolvimento (através de modelo animal), e a resposta ao estresse agudo de indivíduos com transtorno de humor já existente, nesse caso o Transtorno Bipolar tipo I.

3. Objetivos

3.1. Objetivo Geral

Analisar os efeitos neuroimunoendócrinos do estresse durante o desenvolvimento (em modelo animal) e na idade adulta, em mulheres com transtorno bipolar tipo I.

3.2. Objetivos Específicos

Exposição ao estresse no desenvolvimento

1. Analisar a expressão de parvalbumina em interneurônios no cérebro de ratos expostos à separação materna (modelo de estresse na infância).
2. Analisar níveis periféricos de citocinas inflamatórias em animais expostos à separação materna.
3. Analisar os efeitos da administração central de Interleucina 10 (IL-10) no cérebro de ratos expostos à separação materna.

Resposta ao estresse agudo em indivíduos com Transtorno Bipolar tipo I

4. Analisar a reatividade fisiológica ao estresse experimental através da avaliação da frequência cardíaca e níveis salivares de cortisol.
5. Avaliar subtipos linfocitários e marcadores de ativação celular antes e após o protocolo de estresse agudo experimental.
6. Avaliar a sensibilidade das células T periféricas aos glicocorticoides antes e após o protocolo de estresse agudo experimental.

7. Analisar a ativação do fator de transcrição NF-κB através da fosforilação de sua subunidade p65 antes e após o protocolo de estresse agudo experimental.
8. Analisar a fosforilação de proteínas cinases ativadas por mitogenos (MAPKs) antes e após o protocolo de estresse agudo experimental.

4. Hipóteses

1. A exposição ao estresse no desenvolvimento resulta em ativação imune periférica.
2. A ativação imune periférica afeta o correto desenvolvimento e maturação do cérebro.
3. Os linfócitos dos pacientes com transtorno bipolar apresentam uma sensibilidade alterada aos glicocorticoides quando comparados aos indivíduos controle.
4. Os linfócitos de pacientes com transtorno bipolar apresentam um perfil de ativação celular.
5. Pacientes bipolares apresentam alterações funcionais do eixo HPA após o estresse agudo.
6. Pacientes bipolares apresentam respostas imunes exacerbadas após exposição ao estresse agudo.

5. Capítulo 5: Artigo Científico #1

Brain, Behavior, and Immunity 28 (2013) 218–226

Contents lists available at SciVerse ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Evidence for a neuroinflammatory mechanism in delayed effects of early life adversity in rats: Relationship to cortical NMDA receptor expression ☆

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ARTICLE INFO

Article history:
Received 15 October 2012
Received in revised form 26 November 2012
Accepted 26 November 2012
Available online 1 December 2012

Keywords:
Adolescence
Interleukin-10
Inflammation
Parvalbumin
Stress
NMDA

ABSTRACT

Postnatal maternal separation in rats causes a reduction of GABAergic parvalbumin-containing interneurons in the prefrontal cortex that first occurs in adolescence. This parvalbumin loss can be prevented by pre-adolescent treatment with a non-steroidal anti-inflammatory drug that also protects against excitotoxicity. Therefore, the neuropsychiatric disorders associated with early life adversity and interneuron dysfunction may involve neuroinflammatory processes and/or aberrant glutamatergic activity. Here, we aimed to determine whether delayed parvalbumin loss after maternal separation was due to inflammatory activity, and whether central administration of the anti-inflammatory cytokine interleukin (IL)-10 could protect against such loss. We also investigated the effects of maternal separation and IL-10 treatment on cortical NMDA receptor expression. Male rat pups were isolated for 4 h/day between postnatal days 2–20. IL-10 was administered intracerebroventricularly through an indwelling cannula between P30 and 38. Adolescent prefrontal cortices were analyzed using Western blotting and immunohistochemistry for parvalbumin and NMDA NR2A subunit expression. We demonstrate that central IL-10 administration during pre-adolescence protects maternally separated animals from parvalbumin loss in adolescence. Linear regression analyses revealed that increased circulating levels of the pro-inflammatory cytokines IL-1β and IL-6 predicted lowered parvalbumin levels in maternally separated adolescents. Maternal separation also increases cortical expression of the NR2A NMDA receptor subunit in adolescence, which is prevented by IL-10 treatment. These data suggest that inflammatory damage to parvalbumin interneurons may occur via aberrant glutamatergic activity in the prefrontal cortex. Our findings provide a novel interactive mechanism between inflammation and neural dysfunction that helps explain deleterious effects of early life adversity on prefrontal cortex interneurons.

1. Introduction

Exposure of the immature brain to stressful situations affects maturation and results in neuronal dysfunction culminating in psychiatric disorder susceptibility later in life (Andersen and Teicher, 2008; Davey et al., 2008; Heim and Nemeroff, 2001; Kessler et al., 1997; Kohut et al., 2009; Teicher et al., 2006). The delayed emergence of disorders after early life adversity makes it difficult to determine mechanistic cause due to intervening variables found in clinical studies. Animal studies help clarify causality through the use of experimental postnatal stress exposure. Daily removal of rat pups from their mothers (e.g., maternal separation; MS) during the neonatal period is an ethologically-relevant rodent model of early life adversity (Lehmann and Feldon, 2000). We (Brenhouse and Andersen, 2011b) and others (Chocyk et al., 2010; Jahng et al., 2010; Macri et al., 2009) have reported that MS leads to neuronal dysfunction that first manifests in adolescence, which is consistent with the delayed appearance of several disorders after early life adversity (Teicher et al., 2009).

Adolescence is an important period of brain development due to increased neuroanatomical rearrangement (Andersen et al., 2000). The prefrontal cortex (PFC) is a particularly late-maturing region (Alexander and Goldman, 1978), where many stress-induced changes in the PFC have delayed effects due to its late and protracted developmental profile (Alexander and Goldman, 1978; Andersen, 2008). Several of these changes specifically involve the prelimbic region (plPFC) (Diorio et al., 1993; Radley et al., 2009). Recent research shows that consequences of MS in the plPFC typically manifest in adolescence (Chocyk et al., 2010; Helmeke et al., 2008) or adulthood (Stevenson et al., 2008; Wilber et al., 2009).

☆ *Support*: NARSAD Young Investigator Award (2010–2012) to HCB.
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0889-1591/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbi.2012.11.012

The PFC is involved in cognition, decision-making, and behavioral control. We recently observed that working memory is impaired in adolescence after MS, in conjunction with a loss of parvalbumin (PVB)-positive GABAergic interneurons in the plPFC (Brenhouse and Andersen, 2011b). Loss of PVB can impair cognitive processes such as working memory and is related to several psychiatric disorders including schizophrenia (Lewis et al., 2005; Wilson et al., 1994). PVB-containing interneurons are influenced by glutatmatergic N-methyl d-aspartate receptor (NMDAR) activity; for example, PVB expression has been associated with increased susceptibility to NMDAR-mediated neurotoxicity (Hartley et al., 1996). Indeed, aberrant glutamatergic activity is a widely studied mechanism for interneuron dysfunction in schizophrenia (Coyle, 2004; Coyle et al., 2003). It is therefore possible that MS-induced PVB loss involves glutamatergic processes. However, the underlying cause of delayed dysfunction that is set in motion by early life adversity is unclear.

We recently showed that MS increased expression of the inflammatory mediator cyclooxygenase-2 (COX-2) in the adolescent plPFC (Brenhouse and Andersen, 2011b). MS-induced PVB loss and working memory impairment are prevented by systemic COX-2 inhibition during pre-adolescence, suggesting involvement of inflammation in PVB loss. However, COX-2 is a multifunctional signaling molecule that could lead to PVB loss via increased inflammatory cytokine activity, or from oxidative stress or excitotoxicity that are separate from cytokine activity (Madrigal et al., 2003; Schiavone et al., 2009; Takadera et al., 2002). These possible mechanisms for MS-induced PVB loss are all conceivable, given their involvement in neuropsychiatric disorders (Liang et al., 2007; Muller and Dursun, 2011). MS has been proposed to stimulate proinflammatory processes that further sensitize stress and inflammatory responses later in life (Hennessy et al., 2010). We observed a baseline increase in COX-2 expression following MS without any subsequent stress or proinflammatory exposure (Brenhouse and Andersen, 2011b), thus it is possible that the transition through puberty itself could kindle an inflammatory response in previously sensitized subjects. For example, activity of the inflammatory mediator fatty acid amide hydroxylase (FAAH), has been reported to transiently increase in the PFC at P45, with lower activity at P35 and at P50 (Lee et al., 2012). Gonadal hormone changes during puberty have also been suggested to provoke inflammatory activity (Leposavic and Perisic, 2008).

Inflammatory cytokines in the periphery activate microglia, resulting in expression of inflammatory mediators locally in the brain. IL-1β and IL-6 are two main proinflammatory cytokines produced by neurons with an important role in neuroendocrine and behavioral function (Avital et al., 2003; Leonard and Maes, 2012). Effects of such proinflammatory cytokines are counteracted by anti-inflammatory cytokines such as IL-10 (Bachis et al., 2001). Neuroinflammation can cause excessive NMDAR activation (Suyama et al., 2001), which can positively feedback and lead to additional neuroinflammation (Chang et al., 2008; Hennessy et al., 2010; Nair et al., 2006) and neuronal damage (Muller et al., 2009). Uncovering glutamatergic and/or inflammatory mechanisms that may underlie effects of adverse early life events could aid development of preventive treatments against neuronal damage and clinical symptoms observed in adolescence.

Here, we aimed to determine whether MS-induced PVB loss was due to a neuroinflammatory mechanism (e.g., IL-1β and IL-6) that was active during adolescence, and could therefore be prevented with a centrally administered anti-inflammatory cytokine during a critical window of treatment. Secondly, we examined whether MS-induced PVB loss was related to changes in PFC NMDAR expression. If MS alters NMDAR expression in the PFC, then PVB interneurons may be more vulnerable to glutamatergic damage in response to a proinflammatory state.

2. Materials and methods

2.1. Subjects

Pregnant female multiparous Sprague–Dawley rats (250–275 g) were obtained from Charles River Laboratories (Wilmington, MA) on day 13 of gestation. The day of birth was designated as postnatal day 0 (P0). At P2, litters were culled to 10 pups (7 males and 3 females), and litters were randomly assigned to either a maternal separation group (MS Group) or animal facility reared control group (CON Group). Pups in the MS Group were isolated for 4 h per day between P2–20, and kept in a thermoneutral environment at a constant temperature of 35–36 °C maintained by a circulating water bath. From P15 to P20 pups have homeothermic capacity and therefore were kept in small isolated cages for separation. This procedure is identical to procedures used previously by this laboratory (Andersen et al., 1999; Andersen and Teicher, 2004) and similar to others (Plotsky and Meaney, 1993). Pups in the CON Group were not disturbed after P2, except for routine weekly changes in cage bedding, during which all pups were weighed. Rats were housed with food and water available *ad libitum* in constant temperature and humidity conditions on a 12 h light/dark cycle (light period 0700–1900). Rats were weaned on P21–22, and group-housed with same-sex littermates with 3–4 rats/cage until experimentation. Only one rat per litter was used per condition to avoid litter effects. MS condition did not affect growth rate of the pups (Fig. S1). Rats were treated from P30 to P38 and were tested at P40. The treatment age was chosen as a pre-pubescent phase that corresponds to pre- to early adolescence. The testing age was chosen as a solidly adolescent age, since the convergent definition of adolescence in rats is P35–60 (Brenhouse and Andersen, 2011a; McCutcheon and Marinelli, 2009). P40 is an age at which several developmental changes in PFC have been reported, and is an age of onset of sexual maturity, defined by balano-preputial separation in male rats (Brenhouse and Andersen, 2011a; McCutcheon and Marinelli, 2009). Only male rats were used in these studies to directly expand on our previous findings using systemic COX-2 inhibition (Brenhouse and Andersen, 2011b).

These experiments were conducted in accordance with the 1996 Guide for the Care and Use of Laboratory Animals (NIH) and were approved by the Institutional Animal Care and Use Committee at McLean Hospital.

2.2. Cannulation surgery

On P28, rats were anesthetized with ketamine/xylazine (80/12 mg/kg; i.p.) and were implanted with unilateral 26-gauge stainless steel guide cannulae (Plastics One, Roanoke VA) above the right lateral ventricle [stereotaxic coordinates AP: −0.2; ML: −0.8; DV: −2.7 (Sherwood and Timiras, 1970)]. Animals were given 2 full days after surgery to recover before experimental procedures commenced.

IL-10 Microinjection: Every other day from P30 through P38, rats were infused with 1 μL vehicle or IL-10 (Sigma–Aldrich, St. Louis, MO; 50 ng/1 μL), using an injector cut to protrude 1 mm beyond the guide cannula (Plastics One). Injections were completed manually over 1 min, and the injector was left in place for an additional minute to ensure diffusion into the ventricle. This schedule of treatment was identical to the schedule of systemic COX-2 inhibition used in our previous studies (Brenhouse and Andersen, 2011b). The dose of IL-10 was chosen based on previous studies using i.c.v. IL-10 (Hennessy et al.; Perkeybile et al., 2009).

2.3. Western blotting

MS and CON rats were treated with IL-10 or vehicle and were sacrificed by rapid decapitation at P40, with simultaneous trunk-blood collection for use in ELISAs (below). Western immunoblots of the plPFC were analyzed as previously described (Brenhouse and Andersen, 2011b) from rats of each group (n = 6–7/Treatment and Condition of MS and CON). The plPFC was dissected on ice, and was sonicated in 1% SDS solution containing a protease inhibitor cocktail (Pierce, Rockford, IL). Protein content was determined by a Bradford assay (BioRad, Hercules CA). Thirty-five micrograms of protein were loaded into a 4–12% Bis-Tris polyacrylamide gel and subjected to SDS–PAGE. After protein separation, the samples were transferred to a nitrocellulose membrane and probed for PVB protein using rabbit polyclonal anti-PVB IgG (1:500, Thermo-Fisher Scientific, Bellerica MA) and actin (to control for loading protein) using mouse polyclonal anti-actin IgG (1:10,000, MP Pharmaceuticals, Aurora OH). Membranes were then incubated with anti-rabbit and anti-mouse secondary antibodies conjugated with horseradish peroxidase (1: 2000, Sigma). Western immunoblotting was also performed to analyze NR1 and NR2A using mouse monoclonal anti-NR1 (1:500, Millipore, Temecula CA) and rabbit polyclonal anti-NR2A (1:500, Millipore) with anti-mouse and anti-rabbit secondary antibodies (1:2000, Sigma). We have shown the specificity of the PVB antibody in previous reports (Brenhouse and Andersen, 2011b). Specificity of the NR1 and NR2A antibodies is shown in Supplementary Fig. S2.

Immunoreactivity was visualized by enhanced chemiluminescent detection (West Pico Kit; Pierce, Rockford, IL). Optical densities of bands were measured using ImageJ software and normalized with actin. On some occasions, contrast settings were adjusted evenly across entire membrane images (which always included equal representations of all groups), only to more clearly demarcate bands. Images were not digitally processed otherwise. Two–three Western blot runs were completed on all subjects for each protein, and averages were taken of all runs for each subject. Group differences were determined for each protein with 2-way (Condition × Treatment Group) analyses of variances (ANOVA). One-way ANOVAs or LSD post hoc t-tests compared group means after interactions were found.

2.4. Immunofluorescence

To confirm and localize the observed changes in PVB and NR2A protein content, we performed immunohistochemical analysis of the plPFC in a separate cohort of adolescent MS and CON rats that were treated with either IL-10 or vehicle as described above (n = 6/Condition and Treatment Group). At P40, rats were deeply anesthetized and intracardially perfused with ice-cold 4% paraformaldehyde. Tissue was processed with standard immunohistochemical methods (Berretta et al., 2004). Briefly, 40 μm frozen sections were double-labeled with a monoclonal mouse antibody raised against PVB (1:10,000, Sigma, St. Louis MO) and a polyclonal rabbit antibody raised against the NR2A NMDAR subunit (1:1000; Millipore). (Brenhouse and Andersen, 2011b; Liu and Wong-Riley, 2010) Sections were then incubated with anti-mouse Alexa 563-coupled IgG (1:400; Molecular Probes, Grand Island, NY) and anti-rabbit Alexa 488-coupled IgG (1:400, Molecular Probes). All steps were preceded and followed by washes in PBS-Tx. Separate wells were run in the absence of primary antibody to control for non-specific staining. Sections were counterstained with DAPI to visualize cell nuclei, then mounted on gelatin-coated slides and coverslipped with Fluoromount (Thermo Fisher Scientific Inc., Waltham MA). Stereo Investigator Image Analysis System (MBF Bioscience, Williston VT) was used to estimate the density of PVB-positive, NR2A-positive, and PVB + NR2A colocalized cells. The plPFC in 3–4 serial coronal sections (intersection interval 480 μm) per animal were analyzed (Brenhouse and Andersen, 2011b). In each section, the entire plPFC was outlined at 2.5× magnification and the total number of immunoreactive (ir) cells was measured at 20× exclusively within the outlined area. PVB-ir was visualized using a red channel and NR2A-ir was visualized using a green channel. Cells colocalized with both PVB and NR2A were confirmed using an overlay of both channels (see Fig. 6). DAPI-stained nuclei were viewed to aid in verification that individual cells were being counted, when necessary. Investigators were strictly blinded to the conditions for all analyses. Tracings of the plPFC boundaries were used for calculation of the surface area (a) in each section. The density of ir (cells/mm$^{2)}$ was based on the total number of ir cells divided by Σa for each subject (the sum of areas obtained from all outlined regions). Volume of the plPFC was calculated according to the Cavalieri principle (Cavalieri, 1966) as $v = z \times i \times \Sigma a$, where z is the thickness of the section (40 μm) and i is the section interval (12; i.e., number of serial sections between each section and the following one within a compartment). Group differences were determined by 2-way (Condition × Treatment Group) ANOVA. Post hoc t-tests with LSD correction compared group means after interactions were found.

Enzyme-Linked Immunosorbent Assay (ELISA): Trunk blood was centrifuged at 1200 rpm for 10 min (4 °C) in order to separate out plasma. Plasma was collected and IL-6 or IL-1β was measured using commercially available rat ELISA kits (BD Biosciences, San Diego CA). All data are expressed as pg of IL-6/mL or IL-1β/mL plasma. Group differences were determined by 2-way (Condition × Treatment Group) ANOVA. Regression analyses evaluated the relationship between PVB, NR2A, and cytokine levels in subjects where all proteins were measured (SPSS v 17.0; Evanston, IL).

3. Results

3.1. Plasma cytokines

In order to confirm that MS leads to increased immune activation during adolescence, plasma was analyzed for levels of the inflammatory cytokines IL-6 and IL1-β. MS adolescents have significantly more circulating IL-1β (Fig. 1a; Main Effect of Condition: $F[1,20] = 4.54$; $p = 0.046$) and IL-6 (Fig. 1b; Main Effect of Condition: $F[1,22] = 26.8$; $p < 0.001$) compared with control animals. I.c.v. treatment with IL-10 had no effect on plasma IL-1β levels. However, IL-10 treatment did reduce plasma IL-6 levels compared to vehicle treatment (Condition × Treatment Interaction: $F[1,22] = 4.4$; $p = 0.047$; Veh v IL-10 t-test: $t[11] = 2.465$; $p = 0.021$).

3.2. plPFC PVB

3.2.1. Western blotting

A significant Group × Treatment interaction ($F[1,22] = 12.28$; $p = 0.001$) revealed that MS leads to a reduced level of PVB in the adolescent plPFC that is prevented by pre-adolescent treatment with IL-10 (Fig. 2). Post hoc one-way ANOVAs show that vehicle-treated MS animals had lower levels of PVB than both vehicle-treated controls ($F[1,11] = 11.1$; $p = 0.007$) and IL-10 treated MS counterparts ($F[1,12] = 10.7$; $p = 0.007$).

3.2.2. Immunohistochemistry

Protection of PVB neurons by IL-10 treatment was confirmed by comparing PVB-positive cell densities between groups. A main effect of both Group ($F[1,22] = 11.8$; $p = 0.002$) and Treatment ($F[1,22] = 6.6$; $p = 0.018$) was found (Fig. 3). Despite a lack of a significant Group × Treatment interaction, these effects were driven by a reduction in PVB cells in vehicle-treated MS animals (t-test

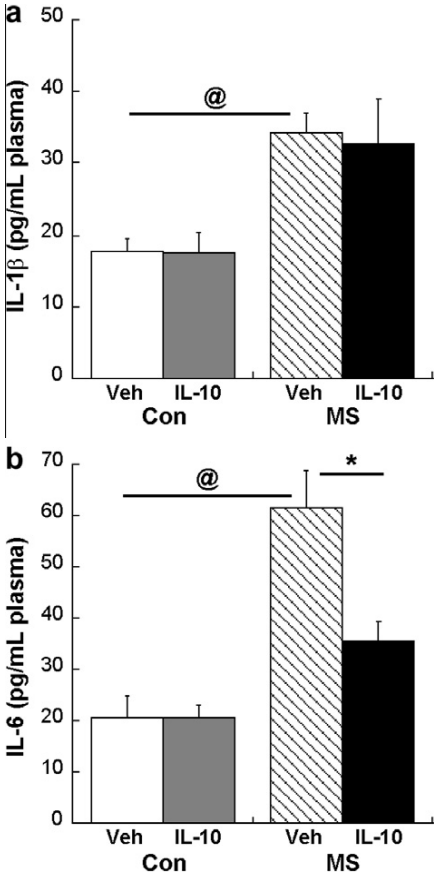

Fig. 1. (a) Circulating plasma IL-1β is affected by maternal separation (MS), but not i.c.v. IL-10 administration. Graphic representation of IL-1β protein levels in two groups after vehicle (Veh) or IL-10 administration i.c.v. during pre-adolescence. (b) Circulating plasma IL-6 is increased in MS adolescents, and IL-10 reduces IL-6 levels after MS. Con: control. Means ± SE are presented. @$p < 0.05$ between Con and MS vehicle-treated groups; *$p < 0.05$ between Veh and IL-10 groups.

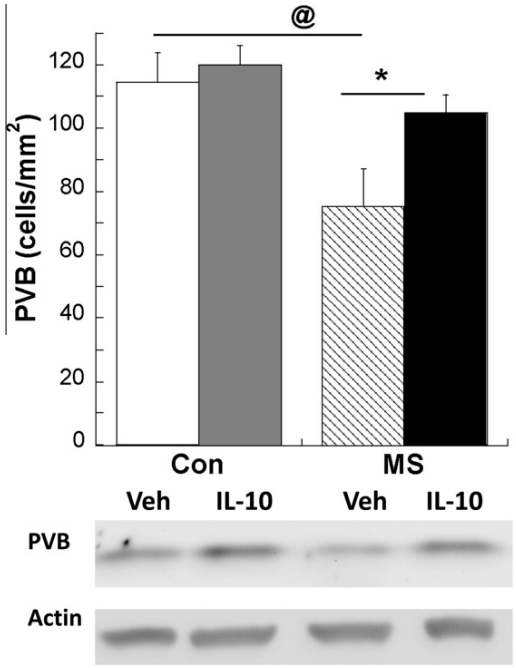

Fig. 2. Effects of pre-adolescent i.c.v. IL-10 administration on parvalbumin (PVB) protein levels in the prefrontal cortex during adolescence after control (Con) or maternal separation (MS) conditions. (a) Means ± SE presented. @$p < 0.05$ difference vehicle-treated Con and MS subjects and *$p < 0.05$ between vehicle (Veh) and IL-10 treated subjects. A representative Western blot is shown below graph. All representative bands were taken from the same membrane.

with LSD correction: $t[12] = 3.62$; $p = 0.004$), but not in IL-10 treated animals ($p = 0.09$).*plPFC NR1*: MS had no effect on NR1 expression in the plPFC in vehicle-treated animals (Fig. 4). Due to tissue availability, IL-10-treated animals were not analyzed for NR1.

3.3. plPFC NR2A

3.3.1. Western blotting

MS caused an increase of NR2A expression in adolescence (Fig. 5; Main Effect of Group: $F[1,22] = 7.32$; $p = 0.013$). IL-10 treatment decreased NR2A expression overall (Main Effect of Treatment: $F[1,22] = 5.56$; $p = 0.028$). The main effect of Treatment appears to be driven by MS subjects rather than CON subjects, as *t*-tests with LSD correction revealed a significant Treatment effect in MS subjects ($t[14] = 2.53$; $p = 0.021$) but not Con subjects ($p = 0.19$).

3.3.2. Immunohistochemistry

Overexpression of NR2A in MS adolescents was confirmed by comparing NR2A-positive cell densities between groups. A Group × Treatment interaction ($F[1,20] = 6.1$; $p = 0.022$; Fig. 6a) revealed that vehicle-treated MS subjects displayed a greater density of NR2A-positive cells compared to CON subjects ($t[10] = 4.54$; $p = 0.001$), while IL-10 reversed the MS-induced increase of NR2A ($t[11] = 2.31$; $p = 0.041$).

In order to determine whether the overexpression of NR2A was occurring on PVB-positive neurons specifically, the density of neurons in the plPFC that were double-labeled with NR2A + PVB, as well as NR2A-positive neurons that were not colocalized with PVB, were compared between groups. Since PVB interneuron density itself was different between groups, we calculated the percentage of PVB-positive cells that co-expressed NR2A for each subject, and compared those percentages between groups. MS effects on NR2A were apparent on both PVB-positive (Main Effect of Group: $F[1,18] = 5.36$; $p = 0.033$) and PVB-negative (Main Effect of Group: $F[1,18] = 7.97$; $p = 0.01$) cells (Fig. 6b and c). Treatment with IL-10 also prevented MS-induced NR2A over-expression on both PVB-positive cells (Group × Treatment interaction: $F[1,17] = 7.8$; $p = 0.012$) and PVB-negative cells (Group × Treatment interaction: $F[1,21] = 4.68$; $p = 0.042$). Therefore, effects of both MS and of IL-10 were not cell-type specific.

3.4. Relationships between circulating cytokines, PVB and NR2A

Direct linear relationships were found between circulating cytokine levels and PVB levels in the PFC. In vehicle-treated MS animals, plasma IL-1β was negatively correlated with PFC PVB levels. MS adolescents with higher IL-1β displayed lower amounts of PVB ($R = 0.838$; $R^2 = 0.702$; $p = 0.019$; Fig. 7a). CON adolescents, however, showed no relationship between PVB and IL1β ($p = 0.60$; Fig. 7b). Pre-adolescent i.c.v. treatment with IL-10 eliminated the relationships between plasma IL-1β and PFC protein levels ($p = 0.521$; not shown).

IL-6 levels did not predict PVB levels in vehicle-treated subjects ($p = 0.216$; not shown). However, IL-10 treatment appeared to precipitate a relationship between IL-6 and PVB in MS subjects ($R = 0.854$; $R^2 = 0.729$; $p = 0.031$ Fig. 7c). In other words, it appears that IL-10 had treatment effects on both IL-6 and PVB in the same

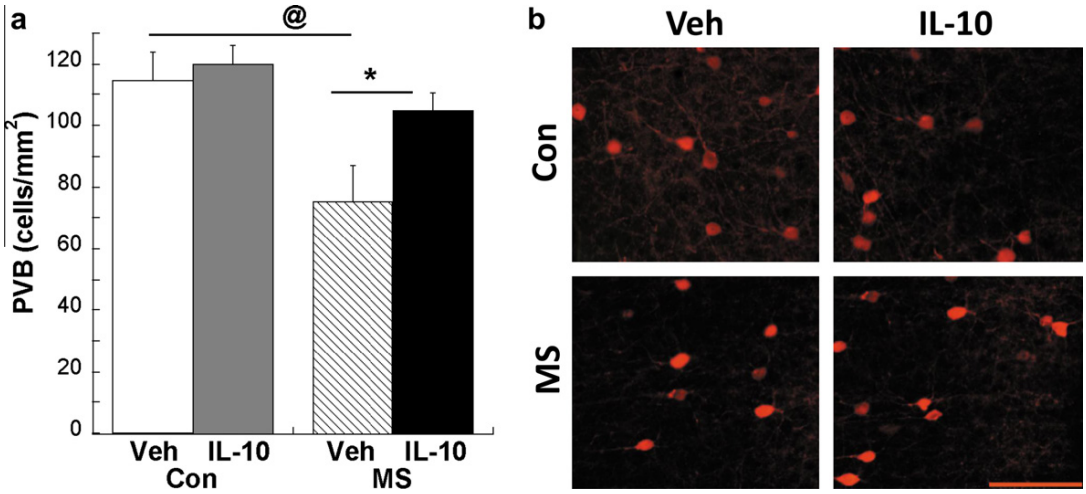

Fig. 3. (a) Graphic representation of parvalbumin (PVB)-immunoreactivity after control rearing (Con) or maternal separation (MS) with pre-adolescent vehicle (Veh) or IL-10 treatment; @$p < 0.05$ difference between Con and MS groups; *$p < 0.05$ difference between Veh and IL-10 groups, although no Group × Treatment interaction was found. Means ± SE presented. (b) Representative PVB-immunoreactive cells within the prefrontal cortex of adolescents in each group. All images taken at 20× from the same location and layer (5) within the prefrontal cortex. bar: 100 μm.

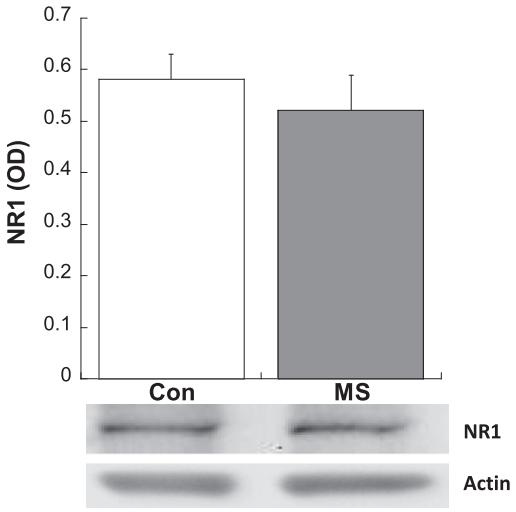

Fig. 4. NR1 subunit protein levels in the prefrontal cortex were unchanged by MS. Means ± SE presented; representative Western blot is shown below graph. All representative bands were taken from the same membrane.

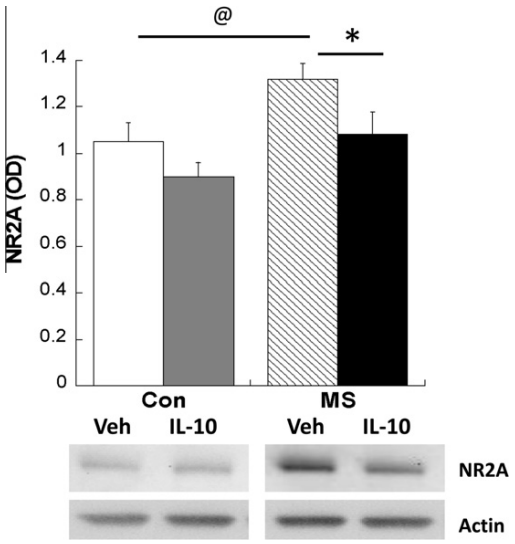

Fig. 5. Effects of pre-adolescent i.c.v. IL-10 administration on NR2A subunit protein levels in the prefrontal cortex during adolescence after control (Con) or maternal separation (MS) conditions. Means ± SE presented. @$p < 0.05$ difference vehicle-treated Con and MS subjects and *$p < 0.05$ between vehicle (Veh) and IL-10 treated subjects, though no Group × Treatment interaction was found. A representative Western blot is shown below graph. All representative bands were taken from the same membrane.

animals. This relationship was not apparent in IL-10 treated CON animals ($p = 0.733$, Fig. 7d). In contrast to their relationships with PVB, circulating IL-1β or IL-6 did not significantly predict NR2A levels in any group (data not shown).

4. Discussion

We demonstrate that pre-adolescent i.c.v administration of the anti-inflammatory cytokine IL-10 after MS prevents delayed loss of PVB and over-expression of the NR2A NMDAR subunit in the plPFC. MS during the first 3 weeks of life decreased PVB protein amounts and PVB-positive cell densities in the plPFC in adolescence, as we previously described (Brenhouse and Andersen, 2011b). Here, we report that NR2A overexpression is a second neural consequence of MS in the plPFC during adolescence. Thirdly, MS animals displayed higher plasma levels of the pro-inflammatory cytokines IL-1β and IL-6, which were directly related to PVB. This suggests that a pro-inflammatory state leads to decreased PVB in the plPFC of adolescent subjects exposed to early adversity.

The effects of MS on circulating cytokines have scarcely been investigated in adolescence. Some animal (Avitsur et al., 2006) and clinical data (Carpenter et al., 2010; Danese et al., 2007) suggest that adults with a history of early adversity exhibit inflammation and vulnerability for inflammatory disease. Here, increased IL-1β and IL-6 in vehicle-treated MS adolescents demonstrates a baseline pro-inflammatory tone during this tumultuous developmental phase. While IL-10 administration directly into the brain did not affect circulating IL-1β levels, i.c.v. IL-10 did prevent circulating IL-1β from predicting PVB loss. This treatment effect was

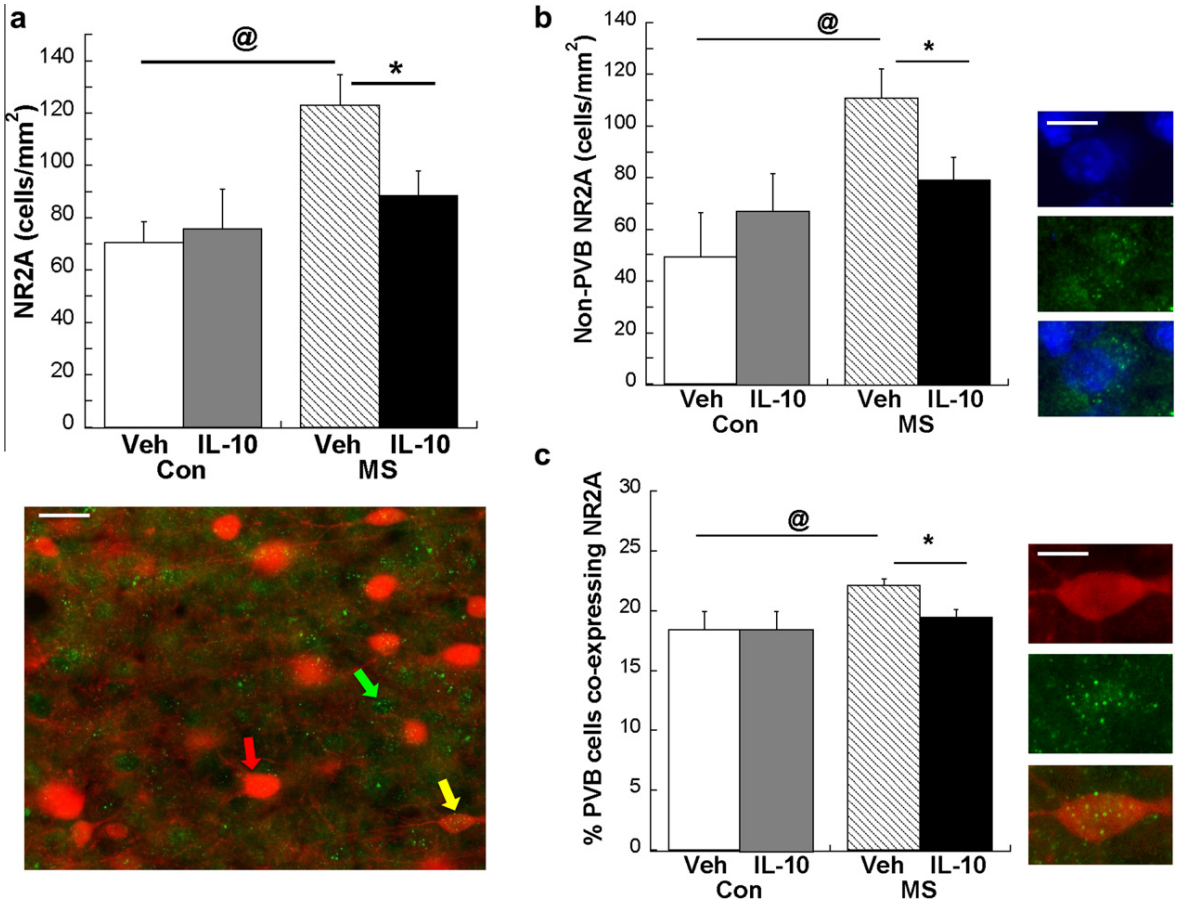

Fig. 6. Graphic representation of (a) density of total NR2A-ir cells, (b) density of NR2A-ir on non-PVB cells, or (c) percentage of parvalbumin (PVB) neurons that were colocalized with NR2A compared between groups. Con: control rearing; MS: maternal separation; Veh: vehicle treatment. @$p < 0.05$ difference between Con and MS groups; *$p < 0.05$ difference between Veh and IL-10 groups. Means ± SE presented. Representative photomicrographs of each cell type counted are presented with each graph. (a) Displays NR2A alone (green arrow), NR2A + PVB (yellow arrow), or PVB alone (red arrow); scale bar: 20 μm. (b) Displays DAPI-stained nucleus (top), NR2A (middle), and an overlay; scale bar: 10 μm. (c) Displays PVB (top), NR2A (middle), and an overlay (bottom); scale bar: 10 μm. All photomicrographs are taken from the prelimbic prefrontal cortex. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

likely due to the prevention of a neuroinflammatory process that may be a consequence of peripheral inflammation. MS adolescents also exhibited increased circulating IL-6, which has been consistently linked to psychiatric disorders such as major depressive disorder and anxiety disorders (Hoge et al., 2009; O'Brien et al., 2004). Central cytokine activity has previously been shown to influence circulating IL-6 (De Simoni et al., 1990); here we observed that manipulating cytokine activity within the brain can potentially mitigate peripheral inflammatory activity after early adversity, since circulating IL-6 was reduced with central administration of IL-10. One possible explanation for this observation may be related to an intrinsic capability of cytokines to modulate HPA-axis activity (Smith et al., 1999; Tu et al., 2007) Given the interplay between immune and neuroendocrine system, IL-10 would stimulate the HPA-axis to increase adrenocorticotropic hormone and corticotropin releasing factor (Smith et al., 1999; Stefano et al., 1998) secretion, leading to systemic immunosuppression affecting circulating IL6-levels. However, we also cannot rule out the possibility that IL-10 entered the peripheral bloodstream from the cerebrospinal fluid, where it could have affected IL-6. Characteristically, inflammatory processes increase adhesion molecule expression, allowing cytokine leakage between CNS and periphery due to increased blood–brain barrier permeability (Banks, 2006; Dantzer, 2009; Elmquist et al., 1997). Notably, microgram levels of systemic IL-10 have previously been used to ablate fever response in LPS treated rats (Cartmell et al., 2003), therefore it appears less likely that the ng levels of IL-10 administered i.c.v in these studies had their effects systemically. While plasma IL-6 did not predict PVB levels in vehicle-treated MS adolescents as seen with Il-1β, MS subjects treated with IL-10 during pre-adolescence displayed a linear relationship between IL-6 and PVB. Taken together, it appears that IL-10 ameliorated MS effects on both IL-6 and PVB in the same animals. Notably, despite statistically significant correlations found between cytokines and PVB, the group sizes in these experiments were fairly small for regression analysis. Therefore confirmatory repetition of these findings will be useful.

Reduced PVB in the plPFC during adolescence may highlight a vulnerability to neuropsychiatric disorders that is produced by childhood adversity (Edwards et al., 2003; Heim and Nemeroff, 2001; Teicher et al., 2006). Clinically, it has been recently reported that schizophrenic patients who were subjected to childhood adversity were more likely to display a proinflammatory phenotype (Dennison et al., 2012). Here, we show empirically how neuroinflammation can be at the root of this vulnerability through a discrete cellular impairment that is consistent with impairments observed in schizophrenia. In addition to PVB deficits, we also observed NMDAR changes that were sensitive to anti-inflammatory treatment, and were themselves reminiscent of disorders such as schizophrenia (Woo et al., 2008) and animal models of antisocial personality (Bortolato et al., 2012).

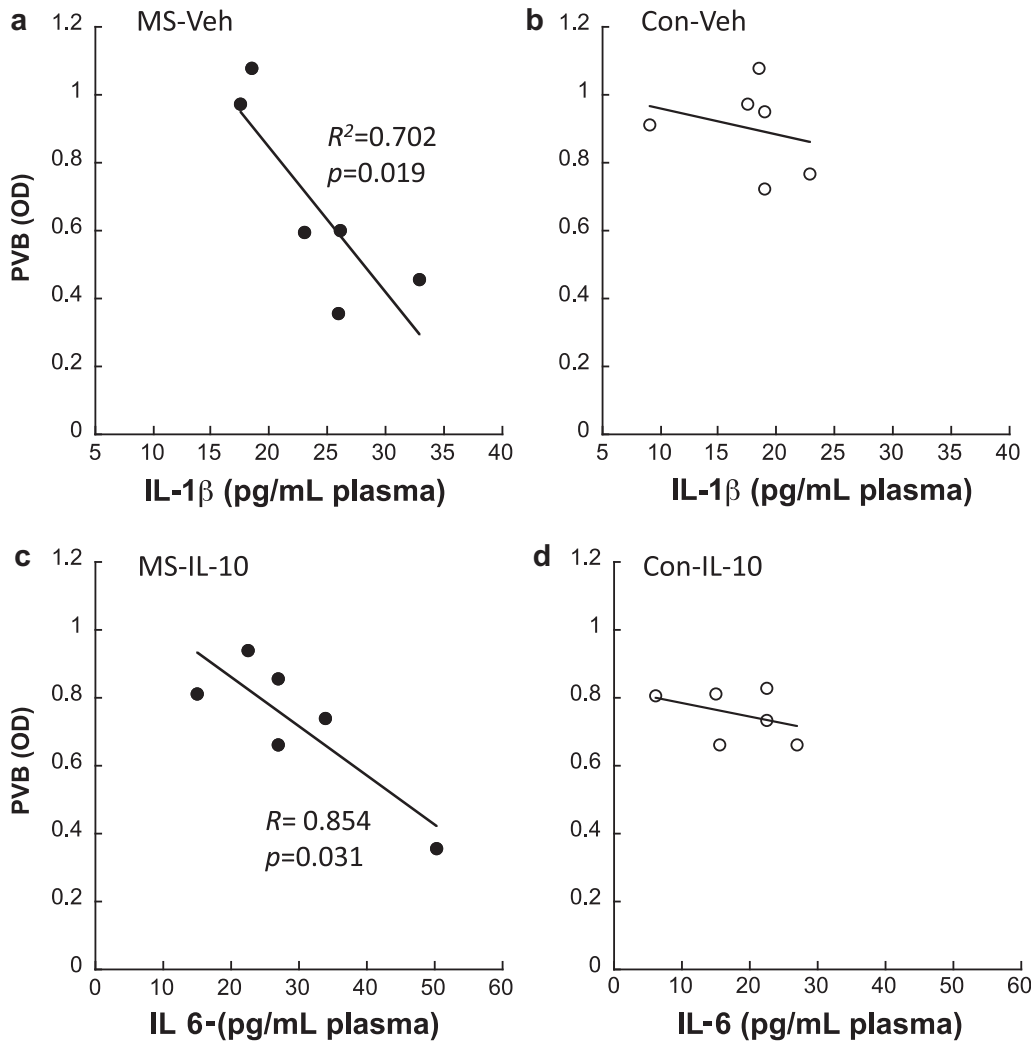

Fig. 7. (a) and (b): Linear regression analysis shows a direct relationship between plasma IL-1β and prefrontal cortex parvalbumin (PVB) levels in vehicle-treated maternal separation (MS; a) but not control (Con; b) adolescents. (c) and (d): Linear regression analysis shows a direct relationship between plasma IL-6 and PFC PVB levels in IL-10 treated MS (c) but not Con (d) adolescents. *Note*: 1–2 subjects per group were not included in regression analyses due to lack of within-subject measurement of both proteins.

Developmental events such as prenatal stress have been shown to affect NMDAR composition in the PFC (Fumagalli et al., 2009). We report here that MS, experienced early in neonatal development, also leads to later altered NMDAR composition in the PFC. Specifically, while NR1 subunit expression was unaltered by MS, NR2A expression was significantly higher in adolescents that had been subjected to MS. Since NR1 is the requisite subunit of NMDAR, these results suggest that NMDAR do not change in number in response to MS exposure, but rather increase in concentration of NR2A. Profound changes in channel kinetics could result from this change in composition, since NR2A subunits confer a shorter channel open time to NMDAR (Monyer et al., 1994) and are therefore predicted to produce a relative 'hypofunction' of the receptor. Further studies will test a working hypothesis that overexpression of NR2A leads to aberrant NMDAR activity within the plPFC, leading to damage of PVB interneurons from neuroinflammation-induced glutamate release during adolescence.

NR2A overexpression occurred on both PVB interneurons and other, non-PVB cells (potentially interneurons, glutamatergic projection neurons, and glia). IL-10 treatment effects on NR2A were also apparent on both PVB-positive and PVB-negative cells. Therefore, more investigation is needed into where exactly these NMDAR changes are having their effects. We hypothesize that cortical NR2A overexpression plays a role in PVB deficits after MS. Indeed, mounting evidence points to atypical NMDAR subunits during development as a potential cause of PVB loss. For example, sub-chronic treatment with agents that decrease NMDAR on PVB cells (Xi et al., 2009) or in general (Schmitt et al., 2003) lead to loss of PVB in the PFC. This susceptibility is consistent with reports that PVB interneurons are especially sensitive to NMDA-mediated damage, relative to other neuron populations in the PFC (Wang et al., 2008). Chronic blockage of the NR2A subunit in particular during a critical period of juvenile development decreases PVB in the neocortex (Zhang and Sun, 2011). While the explicit effects of NR2A *over*-expression have not yet been investigated, these previous reports demonstrate the importance of this subunit in PVB function. Neuroinflammatory processes may play a significant role in this interplay between NMDAR and interneuron health.

Pre-adolescent treatment with IL-10 prevented MS-induced alterations in adolescence. IL-10 regulates the expression and influence of proinflammatory cytokines including those affected during MS (Dantzer, 2004; Perkeybile et al., 2009), and has also been shown to have neuroprotective effects via its regulation of NMDAR signaling (Turovskaya et al., 2012). Here we observed that

this cytokine prevented MS effects on both NMDAR and PVB interneurons. Pro-inflammatory cytokine activity has been shown to augment expression of NR2A (Rai et al., 2012), which together with our data suggests that MS causes inflammation-associated changes in NR2A. However, it is important to note that despite observing IL-10-sensitive effects of MS on both NR2A and PVB, NR2A levels were not reliably predicted by circulating cytokines in regression analyses. Therefore NR2A overexpression does not clearly explain how MS-induced inflammation leads to PVB loss. NMDAR hypofunction has certainly been debated as an explicit cause of PVB loss (Benneyworth et al., 2011). That said, the interactions between IL-10 treatment and MS on both NR2A and PVB suggest that MS-induced neuroinflammation manifests in adolescence as changes in NR2A and PVB, which may be interrelated.

Exposure to adverse, stressful experiences during early postnatal development is known to induce a myriad of alterations in hypothalamic-pituitary axis activity (Rosenfeld et al., 1992), immune responsivity (Dimatelis et al., 2012; Hennessy et al., 2011), and hippocampal anatomy (Lajud et al., 2012) that are immediately apparent. We previously showed that the effect of MS on PFC PVB levels was delayed, as there were no differences between MS and Con subjects any earlier in development (Brenhouse and Andersen, 2011b). The reason why MS-induced neuroinflammation and its deleterious effects on PFC PVB are apparent during adolescence—despite the adverse environmental exposure occurring much earlier in development—is unclear, but is likely due to sensitization from early inflammatory activity (Bilbo and Schwarz, 2012; Hennessy et al., 2011), and may involve epigenetic changes (reviewed by McEwen (2008)). The current study focused on the vulnerable adolescent brain after early life adversity, therefore peripheral inflammation and/or NMDAR changes may first appear earlier in development, leading subsequently to PVB loss in adolescence. It will be important to determine when these changes occur and how they are set in motion by exposure to adverse events early in postnatal life.

Treatment aimed at reducing the negative sequelae of early life stress is sorely lacking. Clinically, we know that individuals that have been exposed to adverse events during development are relatively resistant to pharmacotherapy (e.g., fluoxetine) for depression (Nemeroff et al., 2003). Thus, treatment-resistant schizophrenics may also have an underlying abuse history that renders them less sensitive to standard interventions. Recent clinical reports (Dennison et al., 2012) and the current study point to neuroinflammation as a possible distinction between individuals with a history of early life adversity and those without such history. Targeting the proper mechanistic consequences of early life stress with early intervention in vulnerable individuals could help prevent maladaptive changes during adolescence, and consequentially protect from resulting psychiatric disorders that begin during this phase of development.

5. Financial disclosures

The authors report no competing financial interests or potential conflicts of interest.

Acknowledgments

The authors wish to thank NARSAD and the Shine Initiative for their funding to HCB. We would like to thank Nadja Freund, Ph.D. and Britta Thompson for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2012.11.012.

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6. Capítulo 6: Artigo Científico #2

DIFFERENTIAL NEUROENDOCRINE AND IMMUNE RESPONSES TO ACUTE PSYCHOSOCIAL STRESS IN TYPE 1 BIPOLAR DISORDER

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Conflicts of interest: All authors declare that there are no conflicts of interest.

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Abstract

Bipolar disorder (BD) has been associated with immune imbalance, including lymphocyte activation and increased pro-inflammatory cytokines. Immune activation is part of stress response, and psychosocial stress has been implicated in the pathogenesis of psychiatric disorders. Here, we investigated the neuroendocrine and immune responses to acute psychosocial stress challenge in BD. Thirteen euthymic participants with type 1 BD and 15 healthy controls underwent the Trier Social Stress Test protocol (TSST). Blood samples were collected before and after TSST. Lymphocytes were isolated and stimulated *in vitro* to assess lymphocyte activation profile, lymphocyte sensitivity to dexamethasone, mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signaling by flow cytometry. Heart rate and salivary cortisol levels were monitored across the task. BD participants exhibited blunted stress responses as shown by reduced heart rate and salivary cortisol levels in comparison to healthy controls. BD was also associated with reduction in the percentage of regulatory T cells, but with higher proportion of activated T cells. When compared to controls, patients showed increased lymphocyte MAPK p-ERK and p-NF-κB signaling after the stress challenge, but exhibited a relative lymphocyte resistance to dexamethasone. In conclusion, stress-related neuroendocrine responses are blunted, associated with increased immune activation and lower sensitivity to glucocorticoids in BD. An inability in reducing NF-κB and MAPK signaling following TSST could be underlying the immune imbalance observed in BD.

Keywords: bipolar disorder; psychosocial stress; inflammation; MAPK; NF-kappa B; lymphocyte subsets.

6.1. Introduction

Psychosocial stress is a well-known risk factor for several psychiatric disorders and it has been shown to affect both the onset and course of Bipolar Disorder (BD) (Kapczinski et al., 2008; Post and Leverich, 2006). Consistent with stress-related physiological changes, mood disorders have been associated with dysregulation of the neuroendocrine and immune systems (Kim et al., 2004; O'Brien et al., 2006). Although previous work demonstrated that hypothalamic-pituitary-adrenal (HPA) axis is deregulated in mood disorders (Ahrens et al., 2008; Spiliotaki et al., 2006; Stetler and Miller, 2011), the great majority of studies did not investigate stress reactivity.

Laboratory stress studies provide a unique opportunity to address the underlying mechanisms involved in stress reactivity. The Trier Social Stress Test (TSST), a validated laboratory psychosocial stress task, is commonly used to analyze biological changes due to controlled stress exposure (Kirschbaum et al., 1993). Social evaluative stressors, such as TSST, are capable of eliciting cortisol secretion, which can be used as an objective measure of HPA axis function (Dickerson et al., 2009; Kapczinski et al., 2008). Immune activation is also part of the stress response leading to exacerbation of several chronic inflammatory conditions (Buske-Kirschbaum et al., 2010; Buske-Kirschbaum et al., 2002; Buske-Kirschbaum et al., 2007; Ritz et al., 2011). However, stress reactivity is largely unknown in BD, and failure to mount adequate neuroendocrine responses following stress could be associated with detrimental overshooting immune responses.

A growing body of evidence suggests an immunological imbalance in BD, associated with a pro-inflammatory profile. Higher plasma levels of pro-inflammatory cytokines and soluble receptors have been described during manic (Barbosa et al., 2011; Brietzke et al., 2009; Kim et al., 2010; Kim et al., 2004; O'Brien et al., 2006; Ortiz-Dominguez et al., 2007)

or depressive episodes (Kim et al., 2004; O'Brien et al., 2006; Ortiz-Dominguez et al., 2007) in BD. Altered proportions of activated/regulatory lymphocyte subsets and differential intracellular signaling have been implicated in the immunological imbalance in BD (do Prado et al., 2013). Nuclear factor kappa B (NF-κB) is a pleiotropic transcription factor readily activated following different stimuli (Hayden and Ghosh, 2004), and the phosphorylation of the p65 subunit (p-p65) results in its translocation to the nucleus and consequent transcription of different pro-inflammatory genes. Bierhaus and colleagues (2002) reported increased NF-κB signaling after TSST as a mechanism converting social stress into immune activation in healthy individuals (Bierhaus et al., 2003). In this line, Pace and colleagues (2006) found increased p65 DNA binding major depression after TSST and a consequent increase in IL-6 plasma levels (Pace et al., 2006). Mitogen-activated protein kinase (MAPK) proteins are involved in many cellular processes such as differentiation, proliferation, activation and apoptosis, and may contribute to the immune alterations observed in BD (Johnson and Lapadat, 2002; Raman et al., 2007). Three major MAPK cascades are known, including the extracellular signal-regulated protein kinase (ERK), c-jun amino-terminal protein kinase/stress-activated protein kinase (JNK) and p38 (Raman et al., 2007). While phosphorylation of ERK1/2 is involved in cellular proliferation, differentiation, activation and survival, phosphorylated p38 is related to cellular anergy and pro-apoptotic fate (Johnson and Lapadat, 2002; Raman et al., 2007; Strniskova et al., 2002). Although changes in intracellular signaling events are likely to be involved with immune imbalance, there is no information regarding the role of acute psychosocial stress upon these molecules in BD.

Here, we investigated a comprehensive set of neuroendocrine and immune responses, including activation/regulatory cell profiles, lymphocyte sensitivity to glucocorticoids and intracellular cell signaling (MAPK and NF-κB), of euthymic subjects with BD type 1 and

healthy controls to an acute psychosocial stress challenge. It was hypothesized that blunted neuroendocrine responses to stress could be associated with immune activation in BD.

6.2. Methods

6.2.1. Subjects

Thirteen euthymic female participants with BD type I were recruited by convenience sampling at mental health facility in Porto Alegre, Brazil. Age- and sex-matched healthy controls (n=15) were also recruited to the study. All participants provided their written informed consent before inclusion in the study approved by the Ethical Committee of PUCRS. The BD type 1 diagnosis was based on a psychiatric clinical interview and confirmed with the *Structured Clinical Interview for DSM-IV-Axis I Disorder* (SCID-I) administered by a well-trained clinical psychologist and discussed with an expert psychiatrist. Severity of depressive and manic symptoms was assessed by the *Hamilton Depression Rating Scale* (HDRS) and the *Young Mania Rating Scale* (YMRS), respectively. All individuals were euthymic at the time of procedures. Euthymia was defined by YMRS and HDRS scores < 8 in the last 30 days (Clark et al., 2002). Exclusion criteria to both BD subjects and controls included: a) presence of major axis I psychiatric disorder such as psychotic disorder, mood disorder (for control group), anxiety disorder or substance related disorder according to SCID-I; b) history of a severe medical illness; c) history of brain injury; d) presence of systemic diseases or neurological disorder, e) pregnancy and f) use of any substance that may induce immune or endocrine changes (except psychopharmacotherapy for BD participants).

6.2.2. Trier Social Stress test (TSST)

The TSST is a standardized psychosocial stress protocol that elicit acute stress responses (cortisol and HR) and involves the delivery of a free speech (5 min) concerning

their suitability for employment in a mock job interview and mental arithmetic tasks (5 min) in front of a panel of judges and fake camera and video recorder (Kirschbaum et al., 1993). All recruited participants completed the protocol.

6.2.3. Heart rate

In order to assess the arousal response to TSST the heart rate (HR) was continuously recorded for subsequent 5 min intervals from 5 min before the task until 15 min after cessation of the task using a wireless chest heart rate transmitter (Polar, New York, USA). The HR was also measured 30 and 15 min before and 25 and 40 min after the task.

6.2.4. Cortisol analyses

Salivary cortisol levels were assessed in order to been used as an objective marker of stress-induced activation of the HPA axis (Kirschbaum et al., 1993). Saliva samples were collected with cotton rolls immediately before (5 min) and after (20 min) TSST. In addition, three different samples were collected (30, 15 and 5 minutes before task) during preparation phase as baseline values. During recovery phase salivary cortisol were assessed in two different points (30 and 60 minutes after task). After the protocol all samples were centrifuged and stored in -80^{o}C until analysis. Samples were analyzed in duplicates by radioimmunoassays (Coat-A-Count® Cortisol Kit - Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The sensitivity of this assay was estimated in 0.1 nM. The intra- and inter-assay coefficients of variation were less than 10%. Results from each of the sampling times were expressed in nmol/L. In addition, integrated salivary cortisol levels were estimated by the trapezoidal rule to calculate the area under the curve (AUC) and data were expressed as nM per liter per hour.

6.2.5. Blood collection and cell isolation

Twenty milliliters of peripheral blood were collected by venipuncture pre and post TSST and stored in EDTA tubes prior to analyses. The first sample was taken 30 minutes before the task, during preparation period. The second sample was taken at the end of recovery phase (40 min after stress). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation for 30 min at 900 g. Cells were counted by means of microscopy (100 x) and viability always exceeded 95%, as judged from their ability to exclude Trypan Blue (Sigma, St Louis, MO). PBMCs were resuspended in complete culture medium (RPMI-1640, supplemented with 0.5% gentamicine, 1% glutamine, 1% hepes, 0.1% fungizone, and 10% fetal calf serum; all from Sigma) and adjusted to yield a final concentration of $2x10^5$ cells/well.

6.2.6. Immunophenotyping of lymphocytes

A large panel of lymphocyte subpopulations was identified by multi-color flow cytometry. In order to evaluate specific lymphocyte subsets, cells were stained for 30 min with combinations of the following monoclonal antibodies: anti-CD3 FITC and PECy5 (T cells), anti-CD4 PE and FITC (Th cells), anti-CD8 PE (Tc cells), anti-CD19 PE (B cells), anti-CD56 FITC (NK cells), anti-CD28 FITC (regulatory T cells), anti-CD45RO FITC (memory T cells), anti-CD69 FITC (early activated cells), anti-FOXP3 PECy5 (regulatory T cells), anti-CCR7 Cy7 (memory T cells), anti-CD45RA FITC (naïve T cells), all from BD Biosciences, San Jose, CA, USA. Immediately after staining, cells were washed, resuspended and analyzed by flow cytometry. A minimum of 20,000 lymphocytes were identified by size (FSC) and granularity (SSC) and acquired with a FACS Canto II flow cytometer (BD Biosciences). Data were analyzed using the Flowjo 7.2.5 software (Tree Star Inc., Ashland, Or, USA).

6.2.7. Intracellular activated MAPKs and NF-κB in lymphocytes

Activated MAPKs and NF-κB were assessed by flow cytometry through the analyses of intracellular expression of phospho-p38, phospho-ERK and phospho-p65 (NF-κB) expression in T cells (Human T Cell Activation Kit, BD Biosciences) (do Prado et al., 2013). Cells were stimulated with 40 nM PMA and 1 μM IONO for 15 min (MAPK assay) and 10 μg/mL LPS (NF-κB assay; all from Sigma-Aldrich) for 45 min, harvested and immediately fixed and stored (-80 °C) in Cytofix solution (BD Biosciences). A minimum of 20,000 lymphocytes were identified by size (FSC) and granularity (SSC) and acquired with a FACS Canto II flow cytometer (BD Biosciences). Data were analyzed using the Flowjo 7.2.5 software (Tree Star Inc., Ashland, Or, USA).

6.2.8. Cellular activation and sensitivity to glucocorticoids

Cellular activation profile was analyzed by flow cytometry with cells stained for CD3 and CD25 markers. The lymphocyte glucocorticoid (GC) sensitivity was estimated by functional assays developed to measure the ability of steroids to suppress T-cell activation *in vitro* (Knijff et al., 2006). Briefly, PBMCs were cultured ($1.5x10^5$ cells) in RPMI medium with 10% FCS (Sigma-Aldrich), stimulated with 1% phytohemagglutinin (PHA, from Invitrogen, Carlsbad, CA, USA) and treated with increasing concentrations of dexamethasone (Sigma-Aldrich; 10^{-9} nM,10^{-8} nM, 10^{-7} nM and 10^{-6} nM), for 72h at 37°C and in a 5% CO_2 atmosphere. Activation profile is described by the percentage of CD3+CD25+ stimulated cells minus unstimulated cells. Cellular sensitivity data are represented by basal cell activation, with 100% (basal) corresponding to 1% PHA stimulation without dexamethasone.

6.2.9. Statistical analyses

Differences between continuous variables were analyzed by two-way analysis of variance (ANOVA) (Group X Time) or two-way repeated measures ANOVA when appropriate. Kruskal-Wallis was used when variables assumed a non-normal distribution. Multiple comparisons among levels were checked with Tukey post-hoc test. Differences between variables were also assessed by Student's *t* test or Mann-Whitney test, when indicated. Differences in proportions between groups were compared by means of the chi-square (χ^2) test. Relationships between variables were assessed by means of Pearson's product moment correlations. Statistical analyses were performed using the Statistical Package for Social Sciences, SPSS Statistics 18.0 software (SPSS Inc., Chicago, IL, USA). The significance level was set at $\alpha = 0.05$.

6.3. Results

6.3.1. Blunted sympathetic and neuroendocrine responses to TSST

Demographic and clinical characteristics of the sample are summarized in Table 1. Both groups were homogenous regarding age, gender, ethnicity, BMI and smoking habits. All BD individuals were under a multiple drug regimen (Table 1). Overall HR increased significantly during task (Fig. 1A), indicating that participants were indeed aroused by the psychosocial stress, $F(1,19) = 10.45$, $p<0.0001$. However, when BD subjects were compared to healthy controls the HR response was lower during task phase $F(1,19) = 6.84$, $p=0.01$. Integrated HR analysis (AUC) at baseline did not vary between the two groups. However, HR was found significantly reduced in BD subjects in response to stress ($t=2.28$; $p<0.05$; Fig 1C).

Next, we investigated whether the acute stress response was associated with altered HPA axis activation. Cortisol secretion increased significantly across the stress protocol in

healthy controls, F(1,15) = 4.51, p=0.001 (Fig. 1B) while such alteration was not observed for BD group (F(1,9)=1.27; p=0.29). Integrated cortisol levels (AUC) at baseline did not vary between the two groups (Fig. 1D), (t = 1.54; p=0.14). However, AUC cortisol in response to stress was found significantly reduced in BD subjects compared to healthy controls (t=2.62; p=0.01), indicating a blunted neuroendocrine response.

6.3.2. Differential changes in lymphocyte subsets following acute stress

We investigated different peripheral lymphocyte subsets associated with activation and regulatory cell profiles (Fig. 2). At baseline, increased percentages of activated T cells (CD4+CD25+^{low}) were observed in BD subjects compared to healthy controls, F(3,43)=5.43, p=0.01. In contrast, BD subjects had reduced percentages of regulatory T cells (Tregs: CD4+CD25+FoxP3+; F(3,43) = 19.94, p<0.0001), memory T CD8+ cells (F(3,44) = 14.40, p<0.0001) and naïve T CD8+ cells (F(3,44) = 12.78, p=0.001) as compared to controls. We also observed increased percentages of NKT cells (CD3+CD56+) in BD subjects as compared to controls, although this did not reach statistical significance, F(3,48) = 3.03, p=0.08. No statistically significant differences were observed for other lymphocyte subpopulations analyzed (data not shown).

Opposite effects were observed for major lymphocyte subsets following TSST between the study groups. While BD subjects increased activated T cells after stress, the healthy controls reduced them, F(3,43) = 4.04, p=0.05. Lower regulatory T cells was observed after stress in BD subjects, while controls increased these cells after TSST, F(3,43) = 4.02, p=0.02. No statistically significant differences were observed for the remaining lymphocyte subpopulations.

6.3.3. Differential changes in MAPK phosphorylation after stress

We also sought to investigate intracellular signaling events involved with lymphocyte activation and immune imbalance described in BD. The MAPK ERK and p38 phosphorylation events are good correlates of cell activation and anergy, respectively (Raman et al., 2007). At baseline, we observed increased percentages of p-ERK+CD4+ ($F(3,41) = 20.44$, $p<0.0001$) and p-ERK+CD8+ cells ($F(3,43) = 13.62$, $p=0.001$) in BD subjects compared to healthy controls (Fig. 3C and 3D). The amount of MAPK ERK phosphorylated, as estimated by the mean fluorescence intensity (MFI), was also found increased in CD4+ ($F(3,41) = 20.44$, $p<0.0001$) or CD8+ cells ($F(3,43) = 3.44$, $p<0.0001$) of BD subjects as compared to controls. On average, CD4+ and CD8+ cells of BD subjects had a 3.5X and 2X more intracellular p-ERK at baseline than controls, respectively. No changes were observed for the amount of phosphorylated p38 in T-cell subsets. Because p38 and ERK have opposite cellular effects, we also calculated the p-ERK/p-p38 ratios. We observed an increased CD4+ p-ERK/p-p38 MFI ratio in BD subjects as compared to controls, $F(3,43) = 8.16$, $p=0.007$, Fig. 4E.

After acute stress, both groups had reduced percentages of p-ERK+CD8+ cells (Fig 3C and D), $F(3,42) = 13.71$, $p=0.007$. However, the rate of p-ERK+CD4+ cells was found only decreased in healthy controls after the TSST, $F(3,43) = 4.21$, $p<0.05$; $t = 2.79$, $p=0.01$; Fig 3C. A similar pattern was observed for the relative amount of p-ERK in CD4+ cells of healthy controls only after stress, $F(3,41) = 5.93$, $p=0{,}019$; $t = 2.14$, $p<0.03$; Fig 4C. The CD4+ T cells were particularly targeted following the acute stress and differential signaling events were observed for p-p38 expression between groups (Fig. 4A): while cells of BD subjects increased amounts of p-p38, cells of controls increased these amounts following stress, $F(3,41) = 4.96$, $p=0.031$.

6.3.4. NF-κB intracellular signaling

The NF-κB is a pleotropic transcription factor known to be readily activated after TSST in healthy subjects (Bierhaus et al., 2003). At baseline, we observed increased percentages of p-p65+CD3+ T cells in BD subjects compared to controls ($F(3,38) = 67.01$, $p<0.0001$; Fig. 5A). The amount of p65 phosporylation was also found similarly increased in subjects compared to controls, $F(3,43) = 40.69$, $p<0.0001$; Fig 5B. After stress, increased rates of p-p65+CD3+ T cells were only observed in the control group, $F(3,38) = 4.41$, $p<0.05$; $t = -2,48$, $p<0.05$; Fig 5A. No changes in the relative amount of p-p65 were noted following TSST in both groups (Fig. 5B).

6.3.5. More T-cell activation but less sensitivity to glucocorticoids

Increased proportions of activated T cells were observed in BD subjects following stress exposure ($F(3,43)$-5.03, $p=0.03$; $t= -2.78$, $p=0.01$) compared to healthy controls (Fig. 6A). Glucocorticoids have important immunoregulatory actions upon T-cell activation and proliferation. Cells of subjects with BD showed a relatively resistance to glucocorticoids as compared to controls, $F(1,44) = 5.08$, $p<0.05$ (Fig. 6B and C). No stress effect was noted for glucocorticoid sensitivity.

6.4. Discussion

To the best of our knowledge, this is the first study investigating neuroendocrine and immunological changes elicited by acute psychosocial stressor in BD. It was hypothesized that blunted neuroendocrine responses to stress could be associated with immune activation in BD.

6.4.1. Evidence for immune imbalance at baseline

Data from immune parameters obtained at baseline corroborate previous results indicating an immune imbalance in BD. We investigated lymphocyte subsets, including activated and regulatory cells, which may be implicated in immune imbalance in BD. Reduced proportions of Treg cells were found in BD, supporting a recent study of euthymic type 1 BD subjects (do Prado et al., 2013). Tregs play an important role in the control of immune responses and impairment of this lymphocyte leads to exacerbated immune responses as observed in many chronic inflammatory conditions, allergies and cancer (Sakaguchi et al., 2008). The specific impairment in this Treg cell subset may be also related to the increased incidence of autoimmune diseases, notably autoimmune thyroiditis (Vonk et al., 2007). The study of regulatory T cells in mood disorders is scarce. A recent study from Drexhage and colleagues (2011) did not observe overall alterations in Tregs of male and female individuals with BD type I/II when compared to controls (Drexhage et al., 2011). Variations in disease classification (BD type I versus II), gender and age range may explain the discrepancies. Our data are in agreement with previous works, reporting increased number in circulating activated T lymphocytes in BD (Breunis et al., 2003; Drexhage et al., 2011). We also reported a significant reduction in memory (CD8+CD45RO+) and naïve (CD8+CD45RA+) T cells in BD subjects. The CD8+ repertoire is involved in cellular immunity against viruses, cancer, aloreactive rejection and immunoregulation, however studies analyzing CD8+ memory and naïve cells in psychiatric disorders are scarce.

The immune imbalance observed in BD may also involve changes in intracellular signaling cascades, namely MAPK and NF-κB. The involvement of MAPKs in BD was first described by Padmos and colleagues (2008), suggesting that genes involved in inflammation-

related processes in BD subjects exhibited a specific pro-inflammatory signature (Padmos et al., 2008). In agreement with previous data (do Prado et al., 2013), we also found that BD is associated with increased percentage and phosphorylated protein levels of p-ERK in CD4+ and CD8+ T cells. Increased p-ERK signaling may contribute in different ways to the immune/inflammatory imbalance observed in BD subjects. It is tempting to speculate that lack of appropriate regulatory cells may allow the increase in p-ERK signaling in T-cell subsets, contributing to the immune imbalance (cell activation and pro-inflammatory profile). Previous studies suggest a relevant role for ERK1/2 signaling in behavioral changes in psychiatric disorders (Engel et al., 2009).

Another important intracellular signaling route related to immune response is the NF-κB pathway. Given the role of NF-κB on proliferation, maturation and cell survival (Furuno and Nakanishi, 2006; Koo et al., 2010; Sun et al., 2001), the increased percentage of p-p65 T cell and protein phosphorylation levels could be associated with the cellular activation observed here. Impairment of NF-κB pathway have already been associated with different disorders including PTSD (O'Donovan et al., 2011; Pace et al., 2006), major depression (Pace et al., 2006) and BD (Rao et al., 2010; Sun et al., 2001). The precise involvement of NF-κB in behavioral and cognitive changes is not known.

The stress responsive systems seem to be functioning properly at baseline, as suggested by regular cortisol secretion and HR in BD. The GC-related immunomodulation occurs via membrane and intracellular GC receptors (GR) present in many lymphoid cells, including T cells. Activation of GR normally results in suppression of many inflammatory genes and the GC-mediated negative feedback of immune response is a fundamental mechanism in order to inhibit detrimental inflammatory responses. In accordance with

previous work (Knijff et al., 2006), a potential explanation for the maintenance of the observed immune activation is the finding of reduced cellular sensitivity to GCs in BD.

6.4.2. Under pressure: acute stress response points to allostatic load in BD subjects

In contrast to data obtained at baseline, acute stress exposure reveals blunted stress responses in BD subjects as shown by reduced HR and cortisol secretion in comparison to healthy controls.

The immune imbalance observed in BD subjects at baseline was exacerbated following acute stress exposure. In evolutionary terms, the acute stress responses prepare the organism for 'fight or flight' reactions and such preparatory actions clearly involve immune cell activation and enhanced wound healing (Dhabhar, 2009). Here, we observed blunted neuroendocrine responses following acute stress exposure in BD. Taken together, the blunted HPA axis function following acute stress and reduced cellular sensitivity to GCs indicate a defective neuroendocrine control over the immune system.

Interestingly, the lymphocytes from BD subjects after stress follow opposite directions from those of healthy controls. Two important lymphocytes subsets were altered in BD subjects in response to stress: Tregs and activated T cells. A fine balance of activated/regulatory T cells is pivotal in regulating immune responses. Following TSST, we observed a further reduction in the proportion of Tregs from BD subjects in parallel with increased percentage of activated T cells ($CD4+CD25+^{low}$), with opposite findings for healthy controls. A further stress-related drop in this important regulatory T-cell subset suggests an inability of BD subjects in control immune activation after stress exposure, as suggested by the raised percentage of activated T cells.

Stress exposure triggers MAPKs and NF-κB activation (Bierhaus et al., 2003), suppressing the transcription of several important genes involved in cellular activation. However, this was not observed in PBMCs from BD subjects. We observed an overall reduction in MAPKs signaling in healthy controls following stress. The MAPKs of BD subjects had unaltered responses to stress, except by reduced percentages of p-ERK+CD8+ cells and increased levels of p38 phosphorylation in CD4+ cells. Given ERK and p38 opposite cellular effects, the inability in downregulating ERK1/2 signaling in BD may be supporting the activation profile observed in response to stress. Conversely, increased p38 phosphorylation levels in CD4+ cells may be an attempt to restore the balance by leading CD4+ to death via apoptosis. No previous studies have analyzed MAPK signaling in response to TSST.

Bierhaus and colleagues (2003) described the activation of NF-κB as the mechanism involved in converting psychosocial acute stress (TSST) in immune activation in healthy volunteers (Bierhaus et al., 2003). Although the activation of NF-κB following TSST was replicated here in PBMCs of healthy controls, no changes were reported in BD patients following stress. The absence of alterations in NF-κB signaling in response to stress may be reflecting the attenuated neuroendocrine responses to stress experienced by subjects with BD. Attenuated neuroendocrine responses were represented by defective HPA axis response to stress and by reduced cellular sensitivity to GCs. GC resistance has been related to the pro-inflammatory profile in major depression and has been involved with an inability to suppress immune responses (De Kloet et al., 1998; Lopes et al., 2012). Many of the immune-related inhibitory functions of GCs are due to interference with NF-κB activity, either by inducing expression of its inhibitory protein IκB (Auphan et al., 1995; Scheinman et al., 1995) or by directly interaction with the p65 subunit and thereby repressing NF-κB DNA binding activity

(De Bosscher et al., 1997; De Bosscher et al., 2000; Ray and Prefontaine, 1994). As NF-κB signaling was increased at baseline, blunted cortisol response together with increased GC resistance in PBMCs from BD subjects collaborates to the maintenance of immune activation. This may also explains the increased activation and proliferation of PBMCs from BD subjects once stimulated with mitogen.

There are some limitations in this study to be discussed. One of the major limitations of our study is that all BD subjects were receiving psychotropic drugs (e.g. lithium and valproate) that may modulate immune functions as well as intracellular signaling (Chen and Manji, 2006; Einat et al., 2003; Goldstein et al., 2009; Knijff et al., 2007). In order to verify this possibility, we analyzed the potential effects of psychotropic treatment on immune variables and no significant interactions emerged. The sample size is relatively small and data presented here should be considered preliminary. However, it should be highlighted that both experimental design and power are compatible with previous studies reporting immune data following TSST (Bierhaus et al., 2003; Hill et al., 2009; Pace et al., 2006). In order to avoid confounding effects of the gender related immunological changes (Ghazeeri et al., 2011), only females were recruited in this study. Future studies should thus confirm our results with male BD subjects.

In conclusion, our data suggest that BD subjects have reduced stress reactivity, supporting the immune imbalance demonstrated here by reduced proportion of circulating Tregs, increased proportion of activated T cells, increased MAPK ERK and NF-κB signaling at baseline and relative resistance to GC. Of note, the inability in reducing NF-κB and MAPK signaling after stress may also contribute to the maintenance of immune activation in BD subjects. MAPKs and other intracellular signaling cascades should be further explored to better assess their role to the pathophysiology of BD.

Acknowledgements

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq: MEB, LBR, ALT and RG-O) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES: CHP, AW). CNPq had finance all consumables involved with this paper. CAPES granted scholarships to the cited students.

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Table 1: Characteristics of the studied populations.

	BD	Healthy Controls	P-value
Age, yrs (mean ± SD)	46.36 ± 10.86	48.07 ± 10.63	0.69
BMI (mean ± SD)	26.61 ± 1.54	25.13 ± 3.31	0.40
Years of illness (mean ± SD)	10.56 ± 7	-	-
Age at onset (mean ± SD)	33.54 ± 9.94	-	-
HDRS (mean ± SD)	6.63 ± 3.92	2.07 ± 2.67	0.004
YMRS (mean ± SD)	2.0 ± 3.71	1.57 ± 3.18	0.76
Ethnicity (white/non-white)	10/3	14/1	0.22
Smoking	7	2	0.02
Lithium	6	-	-
Antidepressants	5	-	-
Antipsychotics	8	-	-
Anticonvulsants	8	-	-
Benzodiazepines	2	-	-

Data shown as mean ± standard deviation (SD). Abbreviations: BMI, body mass index; BD, bipolar disorder; HDRS, Hamilton Depression Rating Scale; and YMRS, Young Mania Rating Scale. Data were analyzed by Mann-Whitney.

Figure legends

Figure 1: Neuroendocrine response to stress. **A:** Heart rate across TSST. **B:** Salivary cortisol levels across TSST. **C:** Area under the curve analysis (AUC) of basal and after stress cortisol levels. * $p < 0,05$; light grey highlights anticipatory period of stress task. Dark grey highlights stress period. Dotted lines are BD patients and full lines, healthy controls.

Figure 2. Lymphocytes subsets before (pre) and following acute stress (post) elicited by the Trier Social Stress Test (TSST). **A:** trend for increased NKT (CD3+CD56+) cells in BD patients; **B:** reduction on memory cells (CD8+CD45RO+) in BD patients at baseline (pre-TSST); **C:** reduction of CD8+CD45RA+ naïve cells in BD patients at baseline; **D:** increased percentage of activated T cells (CD4+CD25+) in BD patients at baseline. After stress, increased percentage of CD4+CD25+ cells in BD patients and reduction in controls; **E:** Reduced percentage of Treg cells (CD4+CD25+FoxP3+) in BD patients at baseline. After stress, further reduction in the percentage in BD patients and increase in controls. * $p < 0,05$; ** $p < 0,001$.

Figure 3: Phosphorylated MAPKs in peripheral T cells. **A** and **B**: shows no significant changes in percentages of p38 phosphorylated CD4+ and CD8+ cells. **C:** increased percentage of p-ERK CD4+ cells in BD patients at baseline. After stress, elicited by the Trier Social Stress Test (TSST), it was noticed reduced percentages of p-ERK CD4+ cells only in controls. **D:** Increased percentage of p-ERK CD8+ cells in BD patients with reduction in both groups after stress. * $p < 0,05$; ** $p < 0,001$.

Figure 4: Protein phosphorylation levels evaluated as mean fluorescence intensity (MFI). **A:** stress effect on p-p38 MFI in CD4+ cells. **B:** p38 phosphorylation levels in CD8+ cells. **C:** ERK phosphorylation levels in CD4+ cells. **D:** ERK Phosphorylation levels in CD8+

cells. **E:** Ratio between ERK and P38 Phosphorylation levels in CD4+ cells. * $p < 0,05$; ** $p < 0,001$.

Figure 5: NF-κB phosphorylation analysis. **A:** percentage of T lymphocytes with p-p65. **B:** Levels of p65 NF-κB subunit phosphorylation in total lymphocyte population (CD3+). * $p < 0,05$; ** $p < 0,001$.

Figure 6. T-cell activation and sensitivity to glucocorticoids. Figure **(A)** shows the percentage of activated T cells (CD3+CD25+) following PHA stimulation. Cellular sensitivity to glucocorticoids was estimated by functional assays developed to measure the ability of dexamethasone to suppress T-cell activation *in vitro*. Data are shown as % basal T-cell activation at baseline **(B)** of after stress induced by the Trier Social Stress Test (TSST) **(C)**, with 100% corresponding to 1% PHA stimulation without steroids.

FIGURE 1

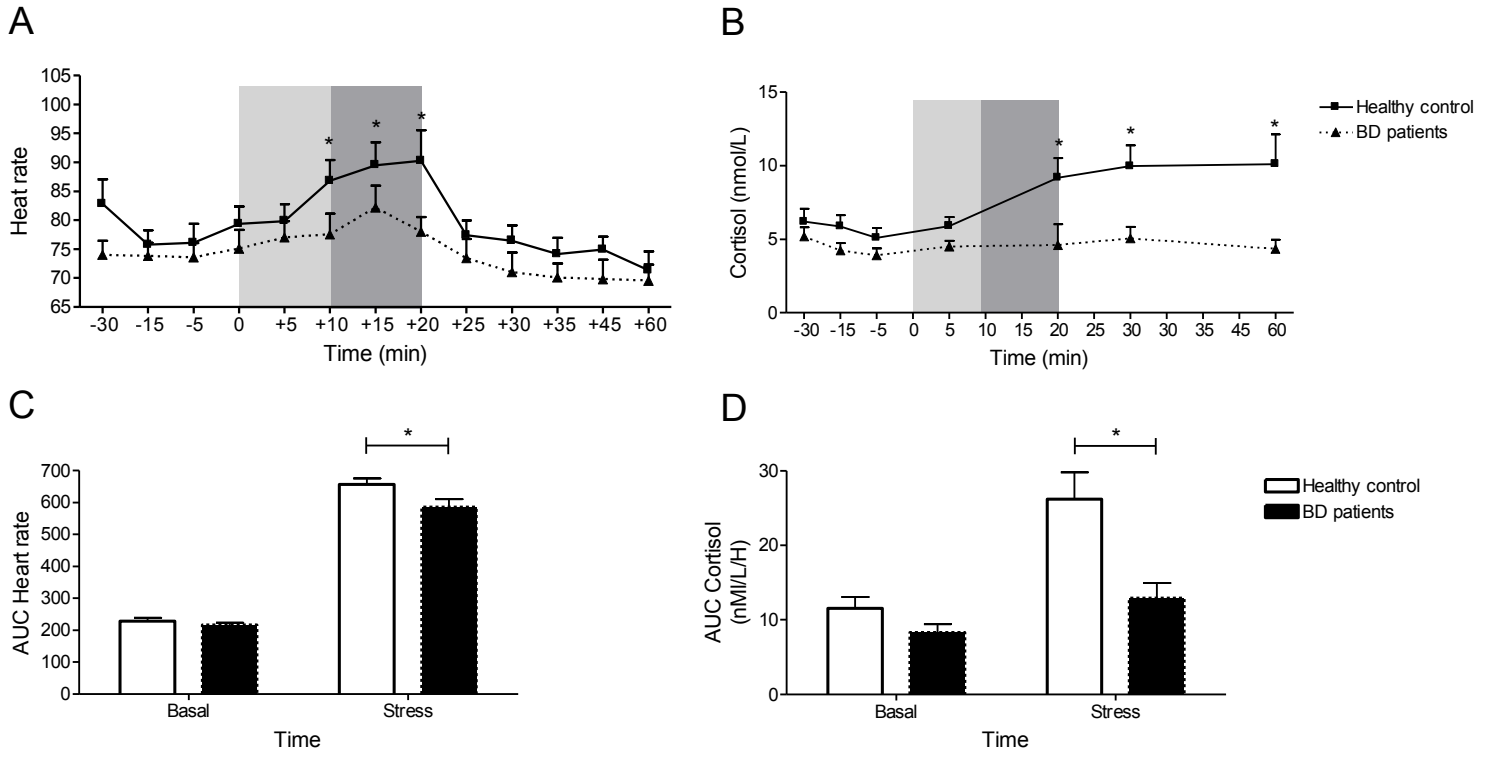


FIGURE 2

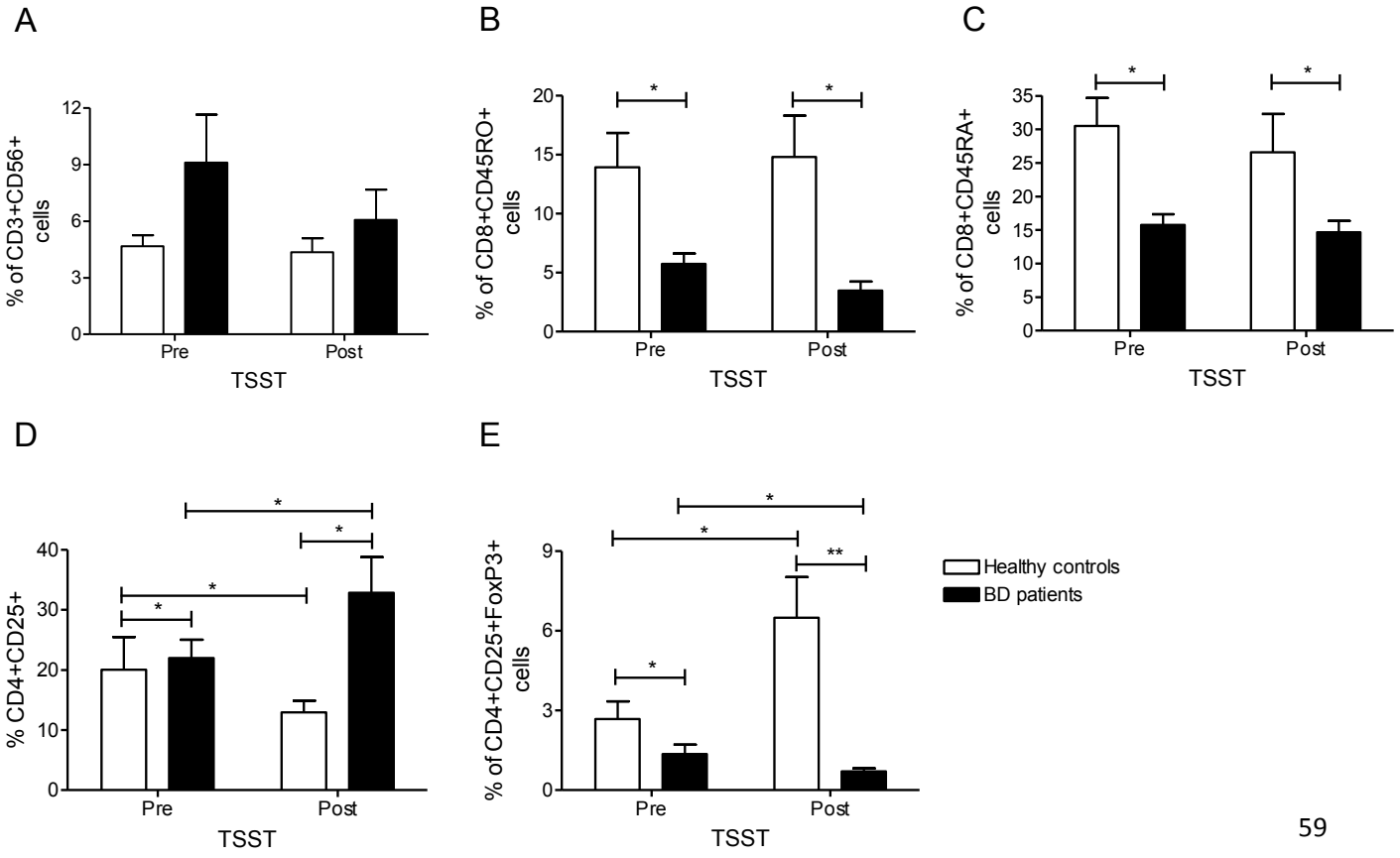
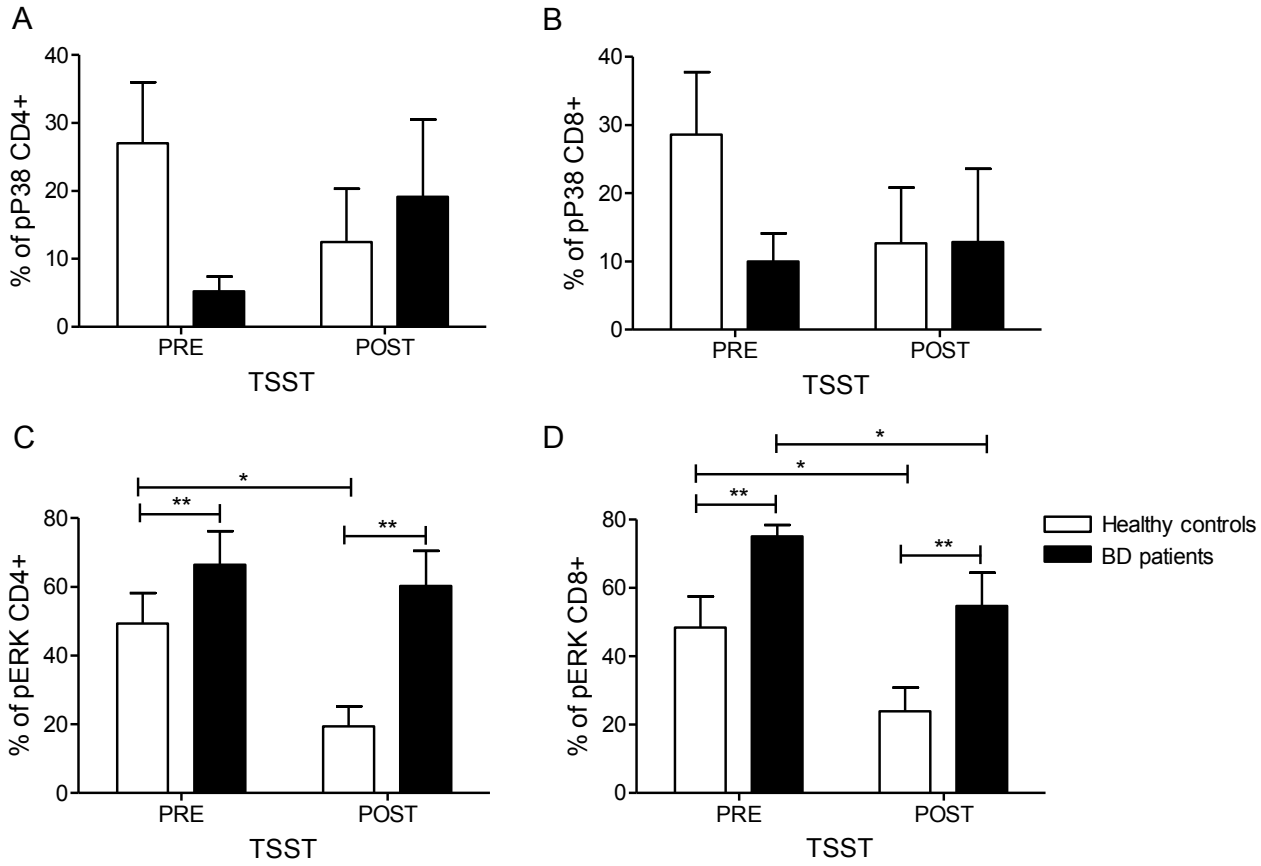


FIGURE 3


A
% of pP38 CD4+
40
30
20
10
0
PRE
POST
TSST
B
% of pP38 CD8+
40
30
20
10
0
PRE
POST
TSST
C
% of pERK CD4+
80
60
40
20
0
*
**
**
PRE
POST
TSST
D
% of pERK CD8+
80
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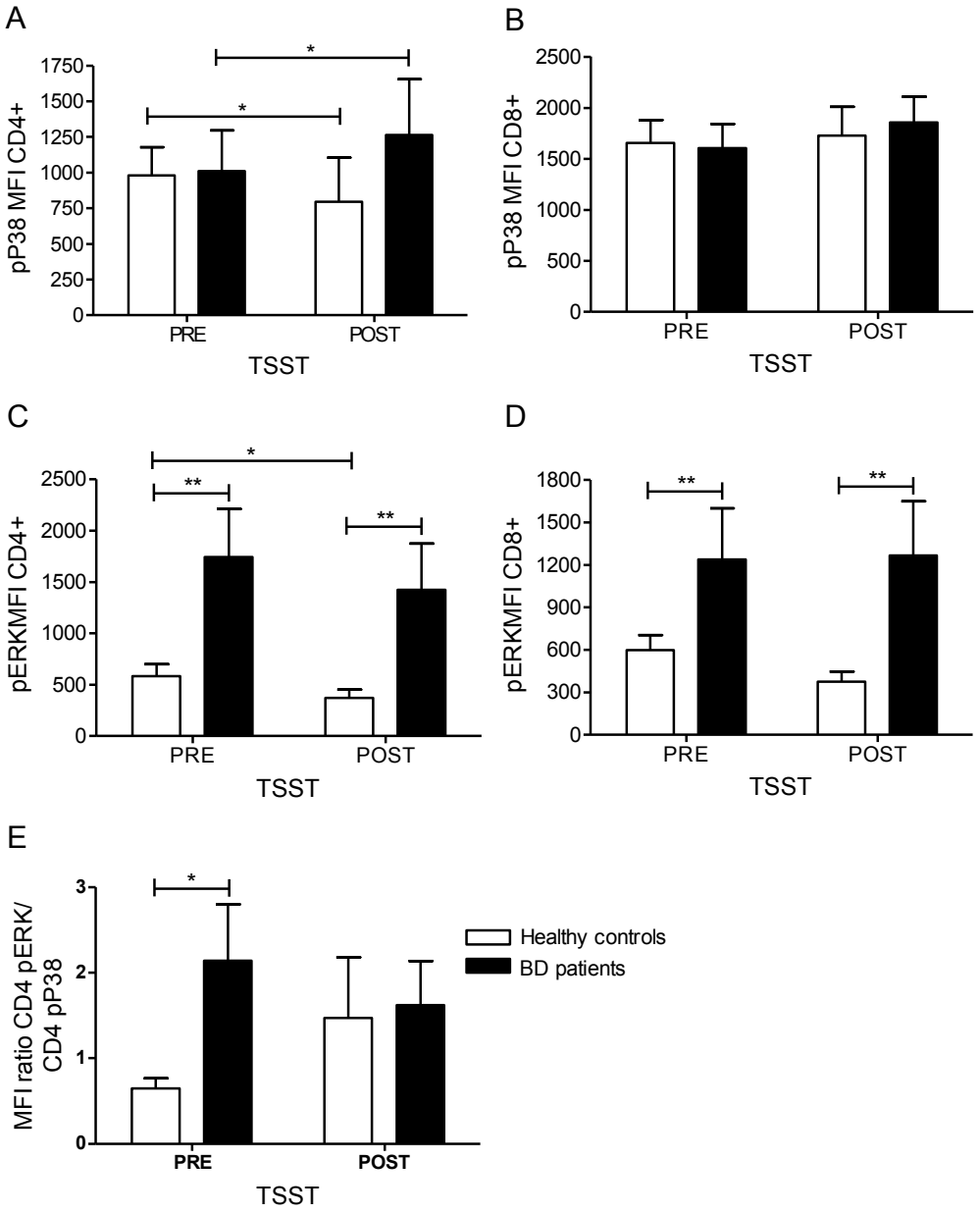
PRE
POST
TSST
Healthy controls
BD patients

FIGURE 4


A
pP38 MFI CD4+
1750
1500
1250
1000
750
500
250
0
*
*
PRE
POST
TSST
B
pP38 MFI CD8+
2500
2000
1500
1000
500
0
PRE
POST
TSST
C
pERKMFI CD4+
2500
2000
1500
1000
500
0
*
**
**
PRE
POST
TSST
D
pERKMFI CD8+
1800
1500
1200
900
600
300
0
**
**
PRE
POST
TSST
E
MFI ratio CD4 pERK/ CD4 pP38
3
2
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PRE
POST
TSST
Healthy controls
BD patients

FIGURE 5

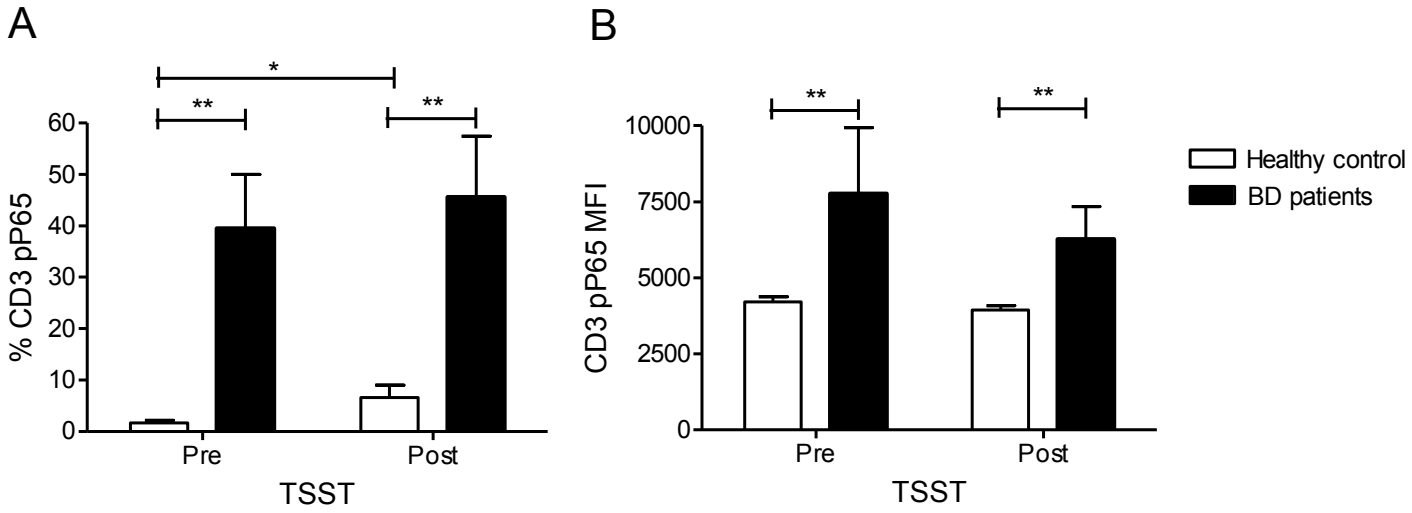
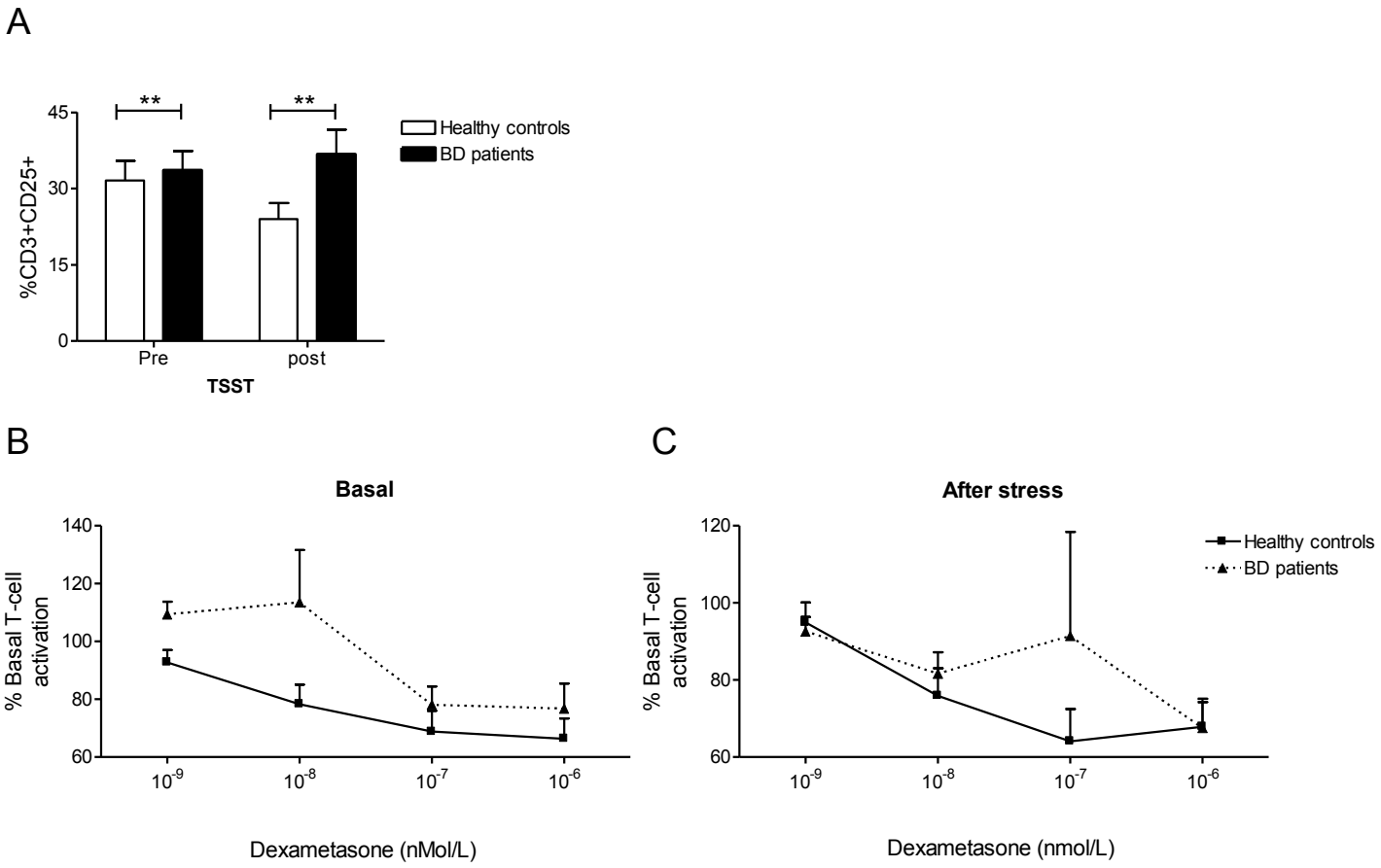


FIGURE 6



7. Conclusões e considerações finais

De forma geral, a saúde de um organismo, depende de um fino balanço entre os diferentes sistemas que o compõem. Considerando a saúde mental do indivíduo, sabe-se que a intercomunicação entre sistema nervoso central, sistema endócrino e sistema imune possui um papel importante na manutenção da homeostase. Qualquer desequilíbrio existente em um desses sistemas pode resultar em uma desregulação do organismo como um todo e, consequentemente, contribuir para o desenvolvimento de diferentes tipos de transtorno de humor.

Um importante fator gerador de desequilíbrio fisiológico é o estresse. Como dito anteriormente, o estresse desencadeia uma série de respostas fisiológicas que, em um indivíduo saudável, irão apenas reestabelecer a homeostase do organismo. Em situações em que o evento estressor passa a ser crônico, a resposta ao estresse deixa de ser adaptativa e passa a ser prejudicial ao organismo resultando em um desbalanço do SNC, SE e SI. Exemplos de tais situações são os casos de cuidadores de indivíduos com demência ou Alzheimer, em que, frequentemente, o cuidador apresenta níveis alterados de cortisol e perfil pró-inflamatório acompanhados de alterações de humor características da depressão.

Os eventos aos quais o indivíduo está exposto ao longo do seu desenvolvimento contribuem para a formação final do mesmo, modulando respostas do SNC e sistema imune e contribuindo para a saúde do indivíduo. Os efeitos da exposição ao estresse talvez sejam mais detrimentais quando o evento estressor ocorre nos anos iniciais do desenvolvimento. A existência de períodos de sensibilidade do desenvolvimento resulta numa maior suscetibilidade ao estresse durante esses períodos. Em estudos animais, o eixo HPA reduz sua atividade durante o desenvolvimento pós-natal (9º ao 15º dia em ratos) com função de

programar as resposta do eixo HPA ao ambiente (119). Durante esse período de hiporesponsividade, os níveis de GC são baixos (secreção reduzida) e o eixo HPA não responde a uma série de estressores que elicitariam a resposta em adultos. Entretanto, alguns eventos estressores são capazes de ativar o eixo HPA hiporesponsivo (120). Um exemplo é a separação materna, em ratos, que resulta em aumento dos níveis de GC levando a uma reprogramação do eixo HPA (121-124). Em humanos, indivíduos que passaram por traumas na infância apresentam aumento nos níveis de ACTH e cortisol durante o TSST, sugerindo uma hipersensibilidade do eixo HPA ao estresse (125). Atualmente tem se discutido se pessoas que sofreram trauma e abuso na infância apresentam hipo- ou hiper (114, 116, 126-128). Estudos prévios demonstram que a exposição ao estresse durante os períodos iniciais do desenvolvimento altera o correto desenvolvimento aumentando o risco para transtornos psiquiátricos como depressão maior, transtorno obsessivo compulsivo, e esquizofrenia (9, 10, 129-132).

A reprogramação do eixo HPA devido a exposição a eventos estressores na infância é refletida não apenas no SNC, mas também no SI (133). Indivíduos que passaram por traumas na infância apresentam alterações imunológicas como aumento nas concentrações séricas de TNF-α , IFN-γ, IL-1β, e IL-6 (134). Pacientes com depressão maior que sofreram abuso na infância apresentam aumento nos níveis do receptor solúvel de TNF em contraste com baixos níveis de IL-2 e da quimiocina RANTES, quando comparados a indivíduos saudáveis (135). Aumento nas proporções de células NK, e redução em células Treg devem ter papel importante na manutenção do perfil inflamatório observado nesses indivíduos (133). É possível que este perfil inflamatório exista desde o momento em que o trauma/abuso ocorreu. Estudos prévios demonstraram a existência de picos no aumento de citocinas pró-

inflamatórias logo após eventos estressores/traumáticos. Por exemplo, em crianças, os níveis de Il-6 foram observados aumentados imediatamente após o trauma, e foram preditivos para o desenvolvimento de TEPT 6 meses após o trauma (136). Os níveis de IL-6, em adultos, também aumentaram logo após acidente automotor (136, 137).

Elevações nos níveis séricos de citocinas pró-inflamatórias decorrentes de diversos processos patológicos estão associadas com alterações comportamentais adaptativas chamadas de "*sickness behavior*". Entretanto, quando os níveis destas citocinas se mantêm elevados por longos períodos de tempo (p.e. tratamento com IFN-α ou doenças inflamatórias crônicas) essas alterações comportamentais deixam de ser adaptativas e passam a ter um papel importante no risco para desenvolvimento de transtornos psiquiátricos (64). O mecanismo pelo qual o sistema imune influencia o risco de desenvolvimento de doenças psiquiátricas ainda não é conhecido. Uma possível explicação envolve o metabolismo central de monoaminas. Citocinas produzidas perifericamente podem atravessar a barreira hemato-encefálica (BHE) de diferentes formas como a expressão de receptores específicos na BHE e aumento da permeabilidade da BHE (característica de processos inflamatórios) (138-142). Uma vez no cérebro, as citocinas ativam células da micróglia, que passam a produzir citocinas localmente, no cérebro (139). As citocinas pró-inflamatórias, por sua vez, irão aumentar a expressão de ciclooxigenase-2 (COX-2) e prostaglandina tipo E_2 (PGE-2), dois importantes mediadores da neuroinflamação (142). O aumento de COX-2 e PGE-2 acabam ativando a rota da indoleamina 2, 3-dioxygenase (IDO), importante enzima do metabolismo de triptofano (38, 143, 144). O Triptofano é a molécula precursora da serotonina, um importante neurotransmissor e principal alvo das terapias para transtorno de humor. O aumento de IDO resulta numa

depleção de triptofano e consequente redução na produção de serotonina (145-150). Além disso, as citocinas pró-inflamatórias regulam a expressão de receptores a transportadores de neurotransmissores, desregulando o balanço correto destas moléculas no cérebro (151). Além disso, citocinas como IL-1β e TNF-α ativam o fator de transcrição NF-κB, induzindo a expressão de moléculas relacionada a estresse oxidativo e levando a neurotoxicidade e morte neuronal (152-155).

Em um dos artigos que compõem a tese demonstramos, num modelo animal, os efeitos detrimentais em longo prazo da exposição ao estresse durante o desenvolvimento. O modelo animal de separação materna é um modelo já validado de estresse na infância que é amplamente utilizado com intuito de demonstrar os efeitos do estresse crônico na infância (156). A exposição ao estresse durante o desenvolvimento, quando diferentes sistemas, incluindo o cérebro, não estão completamente desenvolvidos resulta em alterações neuroquímicas, maturação incorreta de diferentes sistemas de neurotransmissores, perda neuronal e diferentes alterações neuroanatômicas que acabam por comprometer o correto amadurecimento do cérebro afetando a suscetibilidade a doenças psiquiátricas (6, 9, 120, 157-162). Diferentes estudos já demonstraram que a exposição crônica ao estresse precocemente ao longo do desenvolvimento pode resultar em consequências comportamentais e fisiológicas (9, 15, 160, 162, 163). Muitos dos efeitos da exposição ao estresse crônico na infância só se manifestará na adolescência, ou até mesmo na idade adulta, quando o desenvolvimento cerebral se completa (6, 120, 158). Os mecanismos pelos quais o estresse na infância leva a alterações comportamentais e problemas na correta maturação do cérebro ainda não estão totalmente esclarecidos. Um possível mecanismo envolve processos neuroinflamatórios onde o aumento na expressão de marcadores

inflamatórios como citocinas e prostaglandinas podem levar a neurotoxicidade (141, 164, 165).

Dados do presente trabalho demonstraram um aumento nos níveis plasmáticos da citocina pró-inflamatória IL-1β nos animais que foram submetidos à separação materna. O aumento nos níveis periféricos de IL-1β foi correlacionado com as perdas de interneurônios contendo parvalbumina (PVB) no Córtex pré-frontal dos animais expostos a separação materna. Interneurônios contendo PVB são extremamente importantes para o correto desenvolvimento do cérebro, já que são os precursores da formação da árvore serotoninérgica. O Córtex pré-frontal é uma região cerebral que recebe inervações de outras áreas do cérebro como amígdala, tendo papel relevante na cognição, impulsividade e controle do comportamento (166). Alterações neuronais, especialmente nos neurônios contendo PVB, podem resultar em alterações de conectividade do córtex pré-frontal, já observada na esquizofrenia (167). Além disso, a perda de interneurônios contendo PVB pode resultar em comprometimento de processos cognitivos e está relacionada a diferentes transtornos psiquiátricos (167-169).

As alterações cerebrais observadas nos animais expostos à separação materna estão correlacionadas aos processos inflamatórios desencadeados na periferia. Citocinas inflamatórias expressas na periferia se comunicam com o cérebro, ativando células da microglia que passam a produzir marcadores inflamatórios (IL1-β, TNF-α, IL-6, COX2, PGE2) localmente, resultando em processos neuroinflamatórios (170, 171). IL-1β, uma das principais citocinas pró-inflamatórias produzidas no cérebro. É normalmente expressa em níveis baixos por diferentes células neuronais como glia, astrócitos e neurônios, possuindo papel relevante na modulação neural, neuroendócrina e do comportamento durante

processos neuroinflamatórios (172, 173). O aumento na expressão de IL-1β no cérebro já foi associado a outras condições patológicas como Alzheimer e Huntington (173).

O aumento na produção de citocinas pró-inflamatórias acarreta em perda neuronal devido à neurotoxicidade. Um dos possíveis mecanismos envolve alterações em receptores de glutamato do tipo NMDA (N-methyl-D-aspartato). NMDAr são receptores dependentes de atividade com importante papel no desenvolvimento cerebral pois modulam a transmissão sináptica ao regularem a formação, modificação e eliminação sináptica, assim como estão envolvidos com a ramificação dendrítica e plasticidade neuronal (174-176). São heterotetrâmeros compostos por duas subunidades do tipo NR1 e duas subunidades do tipo NR2. O funcionamento incorreto destes receptores, seja por super-estimulação ou sub-estimulação, resulta em neurotoxicidade e morte neuronal (177). Mecanismos neuroinflamatórios resultam em aumento na produção de marcadores de estresse oxidativo, como espécies reativas de oxigênio. A produção excessiva de tais marcadores, como por exemplo, oxido nítrico (NO) leva a um aumento na liberação de L-glutamato, um agonista de NMDAr. Consequentemente, o aumento na liberação de L-glutamato acaba por super estimular os NMDAr induzindo a produção de caspases e culminando em morte neuronal (177-179). A hipofunção destes receptores também ativa a rota de sinalização de caspases resultando em morte neuronal (177-179). Alterações na composição dos NMDAr na região cortical dos animais expostos ao estresse puderam ser observadas. Especificamente, houve um aumento na expressão da subunidade NR2 de NMDAr. De acordo com Monyer e colaboradores (180), a subunidade NR2 de NMDAr confere um menor tempo de abertura dos canais do receptor. Dessa forma, um aumento na expressão desta subunidade, observado no córtex dos animais expostos ao estresse, pode levar a uma sub-estimulação do

receptor, induzindo a morte neuronal. Alterações na expressão de NR2 foram observadas tanto em interneurônios contendo PVB e não contendo PVB. Entretanto, interneurônios contendo PVB são extremamente sensíveis à alterações na função de NMDAr, sendo a expressão de PVB já associada a um aumento na suscetibilidade à neurotoxicidade mediada por NMDAr, justificando a morte destes neurônios observada na região cortical dos animais expostos à separação materna (181, 182). Mais estudos são necessários para esclarecer os mecanismos envolvidos na perda neuronal decorrente da hipofunção de NMDAr.

Os efeitos das citocinas pró-inflamatórias no cérebro são contrabalanceados pelas citocinas anti-inflamatórias, tais como IL-10 (183). No presente trabalho foi possível observar que injeções centrais de IL-10, diretamente na região pré-límbica do córtex pré-frontal, foi capaz de reverter os efeitos cerebrais decorrentes do processo inflamatório desencadeado pela exposição ao estresse como a perda neuronal e o aumento na expressão de NR2 no córtex destes animais, como o processo inflamatório periférico em si.

As alterações cerebrais decorrentes do estresse podem resultar, em última instância, em um desbalanço do eixo HPA, levando a uma carga alostática que acaba por alterar a suscetibilidade a transtornos psiquiátricos. O desbalanço do eixo HPA tem como consequência alterações imunológicas que são refletidas como um perfil inflamatório. A constante exposição a um desbalanço imunológico pode ter resultados detrimentais para a saúde do indivíduo. As consequências psicológicas da exposição ao estresse na infância e consequente perfil inflamatório só se manifestarão no início da vida adulta. Os dados do presente estudo corroboram a hipótese da existência de períodos de sensibilidade do desenvolvimento e que, a exposição a fatores estressores durante esses períodos possui efeitos prejudiciais para o sistema nervoso central e sistema imune.

O TB é uma doença crônica, progressiva, altamente debilitante e cuja prevalência supera a de outros problemas de saúde, como o HIV (0,8%, OMS 2010) e que pode, quando não corretamente tratada, impedir o indivíduo de levar uma vida normal (p.e. estudar, trabalhar, relacionamentos). É considerada uma doença com sintomatologia complexa de etiologia multifatorial, na qual fatores biológicos e ambientais, dentre eles psicossociais, interagem em diversos níveis. Mesmo tendo sido descrito há longo tempo, a etiologia deste transtorno continua obscura. A ausência de marcadores biológicos específicos para o TB é um dos grandes entraves para o correto diagnóstico e tratamento do transtorno. Ainda existem casos em que o TB não é diagnosticado em tempo hábil para um tratamento adequado ou mesmo de forma correta, sendo comum um diagnóstico inicial de depressão maior ou esquizofrenia (184).

O TB é acompanhado de múltiplos sinais de ativação e alterações do sistema imunológico (72, 185), variando de acordo com a fase em que o paciente se encontra (mania, depressão ou eutimia) (31). Existem algumas evidências sugerindo de que o sistema imune, em interação direta com o sistema nervoso central, possui papel importante na patofisiologia do TB (31, 71). Dados do presente trabalho corroboram a hipótese de ativação do sistema imune em pacientes com TB, assim como traz novas informações sobre como é a resposta neuroimunoendócrina destes pacientes. Como mencionado anteriormente, esse é o primeiro estudo a analisar as respostas neuroimunoendócrinas ao estresse agudo em pacientes TB.

A função do eixo HPA e do sistema nervos autonômico (ANS) parecem não estar alteradas em pacientes TB, já que não houve diferença tanto na secreção de cortisol quanto nas frequências cardíacas, no período basal. Entretanto, a resposta ao estresse revelou uma

hiporesponsividade do eixo HPA e do SNA observado pelos níveis de cortisol inalterados, e leve alteração na frequência cardíaca dos pacientes com TB. Os dados relativos a alterações imunológicas corroboram as diversas alterações imunológicas previamente descritas na literatura indicando um perfil imune ativado nestes pacientes (36, 72-76). Diferentes alterações imunes podem levar a este perfil observado em TB. A análise de um grande painel de subtipos linfocitários demonstrou um desbalanço em duas subpopulações crucias para a manutenção da homeostase imunológica, células T ativadas (CD4+ CD25+) e células T regulatórias (Treg). A redução na porcentagem de células Treg observada neste estudo no período basal está de acordo com um estudo previamente publicado por nosso grupo (72). Células Treg são extremamente importantes para a manutenção do correto equilíbrio durante uma resposta imunológica. Alterações na função e quantidade deste subtipo linfocitário já foram observadas em doenças inflamatórias crônicas (186, 187) assim como estão associadas a diversas patologias autoimunes (188-191).

A redução de células Treg seria suficiente para causar um desbalanço imune nos pacientes TB. Porém, concomitante a esta alteração, observamos um aumento nas porcentagens de células T ativadas. Sabe-se que a ativação imune reduz a disponibilidade de triptofano, molécula chave para a produção de serotonina no cérebro, afetando os níveis deste importante neurotransmissor. Dessa forma, é possível que uma ativação imune observada na periferia se comunique com o cérebro e interfira no metabolismo serotonérgico (142-144).

Alterações em rotas de sinalização intracelular também possuem um envolvimento no desbalanço imune observado por nós e previamente descrito por outros. MAPKS são proteínas cinase ativadas por mitógenos. Especificamente, as MAPKs p38 e ERK possuem

relevância ao sistema imune por possuírem papel importante na ativação linfocitária, anergia celular, sobrevivência e morte celular (192, 193). A ativação destas MAPKs se dá através de sua fosforilação. Uma vez fosforiladas, elas migram para o núcleo, onde irão coordenar a expressão de diversos genes envolvidos em ativação e morte celular (192, 193). De acordo com trabalho previamente publicado por nosso grupo, nós observamos um aumento na sinalização via ERK1/2, observado como aumento na porcentagem de células CD4+ e CD8+ contendo ERK1/2 fosforilado, assim como por aumento nos níveis de fosforilação desta proteína em pacientes TB (72). O envolvimento de MAPKs com Tb foi primeiramente descrito por Padmos e colaboradores, que sugeriram que genes envolvidos com processos inflamatórios possuiriam uma assinatura específica no controle de sua transcrição (194). A maior sinalização intracelular via ERK1/2, proteína relacionada à ativação celular, certamente possui um papel importante para o perfil imune ativado observado nos nosso pacientes TB. Estudo de Engel e colaboradores sugeriu uma relação entre sinalização intracelular ERK1/2 e alterações comportamentais em transtornos psiquiátricos (195), porém ainda são dados escassos e futuros estudos são necessários para melhor compreender o papel de ERK1/2 e transtorno de humor.

Outra importante rota de sinalização intracelular que foi observada alterada, no período basal, nos pacientes TB foi a via de sinalização do NF-κB. O NF-κB é um fator de transcrição presente em todas as células do organismos mas com papel crucial no controle de transcrição de genes relacionados a respostas inflamatórias estando envolvido em maturação, sobrevivência e proliferação celular (196-198). O aumento na fosforilação da subunidade p65 do fator de transcrição NF-κB em pacientes TB pode estar diretamente relacionado ao aumento nas porcentagens de células T ativadas (CD4+CD25+). Apesar de já

ter sido relacionado a outros transtornos de humor, como depressão maior, outros trabalhos são necessários para determinar o real envolvimento deste fator de transcrição com alterações comportamentais observadas em TB (108, 193, 198-201).

A manutenção do perfil imune ativado no período basal se justifica não apenas pelas alterações imunes descritas até então, mas também pela redução na sensibilidade à dexametasona. Linfócitos de pacientes TB apresentaram redução na sensibilidade ao glicocorticoide dexametasona. Dessa forma, mesmo a função do eixo HPA estando normal e secretando níveis normais de cortisol, a insensibilidade dos linfócitos circulantes faz com esses níveis não sejam percebidos e as células não respondam ao cortisol, sendo mantidas as rotas de ativação imune observadas.

O desequilíbrio imunológico, consequente de uma inabilidade em manter a homeostase fica mais evidente quando analisamos os dados relativos ao período pós-estresse, quando o evento estressor já foi cessado. Dados neuroendócrinos demonstram reduzida reatividade ao estresse nos indivíduos com TB. A secreção de cortisol foi significativamente inferior a observadas nos indivíduos controle, e não houve alteração na frequência cardíaca dos pacientes TB durante a exposição ao protocolo de estresse agudo.

A resposta imune ao estresse é claramente diferenciada da observada em indivíduo controles. Uma vez expostos ao protocolo de estresse, pacientes TB não são apenas incapazes de suprimir a ativação celular inicial, como aumentam a mesma. Tal incapacidade em suprimir uma ativação imune em reposta ao estresse é resultado da combinação de diversos fatores. O primeiro que nos chama a atenção é a redução de células Tregs em pacientes TB após o estresse. Como dito anteriormente, tais células são extremamente importantes no controle de respostas imunes. Enquanto observamos um aumento destas

células em indivíduos controle, resultando na supressão da resposta imune observada, pacientes TB respondem de forma contrária, com redução nestas células.

Após o estresse ambos os grupos foram capazes de suprimir as rotas de sinalização intracelular, entretanto, essa supressão foi maior nos indivíduos controle do que em pacientes TB, refletindo uma tentativa de controle da ativação imune observada até então. A ativação de NF-κB se manteve estável nesses pacientes, não havendo alterações, enquanto indivíduos controle aumentaram a sinalização via esta rota. NF-κB é o principal fator de transcrição do sistema imune, responsável por coordenar a transcrição de diferentes moléculas do sistema imune envolvidas na resposta inflamatória. Um estudo prévio com indivíduos saudáveis demonstrou que este é o mecanismo ligando exposição ao estresse e ativação imunológica (85).

Outra possível explicação para a manutenção (a até aumento) da ativação imune no período pós-estresse reside na insensibilidade a glicocorticoides. Durante eventos estressores, a ativação do eixo HPA resulta em liberação de cortisol (GC) resultando na imunossupressão observada em uma resposta saudável ao estresse A sensibilidade linfocitária a GC se manteve reduzida nos pacientes TB após a exposição ao estresse. Mesmo não tendo sido alterada, essa insensibilidade a GC pode estar colaborando para a manutenção do perfil de ativação, já que, como dito anteriormente, os linfócitos dos pacientes TB não respondem a secreção de cortisol após ativação do eixo HPA. Não foi observada a ativação do eixo HPA nos pacientes TB, dados os reduzidos níveis de cortisol após e ao longo da exposição ao estresse. Porém, mesmo que esta estivesse inalterada nos pacientes TB, a reduzida sensibilidade aos GC resultaria em uma incapacidade do sistema

imune em responder ao cortisol circulante e consequente inabilidade em suprimir a resposta imune desencadeada pelo estresse.

Nossos dados nos permitem concluir que existe um desequilíbrio nas interações neuroimunoendócrinas em pacientes TB. Tais alterações são evidenciadas por reduzida reatividade ao estresse sugerindo um desequilíbrio do eixo HPA dada sua inabilidade em ser ativado diante de eventos estressores como o protocolo de estresse agudo. Os reduzidos níveis de cortisol observados nos pacientes TB (tanto no período basal, quanto em resposta ao estresse) acabam por colaborar com a ativação imune observada aqui. A manutenção da ativação imune em resposta ao estresse também reflete a maior insensibilidade aos GCs, que também pode ser fruto de uma constante e longa exposição ao desequilíbrio do eixo HPA. Não podemos inferir os motivos que levaram a tais desequilíbrios, mas fica claro a importância de uma correta comunicação entre os diferentes sistemas do organismo para a manutenção da saúde do indivíduo.

De forma geral, podemos concluir que a exposição ao estresse pode resultar em uma importante carga alostática para o organismo. A exposição ao estresse durante as fases iniciais do desenvolvimento resultam em ativação imune na periferia que acarreta na incorreta maturação de regiões cerebrais importantes para o processamento emocional como demonstrado no presente trabalho. Tais alterações influenciam a suscetibilidade ao desenvolvimento de transtornos psiquiátricos. Uma vez presente o transtorno de humor, neste caso TB, a exposição ao estresse evidencia alterações em sistemas chave, como o eixo HPA, que resulta em uma inabilidade de manter o correto equilíbrio imunológico, observado por uma já presente ativação imune (período basal), e levando a consequente exacerbação deste desbalanço após o estresse.

8. Referências

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9. Anexos

9.1. Termo de Consentimento Livre e Esclarecido

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA - CEP - PUCRS

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Sujeito de pesquisa nº____

TÍTULO DA PESQUISA: Alterações neuroimunoendócrinas do estresse agudo experimental na depressão.

JUSTIFICATIVA E OBJETIVOS DA PESQUISA: As experiências traumáticas da infância aumentam o risco para o desenvolvimento para transtornos do humor, como a depressão maior e transtorno de estresse pós-traumático (TEPT). Estas patologias estão associadas com inúmeras alterações emocionais, cognitivas, endócrinas e imunológicas. O entendimento dos efeitos da violência infantil no processo de adoecimento do adulto é muito limitado. Pretendemos com esse estudo: **(1)** determinar características do seu estado emocional que possam estar relacionadas a estresse, depressão, ansiedade e verificar como está a sua memória; **(2)** determinar a quantidade de hormônios presentes na sua saliva (cortisol); **(3)**

analisar se há alteração na atividade dos glóbulos brancos que participam na defesa do seu organismo.

PROCEDIMENTOS: Para a realização deste estudo, necessitaremos da sua colaboração em até quatro (04) dias:

1º DIA - Num dia a ser agendado, será feita uma entrevista utilizando questionários específicos, que irão avaliar suas funções cognitivas e como está sua memória.

DIA:_____HORA:_____LOCAL: Hospital São Lucas – PUCRS

2º DIA – Num dia a ser agendado, a Sra. irá passar aproximadamente 60 min. realizando um teste que nos permitirá avaliar aspectos de sua resposta imunológica e hormonal. Esse teste consiste em realizar um discurso oral seguido de um teste aritmético. Durante este teste sua pressão arterial será monitorada e serão coletadas seis (06) amostras de saliva e duas (02) amostras de sangue. O sangue será processado imediatamente após coleta para ser analisado e descartado logo após análise, não sendo armazenado para manipulações posteriores. A coleta de saliva será efetuada pela Sra. e as coletas de sangue por uma enfermeira. Na hora da punção, a Sra. sentirá um leve desconforto por causa da picada da agulha.

3º DIA – De acordo com os resultados obtidos nos primeiros testes a Sra. será convidada a realizar mais alguns testes. (antes tava assim: Caso a Sra. seja selecionada). A Sra. receberá um kit composto de um comprimido de 1,5 mg de dexametasona e material para fazer a coleta da saliva. Às **23h00min** (onze horas da noite), a Sra. deverá tomar o

comprimido de dexametasona. Às vezes, algumas pessoas podem apresentar um pouco de náusea ou sensação passageira de calor pelo corpo, mas nada disso afetará a sua saúde.

4º DIA – No dia seguinte, às 13:00h, será dado continuidade ao teste que se iniciou na noite anterior. Este teste nos permitirá avaliar aspectos de sua resposta imunológica e hormonal, porém de forma diferente ao já efetuado anteriormente. Será introduzido um cateter na veia do seu braço e a Sra. receberá soro por aproximadamente uma hora (30min) para que se acostume com a presença do cateter. Esse procedimento poderá causar algum desconforto devido à picada da agulha. Posteriormente a Sra. receberá uma solução contendo um hormônio que está presente normalmente no organismo (Hormônio Liberador de Corticotrofina – CRH) durante aproximadamente trinta segundos (30s). O cateter será retirado e a Sra. ficará por mais trinta minutos (30 min.) descansando. Neste teste também serão coletadas seis (06) amostras de saliva e duas (02) amostras de sangue. A coleta de saliva será efetuada pela Sra. e as coletas de sangue por uma enfermeira.

O material obtido não será utilizado para fins comerciais. Fica garantida a privacidade da Sra. quanto aos dados envolvidos na pesquisa. Os resultados obtidos serão armazenados por 5 anos e estarão à inteira disposição da Sra. para acompanhá-los caso assim o deseje. Estes resultados serão divulgados na literatura científica sem nenhuma identificação dos participantes.

Gastos com transporte e alimentação durante o turno em que a Sra. estiver realizando os procedimentos serão devidamente ressarcidos. Para os gastos com transporte será estimado um valor equivalente a 4 passagens de ônibus por dia que a Sra. tiver que se deslocar até os locais do teste e para alimentação, valor equivalente ao de uma refeição de acordo com os valores praticados no local do teste.

Eu, ... fui informado(a) dos objetivos da pesquisa acima de maneira clara e detalhada. Recebi informação a respeito da coleta a ser feita e esclareci minhas dúvidas. Sei que em qualquer momento poderei solicitar novas informações e modificar minha decisão se assim eu o desejar. O pesquisador responsável certificou-me de que todos os dados desta pesquisa serão confidenciais e terei liberdade de retirar meu consentimento de participação na pesquisa, em face destas informações. Declaro igualmente que recebi cópia deste consentimento, de que todos os dados sobre a minha pessoa serão confidenciais e mantidos em sigilo.

_____ _____

Assinatura do voluntário Nome Data

_____ _____

Assinatura do pesquisador Nome Data

Nome do pesquisador para contato: Dr. Moisés Evandro Bauer

Telefone: 0xx51 33203000 / ramal 2725

Endereço: Av. Ipiranga, 6690 – 2º andar, Caixa Postal 1429, CEP 90610-000, Porto Alegre

9.2. Escala Hamilton para avaliação de Depressão

Investigador:..... DATA:..../..../..... No. Paciente:.....

ESCALA DE HAMILTON PARA AVALIAÇÃO DE DEPRESSÃO

Instruções: Em cada item escolha o escore que melhor caracteriza o paciente na última semana. Assinale sua opção no espaço apropriado ( ).

1. Humor deprimido (tristeza, desesperança, desamparo, menos valia)
0 ( ) Ausente.
1 ( ) Sentimentos são relatados somente se perguntados.
2 ( ) Sentimentos são relatados espontaneamente com palavras.
3 ( ) Comunica estes sentimentos não verbalmente, ou seja, na expressão facial, postura, voz e a tendência ao choro.
4 ( ) Paciente comunica quase exclusivamente esses sentimentos, espontaneamente, tanto em seu relato verbal como na comunicação não verbal.

2. Sentimentos de culpa
0 ( ) Ausentes.
1 ( ) Auto-recriminação; acha que decepcionou outras pessoas.
2 ( ) Idéias de culpa ou ruminações sobre erros ou ações do passado.
3 ( ) Acha que a doença atual é um castigo; delírios de culpa.
4 ( ) Ouve vozes que acusam ou denunciam e/ou tem alucinações visuais ameaçadoras.

3. Suicídio
0 ( ) Ausente.
1 ( ) Acha que a vida não vale a pena.
2 ( ) Gostaria de estar morto ou qualquer cogitação sobre possível morte para si mesmo.
3 ( ) Idéias ou gestos suicidas.
4 ( ) Tentativa de suicídio (Qualquer tentativa séria marque 4).

4. Insônia inicial
0 ( ) Sem dificuldade para iniciar o sono.
1 ( ) Queixa-se de dificuldade ocasional para conciliar o sono, ou seja, mais que meia hora.
2 ( ) Queixa-se de dificuldade para conciliar o sono todas as noites.

5. Insônia intermediária
0 ( ) Sem dificuldades.
1 ( ) Queixa-se de ficar com inquietude e perturbação durante a noite.
2 ( ) Acorda durante a noite – qualquer saída da cama marcar 2 (exceto para necessidades fisiológicas).

6. Insônia terminal (madrugada)
0 ( ) Sem dificuldade.
1 ( ) Acorda de madrugada mas, volta a dormir.
2 ( ) Não consegue voltar a dormir se acordar de madrugada ou sair da cama.

7. Trabalho e atividades
0 ( ) Sem dificuldades.
1 ( ) Pensamentos e sentimentos de incapacidade, fadiga ou fraqueza relacionados a atividades, trabalho ou passatempos.
2 ( ) Perda de interesse em atividades, passatempos ou trabalho relatado diretamente pelo paciente ou indiretamente, por meio de falta de iniciativa, vacilação (sente que precisa se forçar para trabalhar ou desenvolver atividades).
3 ( ) Redução do tempo gasto em atividades ou queda de produtividade. Marque 3 se não ocupa pelo menos três horas/dia em atividades (trabalho ou passatempos), exceto as de rotina.
4 ( ) parou de trabalhar devido à doença atual. Marque 4 se o paciente não desenvolve atividades além das de rotina ou deixa de executá-las sem ajuda.

8. Retardo (lentificação do pensamento e discurso, dificuldade de concentração, diminuição da atividade motora)
0 ( ) Pensamento e discurso normal.
1 ( ) Discreta lentificação durante a entrevista.
2 ( ) Óbvia lentificação durante a entrevista.
3 ( ) Entrevista difícil.
4 ( ) Estupor.

9. Agitação
0 ( ) Nenhuma.
1 ( ) Inquietude.
2 ( ) Brinca com as mãos ou cabelos, etc.
3 ( ) Movimenta-se, não consegue sentar-se quieto durante a entrevista.
4 ( ) Retorce as mãos, rói unhas, puxa cabelos, morde lábios.

10. Ansiedade psíquica
0 ( ) Sem problemas.
1 ( ) Tensão e irritabilidade subjetivas.
2 ( ) Preocupação excessiva com trivialidades.
3 ( ) Atitude apreensiva aparente na fisionomia ou no discurso.
4 ( ) Medos expressos espontaneamente.

Investigador:..... DATA:..../..../..... No. Paciente:.....

11. Ansiedade somática (concomitantes fisiológicos da ansiedade: GI: boca seca, flatulência, indigestão, diarréia, cólicas, eructação; CV: palpitação, cefaléias. Resp.: hiperventilação, suspiros,; sudorese ter que urinar frequentemente)
0 ( ) Ausente.
1 ( ) Leve: Sintomas menores relatados quando inquirido.
2 ( ) Moderado: Paciente descreve espontaneamente sintomas não incapacitantes.
3 ( ) Grave: Maior número e freqüência que 2; acompanhado de estresse subjetivo e prejudica o funcionamento normal.
4 ( ) Incapacitante: Numerosos sintomas, persistentes ou incapacitantes na maior parte do tempo; ataques de pânico.

12. Sintomas somáticos (apetite, digestivo)
0 ( ) Nenhum.
1 ( ) Perda de apetite, mas come sem necessidade de encorajamento. Peso no abdome.
2 ( ) Dificuldades para comer sem encorajamento ou insistência. Pede ou requer laxantes ou medicação para sintomas gastrointestinais.

13. Sintomas somáticos (gerais)
0 ( ) Nenhum.
1 ( ) Peso ou lassidão em membros, costas ou cabeça. Dores nas costas, cabeça ou musculares. Perda de energia e fatigabilidade.
2 ( ) Marque 2 para qualquer sintoma bem definido.

14. Sintomas genitais (perda da libido, distúrbios menstruais)
0 ( ) Ausentes ou informação insuficiente.
1 ( ) Leves: redução da libido ou desempenho sexual insatisfatório; tensão pré-menstrual leve.
2 ( ) Graves: desinteresse ou impotência; tensão pré-menstrual grave.

15. Hipocondria
0 ( ) Ausente.
1 ( ) Auto-observação (corporal) aumentada.
2 ( ) Preocupação excessiva com a saúde.
3 ( ) Queixas freqüentes, pedidos de ajuda, etc.
4 ( ) Delírio hipocondríaco.

16. Perda de peso (avalie A ou B)
A. De acordo com o paciente.
0 ( ) Nenhum.
1 ( ) Provável emagrecimento associada à doença atual.
2 ( ) Perda de peso indubitável (de acordo com o paciente).
B. Com base em medidas semanais.
0 ( ) Menos de 0,5 Kg de perda de peso na semana.
1 ( ) Mais de 0,5 Kg de perda de peso na semana.
2 ( ) Mais de 1 Kg de perda de peso na semana.

17. Crítica
0 ( ) Reconhece estar deprimido e doente ou não estar deprimido esta semana.
1 ( ) Reconhece estar doente, mas atribui isso à má alimentação, ao clima, ao excesso de trabalho, ao vírus, à necessidade de descanso, etc.
2 ( ) Nega estar doente.

18. Variação diurna
A. Observar se os sintomas são piores pela manhã ou à tarde. Caso NÃO haja variação, marque "nenhuma".
0 ( ) Nenhuma.
1 ( ) Pior de manhã.
2 ( ) Pior à tarde.
B. Quando presente, aponte a gravidade da variação.Marque "nenhuma" caso não haja variação.
0 ( ) Nenhuma.
1 ( ) Leve.
2 ( ) Grave.

19. Despersonalização e desrealização
0 ( ) Ausente.
1 ( ) Leve.
2 ( ) Moderada.
3 ( ) Grave.
4 ( ) Incapacitante.

20. Sintomas paranóides
0 ( ) Nenhum.
1 ( ) Desconfiança.
2 ( ) Idéias de referência.
3 ( ) Delírios de referência e perseguição.

21. Sintomas obsessivos e compulsivos
0 ( ) Nenhum.
1 ( ) Leves.
2 ( ) Graves.

9.3. Escala de Young para Avaliação da Mania

Investigador:..... DATA:..../..../..... No. Paciente:.....

Escala de Young para Avaliação da Mania

Guia para avaliação dos itens - quando vários sintomas são fornecidos para um grau específico de gravidade, a presença de apenas um dos sintomas é suficiente para a pontuação. Os sintomas são fornecidos como guias.

1. HUMOR EXPANSIVO/ELEVADO
( ) **0** Ausente
( ) **1** Leve ou possivelmente elevado quando questionado
( ) **2** Elevação subjetiva definida; otimista, alegre; autoconfiante; apropriado ao conteúdo
( ) **3** Elevado, inapropriado ao contexto; jocoso
( ) **4** Eufórico; riso inapropriado; cantando

2. Aumento da energia e atividade motora
( ) **0** Ausente
( ) **1** Aumento subjetivo
( ) **2** Animado; aumento de gesticulação
( ) **3** Energia excessiva; às vezes hiperativo; inquieto/impaciente (pode ser acalmado)
( ) **4** Excitação motora; hiperatividade contínua (não pode ser acalmado)

3. Interesse sexual
( ) 0 Normal; sem aumento
( ) 1 Leve ou possivelmente aumentado
( ) 2 Aumento subjetivo bem definido quando questionado
( ) 3 Conteúdo sexual espontâneo; discorre sobre assuntos sexuais; hipersexualizado segundo auto-relato
( ) 4 Atos sexuais evidentes (direcionados a pacientes, equipe ou entrevistador)

4. Sono
( ) 0 Não relata diminuição do sono
( ) 1 Dorme menos do que o habitual (até 1 hora a menos)
( ) 2 Dorme menos do que o habitual (por 1 hora ou mais)
( ) 3 Relata diminuição da necessidade de sono
( ) 4 Nega necessidade de sono

5. Irritabilidade
( ) 0 Ausente
( ) 2 Aumento subjetivo
( ) 4 Irritável em alguns momentos durante a entrevista; episódios recentes de raiva ou importunação na enfermaria
( ) 6 Freqüentemente irritável durante a entrevista.
( ) 8 Hostil, não-cooperativo; entrevista impossível

6. Discurso (velocidade e quantidade)
( ) 0 Sem aumento
( ) 2 Sente-se mais falante
( ) 4 Aumento da velocidade ou quantidade em alguns momentos, prolixo em alguns momentos
( ) 6 Aumento consistente da velocidade e quantidade; difícil interromper
( ) 8 Pressão de discurso; ininterruptível, discurso contínuo

7. Distúrbio de linguagem-pensamento
( ) 0 Ausente
( ) 1 Circunstancial; leve distraibilidade; pensamentos rápidos
( ) 2 Distraído; perde intenção do pensamento; muda de assunto freqüentemente; pensamentos acelerados
( ) 3 Fuga de idéias; tangencialidade; difícil para acompanhar; rimando, ecolalia
( ) 4 Incoerente; comunicação impossível

8. Conteúdo
( ) 0 Normal
( ) 2 Planos questionáveis, novos interesses
( ) 4 Projetos especiais; hiperreligioso
( ) 6 Idéias grandiosas ou paranóides; idéias de referência
( ) 8 Delírios; alucinações

9. Comportamento disruptivo-agressivo
( ) 0 Ausente, cooperativo
( ) 2 Sarcástico; fala alto às vezes, vigilante
( ) 4 Querelante; faz ameaças na enfermaria
( ) 6 Ameaça entrevistador; grita; entrevista difícil
( ) 8 Agressivo; destrutivo; entrevista impossível

10. Aparência
( ) 0 Traje e cuidados pessoais apropriados
( ) 1 Um pouco descuidado
( ) 2 Desleixado; moderadamente desalinhado; trajes exagerados
( ) 3 Desalinhado; parcialmente trajado; maquiagem extravagante
( ) 4 Completamente descuidado; enfeitado; vestes bizarras

11. INSIGHT
( ) 0 Presente; admite doença; concorda com necessidade de tratamento
( ) 1 Possivelmente doente
( ) 2 Admite mudanças de comportamento, mas nega doença
( ) 3 Admite possível mudança de comportamento, mas nega doença
( ) 4 Nega qualquer mudança de comportamento

9.4. Anamnese

Paciente:_____ Idade:_____

Data última menstruação:_____

Data:_____ Responsável:_____

DADOS DE IDENTIFICAÇÃO DO SUJEITO DA PESQUISA

1. INFORMAÇÕES GERAIS:

1.1 Data de nascimento: _____

1.2 Sexo: ( ) Feminino ( ) Masculino

1.3 Etnia: ( ) Branco ( ) Não-branco

1.4 Escolaridade: _____

1.4.1 Anos de estudo:_____

1.4.2 Anos repetidos:_____

1.5 Renda familiar aproximada:_____

1.6 Situação Conjugal: ( ) Casado ( ) Solteiro ( ) Separado/Divorciado ( ) Viúvo

1.7 Pratica atividade física? ( ) Sim ( ) Não Tempo semanal de atividade física?_____

1.8 Endereço:_____

1.9 Telefone: _____

1.10 Tempo de diagnóstico do Transtorno: _____

2. CARACTERÍSTICA DA DOENÇA:

2.1 Idade do primeiro episódio: _____

2.2 Tempo de doença: _____

2.3 Tipo do primeiro episódio:

( ) Mania ( ) Depressão ( ) Misto

( ) Hipomania ( ) Não sabe

2.4 Idade que recebeu o diagnóstico médico: _____

2.5 Idade que usou medicação pela primeira vez: _____

2.6 Primeira crise desencadeada por substância psicoativa? (antidepressivo, maconha, cocaína, álcool, anorexígeno, anfetamina, estimulantes, hormônios).

( ) Sim ( ) Não

2.7 Hospitalizações psiquiátricas: ( ) Sim ( ) Não

2.7.1 Número de hospitalizações: _____

2.7.2 Idade da primeira hospitalização: _____

2.7.2.1 Tipo de episódio:

( ) Mania ( ) Depressão ( ) Misto

( ) Hipomania ( ) Não sabe

2.7.3 Idade na última hospitalização: _____

2.7.3.1 Tipo de episódio:

( ) Mania ( ) Depressão ( ) Misto

( ) Hipomania ( ) Não sabe

2.8 Ciclador rápido (4 ou mais episódio/ano): ( ) Sim ( ) Não

2.9 Realizou ECT (Terapia Eletroconvulsiva): ( ) Sim ( ) Não

2.10 Tentativa de suicídio: ( ) Sim ( ) Não

2.10.1 Número de tentativas: _____

2.10.2 Tipos de tentativas:

( ) Arma de fogo ( ) Enforcamento ( ) Cortar pulsos

( ) Medicação ( ) Pular de local alto ( ) Outro: _____

3. HÁBITOS ATUAIS

3.1 – Cigarro ( ) Sim ( ) Não ( ) Em abstinência

3.2 – Chá ( ) Sim ( ) Não

3.3 – Chimarrão ( ) Sim ( ) Não

3.4 – Café ( ) Sim ( ) Não

3.5 – Bebidas alcoólicas ( ) Sim ( ) Não ( ) Em abstinência

4. HISTÓRIA FAMILIAR DE TRANSTORNOS MENTAIS

4.1 Número de familiares de primeiro grau acometidos por transtorno bipolar: _____

4.2 Número de familiares de primeiro grau acometidos por outros transtornos: _____

5. DESENVOLVIMENTO

5.1 Qual a idade de sua mãe quando você nasceu? _____

5.2 Existiu alguma intercorrência durante a sua gestação (infecções, trauma)?

( ) Sim ( ) Não ( ) Não sei

Se sim, qual? _____

5.3 Durante sua gestação sua mãe fez uso de medicações?

( ) Sim ( ) Não ( ) Não sei

Se sim, qual? _____

5.4 Durante sua gestação sua mãe fez uso de drogas?

( ) Sim ( ) Não ( ) Não sei

Se sim, qual? _____

5.5 Durante sua gestação sua mãe fez uso de cigarros?

( ) Sim ( ) Não ( ) Não sei

Se sim, qual? _____

5.5 Durante sua gestação sua mãe fez uso de álcool?

( ) Sim ( ) Não ( ) Não sei

Se sim, qual? _____

5.6 Seu parto foi

( ) Normal ( ) Cesariana ( ) Não sei

5.7 Existiu alguma complicação durante o seu nascimento como:

5.7.1 Prematuridade ( ) Sim ( ) Não ( ) Não sei

5.7.2 Circular de cordão ( ) Sim ( ) Não ( ) Não sei

5.7.3 Uso de fórceps ( ) Sim ( ) Não ( ) Não sei

5.8 Você teve alguma complicação após o nascimento, necessitando hospitalização?

( ) Sim ( ) Não ( ) Não sei

Se sim, qual? _____

5.9 Você teve algum atraso no desenvolvimento? (sentar, caminhar, falar)

( ) Sim ( ) Não ( ) Não sei

Se sim, qual? _____

6. QUESTIONÁRIO CLÍNICO:

6.1. A Sra. possui diagnóstico de alguma das seguintes doenças?

Alzheimer	( ) Sim	( ) Não
Anemia hemolítica	( ) Sim	( ) Não
Artrite reumatoide	( ) Sim	( ) Não
Bronquite asmática	( ) Sim	( ) Não

Diabete tipo 1 (insulino-dependente)	( ) Sim	( ) Não
Doença Cardiovascular	( ) Sim	( ) Não
Doença da tireóide	( ) Sim	( ) Não
Doença inflamatória do intestino	( ) Sim	( ) Não
Esclerodermia	( ) Sim	( ) Não
Gota ou outra artropatia induzida por	( ) Sim	( ) Não
Hipertensão	( ) Sim	( ) Não
Lúpus eritematoso sistêmico	( ) Sim	( ) Não
Miastenia gravis	( ) Sim	( ) Não
Miosite	( ) Sim	( ) Não
Neoplasia maligna	( ) Sim	( ) Não
Parkinson	( ) Sim	( ) Não
Rinite alérgica	( ) Sim	( ) Não
Vasculite	( ) Sim	( ) Não

6.2. A Sra está tratando alguma doença infecciosa no momento?

( ) Sim ( ) Não

6.3. A Sra esteve tratando alguma doença infecciosa nas últimas duas semanas?

( ) Sim ( ) Não

6.4. A Sra possui alguma doença infecciosa crônica?

( ) Sim ( ) Não

6.5. A Sra faz uso de alguma das seguintes medicações?

Hormônios (da tireóide, TRH)	( ) Sim	( ) Não
Anticoagulantes (Marcoumar ou Marevan)	( ) Sim	( ) Não
Imunossupressores (Azatioprina, Metotrexato, Ciclosporina, Etanercept, Leflunomida, outros)	( ) Sim	( ) Não
Glicocorticóides (Prednisona, Fludrocortisona, Prednisolona, Dexametasona, Hidrocortisona, outros)	( ) Sim	( ) Não

6.6. Outros diagnósticos clínicos conhecidos:

a) _____

b) _____

c) _____

6.7. Outros medicamentos em uso pela paciente:

Nome Farmacológico	Início	mg / dia	Suspensão

7. Exame físico:

    a. Dados antropomórficos:

Peso	Kg
Altura	cm
IMC	

    b. Sinais Vitais:

FC	bpm
PA (1)	mmHg
PA (2)	mmHg
PA (média)	mmHg

8. Exames Laboratoriais:

Análise de Urina (fita)	
Proteinúria	( ) sim ( ) não
Glicosúria	( ) sim ( ) não
ITU	( ) sim ( ) não

Análise de sangue - Laboratório	
Hemograma	
Hct	%
Hb	g/dL
VCM	fL
CHCM	g/dL
Leucócitos	/µL
TGO (AST)	U/L
TGP (ALT)	U/L
Glicemia	mg/dL
Creatinina	mg/dL

10. Anexo II – Demais produções do doutorado

10.1. Artigo Científico

Psychoneuroendocrinology (2012) xxx, xxx–xxx

Available online at www.sciencedirect.com
SciVerse ScienceDirect
journal homepage: www.elsevier.com/locate/psyneuen

Reduced regulatory T cells are associated with higher levels of Th1/TH17 cytokines and activated MAPK in type 1 bipolar disorder

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Received 17 April 2012; received in revised form 16 August 2012; accepted 16 August 2012

KEYWORDS
Bipolar disorder;
Cytokines;
T cells;
Lymphocytes;
MAPK;
Regulatory T cells

Summary Bipolar disorder (BD) has been associated with an immunologic imbalance shown by increased peripheral inflammatory markers. The underlying mechanisms of this phenomenon may include changes in circulating cells and differential activation of mitogen-activated protein kinases (MAPKs). Twenty-seven euthymic female subjects with BD type I (all medicated) and 24 age- and sex-matched controls were recruited in this study. Lymphocytes were isolated and stimulated in vitro to assess Th1/Th17/Th2 cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α) and MAPK phosphorylation. The expression of phospho-MAPKs, a large panel of lymphocyte subsets and cytokines were assessed by multi-color flow cytometry. BD patients had reduced proportions of natural T regulatory cells (CD4+ CD25+ FoxP3+) ($p < 0.01$) in parallel to higher cytokine production (all $p < 0.01$) than healthy controls. In particular, BD was associated with a strong bias to Th1 rather than Th2 profile. There was an expansion of senescence-associated cells (CD8+ CD28−) in BD ($p < 0.0001$). T cells of BD patients had an increased p-ERK signaling ($p < 0.0001$), indicating lymphocyte activation. Our data suggest that multiple molecular and cellular mechanisms may contribute to the immunologic imbalance observed in BD. In addition, our data concur to an early senescence process in these patients.

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http://dx.doi.org/10.1016/j.psyneuen.2012.08.005

Please cite this article in press as: do Prado, C.H., et al., Reduced regulatory T cells are associated with higher levels of Th1/TH17 cytokines and activated MAPK in type 1 bipolar disorder. Psychoneuroendocrinology (2012), http://dx.doi.org/10.1016/j.psyneuen.2012.08.005

Introduction

There is growing evidence suggesting that the immune and inflammatory systems play important roles in the pathogenesis of bipolar disorder (BD). Several studies have investigated the potential role of cytokines in psychiatric disorders, based on their important actions in modulating metabolism of central neurotransmitters, hypothalamic–pituitary–adrenal (HPA) axis and neurotrophic support (Miller et al., 2009). Increased plasma levels of pro-inflammatory cytokines were observed during BD manic episodes, including higher levels of IL-6, IFN-γ, TNF-α, IL-2 and serum soluble IL-6 receptor (sIL-6R) (Kim et al., 2004, 2010; O'Brien et al., 2006; Brietzke et al., 2009; Barbosa et al., 2011). We have recently observed that BD patients in mania had higher sTNFR1 levels than euthymic BD patients and controls (Barbosa et al., 2011). In addition, others have found elevated IL-4 levels (a Th2 cytokine) in patients with manic episodes (Kim et al., 2004; Ortiz-Dominguez et al., 2007). Similar to mania, increased levels of IL-6 and TNF-α were also observed during depressive episodes (Kim et al., 2004; O'Brien et al., 2006; Ortiz-Dominguez et al., 2007). It should be noted that previous studies assessed inflammatory markers in plasma/serum samples. The analysis of biomarkers in cellular supernatants is advantageous to serum/plasma sampling as it can precise the cellular source of cytokines. The underlying mechanisms of the immunologic imbalance observed in BD are largely unknown, and may include changes in circulating lymphocytes and the differential expression of intracellular signaling cascades.

Changes in circulating leukocytes may contribute to the immunologic imbalance observed in BD. The analyses of lymphocyte subsets, particularly T, B and NK cells are very scarce in BD though. Increased percentages of activated T cells (i.e. CD3+ HLADR+, CD3+ CD25+ and CD3+ CD71+) and B cells (CD19+ CD20+) were observed in BD compared to healthy controls (Breunis et al., 2003). This activation state could be theoretically due to a lack of peripheral regulatory cells. A recent study did not observe changes in regulatory T cells (Tregs) in BD patients as compared to controls (Drexhage et al., 2011). Tregs (CD4+ CD25+ Foxp3+) play key roles in suppressing excessive or misguided immune responses that can be harmful to the host. In particular, they are responsible for turning off immune responses against self-antigens in autoimmune diseases, allergies or commensal microbes in certain inflammatory diseases (Sakaguchi et al., 2008). To date, the roles of CD8+ regulatory T cells (CD8+ CD28− and CD8+ CD103+) in BD are largely unknown.

The immunologic imbalance observed in BD could be also explained by the differential expression of intracellular signaling cascades. Mitogen-activated protein kinases (MAPKs) are important intracellular signal transduction systems and participate in a series of physiological and pathological processes, including cell growth, differentiation and apoptosis (Strniskova et al., 2002; Sosa et al., 2011). Three major MAPK cascades are known, including the extracellular signal-regulated protein kinase (ERK), c-jun amino-terminal protein kinase/stress-activated protein kinase (JNK/SAPK) and p-38. Studies based in animal models found impaired central MAPKs signaling associated to behavioral changes and cognitive deficits related to mood disorders, suggesting an important role of this cascade in psychiatric disorders (Mazzucchelli et al., 2002; Einat et al., 2003a; Thomas and Huganir, 2004; Yuan et al., 2010). Spiliotaki et al. (2006) found reduced levels of JNK in lymphocytes from depressed BD patients when compared to euthymic patients and control individuals (Spiliotaki et al., 2006). Yuan et al. (2010) found reduced ERK levels in pre-frontal cortex of BD, major depression and schizophrenic patients compared to controls. The ERK pathway regulates gene expression including proteins associated to apoptotic processes as well as associated with cell proliferation and differentiation. Phosphorylation of ERK correlates with ERK activation (Yuan et al., 2010). While p38 is associated to a pro-apoptotic pathway, ERK is activated by mitotic stimuli, been associated to cell proliferation (Raman et al., 2007; Furler and Uittenbogaart, 2010; Xie et al., 2010). Also, p38 is often linked to inflammation and cellular non-responsiveness (anergy) (Raman et al., 2007; Furler and Uittenbogaart, 2010). Interestingly, these two enzymes have reciprocal antagonistic actions (Raman et al., 2007).

Here, we assessed cellular and molecular mechanisms that may influence the inflammatory state observed in BD patients. Specifically, we determined (a) Th1/Th2/Th17 cytokines in supernatants and addressed the distribution of (b) regulatory T cells and various lymphocyte subsets, as well as (c) the intracellular expression of activated MAPKs p38 (p-p38) and ERK (p-ERK) in euthymic type 1 BD patients and healthy controls.

Methods

Subjects

Twenty-seven euthymic female subjects with BD type I were recruited by convenience sampling at the Outpatient Psychiatric Clinic for Women with bipolar disorder of the Presidente Vargas Hospital, Porto Alegre, Brazil. Only women took part in this study to avoid immunological differences associated with sexual dimorphism (Ghazeeri et al., 2011). Age- and sex-matched healthy controls also took part in this study. All subjects provided their written informed consent before inclusion in the study approved by the Ethical Committee of the institution. The BD type 1 diagnosis was based on clinical interview and confirmed with the *Structured Clinical Interview for DSM-IV-Axis I Disorder* (SCID-I) administered by an expert and well-trained psychiatrist. Severity of depressive and manic symptoms was assessed by the *Hamilton Depression Rating Scale* (HDRS) and the *Young Mania Rating Scale* (YMRS), respectively. All patients were euthymic at the time of blood collection. Therefore, this study was designed to examine more enduring immunological changes not necessarily associated to mood episodes. Euthymia was defined by YMRS and HDRS scores < 8 (Clark et al., 2002). Exclusion criteria to both patients and controls included: (a) presence of major axis I psychiatric disorder such as psychotic disorder, mood disorder (for control group), anxiety disorder or substance related disorder according to SCID-I; (b) history of a severe medical illness; (c) history of brain injury; (d) presence of systemic diseases (including hypertension, inflammatory diseases, such as rheumatoid arthritis or infection) or neurological disorder, and (e) use

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of any substance that may induce immune or endocrine changes (exception of psychopharmacotherapy for BD patients).

Blood collection and cell isolation

Twenty milliliters of peripheral blood were collected by venipuncture between 1000 h and 1200 h and stored in EDTA tubes prior to analyses. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation for 30 min at 900 × *g*. Cells were counted by means of microscopy (100×) and viability always exceeded 95%, as judged from their ability to exclude Trypan Blue (Sigma). PBMCs were resuspended in complete culture medium (RPMI-1640, supplemented with 0.5% gentamicine, 1% glutamine, 1% hepes, 0.1% fungizone, and 10% fetal calf serum, FCS; all from Sigma) and adjusted to yield a final concentration of 2×10^5 cells/well.

Immunophenotyping

A large panel of lymphocyte subpopulations was identified by multi-color flow cytometry in freshly isolated PBMCs. Briefly, PBMCs were washed in flow cytometry buffer (PBS containing 1% FCS and 0.01% sodium azide) and treated with Fc Block solution for 20 min. In order to evaluate specific lymphocyte subsets, cells were stained for 30 min with combinations of the following monoclonal antibodies: anti-CD3 FITC, anti-CD3PECy5, anti-CD4 PE, anti-CD4 FITC, anti-CD8 PE, anti-CD19 PE, anti-CD56 FITC, anti-CD28 FITC, anti-CD45RO FITC, anti-CD69 FITC, anti-FOXP3 PECy5, anti-CD103 FITC, anti-CCR7 Cy7, anti-CD45RA FITC (all from BD Biosciences, San Jose, CA, USA). Immediately after staining, cells were washed, resuspended and analyzed by flow cytometry. A minimum of 20,000 lymphocytes were identified by size (FSC) and granularity (SSC) and acquired with a FACS Canto II flow cytometer (BD Biosciences). The instrument has been checked for sensitivity and overall performance with Cytometer Setup and Tracking beads (BD Biosciences) prior to data acquisition. Data were analyzed using the Flowjo 7.2.5 software (Tree Star Inc., Ashland, OR, USA).

Quantification of cytokines

To determine cytokine production, PBMCs were cultured (1.5×10^5 cells) in RPMI medium with 10% FCS (Sigma–Aldrich) and 1% phytohemagglutinin (PHA, from Invitrogen, Carlsbad, CA, USA), for 72 h at 37 °C and in a 5% CO_2 atmosphere. The supernatants were collected and stored at −80 °C for later analysis. The samples were thawed in the same day and processed together. Multiple soluble cytokines (IL-2, IL-10, IL-4, IL-6, IFN-γ, TNF-α and IL-17) were simultaneously measured by flow cytometry using the Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Kit (BD Biosciences). Acquisition was performed with a FACSCanto II flow cytometer (BD Biosciences). The instrument has been checked for sensitivity and overall performance with Cytometer Setup and Tracking beads (BD Biosciences) prior to data acquisition. Quantitative results were generated using FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary). The detection limits for these assays ranged from 2.4 to 4.9 pg/mL (IL-2, IL-10, IL-4, IL-6, IFN-γ, and TNF-α) and 18.9 pg/mL for IL-17.

Analysis of intracellular activated MAPKs in lymphocytes

Activated MAPKs in lymphocytes were assessed by flow cytometric evaluation of intracellular phospho-p38 and phospho-ERK expression in CD3+ CD4+ and CD3+ CD8+ cells (Human T Cell Activation Kit, BD Biosciences). Isolated PBMCs were cultured in RPMI medium with 10% FCS and stimulated with 40 nM phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin (IONO, all from Sigma–Aldrich) for 15 min at 37 °C and in a 5% CO_2 atmosphere. Cells were harvested and immediately fixed and stored frozen (−80 °C) in Cytofix solution (BD Biosciences) for later analysis. All samples were thawed in the same day and processed together to reduce variation. Cells were permeabilized on ice for 30 min with Phosflow Perm Buffer III (BD Biosciences). Cells were washed (600 × *g*, 6 min), stained for 60 min at room temperature, washed (600 × *g*, 6 min) and resuspended in final concentration of 4.5×10^5 cells/200 μL, all in Pharmingen Staining Buffer (BD Biosciences). The 4-color immunofluorescent staining procedure was performed combining the following monoclonal antibodies from BD Biosciences: anti-CD3 PerCP (clone SK7), anti-CD4 FITC (clone SK3) and anti-CD8 PE (clone SK1) with anti-phospo ERK1/2 Alexa Fluor 647 (clone 20A, which recognizes the phosphorylated threonine 202, pT202, and tyrosine 204, pY204 of ERK1 and the pT184/pY186 of ERK2) or anti-phospho p38 Alexa Fluor 647 (clone 36/p38, which recognizes the conserved dual phosphorylated site pT180/pY182 of p38α, β, γ and δ). Lyophilized human control cells (BD Biosciences) were used as positive and negative controls due to the known presence of mitogen-activated (upregulation of phosphorylated MAPKs) or non-activated (basal levels of phospho-MAPKs) T cells in each control, respectively. A minimum of 20,000 lymphocytes were identified by size (FSC) and granularity (SSC) and acquired with a FACS Canto II flow cytometer (BD Biosciences). The instrument has been checked for sensitivity and overall performance with Cytometer Setup & Tracking beads (BD Biosciences) prior to data acquisition. Data were analyzed using the Flowjo 7.2.5 software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

All variables were tested for homogeneity of variances and normality of distribution by means of the Levene and Kolmogorov–Smirnov tests, respectively. For continuous variables, differences between groups were analyzed by Student's *t*-test or Mann–Whitney *U* test when appropriate. Statistical interactions between categorical variables were compared by means of the chi-square (χ^2) test. Statistical analyses were performed using the Statistical Package for Social Sciences, SPSS Statistics 17.0 software (SPSS Inc., Chicago, IL, USA). The significance level was set at $\alpha = 0.05$ (two-tailed).

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Results

Characteristics of the studied populations

Demographic and clinical characteristics of the samples are summarized in Table 1. Both groups were homogenous regarding age, gender, ethnicity, BMI and smoking habits. Most patients were under a multiple drug regimen (Table 1). However, 12 patients were under lithium monotherapy, one under antidepressant only, one with antipsychotic only and none with anticonvulsant only.

Lymphocyte subsets

We screened a large panel of circulating lymphocyte subpopulations by multicolor flow cytometry, including activated, regulatory and immunosenescence markers (Table 2). Cells were immunophenotyped prior to cell cultures. The studied groups were homogenous regarding most lymphocyte markers. However, BD patients had altered proportions of regulatory T cells (Fig. 1). In particular, lower percentages of natural Treg cells (CD4+ CD25+ FoxP3+) were observed in BD patients, as shown in Fig. 1C and D ($U = 100.00$; $p < 0.01$). Furthermore, BD patients had a reduced FoxP3 expression (−31.2%) in total CD4+ cells as compared to controls (1576.08 ± 363.46 vs. 2292 ± 374.39, respectively), as estimated by the mean fluorescence intensity ($U = 75$, $p < 0.05$). In contrast, BD patients had higher frequencies of CD8+ CD28− T cells as compared to controls ($U = 165.00$; $p < 0.0001$). With respect to possible effects of pharmacotherapy, no significant associations were found with the immunological measures (all $p > 0.05$, assessed by Mann−Whitney Tests).

Cytokine production

Multiple Th1/Th2/Th17 cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α) were assessed in culture supernatants by CBAs. Table 3 shows the cytokine profiles following polyclonal T-cell stimulation. All cytokines were found significantly increased in BD when compared with healthy controls (all $p < 0.01$). To further investigate the cytokine profiles we compared the cytokine ratios between the two groups. BD patients showed higher IL-6/IL-4 ($U = 195.00$; $p < 0.0001$), TNF-α/IL-4 ($U = 91.00$; $p < 0.0001$), IFN-γ/IL-4 ($U = 79.00$; $p < 0.0001$) and IFN-γ/IL-10 ($U = 161.00$; $p = 0.009$) ratios compared to controls (Fig. 2), suggesting a strong bias to Th1 rather than Th2 profile. There were no statistical differences regarding the remaining cytokine ratios (all $p > 0.05$). No significant associations were found between pharmacotherapy use and the immunological measures (all $p > 0.05$, assessed by Mann−Whitney Tests).

Analysis of intracellular phospho-MAPKs in peripheral lymphocytes

We have also analyzed the expression of phosphorylated p-38 and p-ERK MAPKs in lymphocytes following stimulation with PMA and IONO. Fig. 3 shows the unstimulated (solid line) and activated (dotted line) profiles of a representative sample. The expression of p-ERK MAPK in T CD8+ ($U = 17.00$; $p = 0.001$) and CD4+ cells ($U = 31.00$; $p = 0.018$), as estimated by the mean fluorescence intensity (MFI) was found increased in patients in comparison with controls (Fig. 4C). This was not observed for the p-p38 expression (Fig. 4D). There were no significant differences in the percentages of T cells expressing p-ERK or p-p38 between groups (Fig. 4A and B). No significant associations were found between pharmacotherapy use and the immunological measures (all $p > 0.05$, assessed by Mann−Whitney Tests).

Discussion

Data presented here are in accordance to previous studies suggesting an immune/inflammatory imbalance in BD. Briefly, patients had lower proportions of natural regulatory T cells (CD4+ CD25+ FoxP3+) in parallel to higher cytokine production when compared to healthy subjects (with strong bias to Th1). We also observed an increased p-ERK signaling in peripheral T-cell subsets of BD patients, suggesting increased lymphocyte activation.

PBMCs of BD patients produced significantly higher amounts of IL-2, IL-4, IL-5, IL-10, IL-17, IFN-γ and TNF-α

Table 1 Characteristics of the studied populations.

	BD	Healthy Controls	*p*-Value
N	27	24	–
Age, years (mean ± SD)	45.72 ± 9.22	40.48 ± 13.24	0.13
BMI (mean ± SD)	27.92 ± 4.20	26.48 ± 3.26	0.23
Years of illness (mean and interval)	10.44 (1−46)	–	–
Age at onset (mean ± SD)	34.95 ± 12.28	–	–
HDRS (mean ± SD)	4.96 ± 2.21	–	–
YMRS (mean ± SD)	1.70 ± 2.09	–	–
Ethnicity (white/non-white)	21/6	23/1	0.09
Smoking	5	2	0.29
Lithium	18	–	–
Antidepressants	11	–	–
Antipsychotics	11	–	–
Anticonvulsants	3	–	–

Data shown as mean (*M*) ± standard deviation (SD). *Abbreviations*: BMI, body mass index; BD, bipolar disorder; HDRS, Hamilton Depression Rating Scale; and YMRS, Young Mania Rating Scale. Data were analyzed by Student's *t*-test or χ^2 test.

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Table 2 Immunophenotyping of lymphocyte subsets.

Markers	Cell type	BD (%)	Healthy controls (%)	*p*-Value
CD3+ CD4+	Th	48.51 ± 6.83	48.19 ± 6.98	0.50
CD3+ CD8+	Tc	24.96 ± 6.92	25.09 ± 6.11	0.68
CD3− CD19+	B	8.59 ± 4.61	7.40 ± 2.30	0.30
CD3− CD56+	NK	11.18 ± 11.47	13.02 ± 8.51	0.22
CD3+ CD56+	NK T	6.55 ± 6.08	8.46 ± 20.13	0.24
CD4+ CD45RO+	Memory (Th)	27.86 ± 9.73	27.40 ± 5.10	0.94
CD8+ CD45RO+	Memory (Tc)	10.52 ± 6.60	9.99 ± 3.01	0.94
CD4+ CD25+	Activated T cell	1.34 ± 0.72	1.97 ± 1.66	0.50
CD3+ CD69+	Activated T cell	2.32 ± 1.33	2.27 ± 1.23	0.34
CD8+ CD28+	Activated T cell	14.24 ± 7.96	15.68 ± 5.08	0.25
CD8+ CD28−	Regulatory T Cell	17.92 ± 6.76	12.55 ± 5.08	0.002**
CD4+ CD25+ FOXP3+	Regulatory T cell	2.33 ± 2.46	7.18 ± 10.14	0.014*
CD8+ CD103+	Regulatory T cell	0.695 ± 0.31	1.05 ± 0.68	0.12
CD4+ CCR7+ CD45RA−	Central memory (Th)	44.51 ± 11.22	45.40 ± 9.97	0.97
CD4+ CCR7− CD45RA−	Effector memory (Th)	16.42 ± 5.68	14.42 ± 5.21	0.29
CD4+ CCR7+ CD45RA+	Naïve T cell (Th)	34.47 ± 13.75	34.86 ± 11.56	0.94
CD8+ CCR7+ CD45RA−	Central memory (Tc)	24.81 ± 9.74	23.11 ± 6.95	0.68
CD8+ CCR7+ CD45RA+	Naïve T cell (Tc)	40.85 ± 12.15	44.68 ± 9.65	0.38
CD8+ CCR7− CD45RA+	TEMRA (Tc)	18.39 ± 6.67	16.33 ± 6.01	0.28
CD8+ CCR7− CD45RA−	Effector memory (Tc)	15.88 ± 6.24	15.19 ± 5.26	0.81

Abbreviations: CM, central memory; EM, effector memory; TEMRA, T effector memory RA+; Th, T helper cell; and Tc, T cytotoxic cell. Percentages of Tregs refers to CD4+ cells.

* Statistical significant differences are indicated (Mann–Whitney Test): $p < 0.05$.

** Statistical significant differences are indicated (Mann–Whitney Test): $p < 0.01$.

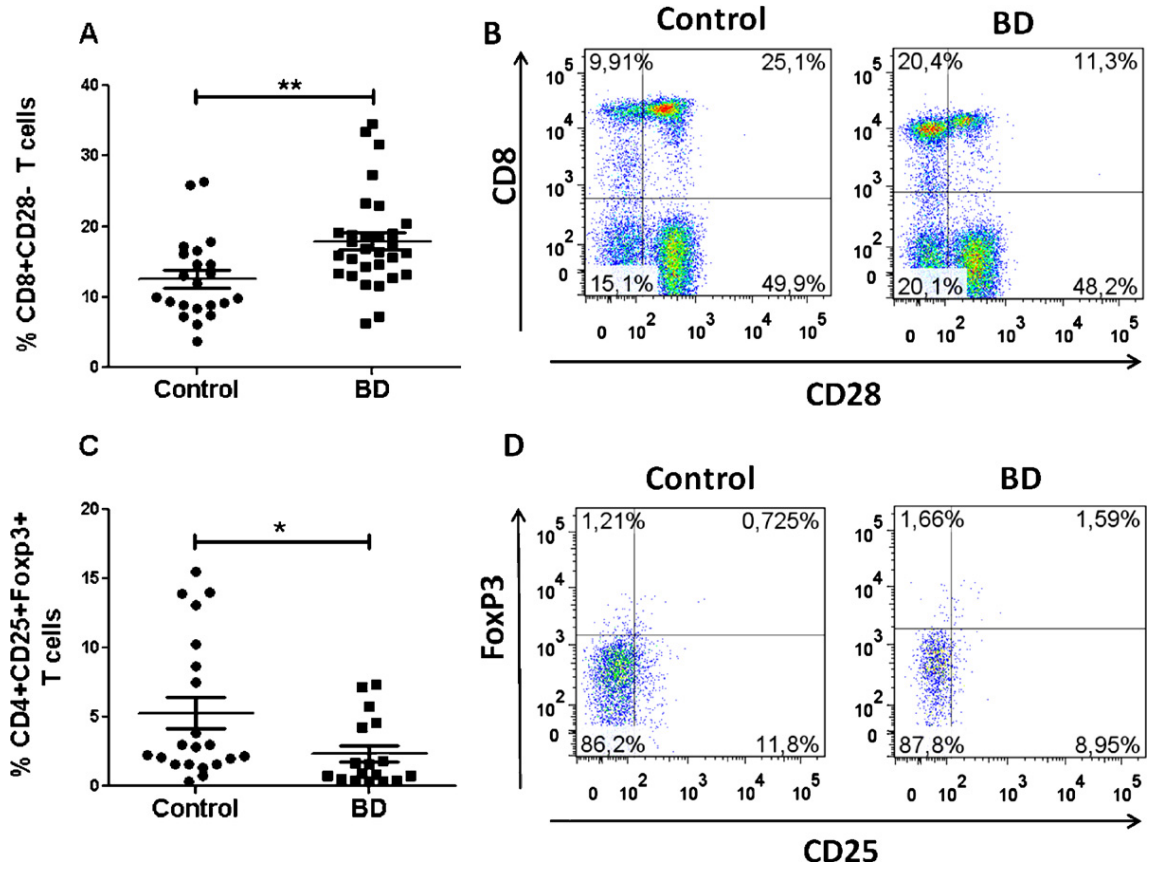

Figure 1 Major regulatory T cells in BD and healthy controls. Figures show the percentages (A and C) and representative dot plots (B and D) of regulatory T CD8+ and CD4+ cells of gated peripheral lymphocytes. Statistical significant differences are indicated: **$p < 0.0001$ and *$p < 0.01$. Data were analyzed by Mann–Whitney Test.

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Table 3 In vitro production of Th1/Th2/Th17 cytokines by PBMCs.

	BD (pg/ml)	Healthy controls (pg/ml)	*p*-Value
IL-2	978.86 ± 1862.96	92.703 ± 230.38	0.005**
IL-4	60.04 ± 123.43	36.65 ± 81.32	0.015*
IL-6	9548.51 ± 3442.92	3325.25 ± 5214.56	0.001**
IL-10	3359.06 ± 2524.33	1293.92 ± 2116.79	0.002**
TNF-α	2678.99 ± 2913.88	566.51 ± 1076.69	0.001**
IFN-γ	26,572.7 ± 16,014.6	9168.61 ± 17,420.08	0.001**
IL-17	275.9 ± 251.24	167.08 ± 217.54	0.028*

Peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with PHA for 72 h. Supernatants were collected and cytokines measured by CBA (Cytometric Bead Array, BD). Data are shown as mean (pg/ml) ± S.D.
* Statistical significant differences are indicated (Mann–Whitney Test): $p < 0.05$.
** Statistical significant differences are indicated (Mann–Whitney Test): $p < 0.01$.

compared to controls, providing further support to the immune/inflammatory imbalance described in BD. This method is advantageous to serum/plasma sampling because it can precise the cell source of cytokines. In order to better understand the increased rates of cytokines, we analyzed the pro-inflammatory/anti-inflammatory cytokine ratios. BD was associated with a strong bias to Th1 (pro-inflammatory) rather than Th2 profile. There is scarce immune data regarding euthymic BD patients (Brietzke et al., 2009; Guloksuz et al., 2010; Kunz et al., 2011). Previous studies have observed increased plasma levels of pro-inflammatory cytokines during manic (Kim et al., 2004, 2010; O'Brien et al., 2006; Brietzke et al., 2009; Barbosa et al., 2011) or depressive episodes (Kim et al., 2004; O'Brien et al., 2006; Ortiz-Dominguez et al., 2007), suggesting this immune/inflammatory imbalance could be a trait phenomenon in BD and may contribute to the behavioral alterations observed in these patients. Indeed, we have recently shown that plasma TNF-α and sTNFR2 levels were negatively correlated to cognitive (executive) functions in BD type I euthymic patients (Barbosa et al., 2012).

Specific changes in circulating lymphocytes may also contribute to the immune imbalance observed in BD. We investigated a large panel of lymphocyte subpopulations, including activated and regulatory cells as well as immunosenescence markers. Lower proportions of natural Tregs in BD patients (−55.91%) were observed when compared to healthy subjects. Tregs cells are responsible for the control of immune responses and the absence of such important regulatory cells can lead to exacerbated immune responses (Sakaguchi et al., 2008). Indeed, the lack of regulatory T cells has been observed in several chronic inflammatory conditions (Sakaguchi et al., 2008). The study of the Tregs cells in mood disorders is scarce. Our study is in accordance with recent studies reporting low proportions of Tregs in major depression (Li et al., 2010; Chen et al., 2011). However, Drexhage et al. (2011), in a recent study, did not observe overall changes in Tregs of female and male patients with BD type I/II as compared to controls (Drexhage et al., 2011). However, when data were co-variated for age, increased percentages of Tregs were observed in BD patients younger than 40 years old compared to healthy individuals within the same age range (Drexhage et al., 2011). Differences in disease classification (BD types I and II), gender and age range could explain the discrepancy in Tregs between these two studies. Future studies are necessary to address the functional activity of these cells and to give further support to the data presented here.

Breunis et al. (2003) reported increased activated T cells in BD, as suggested by higher levels of CD3+ cells expressing CD25, CD71 and MHC II (Breunis et al., 2003). We found similar percentages of T cells expressing early activation markers (CD25, CD28 and CD69) between BD patients and healthy controls. However the CD25 marker was only measured on CD4+ T cell subset, which can possibly explain such discrepancy. Our study is in agreement with Breunis' findings regarding the absence of changes in circulating CD4+ CD69+ cells. It should be notice that Breunis' work evaluated a pool of manic, depressive and euthymic BD patients, which could possibly be a confounding factor. Interestingly, BD patients

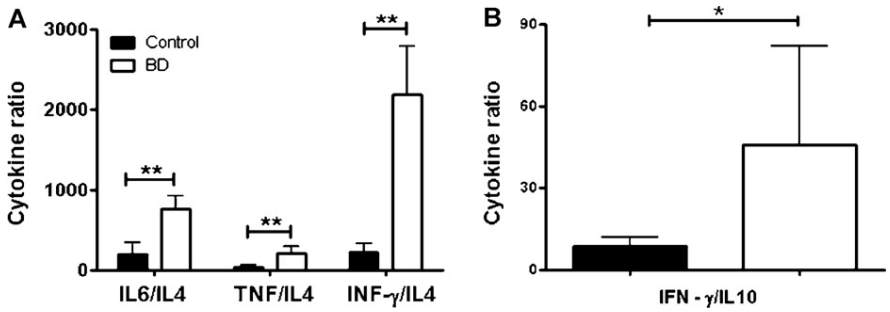

Figure 2 Th1/Th2 cytokine ratios between BD patients and healthy controls. Statistical significant differences are indicated: *$p < 0.01$, **$p < 0.0001$. Data were analyzed by Mann–Whitney Test.

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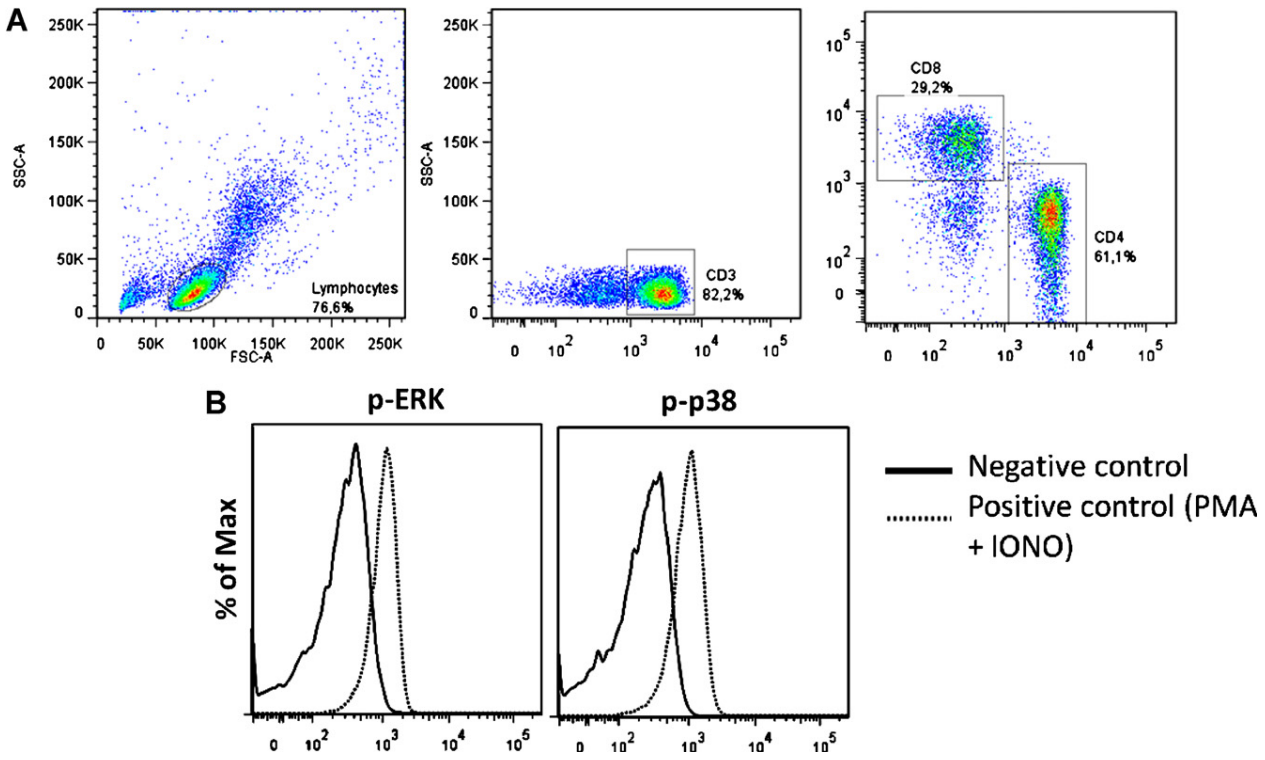

Figure 3 Gating strategy (A) and representative flow cytometry graphs (B) of intracellular MAPK expression. Cells were stimulated (dotted line) with 40 nM PMA and 1 μM ionomycin (IONO) for 15 min to assess phosphorilated MAPK in major T-cell subsets. Bold line represents non-stimulated negative controls.

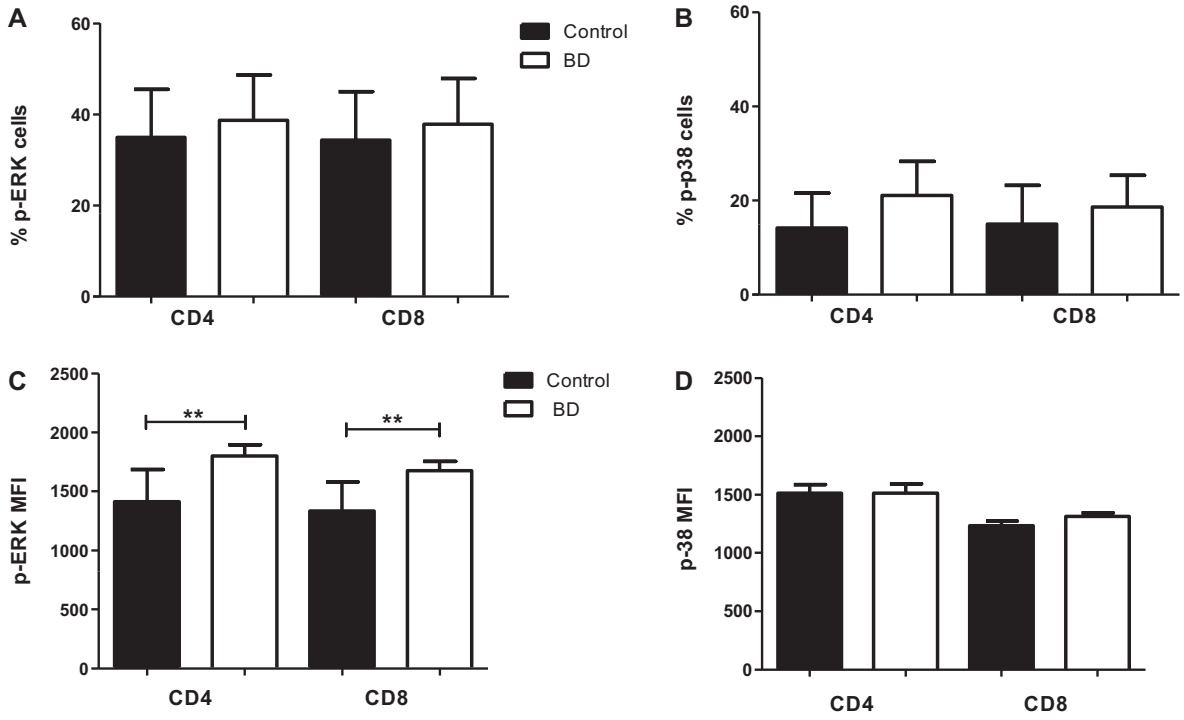

Figure 4 Analysis of intracellular activated MAPKs in peripheral T-cell subsets. Data show the expression of MAPK profiles of lymphocytes following stimulation with 40 nM PMA and 1 μM ionomycin (IONO) for 15 min. (A and B) Percentages of T cells expressing phospho-p38 and phospho-ERK. (C and D) Mean fluorescence intensity (MFI) of p-pERK and p-p38 expression in T-cell subsets. Statistical significant differences are indicated: $^{**}p < 0.0001$. Data were analyzed by Mann–Whitney Test.

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Please cite this article in press as: do Prado, C.H., et al., Reduced regulatory T cells are associated with higher levels of Th1/TH17 cytokines and activated MAPK in type 1 bipolar disorder. Psychoneuroendocrinology (2012), http://dx.doi.org/10.1016/j.psyneuen.2012.08.005

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Please cite this article in press as: do Prado, C.H., et al., Reduced regulatory T cells are associated with higher levels of Th1/TH17 cytokines and activated MAPK in type 1 bipolar disorder. Psychoneuroendocrinology (2012), http://dx.doi.org/10.1016/j.psyneuen.2012.08.005

10.2. Artigo de Revisão

Curr Tran Geriatr Gerontol Rep
DOI 10.1007/s13670-012-0010-9

Biomarkers in Mood Disorders Among the Elderly: Can They Contribute to Diagnosis and Prognosis?

Antonio Lucio Teixeira · Andrea Wieck · Breno Satler Diniz · Moises Evandro Bauer

Abstract Late-life depression is one of the most common neuropsychiatric disorders in the elderly population. Its clinical presentation is heterogeneous and has some distinctive features from depression in adults. In recent years, it has been demonstrated that patients with late-life depression present significant abnormalities in several neurobiological cascades. Among them, inflammatory, neuroendocrine, and neurotrophic cascades are of paramount importance. In this review, we revise the evidence of involvement of these cascades in the pathophysiology of late-life depression and the potential of the associated molecules (such as cytokines, neurotrophic factors, and hormones) as diagnostic and prognostic biomarkers. Despite the unequivocal advance in the understanding of its neurobiological basis, to date there is no sufficient evidence to support any biomarker of late-life depression. The search for valid biomarkers of late-life depression is warranted because they may contribute to correct diagnostic classification and to predict clinical outcome.

Keywords Aging · Major depression · Late-onset depression · Immunosenescence · Inflammaging · Cytokines · Adhesion molecules · Lymphocytes · Cortisol · Estrogen · Brain-derived neurotrophic factor · GSK-3beta

Introduction

Late-life depression (LLD) is one of the most common neuropsychiatric disorders in the elderly population [1]. LLD is a debilitating disorder and has a major impact on the patients' life. It is associated with worsened quality of life, loss of productivity, increased medical comorbidity, health service use, and higher risk of death [2]. The prevalence of LLD is variable and depends on several factors such as study setting, definition of depressive episode, measurement scales, and assessment of psychiatric and medical comorbidities. In community-dwelling elderly patients, prevalence of major depressive episode ranges from 4 % to 22 % [1, 3]. The prevalence of subsyndromal or minor depression and clinically relevant depressive symptoms are much more frequent than major depressive episode, ranging from 20 % to 40 % [4–6]. The most consistent risk factors for LLD episodes are older age, low educational attainment, presence of multiple medical and neurological comorbidities, living alone, and lack of social support [7].

In this article, we review recent advances in the understanding of biomarker changes in LLD and how they can inform about diagnosis, prognosis, and the neurobiological substrates for LLD.

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Published online: 28 March 2012

Clinical Presentation of Late-Life Depression: Does Age of Onset Matter?

The clinical presentation of LLD is heterogeneous and has some distinctive features from depressive episodes in adults. Complaints about sadness or downcast mood are less common in elderly persons. On the other hand, lack of energy and apathy along with somatic complaints and psychomotor retardation are prominent features of LLD. Psychotic phenomena, in particular nihilistic and guilty delusions, are also common in LLD [8, 9]. Patients with LLD show significant global cognitive impairment [10, 11]. Specific cognitive domains, such as executive function, short-term episodic memory, and processing of information speed, are particularly affected [11, 12].

Age of onset of LLD is an important feature of this condition and has a significant impact on clinical presentation. Patients are classified in early-onset LLD (EOD) if their first episode of major depression occurs at younger age or in late-onset LLD (LOD) if the first major depression episode occurs after age 65 years. Patients with LOD tend to have worse long-term prognosis and with increased comorbid cardiovascular disease as compared to EOD [13, 14]. Patients with LOD depict more severe and generalized cognitive deficits compromising most cognitive domains [11]. Executive functioning is particularly affected in LOD and may mediate the significant disability and functional impairment observed in these patients [15, 16, 17•]. Patients with EOD also may present significant cognitive impairment, notably of short-term episodic memory and information processing speed [11].

The heterogeneity of clinical manifestations and cognitive impairment patterns according to age of onset in LLD raised the question of whether this may reflect distinct underlying neurobiological substrates. Structural and functional neuroimaging studies have been shedding light on cerebral changes related to LLD. A large body of evidence suggests that patients with LLD show a significant higher frequency of cerebrovascular lesions (mainly periventricular and deep white matter hyperintensities and lacunar infarcts) and cerebral atrophy as compared to normal elderly control patients [18–21]. However, the pattern of cerebral structural changes is dependent on the age of onset of LLD. LOD patients usually show more significant cerebrovascular lesions as compared to patients with EOD [22–25]. These changes are most commonly located in the basal ganglia and in the fronto-subcortical white matter and seem to be associated with cognitive impairment, in particular executive dysfunction [25]. Given this close association between cerebral and cognitive changes, the term "vascular depression" has been coined to describe a subgroup of patients who presents LOD with history of cardio- and cerebrovascular diseases, accompanied by cerebrovascular lesions on the MRI and significant executive dysfunction [26–28, 29•].

Conversely, patients with EOD frequently show a significant regional cerebral atrophy, mostly in the hippocampal formation [30, 31]. Hippocampal atrophy correlates with the duration of the index depressive episode and the number of recurrent episodes [32, 33], and might be a harbinger of future dementia in some patients [34]. Such structural changes are in parallel with progressive short-term episodic memory decline in EOD patients. The exact neurobiological mechanisms that lead to hippocampal atrophy in these patients are unknown, but is possibly related to the sum of multiple mechanisms including hypothalamic-pituitary-adrenal (HPA) axis dysfunction, high cortisolemia, and reduced neurotrophic support [32, 35].

Inflammaging as a Pathogenic Mechanism in Late-Life Depression

In the past two decades a growing body of evidence emerged suggesting that a deregulation of inflammatory control, with increased proinflammatory status, plays a significant role in the pathophysiology of depression across the lifespan.

Chronic low-grade inflammation (called "inflammaging") has been observed during human aging (particularly in unhealthy populations), and it has been associated with frailty, morbidity, and mortality in elderly patients [36]. Indeed, chronic inflammation is considered to be involved in the pathogenesis of major age-related diseases, including Alzheimer's disease (AD), atherosclerosis, diabetes, sarcopenia, cancer, and major depression [37]. Cytokines are well known to mediate central effects of peripheral inflammation, including sickness behavior and fever.

There are multiple mechanisms through which cytokines may lead to depression. One mechanism involves the metabolism of certain neurotransmitters, such as serotonin, dopamine, and glutamate [38••]. Tryptophan is the main component of serotonin synthesis. Once inflammatory cytokines reach the brain, the activation of various transcription factors (eg, mitogen-activated protein kinase and nuclear factor-κB) takes place, leading to the activation of the enzyme indoleamine 2,3 dioxygenase (IDO). IDO is capable of metabolizing tryptophan into kynurenine, resulting in decreased synthesis of serotonin. Interestingly, patients undergoing interferon-alpha therapy for Hepatitis C virus infection or melanoma had decreased peripheral tryptophan levels and increased kynurenine levels concomitant with depressive symptoms development. Otherwise, kynurenine is preferentially converted into kynurenic acid in the brain, which interferes with the release of glutamate and dopamine. Dopamine also can be affected by inflammatory cytokines in a second way: cytokines reduce the levels of tetrahydro-biopterin (BH_4), which is an important co-factor

for tyrosine hydroxylase, a rate-limiting enzyme for dopamine synthesis, resulting in decreased dopamine levels. Another mechanism involves the metabolism of neurotrophic factors like brain-derived neurotrophic factor (BDNF). Cytokines induce glutamate release by astrocytes and reduce the expression of glutamate transporters, reducing glutamatergic reuptake. The glutamate released by astrocytes has preferential access to extra-synaptic N-methyl-D-aspartate receptors, which will reduce BDNF expression and decrease neurotrophic support. This reduced neurotrophic support will lead to increased neuronal susceptibility to oxidative stress.

Therefore, by modulating brain metabolism, "inflammaging" may be a relevant factor for the development of LLD. In addition, low-grade increases in levels of circulating tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, soluble IL-2 receptor, and C-reactive protein (CRP) are strong predictors of all-cause mortality risk in several longitudinal studies of elderly cohorts. It is worth mentioning, however, that low-grade inflammation was not observed in strictly healthy elderly persons or centenarians, suggesting that "inflammaging" is more likely a feature of unsuccessful aging [39].

Proinflammatory Profile in Late-Life Depression

Cytokines and Acute-Phase Proteins

Cytokines and acute-phase proteins are important mediators of inflammatory response. These proteins can be readily assessed in different biological matrices and, thus, can be reliable biomarkers of inflammatory activity in an individual patient. Cytokines can be produced in the periphery and by central nervous system cells, such as activated microglia, exerting active biological effects in glial and neuronal functions [40]. Some of these effects are directly related to the physiopathology of depression, as previously mentioned, presenting also long-term consequences such as the emergence of neurodegenerative changes in the brain [41, 42].

Several studies have examined peripheral levels of cytokines and acute-phase proteins in patients with LLD. IL-1β, a potent proinflammatory cytokine, was found significantly elevated in patients with LLD [43]. In a study carried out in our group, IL-1β was also significantly elevated in patients with LLD. Nonetheless, patients with EOD showed the highest plasma levels of IL-1β [44]. It is noteworthy that recurrent depressive episodes in adults was also associated with increased circulating levels of inflammatory markers like CRP [45], suggesting that recurrent depression is associated with cumulative proinflammatory burden.

TNF-α is the prototype proinflammatory cytokine and has been involved in the physiopathology of several chronic inflammatory disorders [46]. Despite studies in adult patients showing that TNF-α levels are significantly increased in patients with major depression [47••], the only study in LLD did not find significant differences between depressed patients and elderly control patients [48•]. Nonetheless, patients with LLD presented significant higher levels of soluble TNF-α receptor 2 (sTNF-R2, or p75), with no significant change in the levels of soluble TNF-α receptor 1 (sTNF-R1, or p55). These findings suggest that, despite patients with LLD not having significant changes in TNF-α levels, they present with an abnormal regulation of the TNF-α signaling system during depression [48•]. In line with this, a recent study found that elevated serum levels of sTNF-R1 were associated with higher depressive symptoms, as measured by the Geriatric Depression Scale, in elderly patients 1 year after hip fracture [49]. Other proinflammatory cytokines and acute-phase proteins, such as IL-6, CRP, and α1-antichymotrypsin, are also increased in patients with LLD [49–51].

Overall, current research findings suggest that LLD is characterized by a deregulation of inflammatory control with increased proinflammatory status. Such changes tend to correlate with the severity of depressive symptoms in most studies and recurrent depressive episodes may have a cumulative proinflammatory effect. These proinflammatory changes are in excess of those expected during the senescence process, suggesting that abnormalities in the inflammatory control may play a significant role in the physiopathology of LLD.

Despite that these findings are relevant for the understanding of LLD neurobiological basis, they are nonspecific and do not help the diagnostic process for this disorder. Elevated inflammatory markers also have been reported in other major psychiatric disorders, such as bipolar disorder, schizophrenia, and obsessive-compulsive disorder [52–56]. Neurodegenerative disorders common in older patients, such as AD and Parkinson's disease, also show increased levels of proinflammatory cytokines [57, 58]. It is worth highlighting the overlap of the profile of circulating biomarkers in LLD and AD, preventing any differentiation between these two conditions based on them (see Table 1). In this context, CSF biomarkers may be of great value. Patients with other neurological diseases, such as multiple sclerosis, in which depressive symptoms are very common, also present with high levels of proinflammatory cytokines [59]. Nonetheless, a recent study combining nine serum biomarkers related to inflammatory, neurotrophic, and endocrine-metabolic cascades (α1-antitrypsin, apolipoprotein CIII, BDNF, cortisol, epidermal growth factor, myeloperoxidase, prolactin, resistin, and sTNF-R2) showed a high accuracy for the diagnosis of major depression in younger adults [60••]. This promising result needs to be confirmed by independent studies and it is uncertain whether it is valid

Table 1 Peripheral biomarkers during aging, late-life depression, and Alzheimer's disease

Biomarkers	Aging	Late-life depression	Alzheimer's disease
IL1-α	↑	↑	—
IL1-β	↑	↑	↑
IL6	↑	↑	↑
TNF-α	↑	↑	↑
NK cells	↑	↑	—
CD45RA+ (naive T cell)	↓	↓	↓
CD45RO+ (memory T cell)	↑	↑	—
CD8+CD28- (senescent cells)	↑	↑	↓
CD4/CD8 ratio	↓	↑	—
Adhesion molecules	↑	↑	↑
Sexual hormones	↓	↓	↓
BDNF	↓	↓	↓
Cortisol	↑	↓↑	↑

↑ increased; ↓ decreased; *IL* interleukin; *TNF-α* tumor necrosis factor alpha; *NK* natural killer; *BDNF* brain-derived neurotrophic factor

for LLD. The prognostic value of inflammation-related molecules in LLD has not been investigated yet.

Adhesion Molecules

Adhesion molecules play an important role in the inflammatory process. Once inflammation is triggered, upregulation of adhesion molecule genes takes places in endothelial and immune cells to facilitate leukocyte adhesion and migration to sites of inflammation. Data regarding levels of soluble adhesion molecules such as soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular adhesion molecule-1 (sVCAM-1) are scarce and contradictory. Levels of sICAM-1 and sVCAM-1 were found elevated in the periphery of depressed elderly patients [61]. Postmortem studies also found increased expression of ICAM-1 and VCAM-1 in dorsolateral prefrontal cortex of depressed elderly patients [62]. Conversely, Thomas and colleagues [63] did not find any association between peripheral levels of sICAM-1 or sVCAM-1 and depression in elderly patients.

As observed for cytokines, sICAM-1 levels were also found increased in patients undergoing interferon-α treatment who developed major depression [64]. However, given the high frequency of ischemic changes during the aging process, it is difficult to define whether elevated levels of adhesion molecules are due to depression or not. Vascular depression theory postulates that LOD is associated with vascular and ischemic diseases to which adhesion molecules are considered good markers [61]. Accordingly, sICAM-1 levels were found elevated in individuals who developed major depression after an episode of acute coronary syndrome [65]. Interestingly, these levels were significantly higher in patients with no past history of depressive disorder than those who had [65]. Adhesion molecules are widely expressed on blood–brain barrier endothelial cells and can be related to its increased permeability during inflammatory processes, allowing cytokines to cross this barrier and exert its effects in the brain [61]. Together, these data point to a possible mechanism of action through which low-grade inflammation is involved in the development of LOD. The absence of studies assessing adhesion molecules levels in elderly patients diagnosed only with major depression (with no comorbidity) makes difficult to establish the precise role of these molecules as biomarkers and in the development of depressive states.

Leukocyte Subsets

Data regarding immune cell subsets in LLD are scarce. Changes in number and function of these cells would not be surprisingly, as they are responsible for the production of many cytokines observed altered in this context. However, cellular alterations are also common to the aging process itself, being hard to address the precise role of these putative biomarkers in LLD. Quantitative changes in leukocytes such as decreased in naïve T cells ($CD45RA^+$), increased number of memory T cells ($CD45RO^+$), expansion of $CD8^+CD28^-$ T cells (known as "senescent cells"), and increased natural killer (NK) cells have been observed during aging, particularly in elderly patients with increased depressive symptoms [66, 67]. Increased T cell counts have been found in elderly depressed patients with no antidepressant treatment. NK-T cells, $CD8^+$ cytotoxic T cells, and CD4/CD8 ratio also have been found increased in this population [68]. Interestingly, when analyzing cell subsets from elderly depressed patients undergoing antidepressant treatment, the numbers of $CD8^+$ and NK-T cells did not differ from healthy individuals, while the CD8/CD4 ratio is unaltered by antidepressant treatment [12, 13, 68, 69]. More studies are needed to better understand the role of lymphoid subsets during aging and their possible role in LLD.

Neuroendocrine Changes in Late-Life Depression

Hypothalamic-Pituitary-Adrenal Axis

Current literature suggests that both major depression and aging are associated with significant activation of the HPA axis. LLD seems to follow the same scenario. Adults with major depression have shown increased plasma cortisol levels and enlarged anterior pituitary and adrenal glands, as well as failure to suppress cortisol levels following dexamethasone administration [70, 71]. The increased HPA axis

activity is thought to be related, at least in part, to diminished feedback regulation by endogenous glucocorticoids. Remarkably, successful antidepressant treatment is associated with normalization of feedback regulation of the HPA axis induced by glucocorticoids. Increased circulating cortisol might contribute to depression pathogenesis, as correction of hypercortisolemia with cortisol synthesis inhibitors also has been reported to ameliorate depression [72].

The HPA axis activation is regarded to be fundamental for the body to deal with changing environmental demands by increasing circulating energy substrates such as glucose and fatty acids. However, long-term increase in cortisol levels will negatively impact key brain areas involved with HPA axis feedback regulation (hippocampus and hypothalamus), impairing cognitive functions as well as leading to poorer cell-mediated immune responses. We have previously observed that lymphocytes of depressed patients are resistant to glucocorticoid treatment in vitro or in vivo [73, 74]. Ineffective action of glucocorticoids on target tissues could lead to immune activation as shown by chronic low-grade inflammation. Conversely, inflammation can stimulate the HPA axis via both a direct action of cytokines on the brain and by inducing glucocorticoid resistance [75]. Hypercortisolemia may have important long-term consequences for health, including higher allostatic load and accelerated aging. Indeed, depression has been associated with features of premature aging, and depressed individuals have a higher incidence of various age-related diseases, including cardiovascular and cerebrovascular diseases, metabolic syndrome, and dementia [76].

Aging is also associated with significant activation of the HPA axis. We have observed that strictly healthy elderly persons had remarkably higher salivary cortisol but low dehydroepiandrosterone (DHEA) levels throughout the day compared to young adults [4]. These hormonal changes were found in parallel to age-related psychological distress, including increased depressive symptoms. DHEA is produced by the adrenal glands and is under the regulation of the HPA axis. It has been suggested that DHEA may antagonize many physiologic changes of cortisol, including enhancing immune functions [77]. The lack of appropriate DHEA levels could be a detrimental factor during aging. Interestingly, it has been shown that low DHEA levels were associated with depressed mood in older women [78] and DHEA supplementation significantly improved memory performance and depression ratings in elderly patients with depression [79].

The presence of depression seems to amplify the changes of the adrenal secretory pattern, already present in the physiological aging. Elderly patients with major depression or patients reporting increased self-reported ratings of depressive symptoms had increased nocturnal cortisol levels compared to healthy control patients [80, 81]. In a recent large population-based study, it was observed that LLD is associated with both hypo- and hypercortisolemia [82]. Nevertheless, only hypercortisolemic depression was associated with older age, cardiovascular diseases, and cognitive impairment [82].

Taken together, these studies suggest that changes in HPA axis molecules are not reliable biomarkers of LLD, but rather common phenomena observed during aging and depressive disorder.

Sexual Hormones

The role of sexual hormones in depression has long been addressed in an attempt to explain the higher susceptibility carried by women to develop mood disorders. Because depressive symptoms are common to women experiencing the low-estrogen phase of menstrual cycle, after childbirth, and during climacteric and menopause, it is believed that these hormones are related to physiopathology of depression in women [83–85].

During the climacteric phase (ie, the transition to menopause) the gradual decline in ovarian function leads to a reduction in sexual hormones production, including estrogen, testosterone, and progesterone. Estrogen reduction or deprivation can lead to many physiological changes, such as alterations in neuronal plasticity and neurotransmission [83]. More precisely, estrogen can modulate the serotonergic system, which is greatly involved in mood control. There is a high risk of depression during this phase, but several studies failed to establish any association between the levels of sexual hormones and development of depressive symptoms [83–88, 89•]. Therefore, sexual hormones do not seem useful as biomarkers in this context. Interestingly the longer climacteric phase, the higher risk of developing major depression [87, 89–91]. Moreover, studies described reduction in depressive symptoms after hysterectomy and oophorectomy in climacteric women, supporting the idea of hormonal fluctuations having worse effect on mood than low estrogen levels itself [28, 29•].

Hormonal replacement has been reported as increasing well-being in climacteric women, but its effects in ameliorating depressive symptoms remain controversial [84]. While women in estrogen therapy show a better response to fluoxetine treatment [84], postmenopausal women demonstrated increased risk to develop depressive disorders during hormonal replacement [85]. The development of major depression in menopausal state seems to be more common in women with a previous history of depressive disorder and to be related with psychosocial stress instead of sexual hormones [84–86].

Neurotrophic Factors in Late-Life Depression

Neurotrophic factors are a broad family of proteins that play several roles in the central nervous system, mainly

maintenance of neuronal homeostasis, neuroprotection against insults, neuronal repair and regeneration, and synaptic formation and strengthening [92]. BDNF is the most abundant neurotrophic factor in the brain. Several studies found significant lower circulating levels of BDNF in patients with LLD as compared to nondepressed controls [93, 94]. When studies stratified LLD according to the age of onset, patients with LOD had lower BDNF levels than those with EOD [95]. In addition, a recent study reported that older patients with subsyndromal depression showed levels of BDNF that were intermediate between patients with major depression and nondepressed control patients, suggesting a gradient effect [94]. In contrast, a community-based study failed to find significant changes in BDNF levels in LLD [96]. Differences in samples, assessment of depressive symptoms, and severity of depressive symptoms may help to explain such conflicting results.

The dynamics of other neurotrophic factors have not been extensively explored in LLD. The glial cell line–derived neurotrophic factor (GDNF) plays a major role in the protection of catecholaminergic, dopaminergic, and cholinergic neurons [97] and axonal regeneration after injury [98]. Studies in LLD have reported contradictory results showing either elevated [99] or reduced GDNF levels [100] in LLD when compared to age- and sex-matched control patients. Likewise, studies with the nerve growth factor (NGF) also have reported contradictory findings in LLD, with one study reporting nonsignificant differences [96] and another reporting a significant reduction in LLD [101]. In the latter study, we found that older patients with previous history of depression, but who were euthymic and under antidepressant treatment at the time of laboratory assessment, also showed a significant reduction in NGF levels, comparable to those observed in patients with current depressive episode [101]. In light of these results, we hypothesized that lower NGF levels may represent a state marker of depressive disorder in elderly patients and also may indicate a significant disruption in the neurotrophic regulatory mechanism that takes place during the depressive episode and does not completely recover despite clinical improvement after treatment.

Other Peripheral Biomarkers in Late-Life Depression

Changes in other neurobiological cascades that may have physiopathologic and clinical relevance in LLD have been recently reported. Increased oxidative stress markers have been consistently reported in adult patients with major depression and bipolar disorder [102, 103]. In LLD, one study so far reported a significant increase in the peripheral levels of plasma 8-iso-prostaglandin F2-α (8-iso-PGF2-α), a marker of oxidative damage, in patients with LLD [50]. Such changes were correlated to increased proinflammatory status in these patients, suggesting a significant crosstalk between oxidative damage and inflammatory status in LLD patients.

The glycogen synthase kinase-3β (GSK-3β) is an intracellular enzyme that is involved in many cellular functions such as energy metabolism, structural plasticity, neurogenesis, and resilience to cellular injury [104]. Its activity is regulated by the phosphorylation of serine 9 epitope, rendering the enzyme inactive. As GSK-3β is involved in diverse cellular functions, it is plausible to hypothesize that this enzyme may be related to the physiopathology of mood and neurodegenerative disorders [105]. A recent study showed patients with LLD had lower levels of phosphorylated GSK-3β with no changes in total GSK-3β in platelets, suggesting that GSK-3β is possibly overactive in patients with LLD [106]. Moreover, these changes were markedly pronounced in patients with more severe cognitive impairment and depressive symptoms, indicating that GSK-3β overactivation is a state marker of more severe depressive episodes in older patients. Further studies are warranted to confirm this finding.

Cerebrospinal Fluid Biomarkers in Late-Life Depression

Few studies addressed changes in cerebrospinal fluid (CSF) biomarkers in LLD. Most studies focused on the accuracy of AD-related biomarkers (amyloid-β_{42}, total Tau, and phosphorylated Tau proteins) to differentiate between LLD and AD disease. In general, patients with LLD showed a pattern similar to those observed in elderly control patients when compared to AD profile that is characterized by low levels of total and phosphorylated Tau proteins and high levels of amyloid-β_{42} [107–109]. Schneider and colleagues [110•] recently proposed that the only feasible tools to discriminate LLD and early AD (which is commonly associated with depressive symptoms) are the CSF biomarkers for AD. Their high negative predictive value could be regarded as inverse evidence ("negative depression biomarker") that LLD is the sole cause of cognitive symptoms in depressed elderly patients as opposed to prodromal or early Alzheimer's disease.

In a small study that included LLD patients and nondepressed elderly control patients, the former group showed higher levels of amyloid-β_{42} and no difference in total and phosphorylated Tau proteins as compared to the latter group [111]. Nonetheless, LLD patients showed a higher CSF/serum albumin ratio, suggesting dysfunction of the blood–brain barrier possibly due to vascular processes. Another study showed increased CSF levels of a nonspecific marker of neurodegeneration, the neurofilament light protein, in LLD as compared to healthy control patients [112]. These results suggest that patients with LLD may develop nonspecific neurodegenerative and vascular changes during mood episodes that may render these patients more vulnerable to the development of dementia [113, 114].

Conclusions and Perspectives

A growing body of evidence suggests that LLD patients present significant abnormalities in several neurobiological cascades, determining changes in peripheral and central nervous system biomarkers. These studies contributed to the understanding of the physiopathological features of LLD and its relationship with medical comorbidities and neurodegenerative and cerebrovascular disorders. However, they are much less informative regarding diagnosis, prognosis, and treatment selection for individual patients. To date, there are no sufficient data to support any biomarker as diagnostic or prognostic of LLD.

Advances in this field will be possible by integrating distinct approaches and taking into consideration diverse biomarkers derived from several neurobiological cascades involved in LLD. New strategies for biomarkers discovery and development, including the "-omics" (genomics, proteomics, metabolomics), new structural neuroimaging (iron imaging, microbleeds, tractography), and functional and molecular imaging techniques should be incorporated to long-term clinical and epidemiological studies to determine the diagnostic and prognostic values of different biomarkers. Moreover, these strategies should be systematically included in clinical trials to provide more specific (neurobiological-based) selection criteria for patients, and to predict responsiveness or refractoriness to treatment.

The identification of LLD biomarkers may contribute to the development of more specific and personalized interventions aiming not only at the treatment of current depressive episodes but also the prevention of adverse outcomes, mainly functional and cognitive decline that ultimately lead to clinical diagnosis of dementia.

Acknowledgments This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq [ALT and MEB]) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES [AW]), Brazil.

Disclosures No potential conflicts of interest relevant to this article were reported.

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10.3. Artigo de revisão

EXPERT OPINION

Novel biomarkers for bipolar disorder

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Introduction: Bipolar disorder is diagnosed on the basis of patient and/or family reports and behavioral observation. Traditionally regarded as an affective disorder involving behavioral changes, bipolar disorder has been reconceptualized as a multisystem disease associated with mood, cognitive, metabolic, autonomic and sleep/wake dysfunctions. Accordingly, recent studies have focused on the identification of biomarkers related to the pathophysiological mechanisms underlying the development, clinical presentation and course of bipolar disorder.
Areas covered: This article provides an overview of the available literature regarding circulating peripheral and neuroimaging biomarkers in bipolar disorder. Neurotrophic factors, immune parameters, oxidative stress parameters, hormones and neuroimaging findings were taken into consideration.
Expert opinion: Biomarkers research in bipolar disorder is a new field with an expanding knowledge. Current evidence suggests that a single biomarker will not be able to cover the biological and clinical complexity of bipolar disorder. Alternatively, a composite of biomarkers, including neurotrophic factors, cytokines and oxidative stress molecules, may be promising to identify altered mood states and neuroprogression in bipolar disorder.

Keywords: biomarkers, bipolar disorder, cytokines, neuroimaging, neurotrophic factors, oxidative stress

Expert Opin. Med. Diagn. [Early Online]

1. Introduction

Bipolar disorder is a prevalent, severe, disabling and chronic medical illness. The lifetime prevalence of bipolar disorder is estimated at 1 – 5%, depending on only type 1 or both type 1 and type 2, while the mean age of onset is around 20 years. Lifetime expectancy is reduced in bipolar disorders as a result of high rates of suicide and increased comorbidity of medical diseases. A recent meta-analysis estimated the prevalence of suicide attempts in bipolar disorder patients between 32.4 and 36.3% [1] and the mortality by suicide is around nine times higher than in the general population [2]. Bipolar disorder is frequently comorbid with anxiety disorders, substance and alcohol abuse, endocrine and metabolic diseases (particularly diabetes mellitus and obesity) and cardiovascular diseases [3]. Therefore, bipolar disorder cannot be regarded solely as an affective or mood disorder, but a multisystem condition involving mood, cognitive, endocrine, autonomic and sleep/wake dysfunctions.

The diagnosis of bipolar disorder is still based on the report and clinical observation of the patient. Apart from complementary exams to exclude secondary causes of mood symptoms, to date no laboratory or neuroimaging tests are currently available to support the diagnosis of bipolar disorder. Based on the complexity of the diagnosis and clinical presentation of bipolar disorder, which is associated with a polygenic biological basis and psychosocial factors, putative diagnostic biomarkers so far have

10.1517/17530059.2013.734807 © 2012 Informa UK, Ltd. ISSN 1753-0059, e-ISSN 1753-0067

Article highlights.

- Bipolar disorder is associated with an imbalance of diverse biological systems, including immune system, neurotrophins, neuroendocrine axis and oxidative stress.
- Peripheral molecules related to the pathophysiological mechanisms underlying bipolar disorder may be putative biomarkers of the disease.
- Bipolar disorder is associated with structural and functional neuroimaging changes associated with brain atrophy, notably involving the enlargement of lateral ventricles and decrease in prefrontal cortex and amygdala volumes.
- Bipolar disorder peripheral and neuroimaging findings may reflect neuroprogressive processes of the illness.
- A single candidate biomarker seems unlikely to tap clinical and biological heterogeneity of bipolar disorder.

This box summarizes key points contained in the article.

failed to provide consistent reliability. Alternatively, the identification of mood state (i.e., depression, mania and euthymia) biomarkers may contribute to the clinical management of bipolar disorder. For instance, changes in biomarkers profile could guide therapeutic interventions before the complete development of depressive or manic relapses. Moreover, the identification of biomarkers in bipolar disorder may help to understand the pathophysiological mechanisms underlying the development and progression of the disease [4]. The pathophysiology of bipolar disorder is largely unknown, but recent evidence has pointed out that it may arise from the complex interaction among multiple genes, environmental factors and dysfunction in several brain circuits and mechanisms of neuroplasticity.

In the present paper, the authors aim to review putative biomarkers of bipolar disorder and their potential application in mood state diagnosis and disease stratification. They used the following search strategy: online search of the database MEDLINE and SCOPUS from 1990 were performed in June 2012 using the keywords (MESH criteria): 'bipolar disorder' AND 'neurotrophic factor' AND 'cytokine' AND 'adipokine' AND 'tumor necrosis factor' AND 'leukocyte' AND 'biomarker' AND 'imaging'.

2. Potential candidate biomarkers for bipolar disorder

2.1 Candidate biochemical biomarkers

2.1.1 Neurotrophic factors

Neurotrophic factors are a family of proteins that are essential for the development, differentiation and survival of neurons. Neurotrophic factors also exert an important role in the modulation of neuronal excitability and synaptic transmission [5]. Neurotrophic factors belong to three major families: neurotrophins, glial cell-derived neurotrophic factor (GDNF) and neuropoietic cytokines. The distinction between these families is based on the molecular structure, receptor interaction, pattern of expression after neural injury and cellular effects.

The neurotrophin family comprises: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4/5). BDNF is the most abundant neurotrophic factor in the central nervous system (CNS), being particularly abundant in the amygdala, hippocampus and prefrontal cortex, brain areas directly involved in emotional regulation and several aspects of cognition such as attention, memory and executive functioning [6].

A relevant issue is whether circulating levels of neurotrophic factors correlate with the CNS concentration of the respective molecule. Evidence from experimental studies suggests that peripheral levels of BDNF are correlated with BDNF levels in the brain [7]. Nevertheless, studies addressing this issue in humans are lacking. Of note, as the concentration of BDNF in the cerebrospinal fluid (CSF) is very low, CSF does not seem to be a useful source to investigate BDNF levels [8].

In animal models of depression, a significant decrease in BDNF levels has been found in the hippocampus. The administration of antidepressant drugs increases hippocampal BDNF levels and the infusion of BDNF into rat hippocampus results in antidepressant-like effects [9]. Decreased BDNF levels in hippocampus and amygdala have also been shown in animal models of mania and an increase in BDNF levels was observed following the infusion of mood stabilizers [10,11].

In line with these experimental data, two recent meta-analyses demonstrated that bipolar disorder patients exhibited decreased circulating (plasma or serum) levels of BDNF in comparison with controls (**Table 1**) [12,13]. When these meta-analyses stratified bipolar disorder patients according to mood state, bipolar disorder patients in mania or in depression exhibited decreased BDNF levels when compared with controls [12,13]. BDNF levels in bipolar disorder patients in euthymia did not differ from controls [12,13]. These results suggest BDNF as a state-dependent (mania or depression) biomarker of bipolar disorder. Moreover, the severity of manic and depressive symptoms has been negatively correlated with BDNF levels [12]. However, longitudinal studies assessing BDNF levels across different mood states in the same patients are lacking as well as studies across the lifespan. Studies involving patients with long-term bipolar disorder are also warranted to confirm whether the pattern of circulating BDNF is the same for patients with a more recent illness. In opposition with these meta-analytic findings, the authors recently reported increased plasma levels of BDNF in bipolar disorder patients with more than 10 years of disease [14]. There is evidence of increased BDNF levels in other chronic neuropsychiatric disorders such as long-term schizophrenia [15]. One hypothesis for this finding involves the effect of the treatment with mood-stabilizing drugs, that is, lithium and valproate, on BDNF levels, determining increase in its circulating levels [10,11].

Table 1. Non-immune peripheral biomarkers in bipolar disorder patients.

Peripheral biomarker	Biological fluid	Mood state	Findings	Concluding remarks
Neurotrophic factors				
BDNF	Serum/Plasma	Mania	↓ [12,13]	↓ in mania, and depression
		Euthymia	↔ [12,13]	↔ in euthymia
		Depression	↓ [12,13]	
NGF	Plasma	Mania	↓ [16]	↓ in mania
		Euthymia	↔ [16]	↔ in euthymia
NT-3	Serum	Mania	↑ [18,19]	↑ in mania, and euthymia
			↔ [20]	↔ in euthymia
		Euthymia	↔ [19,20]	
		Depression	↑ [18-20]	
NT-4/5	Serum	Mania	↑ [21]	↑ in mania, euthymia and depression
		Euthymia	↑ [21]	
		Depression	↑ [21]	
GDNF	Serum/Plasma/Whole blood	Mania	↑ [24]	Not conclusive in all mood states
			↔ [23]	
			↓ [25]	
		Euthymia	↑ [23]	
			↔ [24,26]	
		Depression	↑ [24]	
			↓ [25]	
Cortisol and HPA measurements				
Basal cortisol		Mania	↑↑ [59]	↑↑ in mania and depression
		Euthymia	↑ [59]	↔ in euthymia
			↔ [60-63]	
		Depression	↑↑ [59]	
			↔ [60]	
Basal ACTH		Mania	↑ [76]	↑ in mania
		Euthymia	↑ [76]	↔ in euthymia
			↔ [60-63]	
CRH infusion test		Mania	Secretory activity ↑↑ [59]	↑ Secretory activity in all mood states
		Euthymia	Secretory activity ↑ [59,60]	
		Depression	Secretory activity ↑↑ [59,60]	
DEX suppression test		Euthymia	↔ [60]	↔ in euthymia
Oxidative stress markers				
SOD	Serum	Mania	↑ [72,74,78]	↑ in mania, and depression
		Euthymia	↔ [72,74,78]	↔ in euthymia
			↑ [76]	
		Depression	↑ [72,74]	
GPx	Serum	Mania	↔ [72]	↔ in mania, and depression
		Euthymia	↑ [72]	↑ in euthymia
		Depression	↔ [72]	
SOD/GPx ratio	Serum	Mania	↑ [72]	↑ in mania and depression
		Euthymia	↔ [72]	↔ in euthymia
		Depression	↑ [72]	
TBARS	Serum	Mania	↑ [72,74]	↑ in mania, euthymia and depression
		Euthymia	↑ [72,74]	
		Depression	↑ [72,74]	
CAT	Serum	Mania	↓ [72]	Not conclusive in all mood states
			↑ [78]	
		Euthymia	↓ [72]	
		Depression	↔ [72]	
			↑ [78]	
NO	Serum	Euthymia	↑ [76]	↑ in euthymia

↓: Decreased levels; ↔: Not altered levels; ↑: Increased levels; ACTH: Adrenocorticotropic hormone; BDNF: Brain-derived neurotrophic factor; CAT: Serum catalase; CRH: Corticotrophin-releasing hormone; DEX: Dexamethasone; GDNF: Glial cell-derived neurotrophic factor; GPx: Glutathione peroxidase; HPA: Hypothalamic–pituitary–adrenal; NGF: Nerve growth factor; NT-3: Neurotrophin-3; NT-4/5: Neurotrophin-4/5; NO: Nitric oxide SOD: Superoxide dismutase; TBARS: Thiobarbituric reactive substances.

Other neurotrophic factors have been less well studied in bipolar disorder. NGF was the first neurotrophin discovered in 1951 by Rita Levi-Montalcini, but there is only one study evaluating its level in bipolar disorder patients [16]. Bipolar disorder patients had reduced plasma levels of NGF, and its levels were negatively correlated with the severity of manic symptoms. In the CNS, NGF can promote neuronal survival, protecting sympathetic and cholinergic neurons against neurodegeneration, and also mediates cognitive functions like learning and memory [17]. In light of these results, it was hypothesized that lower NGF levels may reinforce the theory of cholinergic system dysfunction as a contributing element to cognitive deficits in bipolar disorder. NT-3 is likely to be a key regulator of neurogenesis and neuron differentiation in both the central and peripheral nervous systems. Three studies demonstrated increased NT-3 levels in bipolar disorder patients [18-20]. Only one study evaluated NT-4/5 in bipolar disorder, finding increased circulating levels in comparison with controls, but no difference across mood states [21]. The function of NT-4/5 is not clear in CNS, but it may play a role in facilitating glutamatergic transmission in the hippocampus and protecting dopaminergic neurons [21].

The GDNF plays an important role in the development and maintenance of the nigrostriatal system. Increased production on GDNF by astrocytes or microglial cells is associated with a local mechanism to limit neuronal loss and promote regeneration under stress [22]. Studies with bipolar disorder patients have reported contradictory results showing either elevated [23,24] or reduced levels of circulating GDNF [25,26].

2.1.2 *Circulating immune molecules*

The consistent clinical observation of cycle shortening and cognitive impairment with the recurrence of mood episodes along with increased prevalence of metabolic and cardiovascular diseases corroborate the view of bipolar disorder as a 'neuroprogressive disorder' [27]. Mood episodes seem to play a pivotal role in this neuroprogression and one of the involved mechanisms is an exacerbated pro-inflammatory response during mania and bipolar depression acting as a major 'toxic player' [28-31]. Indeed, an increasing body of evidence indicates that inflammation may be relevant to bipolar disorder since inflammatory pathways interact with several systems in the brain and body periphery is believed to be involved in mood disorder physiopathology.

Under physiological conditions, inflammatory mediators are not able to freely cross the blood–brain barrier. Nevertheless, these mediators can influence the CNS through alternative routes, including: i) entry through leaky regions in the blood–brain barrier; ii) active transport via saturable transport molecules; iii) activation of endothelial cells and other cell types lining the cerebral vasculature and iv) binding to cytokine receptors associated with peripheral afferent nerve fibers (e.g., the vagus nerve) that then relay cytokine signals to relevant CNS regions [32]. After the cytokine signals reach the CNS, a complex network composed of neurons and glial elements amplifies the effect through production of cytokines and increased expression of cytokine receptors.

There are multiple mechanisms through which cytokines may lead to mood symptoms. One mechanism involves the metabolism of certain neurotransmitters, such as serotonin, norepinephrine, dopamine and glutamate, in brain regions, like limbic system (amygdala, hippocampus and *nucleus accumbens*), essential to the regulation of emotion, reward and psychomotor functions [32]. Cytokines may also influence the hypothalamic–pituitary–adrenal (HPA) axis through effects on its negative feedback regulation and on the glucocorticoid receptor (GR) function [32]. Moreover, cytokines can directly affect neuronal activity, inducing neuronal excitability and plastic changes [32].

TNF-α is the prototype of the pro-inflammatory cytokine and it is produced by neutrophils, macrophages and other cell types like glia cells and neurons in response to injury or infection. TNF-α can bind to two types of receptors, TNFR1 (p55) and TNFR2 (p75), which are responsible for its biological effects. The extracellular portions of these receptors may constitute soluble forms (sTNFR1 and sTNFR2) and can be measured in the circulation. Measurement of the circulating levels of the two sTNFRs is useful to determine the overall production of TNF-α, being regarded as more reliable markers of inflammatory activity than TNF-α concentration itself, as TNF-α is degraded soon after its release. Several studies have consistently demonstrated increased levels of TNF-α and/or sTNFR1 in bipolar disorder [29-31,33-35]. A pro-inflammatory status associated with bipolar disorder is corroborated by the finding of increased circulating levels of other pro-inflammatory cytokines, such as IL-6 [29,31,35,36]. A recent study showed increased IL-1β in the CSF of subjects with bipolar disorder, indicating a pro-inflammatory status not only in the periphery, but also in the CNS [37].

Other molecules related to immune response are altered in bipolar disorder. Preliminary data indicate that bipolar disorder patients present: i) increased endothelial cell activation, suggested by increased plasma levels of von Willebrand factor [29,38]; ii) increased stimuli to leukocyte rolling and adhesion, suggested by increased VEGF plasma concentration [39] and chemokine levels [40,41]; iii) increased adipokine levels (mediators produced by the adipose tissue) [42,43].

Table 2 summarizes the studies investigating circulating immune biomarkers in bipolar disorder.

2.1.3 *Leukocyte subsets*

Data regarding immune cell subsets in bipolar disorder patients are scarce. Two studies evaluated total leukocyte count in bipolar disorder patients showing conflicting results: one study showed decreased count [44], while another found no difference [45]. Darko *et al.* [46]. demonstrated a trend toward increased neutrophil count in bipolar disorder patients. Nevertheless, neutrophil activity seems to be reduced in these patients [47].

Table 2. Immune peripheral biomarkers in bipolar disorder patients.

TNF-α	Serum/Plasma	Mania	↑ [20,30,31]	↑ in mania.
			↔ [36]	↔ in euthymia and depression
		Euthymia	↔ [20,33-36,42,48,107,108]	
		Depression	↑ [30,31]	
			↔ [20,36,109]	
sTNFR1	Plasma	Mania	↑ [29,34]	↑ in mania and depression
			↔ [110]	Not conclusive in euthymia
		Euthymia	↑ [34,42]	
			↔ [29,33,110]	
		Depression	↑ [29]	
sTNFR2	Plasma	Mania	↔ [34]	↔ in mania and euthymia
		Euthymia	↔ [33,34,42]	
C-reactive protein	Plasma	Mania	↔ [29,110]	↔ in all mood states
		Euthymia	↔ [29,110]	
		Depression	↔ [29,109]	
IL-6	Serum/Plasma	Mania	↑ [31,36]	↔ in all mood states
			↔ [20,29,111]	
		Euthymia	↔ [20,35,36,48,108]	
			↑ [29]	
		Depression	↑ [36]	
			↔ [20,29,31,109]	
	CSF	Euthymia	↓ [37]	↓ in euthymia
IL-4	Serum	Mania	↑ [30,36,112]	↑ in mania
		Euthymia	↑ [36]	↔ in euthymia.
			↔ [48,107]	Not conclusive in depression
		Depression	↔ [36]	
			↓ [30]	
IL-1β	Serum	Mania	↔ [30]	↔ in all mood states
		Euthymia	↔ [48]	
		Depression	↔ [30]	
	CSF	Euthymia	↑ [37]	↑ in euthymia
IL-1RA	Plasma	Mania	↔ [29,110]	↔ in all mood states
		Euthymia	↔ [29,110]	
		Depression	↔ [29]	
CCL2	Serum/Plasma	Mania	↔ [41]	↔ in mania and euthymia
		Euthymia	↔ [40,41,48]	
CCL3	Serum/Plasma	Mania	↔ [41]	↔ in mania and euthymia
		Euthymia	↔ [40,41]	
CXCL8	Serum/Plasma	Mania	↑ [31]	Not conclusive in all mood states
			↓ [41]	
		Euthymia	↔ [48]	
			↓ [41]	
		Depression	↑ [31]	
	CSF	Euthymia	↔ [37]	↔ in euthymia
CXCL10	Serum/Plasma	Mania	↑ [41]	↑ in mania and euthymia
		Euthymia	↑ [40,41]	
CXCL11	Serum/Plasma	Mania	↑ [41]	↑ in mania.
		Euthymia	↔ [40]	Not conclusive in euthymia
			↑ [41]	
CXCL24	Serum/Plasma	Mania	↔ [41]	↔ in mania.
		Euthymia	↓ [40]	Not conclusive in euthymia
			↑ [41]	
Adiponectin	Plasma	Euthymia	↑ [42]	Not conclusive in euthymia and depression
		Depression	↓ [109]	
Leptin	Serum/Plasma	Mania	↔ [110]	↔ in mania and euthymia
		Euthymia	↔ [110,113]	
			↑ [42]	
Resistin	Plasma	Euthymia	↔ [42]	↔ in euthymia
VEGF	Plasma	Mania	↑ [39]	↑ in mania

↓: Decreased levels; ↔: Not altered levels; ↑: Increased levels; CSF: Cerebrospinal fluid; IL-1RA: Interleukin-1 receptor antagonist.

Bipolar disorder patients did not present changes in monocyte ($CD14^+$) count [48,49]. However, McAdams and Leonard [47] demonstrated an increased monocyte phagocytic activity in bipolar disorder. Corroborating this finding, Knijff *et al.* [50]. showed that monocytes from bipolar disorder patients presented an altered pro-inflammatory response, that is, higher production of IL-6 and decreased production of IL-1β, following lipopolysaccharide (LPS) stimulus in comparison with monocytes from controls. Higher monocyte activation was also demonstrated in children with bipolar disorder [51]. There are some hypotheses to explain the association between bipolar disorder and a state of monocyte hyperactivation: i) bipolar disorder or the stress associated with mood episodes as responsible for inducing a state of monocyte hyperactivity; ii) the state of hyperactivity of monocytes as the trigger of the mood disorder (as suggested by Smith [52] in the 'macrophage theory of depression'); iii) a common underlying factor to bipolar disorder and monocyte hyperactivity; iv) two independent underlying factors, sharing the same environment and leading to bipolar disorder and the activation of monocytes [53].

There are conflicting data regarding total lymphocyte count. Abeer *et al.* [45]. showed increased total lymphocyte count in bipolar disorder, which was not confirmed by other studies [48]. T cells from bipolar disorder patients present resistance to dexamethasone (DEX) [50]. This result reinforces data supporting a state of glucocorticoid resistance of the HPA axis in bipolar disorder patients (see below). It seems that bipolar disorder patients do not present differences in cytotoxic ($CD8^+$) cells or natural killer (NK) cells [49,54,55]. The results are controversial regarding $CD4^+$ T-helper cells [48,49,55]. Recent studies investigated regulatory T lymphocytes (Tregs), $CD4^+$ T cells expressing the transcription factor FOXp3 ($CD4^+CD25^+FOXp3^+$), due to their properties of suppressing the proliferation of other lymphocytes *in vitro* as well as inhibiting the development of autoimmune diseases *in vivo* [56]. A reduction in the frequency of Tregs was reported in these patients [48] and may be one of the factors associated with a greater prevalence of autoimmune diseases observed in bipolar disorder [57].

2.1.4 Hypothalamic–pituitary–adrenal axis

The HPA axis is the main system activated in response to physical or psychological stress, leading to an increase in the production and release of corticotrophin-releasing hormone (CRH) and vasopressin from the hypothalamus. Both hormones stimulate the anterior pituitary to produce adrenocorticotropic hormone (ACTH), which activates the adrenal glands to release glucocorticoids (cortisol in humans). Glucocorticoids, in turn, exert inhibitory feedback effects mainly at the hypothalamus and pituitary gland to inhibit the synthesis and secretion of CRH and ACTH, respectively. Different measures of HPA axis function have been assessed in several psychiatric disorders, including basal cortisol levels, dexamethasone suppression test (DST) and DEX/CRH test. It is well known that the HPA axis is altered in a wide range of neuropsychiatric disorders, including bipolar disorder.

Several studies have consistently demonstrated increased activity of the HPA axis and basal cortisol levels during manic and depressive episodes in bipolar disorder patients (**Table 1**) [58,59]. Data concerning HPA function in euthymia are controversial, but there is a trend to persistent increased levels of cortisol in response to the DEX/CRH test [59-61], indicating that HPA axis dysregulation may persist even after symptom remission and can possibly contribute to the pathophysiology of bipolar disorder.

It has been hypothesized that the HPA axis dysregulation may contribute to the pathophysiology of bipolar disorder and mood swings. Vieta *et al.* [62]. found that a poor ACTH response after CRH challenge test was predictive for depressive relapse within 6 months, while an enhanced ACTH response after CRH challenge test was predictive of manic relapse in euthymic patients [63]. Offspring of parents with bipolar disorder exhibit increased levels of salivary cortisol when compared with offspring of parents with no mental disorder, suggesting that the HPA dysfunction may precede bipolar disorder onset [64].

2.1.5 Hormones and neuropeptides

2.1.5.1 Melatonin

The pineal hormone melatonin is one of the main regulators of the circadian cycle. Melatonin is regulated by the light/dark cycle. As light inhibits melatonin secretion, its levels rise before bedtime, stay high during nocturnal sleep period, decrease quickly around wake time and is almost undetectable during daytime.

Based on the clinical observation of dysregulation of circadian rhythm in bipolar disorder patients, Lewy *et al.* [65] investigated in a seminal study melatonin plasma levels in bipolar disorder patients and showed increased suppression of melatonin secretion after light exposition in comparison with controls. Other studies confirmed increased suppression and/or lower levels of melatonin in bipolar disorder patients [66,67]. Recent studies have proposed the melatonin dysregulation as a possible target for pharmacotherapy. Indeed, recent trials with melatonin agonist receptors (M1 and M2), like agomelatine, had promising results. Calabrese *et al.* [68] reported that 81% of bipolar disorder patients presented a significant improvement of depression scores under agomelatine treatment [68]. Moreover, melatonin can be used as adjuvant therapy, reducing depressive and manic symptoms, and improving sleep quality and quantity [69].

2.1.5.2 Thyroid hormones

Bipolar disorder is traditionally associated with abnormalities in the hypothalamic–pituitary–thyroid (HPT) axis. There is an elevated prevalence of thyroid dysfunction, mainly hypothyroidism, in bipolar disorder patients. Amsterdam *et al.* [70] showed lower levels of thyroxin (T4) and an increased

proportion of abnormal levels of thyroid-stimulating hormone (TSH) in response to thyrotropin-releasing hormone (TRH) challenge in bipolar disorder patients [70].

Actually, it seems that thyroid gland presents a reciprocal connection with mood. Dysfunction of the thyroid is capable of influencing mood, being related to mixed states and rapid cycling. Conversely, depressive state is associated with increased TSH levels and mania with decreased TSH levels [71]. It is worth mentioning that this bidirectional connection can be influenced by lithium, a well-known drug with the capacity to inhibit T4 release from the thyroid gland.

2.1.6 *Oxidative stress markers*

Altered oxidative stress has been considered an important biological finding in bipolar disorder pathophysiology. The brain requires high levels of oxygen, demanding a perfect balance between reactive oxygen species (ROS) production and anti-oxidant defense. ROS are physiologically produced by different cells and are eliminated by enzymatic (superoxide dismutase (SOD) and glutathione peroxidase (GPx)) and non-enzymatic antioxidant processes. Nitric oxide (NO), an important oxidative stress marker, has multiple cellular and molecular targets, presenting cytotoxic or even cytoprotective functions depending on its target, and also participates in neurosecretory processes, especially in the CRH system. Any imbalance between ROS production and elimination induces an oxidative cell stress with consequent peroxidation of lipids, proteins and DNA damage. Several studies have investigated the role of oxidative stress markers, and changes in antioxidant enzymes, lipid peroxidation and NO levels in bipolar disorder [72-74].

Bipolar disorder patients present increased NO levels regardless of mood state [75,76]. Andreazza *et al.* [72] found an increase in SOD activity in serum and erythrocytes from bipolar disorder patients in mania and depression. In the same study, a reduced activity of serum catalase (CAT) and normal levels of GPx was observed in bipolar disorder patients in mania, suggesting an enhanced oxidative state [72]. Accordingly, increased DNA damage was reported in bipolar disorder patients, being associated with the severity of manic and depressive symptoms [73]. DNA damage due to increased oxidative stress is related to a decreased telomeric length in lymphocytes of bipolar disorder patients, an index of early immunosenescence [77]. Other oxidative stress-related molecules, such as thiobarbituric reactive substances (TBARS), are also increased in bipolar disorder [73,74].

It is important to mention that the therapeutic effects of mood stabilizers may be related to their regulatory effects on oxidative stress pathways. Bipolar disorder patients treated with lithium exhibit decreased levels of TBARS and SOD, and increased CAT levels [78], while healthy subjects exposed to lithium do not exhibit the same changes, suggesting that these oxidative stress changes may be associated with the therapeutic effects of lithium in bipolar disorder [79].

2.2 Candidate neuroimaging biomarkers

One of the most replicated findings in neuroimaging studies in bipolar disorder is the presence of white matter hyperintensities [80]. Other structural findings include the enlargement of the lateral ventricle which was described in most studies, while enlargement of the third ventricle was reported in approximately 70% of studies. Decreased subgenual prefrontal cortex has been described in bipolar disorder patients by several independent groups, which is associated with therapeutic response [81-83]. Similarly, decreased gray matter volume in the ventral/orbitomedial prefrontal cortex has been described [84,85]. The results of magnetic resonance imaging (MRI) studies evaluating hippocampus, amygdala and anterior cingulate cortex have been heterogeneous, which may be related, at least in part, to the use of mood stabilizers, especially lithium [86,87].

In the last decade, studies demonstrated deep white matter abnormalities in individuals with bipolar disorder patients using a technique called diffusion tensor imaging, which may provide evidence of microstructural abnormalities in myelinated tracts [88,89]. Interestingly, microstructural white matter changes are associated with poor cognitive outcome in bipolar disorder [90]. Altered energy parameters in brain areas associated with mood regulation and cognitive processing have also been described in magnetic resonance spectroscopy in bipolar disorder. Specifically, bipolar disorder patients present state-dependent higher myoinositol levels [91]. Also, decreased *N*-acetyl aspartate (NAA) and elevated choline levels have been described in bipolar disorder patients [92,93], which has been suggested to result from dysfunction in mitochondrial-mediated changes in oxidative stress, apoptosis and disruption in gene expression [94].

Lithium was found to increase NAA, the second most abundant amino acid in the brain and a marker of neuronal integrity and viability, which is directly associated with mitochondrial energy metabolism [84,87]. In line with this finding and corroborating the concept of neuroprotection in bipolar disorder, several structural neuroimaging studies have consistently described an association between lithium treatment and increased gray matter volume in brain areas implicated in emotional processing and cognitive control [80,95]. A recent meta-regression and meta-analysis analyzing 98 structural studies in bipolar disorder showed a significant increase in the gray matter volume induced by lithium [80].

Bipolar disorder has been associated with altered cerebral connectivity in diffusion tensor imaging (morphological connectivity) and functional MRI (functional connectivity) studies. More specifically, cortical-limbic dysregulation has been consistently reported in bipolar disorder. A significant hyperactivity of subcortical limbic structures involved in automatic emotion processing is described in bipolar disorder patients, especially when subjected to emotional tasks [96,97]. In this scenario of altered frontal/cortical-limbic activation, there is attenuation in inferior frontal cortex or ventrolateral prefrontal cortex activation. The activation of dorsal brain structures

is also decreased in bipolar disorder, reducing the inhibition of the ventral-limbic network and enhancing emotional responses [98]. Therefore, the finding of hypoactivation of frontal lobe with associated disinhibition of limbic structures [99,100] suggest that corticolimbic dysregulation may underlie the emotional dysregulation and cognitive impairments associated with bipolar disorder.

Positron emission tomography (PET) studies in resting state in depressed and manic subjects have demonstrated higher amygdala and ventral striatal limbic subcortical activity in comparison with healthy controls [90,101,102]. Severity of depressive symptoms was negatively correlated with prefrontal metabolism, while positively associated with paralimbic subcortical metabolism [103]. In mania, it has been reported an enhanced metabolism in the dorsal cingulate cortex, striatal regions and the nucleus accumbens, as well as in limbic structures of the temporal lobes. Regarding specific PET ligands, dopamine 1 receptor binding is reduced in the frontal cortex of subjects with bipolar disorder. Psychotic bipolar disorder patients had higher dopamine 2 receptor density in the caudate, which is associated with the severity of psychotic symptoms. In relation to serotonin (5-HT) ligands, serotonin transporter density was increased in the thalamus, dorsal cingulate cortex, medial prefrontal cortex and insula in bipolar depression [104].

Despite the current limitations, including the size and heterogeneity of the studied samples, the use of neuroimaging tools will continue to generate knowledge on the physiopathology of bipolar disorder that will ultimately lead to definition of biomarkers to guide diagnostic process and therapeutic interventions.

3. Conclusion

Bipolar disorder is associated with significant changes in brain structures and several biological cascades involving neurotrophic factors, cytokines, hormones and oxidative stress molecules. However, to date there are no sufficient data to support any of these biomarkers as diagnostic or prognostic in bipolar disorder.

Advances in this very important area depend on the integration of different approaches, including the 'omics' (genomics, proteomics, metabolomics) and new structural, functional and molecular neuroimaging techniques, to longitudinal studies with bipolar disorder patients presenting the wide clinical spectrum of the disease.

4. Expert opinion

The diagnosis of bipolar disorder is based on the patients' symptoms and clinical observation according to standardized criteria laid out in the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and the tenth edition of the International Classification of Diseases (ICD-10). Based on the complexity of the clinical presentation of bipolar disorder, no laboratory or neuroimaging test is currently available to diagnosis the disease.

It is worth mentioning that the identification of several changes in peripheral and neuroimaging markers contributed significantly to the understanding of the physiopathology of bipolar disorder. Stress and impaired cellular resilience seems to impact significantly on the development and clinical course of the disease. Stress influences the brain and it is translated by disturbances in several peripheral parameters and pathways (i.e., imbalance in neurotrophic factors, activation of a pro-inflammatory state and abnormalities in oxidative energy generation with accumulation of oxidative damage). The imbalance in these peripheral parameters may directly affect growth and activity of neurons and glia cells, resulting in increased brain sensitivity and decreased cellular resilience, especially during mood episodes. Neuroimaging studies support this model, in which all these processes are translated into morphological and biochemical abnormalities in the brain, including enlargement of lateral ventricles and decrease on cortical volume. In this context, it has been hypothesized that bipolar disorder is a neuroprogressive disease [27]. Progressive changes in the CNS take place in the course of bipolar disorder turning the patient more prone to pathological mood states since neuroprogression is related to brain tissue damage and structural/functional abnormalities involving the neural substrate of mood regulation.

Taking into consideration its complex neurobiological basis, it will not be possible to find out a single specific biomarker for the diagnosis of bipolar disorder. Moreover, it is uncertain whether these putative biomarkers present the same profile across the lifespan. Instead, studies in this field should focus on the development of state biomarkers, also evaluating potential predictors of therapeutic response and surrogate outcomes. This redefinition of biomarkers research in bipolar disorder is of paramount importance as its treatment usually requires polypharmacy, and even under combined treatment, patients have several relapses and high rates of treatment-resistant cases are reported.

It is worth mentioning that these peripheral biomarkers, when considered individually, are not specific for bipolar disorder, since they have been described in other neuropsychiatric disorders like major depressive disorder, schizophrenia and Alzheimer's disease. Taking this into consideration, a composite of biomarkers, including neurotrophic factors, cytokines and oxidative stress molecules, may be a promising tool to identify altered mood states and neuroprogression in bipolar disorder. Kapczinski *et al.* [20,28]. proposed a composite measure involving neurotrophins (BDNF, NT-3), oxidative stress markers (protein carbonyl content, thiobarbituric acid reactive substances, total reactive antioxidant potentials) and inflammatory molecules (IL-6, IL-10 and TNF-α) capable of separating acute mood states (mania or depression) from controls. This interesting result must be confirmed by independent longitudinal studies. Of note, a similar approach to biomarkers has been recently reported in Alzheimer's

disease [105] and depression [106]. A study combining nine serum biomarkers related to inflammatory, neurotrophic and endocrine-metabolic cascades (α1-antitrypsin, apolipoprotein CIII, BDNF, cortisol, epidermal growth factor, myeloperoxidase, prolactin, resistin and sTNFR2) showed a high accuracy for the diagnosis of major depression [106].

Several peripheral biomarkers are altered in bipolar disorder, but it is uncertain what comes first, that is, the peripheral changes or the disease. It seems that a genetic vulnerability turns the subject more prone to environmental stresses (i.e., childhood maltreatment, drug abuse and sleep deprivation). Stress has been recognized as one of the major triggers of relapses in bipolar disorder. The biological translation of stress is complex, affecting several molecules and pathways, determining imbalance in neurotrophic factors, activation of a pro-inflammatory state and abnormalities in oxidative energy generation with accumulation of oxidative damage, leading to changes in brain circuits and, therefore, in neuroimaging studies. It is also unknown whether there is a specific pathway responsible for initiating the process. It is clear however that these pathways interact with each other. For instance, the activation of a pro-inflammatory state affects the release of neurotrophic factors. Finally, to date it is not possible to suggest any of these parameters or pathways as 'more relevant' to the physiopathology of bipolar disorder.

Declaration of interest

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Fundação de Amparo à Pesquisa de Minas Gerais (Fapemig), Brazil.

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10.4. Artigo de Revisão

NeuroImmunoModulation

Neuroimmunomodulation 2010;17:192–195
DOI: 10.1159/000258721

Published online: February 4, 2010

Interplay between Neuroimmunoendocrine Systems during Post-Traumatic Stress Disorder: A Minireview

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Key Words
Post-traumatic stress disorder · Cortisol · Hypocortisolism · Cytokines · Inflammation · Lymphocytes

Abstract
Early life stress has been suggested to mediate vulnerability to affective disorders. Traumatic events experienced in childhood such as sexual abuse and/or physical neglect may lead to psychiatric diseases in adult life, including post-traumatic stress disorder (PTSD). Previous studies have focused on adult traumatic events and very little is known regarding the long-term physiological effects of early life stress. Here, we review the complex interplay between most important cognitive, neuroendocrine and immunological changes reported in PTSD, focusing on long-term implications of childhood maltreatment. PTSD has been associated with significant biological changes related to impaired cognitive functions, attenuated hypothalamic-pituitary-adrenal (HPA) axis function (hypocortisolism) and activation of innate immune responses (low-grade inflammation).

Introduction

Early life stress has been suggested to mediate vulnerability to psychopathology such as major depression and post-traumatic stress disorder (PTSD). Previous studies have mainly focused on adult traumatic events and very little is known regarding the long-term physiological effects of early life stress. Here, we review the complex interplay between cognitive, neuroendocrine and immunological changes reported in PTSD, focusing on long-term implications of childhood maltreatment. We discuss that many biological changes observed during PTSD could be attributable to insufficiency of glucocorticoid signaling.

Impaired HPA Axis Function

There is a general consensus that PTSD is associated with hypocortisolism. This finding may constitute a paradox because ever since the seminal studies by Selye (1936), stress has been associated with activation of the HPA axis with increasing cortisol levels. Indeed, cortisol hypersecretion has widely been used to define states of stress in human studies. Recently, hypocortisolism has

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1021–7401/10/0173–0192$26.00/0

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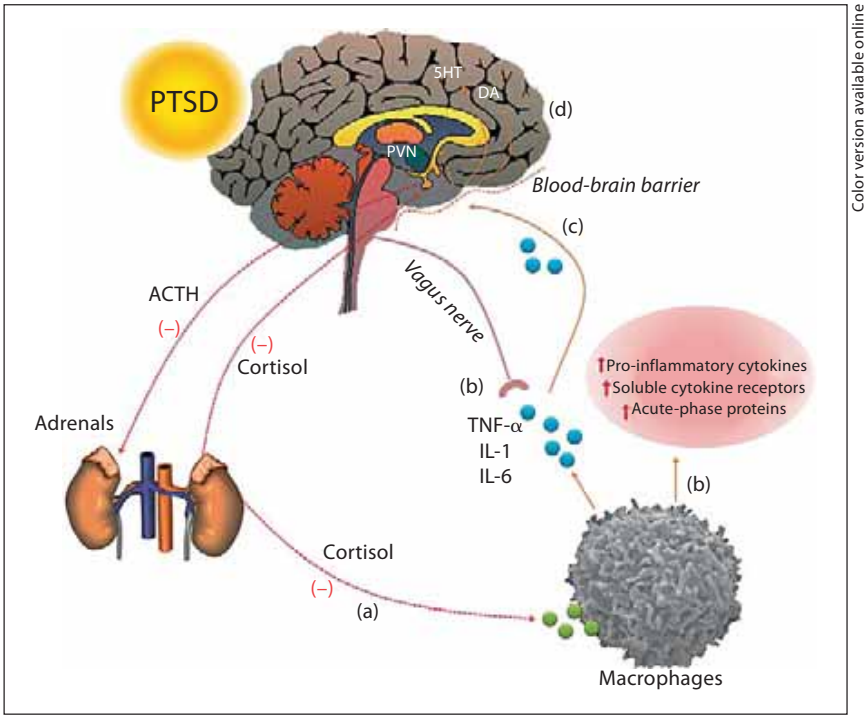

Color version available online

Fig. 1. Neuroimmune interactions during PTSD. Hypocortisolism is associated with lack of adequate control of the immune system (a), leading to low-grade inflammation as indicated by increased levels of pro-inflammatory cytokines (b). These cytokines, in turn, access the brain via afferent fibers (e.g. vagus nerve) (b) or through leaky regions of the blood-brain barrier (c) or through active transport molecules. Once in the brain (d), cytokine signals participate in pathways known to be involved in the development of depression, including: (1) altered metabolism of relevant neurotransmitters such as serotonin (5HT) and dopamine (DA), and (2) activation of CRH in the paraventricular nucleus (PVN) and the subsequent production and/or release of ACTH and glucocorticoids (cortisol).

also been observed in patients with burnout, physical complaints, chronic fatigue syndrome, fibromyalgia, chronic pelvic pain, asthma and others [1]. These data suggest that hypocortisolism is not a specific phenomenon of PTSD.

However, chronically elevated levels of cortisol seem to exist in children who are currently living in adverse situations. Studies performed with maltreated children or who are diagnosed with PTSD show hypercortisolemia [2]. Girls exposed to sexual abuse show an impaired HPA axis function following pharmacological or nonpharmacological challenge tests. In line with such results, Heim et al. [3] found that female adults with childhood sexual abuse had significantly higher cortisol and adrenocorticotropic hormone (ACTH) levels following exposure to acute psychosocial stress (Trier Social Stress Test, TSST) compared to controls. On the other hand, women with early childhood sexual abuse and PTSD had lower concentrations of cortisol during the afternoon hours (noon to 8 p.m.) compared with women with abuse without PTSD and women without abuse or PTSD [4]. It has been hypothesized that an early chronic increase of CRH would lead to downregulation of pituitary CRH receptors during life. This would be thus associated with adrenal insufficiency ('functional adrenalectomy'), and would ultimately explain the blunted cortisol levels found in women with a history of childhood abuse and PTSD.

Low-Grade Inflammation

The immune system is critically regulated by the glucocorticoids. In line with the lack of adequate glucocorticoid-mediated inhibition of immune responses, increasing evidence of immune activation has been report-

ed in stress-related disorders characterized by hypocortisolism. There is growing evidence supporting the link between traumatic stress to a pro-inflammatory profile [5], with increasing serum concentrations of TNF-α, IFN-γ, IL-1β and IL-6. Recently, we observed that depressed patients with or without PTSD symptoms had higher soluble TNF receptor 2 levels in contrast to lower IL-2 and chemokine RANTES levels compared to healthy controls [6]. The low-grade inflammatory response has been linked to disease severity. PTSD patients seem to respond to mild everyday stressors with exaggerated anxiety responses that could potentially trigger the production of pro-inflammatory cytokines. It is also known that experimental psychosocial stress increase pro-inflammatory cytokines. The involvement of cytokines in the pathophysiology of PTSD may involve changes of synaptic transmission, especially in hippocampal-amygdala structures, influencing various aspects of memory related to trauma.

The phenomenon of low-grade inflammation could be also involved with increased morbidity in PTSD. Peripheral administration of pro-inflammatory cytokines or increased levels observed during infections has been associated with changes in the patient's behavior known as 'sickness behavior'. The patient becomes irritable and exhibits increased sleep, depression, fatigue, decreased appetite and sexual drive. Pro-inflammatory cytokines have been implicated in this phenomenon. One clinical example of the role of cytokines in determining behavioral changes comes from studies evaluating the biological effects of IFN-α. Depression has been reported in up to 60% of patients with hepatitis C under treatment with IFN-α. The HPA axis may also have its activity enhanced by pro-inflammatory cytokines; however, a positive relationship between increased HPA axis activity and markers of immune activation is not well established.

Increased Cell-Mediated Immunity and Activation Phenotype

In addition to low-grade inflammation, patients with PTSD also reported important changes in cell-mediated immunity. For instance, NK cells, lymphocytes, T cells have been found particularly increased [7]. The relative balance between Th1/Th2 immune responses seems to be balanced towards a potent Th1 immunity. Indeed, delayed-type hypersensitivity (DTH) was found enhanced in women with PTSD due to childhood sexual or physical abuse [8].

The phenotype of lymphocytes is also altered in PTSD, suggesting an activation profile. Increased counts of activated T ($CD2^+HLA^-DR^+$), B ($CD20^+CD23^+$) and NK ($CD16^+CD71^+$) cell subpopulations in women with PTSD due to war displacement have been reported [9]. Lymphocytes expressing the late ($CD71^+$) but not the early ($CD25^+$) activation marker were also elevated in displaced women. Another phenotype alteration is regarding the T cell memory profile, especially those associated with a history of childhood sexual abuse. An increased percentage of both central memory ($CD45RA^-CCR7^+$) and effector memory ($CD45RA^-CCR7^-$) T cell subsets has been observed in PTSD patients [10]. Interestingly, Sommershof et al. [10] also observed a ~50% drop of regulatory T cells ($CD4^+CD25^+FoxP3^+$) in PTSD patients as compared to healthy controls. This substantial decline of regulatory T cells (Tregs) could bear the risk of excessive inflammation due to suboptimum control of immune responses and provide further support to the pro-inflammatory profile observed in patients with PTSD. Therefore, PTSD patients could be at risk for inflammatory disorders. Indeed, deficiency or dysfunction of Tregs in humans has been linked to several inflammatory and auto-immune diseases including multiple sclerosis, asthma, type 1 diabetes, psoriasis, and rheumatoid arthritis.

Altered Peripheral Sensitivity to Glucocorticoids

The effects of glucocorticoids on the immune system are mediated via both intracellular and membrane-bound glucocorticoid receptors (GRs). However, the functional effect of a stress hormone will depend on the sensitivity of the target tissue for that particular hormone. A reliable way of assessing the cross talk between peripheral hormones and the immune system is to determine the functional hormone action in specific target cells. We have recently observed that dexamethasone (GR agonist) was less capable to suppress T cell proliferation of depressed women with long-standing PTSD symptoms due to childhood maltreatment, suggesting acquired steroid resistance. Conversely, it has also been shown that mononuclear cells from Bosnian war refugees with PTSD symptoms required less dexamethasone concentrations to inhibit cellular LPS-induced IL-6 and TNF-α secretion, suggesting increased sensitivity to glucocorticoids [11].

The magnitude of the biological effects of glucocorticoids is determined, among other factors, by the

number and functionality of GRs. Yehuda et al. [12] reported increased densities of GR in peripheral lymphocytes of combat Vietnam veterans, without changes in cortisol levels. However, there are contradictory findings in the literature reporting lower GR densities with unaltered affinity for glucocorticoids. To what extent changes in glucocorticoid signaling in the immune system are related to fluctuations in cortisol levels or immune mediators (cytokines) remains to be determined.

Concluding Remarks

PTSD is associated with impaired homeostatic forces, leading to a significant allostatic load across the nervous, endocrine and immune systems. Hypocortisolism, impaired central and peripheral glucocorticoid signaling, low-grade inflammation and activation of cell-mediated immunity are commonly observed changes reported in PTSD. The constant stress experienced by PTSD patients may have important deleterious consequences and predispose the patient to stress-related disorders.

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